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Research Article

Cancer Prevention Research

Chemoprevention of Azoxymethane/Dextran Sodium Sulfate–Induced Mouse Colon Carcinogenesis by Freeze-Dried Yam *Sanyaku* and Its Constituent Diosgenin

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Abstract

The effects of *sanyaku*, a traditional Chinese medicine [freeze-dried powder of the yam tuber (*Dioscorea*)], and its major steroidal saponin constituent, diosgenin, on colon carcinogenesis were investigated. Male ICR mice were subjected to a single intraperitoneal injection of azoxymethane (AOM; 10 mg/kg body weight) followed by administration of 1.5% dextran sodium sulfate (DSS) in drinking water for 7 days to establish carcinogenesis. Commercial diosgenin or *sanyaku*, which contained diosgenin at 63.8 ± 1.2 mg/kg dry weight, was given in the diet at 20, 100, or 500 mg/kg for 17 weeks. Groups of mice that received diosgenin or *sanyaku* at all doses yielded significantly less number of colon tumors compared with the AOM/DSS-treated mice. Occurrence of colonic mucosal ulcer and dysplastic crypt induced by AOM/DSS treatment was also significantly decreased by the administration of diosgenin and *sanyaku*, which was in accordance with the significant reduction of AOM/DSS-mediated increases in expression of inflammatory cytokines such as *IL-1β* by diosgenin and *sanyaku*. Furthermore, elevated levels of serum triglyceride in the AOM/DSS-treated mice tended to be reduced in mice given diosgenin and *sanyaku*. Microarray and real-time reverse transcriptase PCR analyses revealed that diosgenin administration increased 12-fold the expression of lipoprotein lipase, which may contribute to reduced serum triglyceride levels. Other genes altered by diosgenin included those associated with antioxidative stress responses and apoptosis, such as heme oxygenase-1, superoxide dismutase-3, and caspase-6. Our results imply that the Chinese medicine *sanyaku* and the tubers of various yams containing diosgenin as food could be ingested to prevent colon carcinogenesis in humans. *Cancer Prev Res; 4(6); 924–34. ©2011 AACR.*

Introduction

Colorectal cancer is one of the most common cancers worldwide and has high rates of morbidity and mortality. The International Agency for Research on Cancer reported that colorectal cancer follows a sporadic pattern of occurrence, and only 5% of cases are inherited (1). Several risk factors for colorectal cancer have been reported, including age more than 50 years, formation of colorectal polyps, family history of colorectal cancer, and alteration of certain genes, such as *Apc*, *Madh4*, *Smada*, *Bmpr1a*, and *Lkb1* (2, 3). Epidemiologic studies have shown convincing evidence that a diet high in calories and rich in animal fats, and poor in fruits, vegetables, and fiber is associated with an increased risk of colorectal cancer. Moreover, recent studies have shown that obesity and related metabolic abnormalities, including hyperglycemia, hyperlipidemia, and hyperleptinemia, are associated with an increased risk of colorectal cancer (4, 5). Conversely, a diet low in fat, high in vegetables, and, possibly, high in fiber has a protective effect. Persons with an increased intake of vitamin D and calcium also have a reduced risk of colon cancer (1). Several functional food components and other chemicals, such as curcumin, epigallocatechin gallate, and folate have been reported to suppress colon carcinogenesis in several animal models (6, 7). Thus it has been estimated that 70% of colorectal cancers could be prevented by nutritional intervention, because diet is the most important exogenous lifestyle-related factor in the etiology of this disease (1).

Yams are perennial trailing rhizome plants of the *Dioscorea* genus belonging to the Dioscoraceae family. Yam tubers (*Dioscorea* spp.) are rich in many nutrients,

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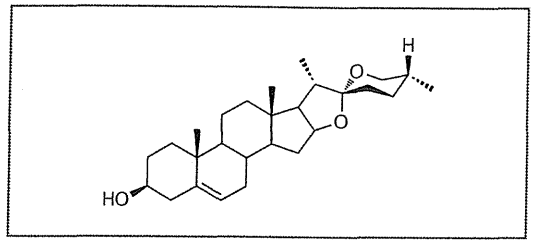

Figure 1. Chemical structure of diosgenin.

including carbohydrates, essential amino acids, vitamin C, minerals, and physiologically active components such as mucin (glycoprotein), polysaccharides, and steroidal saponins, and are consumed as a food in Africa, Asia, Latin America, and Oceania (8–17). Freeze-dried powder from yams has been widely used as a traditional Chinese medicine (*sanyaku*) whose benefits include nutritional fortification, tonic, and antitussive effects, as well as antidiarrheal, expectorant, and hypoglycemic effects (18). Diosgenin (Fig. 1) is an aglycone of the steroidal saponin, dioscin, which is present at relatively high concentrations in the tubers of wild yams (*Dioscorea villosa* Linn) and the seeds of fenugreek (*Trigonella foenum graecum* Linn; refs. 19, 20). Diosgenin is used for the commercial synthesis of steroid products, such as cortisone, pregnenolone, and progesterone. Diosgenin is neither synthesized nor metabolically converted into steroid by-products in the mammalian body, and hence is considered safe (21). The health benefits of diosgenin have been shown in human preclinical studies, and include its efficacy against hyperglycemia (22), hypercholesterolemia (23, 24), and hypertriacylglycerolemia (25).

Several studies have shown that diosgenin possesses anticancer properties. Mechanistic *in vitro* studies have been conducted to understand the role of diosgenin as a chemopreventive agent against several types of cancer cells. These studies have shown that diosgenin exerts its anticancer effects through the modulation of multiple cell signaling pathways associated with growth, differentiation, apoptosis, and oncogenesis (21). *In vivo* research showing the cancer chemopreventive efficacy of diosgenin has been limited. Diosgenin and fenugreek seed powder have been reported to inhibit the formation of colonic aberrant crypt foci (ACF), putative precancerous lesions of the colon in the azoxymethane (AOM)-induced rat colon carcinogenesis model (26). The total number of ACF was decreased by the administration of 0.1% or 0.05% diosgenin either during initiation/postinitiation or promotion stages; the lower dose (0.05%) of diosgenin was as effective as the higher dose (0.1%) in blocking ACF formation (26). Altogether, these preclinical and mechanistic findings strongly implicate the use of diosgenin as a novel, multitarget-based chemopreventive or therapeutic agent against several cancer types (21), although the amounts of diosgenin used in these studies were much higher than the amount that can be obtained in the human diet.

In this study, we investigated the effects of diosgenin and *sanyaku* on colon carcinogenesis induced by AOM/dextran sodium sulfate (DSS) in mice. We found for the first time that diosgenin and *sanyaku* significantly inhibited AOM/DSS-induced colon carcinogenesis, even when low doses (20 ppm) were examined. We also studied possible mechanisms for the chemoprevention of colon carcinogenesis by diosgenin, and found that diosgenin suppressed colon carcinogenesis by decreasing colonic inflammation and serum triglyceride levels, upregulating lipoprotein lipase and modulating multiple signaling pathways.

Materials and Methods

Chemicals

Diosgenin (~95%) and AOM (≥90%) were purchased from Sigma-Aldrich. DSS (molecular weight of 36,000–50,000 Da; catalog no. 160110) was obtained from MP Biomedicals, LLC. DSS for induction of colitis was dissolved in water at a concentration of 1.5% (w/v). *Sanyaku*, a freeze-dried powder preparation of Chinese yam, was obtained from a local pharmacy and stored at −20°C until use. Diosgenin was present in the *sanyaku* sample at 63.8 ± 1.2 mg/kg dry weight (0.0064% w/w) determined by gas chromatography mass spectroscopy (GC-MS) analyses according to the method of Taylor and colleagues (27) and Shah and colleagues (28) with slight modifications.

Animal study for colon cancer chemoprevention

The protocols for the present animal experiments were approved by the Committee of Institutional Animal Experiments. All handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines. Five-week-old male ICR mice were purchased from Charles River Laboratories, Inc. All animals were housed in plastic cages (4–5 mice/cage) and had free access to drinking water and a basal diet (CRF-1; Oriental Yeast Co. Ltd.) *ad libitum*, under controlled conditions of humidity (50 ± 10%), light (12/12 hours light/dark cycle), and temperature (23 ± 2°C). Animals were quarantined for 7 days, and then randomized by body weight into 12 groups. Experimental diets were prepared by mixing diosgenin or *sanyaku* in powdered CRF-1 at dose levels (w/w) of 20, 100, or 500 mg/kg (ppm). The level of diosgenin in CRF-1 (before adding diosgenin) was less than the detection limit (<4 ppm), which was confirmed by the GC-MS method described earlier in the text. Animals had free access to food and water, which was replenished every day. As shown in Supplementary Figure S1, mice in groups 1 to 7 were injected with a single intraperitoneal dose of AOM (10 mg/kg body weight) at the age of 6 weeks. One week after the AOM injection, animals started to receive 1.5% DSS in drinking water for 7 days. After DSS administration was stopped, the mice received a CRF-1 diet for 7 days. Subsequently, they were fed diets containing 0, 20, 100, and 500 ppm diosgenin or *sanyaku* in the CRF-1 diet for 17 weeks.

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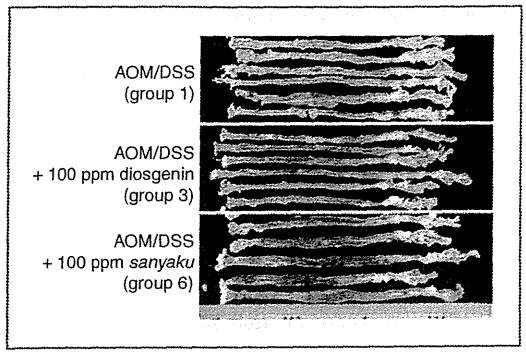

Figure 2. Representative macroscopic view of the colon of mice treated with AOM/DSS (group 1), AOM/DSS + 100 ppm diosgenin (group 3), and AOM/DSS + 100 ppm *sanyaku* (group 6).

Mice in groups 8 and 9 were given either AOM or DSS alone, respectively. Mice in group 10 or 11 were fed a diet containing 500 ppm diosgenin or *sanyaku* without AOM and DSS. Group 12 was an untreated control. All mice were sacrificed at the age of 27 weeks by using excess ether. Prior to termination, animals were starved overnight. On killing, blood samples were obtained with a 1-mL syringe from the inferior vena cava and analyzed for serum lipids. The large intestines were flushed with saline and excised. Other organs such as liver, kidney, and spleen were also collected. After measuring the length of the large intestines from the ileocecal junction to the anal verge, they 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 and fixed in 10% buffered formalin for at least 24 h (Fig. 2). The large intestines were cut into 4 parts from the anus along a vertical axis and 3 histologic sections were made from each part, so that a total of 12 longitudinal sections per colon were made. These were subjected to histopathologic examination, performed on hematoxylin and eosin–stained sections. Pathologic lesions, such as mucosal ulceration, dysplasia, and colonic tumors (tubular adenoma and adenocarcinoma) were diagnosed on all the histologic sections from a colon, and the total number of lesions per colon was calculated.

Analyses for inflammatory gene expression in colonic mucosa

Six-week-old male ICR mice were divided into 3 experimental and 1 control groups ($n = 3$ in each group) corresponding to groups 1, 2, 5 and 12 in the experiment described earlier. After treatment with AOM/DSS, mice were fed with the basal diet or that containing 20 ppm diosgenin or 20 ppm *sanyaku* for 3 weeks. Total RNA was extracted from the scraped colonic mucosa by using TRIZOL reagent (Invitrogen). Real-time reverse transcriptase (RT)-PCR was carried out by SuperScript III reverse transcriptase (Invitrogen) and SYBR Premix (Takara Bio Inc.). The cycle threshold values of each gene and β-actin detected by real-time RT-PCR were converted to signal intensities by the delta-delta method. The sequences of the PCR primer pairs are as follows: *TNF-α*, 5′-GATTATGGCTCAGGGTCCAA-3′ and 5′-CCCAGCATCTTGTGTTTCTG-3′; *IL-1β*, 5′-TCTTCCTAAAGTATGGGCTGGA-3′ and 5′-AAAGGGAGCTCCTTAACATGC-3′; *IL-6*, 5′-CGCTATGAAGTTCCTCTCTGC-3′ and 5′-TTGGGAGTGGTATCCTCTGTG-3′; *IL-12b*, 5′-GCTTCTTCATCAGGGACATCA-3′ and 5′-CTTGAGGGAGAAGTAGGAATGG-3′; and β-actin, 5′-CAGCTTCTTTGCAGCTCCTT-3′ and 5′-CTTCTCCATGTCGTCCCAGT-3′.

Hepatic gene expression profile in diosgenin-administered mice

For microarray analyses and real-time RT-PCR, additional sets of animals were prepared. Nine-week-old ICR mice were fed with a basal diet (CRF-1) or one containing 500 ppm diosgenin for 4 weeks ($n = 3$ in each group). The mice were sacrificed and organs collected for gene expression analyses were stored in RNAlater solution (Ambion). Total RNA was extracted from the liver by using an RNeasy mini kit (Qiagen). Aliquots (5 μg) of total RNA pooled from 3 mice were converted to cRNA and labeled with biotin by using a one-cycle labeling kit (Affymetrix) according to the manufacturer's instructions. Aliquots (20 μg) of biotin-labeled cRNA were hybridized to a Mouse Genome 430 2.0 Array (Affymetrix). After washing steps, the microarray plates were analyzed with a GeneChip Scanner 3000 (Affymetrix). Data analysis was carried out by using the GeneChip Operating System (GCOS; Affymetrix) and Excel (Microsoft). Variable spots detected by the algorithm in GCOS in both plates were defined as nonexpressed genes and removed. Normalization of biotin-labeled signals was carried out by global median normalization. Data were represented by base 2 logarithms. The microarray data were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE24580. Biological reproducibility was confirmed by real-time RT-PCR as described earlier in the text, using the following primers: HMG-CoA synthase 1 (*Hmgcs1*), 5′-CTAGCTCGGATGTTCCTGAATG-3′ and 5′-GACGCCTTTGTTTTCTGGTTG-3′; HMG-CoA reductase (*Hmgcr*), 5′-CCGTCGTGACCTCAAAGAAAG-3′ and 5′-ACAGAAGCCCCAAGCACAA-3′; Squalene epoxidase (*Sqle*), 5′-TTCTACGCTCCCGACTCCTT-3′ and 5′-AACGGCTCCTGATTACACACTTC-3′; Cytochrome P450 family 51 (*Cyp51*), 5′-TGGGCGTCATCGTTGTGT-3′ and 5′-CTGGGTTTTCTGGGGTGTG-3′; Cytochrome P450, family 7, subfamily a, polypeptide 1 (*Cyp7a1*), 5′-TGGTGGTGAGAGCTTGAAAATG-3′ and 5′-TGGTGTGGTTCTTGGAGGTG-3′; lipoprotein lipase (*Lpl*), 5′-CCAGGATGCAACATTGGAGA-3′ and 5′-CAACTCAGGCAGAGCCCTTT-3′; and β-actin, 5′-CAGCTTCTTTGCAGCTCCTT-3′ and 5′-CTTCTCCATGTCGTCCCAGT-3′.

Statistical analysis

The incidences among the groups were compared by using Fisher's exact probability test. Other measurements

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Table 1. Effect of diosgenin and *sanyaku* on the development of colonic mucosal ulcer (UI) and dysplasia (DYS)

Group no.	Treatment	No. of mice examined	Incidence (%)		Multiplicity	
			UI	DYS	UI	DYS
1	AOM/DSS	15	93.3	80.0	1.40 ± 0.83	2.13 ± 1.81
2	AOM/DSS + 20 ppm diosgenin	15	53.3[a]	46.7	0.80 ± 0.86	1.13 ± 1.41[c]
3	AOM/DSS + 100 ppm diosgenin	15	46.7[a]	40.0[a]	0.53 ± 0.64[b]	0.87 ± 1.46[c]
4	AOM/DSS + 500 ppm diosgenin	15	40.0[a]	46.7	0.47 ± 0.64[b]	0.73 ± 1.03[c]
5	AOM/DSS + 20 ppm *sanyaku*	13	53.8[a]	38.5[a]	0.62 ± 0.65	0.69 ± 1.03[c]
6	AOM/DSS + 100 ppm *sanyaku*	15	46.7[a]	40.0[a]	0.47 ± 0.52[b]	0.53 ± 0.83[d]
7	AOM/DSS + 500 ppm *sanyaku*	15	40.0[a]	33.3[a]	0.47 ± 0.64[b]	0.87 ± 1.41[c]
8	AOM	8	0	0	0	0
9	DSS	8	0	0	0	0
10	500 ppm diosgenin	8	0	0	0	0
11	500 ppm *sanyaku*	8	0	0	0	0
12	Untreated	8	0	0	0	0

NOTE: All data shown as the mean ± SD were from histopathologic analysis. Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ([a]$P < 0.05$). Significantly different from the AOM/DSS group (group 1) by Tukey–Kramer multiple comparison posttest ([b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$).

expressing mean ± SD were statistically analyzed by using Tukey–Kramer multiple comparison posttest or Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

General observations

Mean body weight, liver weight, relative liver weight [liver weight (g)/100 g body weight], and colon length of mice administered diosgenin or *sanyaku* at the age of 27 weeks was not significantly different when compared with that of group 1 (AOM/DSS) or group 12 (untreated; Supplementary Table S1). The amounts of food consumed were not significantly different among the groups (data not shown).

Incidence and multiplicity of colonic mucosal ulcer and dysplasia

Macroscopic views of the colon of mice treated with AOM/DSS and also those given diosgenin and *sanyaku* are shown in Figure 2. Table 1 summarizes the incidence and multiplicity of colonic mucosal ulcer (Fig. 3A) and colonic dysplasia (Fig. 3B), respectively. The AOM/DSS treatment induced mucosal ulcers in 93% of mice; the incidences of these ulcers were significantly reduced by the administration of diosgenin or *sanyaku* to 40%–53% and 40%–54%, respectively, depending on dose. Even when the mice were administered the lowest dose (20 ppm) of diosgenin or *sanyaku*, the incidences of mucosal ulcer were significantly decreased in groups 2 and 5 (to 53% and 54%, respectively; $P < 0.05$) as compared with group 1. Moreover, oral administration of diosgenin and *sanyaku* seemed to inhibit the incidence and multiplicity of dysplasia (Table 1). When the AOM/DSS-treated mice were given the lowest dose (20 ppm) of diosgenin or *sanyaku*, the multiplicity of dysplasia was significantly decreased in groups 2 (1.1 ± 1.4, $P < 0.01$) and 5 (0.7 ± 1.0, $P < 0.01$), compared with group 1 (AOM/DSS, 2.1 ± 1.8). Mucosal ulcer and dysplastic crypts were not observed in mice treated with either AOM or DSS alone.

Incidence and multiplicity of large bowel neoplasms

Table 2 summarizes the incidence and multiplicity of large bowel neoplasms. The incidences of adenoma (Fig. 3C) and adenocarcinoma (Fig. 3D) in mice treated with AOM/DSS were 47% and 53%, respectively. In contrast, mice treated with AOM/DSS and given diosgenin or *sanyaku* (groups 2–7) developed adenoma and adenocarcinoma less frequently than those in group 1 (Fig. 2; Table 2). The multiplicity of adenoma in the diosgenin or *sanyaku*-treated mice (groups 2–7) tended to also be less than that of group 1, although the difference was not statistically significant (Table 2). Administration of diosgenin and *sanyaku* at all doses resulted in a significant reduction of the multiplicity of adenocarcinoma and of total tumors (adenoma + adenocarcinoma; Table 2). Even when the lowest dose (20 ppm) of diosgenin or *sanyaku* was administered, the total tumor multiplicity in group 2 (1.6 ± 2.4) and group 5 (1.4 ± 2.2) was significantly less ($P < 0.05$) than that in group 1 (AOM/DSS, 4.3 ± 5.4). Adenoma and adenocarcinoma were not observed in mice treated with either AOM or DSS alone.

Effect of oral administration of diosgenin or sanyaku on gene expression levels of inflammatory cytokines in the colonic mucosa

To examine the anti-inflammatory activity of diosgenin and *sanyaku*, we analyzed expression levels of inflammatory

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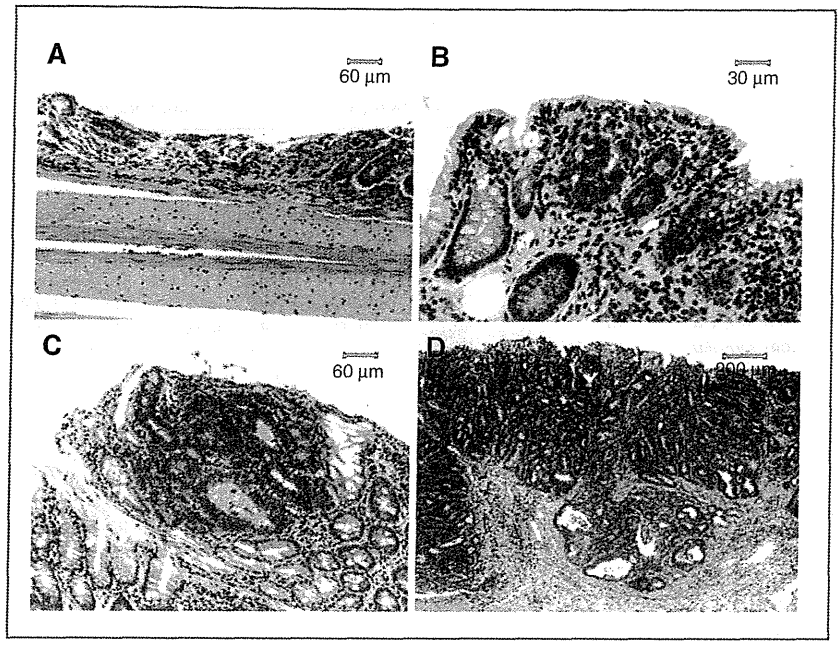

Figure 3. Histopathology of colonic lesions induced by AOM/DSS (group 1). A, mucosal ulcer; B, dysplastic crypts; C, tubular adenoma; and D, invasive ductal adenocarcinoma. Bars in the images represent the distances shown as an indication of magnification.

cytokines in the colonic mucosa. Real-time RT-PCR analyses revealed that treatment with AOM/DSS significantly increased expression levels of inflammatory cytokines (Fig. 4). However, these elevated levels of *IL-1β*, *IL-6*, and *IL-12b* were significantly reduced by treatment with diosgenin and/or *sanyaku* (Fig. 4). *TNF-α* levels were also reduced by treatment with diosgenin and *sanyaku*, to 33% and 22%, respectively, of that induced by AOM/DSS, although these changes were not statistically significant (Fig. 4). These results suggest that the oral

Table 2. Effect of diosgenin and *sanyaku* on the development of colonic adenoma (AD) and adenocarcinoma (ADC)

			Incidence (%)			Multiplicity		
Group no.	Treatment	No. of mice examined	AD	ADC	Total tumor	AD	ADC	Total tumor
1	AOM/DSS	15	46.7	53.3	53.3	1.80 ± 2.21	2.53 ± 3.54	4.33 ± 5.35
2	AOM/DSS + 20 ppm diosgenin	15	40.0	33.3	46.7	0.80 ± 1.15	0.80 ± 1.37[b]	1.60 ± 2.41[b]
3	AOM/DSS + 100 ppm diosgenin	15	33.3	26.7	33.3	0.60 ± 0.99	0.47 ± 0.92[c]	1.07 ± 1.79[c]
4	AOM/DSS + 500 ppm diosgenin	15	40.0	26.7	53.3	0.53 ± 0.74	0.73 ± 1.67[b]	1.27 ± 1.71[b]
5	AOM/DSS + 20 ppm *sanyaku*	13	46.2	23.1	46.2	0.77 ± 1.01	0.62 ± 1.33[b]	1.38 ± 2.22[b]
6	AOM/DSS + 100 ppm *sanyaku*	15	26.7	6.7[a]	26.7	0.47 ± 1.06[b]	0.07 ± 0.26[d]	0.53 ± 1.13[c]
7	AOM/DSS + 500 ppm *sanyaku*	15	26.7	28.6	35.7	0.53 ± 1.06	0.43 ± 0.76[c]	1.00 ± 1.80[c]
8	AOM	8	0	0	0	0	0	0
9	DSS	8	0	0	0	0	0	0
10	500 ppm diosgenin	8	0	0	0	0	0	0
11	500 ppm *sanyaku*	8	0	0	0	0	0	0
12	Untreated	8	0	0	0	0	0	0

NOTE: All data shown as the mean ± SD were from histopathologic analysis. Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ([a]$P < 0.05$). Significantly different from the AOM/DSS group (group 1) by Tukey–Kramer multiple comparison posttest ([b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$).

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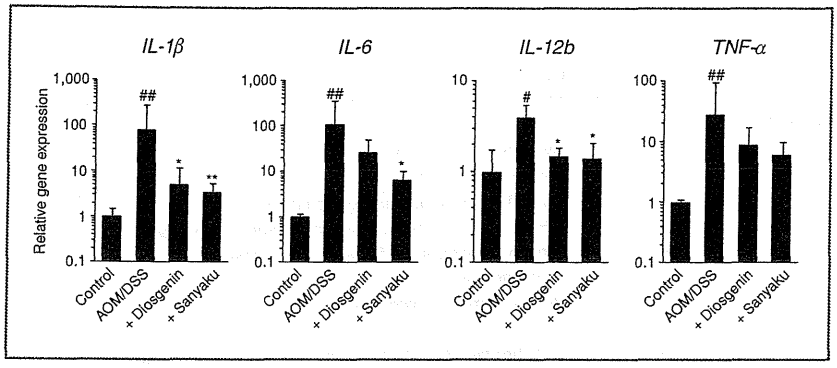

Figure 4. Effects of diosgenin on AOM/DSS-induced inflammatory cytokine gene expression in colonic mucosa. Total RNA was extracted from scraped colonic mucosa of mice treated with AOM/DSS, AOM/DSS followed by 20 ppm diosgenin or *sanyaku* for 3 weeks, and control as described in the Materials and Methods section ($n = 3$ in each group). Real-time RT-PCR analyses were carried out by using specific gene primers. Data are mean ± SD on a $\log_{10}$ scale ($n = 3$). Statistical significance was determined by Student's t test. #, $P < 0.05$; ##, $P < 0.01$, when compared between control and AOM/DSS groups, and *, $P < 0.05$; **, $P < 0.01$, when compared between AOM/DSS and diosgenin- or *sanyaku*-administered groups.

administration of diosgenin and *sanyaku* effectively inhibits AOM/DSS-induced colonic inflammation by reducing the expression of pro-inflammatory cytokines.

Effect of oral administration of diosgenin or sanyaku on serum lipid levels

Because it has been reported that abnormalities of lipid metabolism are involved in the mechanism of colon carcinogenesis (29, 30), we analyzed the levels of serum lipids (Table 3). Mice that developed colon tumors in group 1 exhibited an approximately 2-fold increase in the levels of triglyceride compared with untreated control mice in group 12, although the difference was not statistically significant (Table 3). Administration of 500 ppm diosgenin and *sanyaku* tended to reduce the levels of triglycerides to approximately 79% and 68%, respectively, compared with those of group 1 (AOM/DSS). However, this decrease in triglyceride levels was not observed in mice given 20 ppm diosgenin, or 20 or 100 ppm *sanyaku*. In addition, no statistical differences in the levels of total cholesterol were observed among the groups of mice treated with AOM/DSS with or without diosgenin and *sanyaku*.

Microarray analysis and real-time RT-PCR

To examine the effect of diosgenin administration on mRNA expression, we carried out microarray analysis by using the liver of mice given 500 ppm diosgenin for 4 weeks without AOM/DSS treatment. Microarray analyses revealed

Table 3. The levels of serum triglyceride, cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol

Group no.	Treatment	No. of mice examined	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	LDL cholesterol (mg/dL)
1	AOM/DSS	5	95.2 ± 25.9	121.2 ± 22.1	81.8 ± 12.7	7.4 ± 1.1
2	AOM/DSS + 20 ppm diosgenin	5	103.6 ± 65.3	118.2 ± 26.6	80.8 ± 20.5	7.4 ± 1.7
3	AOM/DSS + 100 ppm diosgenin	5	74.0 ± 45.1	108.4 ± 20.2	73.6 ± 18.4	8.0 ± 2.5
4	AOM/DSS + 500 ppm diosgenin	5	75.6 ± 37.8	111.6 ± 28.7	76.6 ± 17.8	6.2 ± 2.9
5	AOM/DSS + 20 ppm *sanyaku*	5	156.6 ± 95.1	104.2 ± 8.8	58.8 ± 8.9	9.6 ± 3.4
6	AOM/DSS + 100 ppm *sanyaku*	5	101.2 ± 73.8	135.8 ± 26.4	89.8 ± 14.2	8.0 ± 3.2
7	AOM/DSS + 500 ppm *sanyaku*	5	64.8 ± 21.0	126.4 ± 12.1	86.8 ± 9.9	7.2 ± 1.3
8	AOM	5	61.2 ± 22.2	103.4 ± 22.1	66.4 ± 15.8	7.0 ± 1.9
9	DSS	5	62.8 ± 28.8	140.4 ± 19.5	91.8 ± 12.5	7.6 ± 1.8
10	500 ppm diosgenin	5	79.8 ± 40.9	152.2 ± 31.0	91.8 ± 17.3	9.0 ± 2.9
11	500 ppm *sanyaku*	5	52.2 ± 16.0	115.2 ± 27.5	75.4 ± 15.6	6.0 ± 1.9
12	Untreated	5	50.6 ± 9.4	124.6 ± 23.3	79.0 ± 15.0	6.8 ± 1.3

NOTE: All data shown as the mean ± SD.

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Table 4. DNA microarray results for gene-related lipid metabolism, inflammation, cell growth

GenBank	Symbol	Gene name	Fold change (log)
Cholesterol biosynthesis			
NM_020010	*Cyp51*	Cytochrome P450, family 51	0.69
AB016248	*Sc5d*	Sterol-C5-desaturase	0.52
BC004801	*Idi1*	Isopentenyl-diphosphate delta isomerase	0.45
BB705380	*Hmgcs1*	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1	0.25
NM_010191	*Fdft1*	Farnesyl diphosphate farnesyl transferase 1	0.24
NM_009270	*Sqle*	Squalene epoxidase	0.22
AK005441	*Sc4mol*	Sterol-C4-methyl oxidase-like	0.21
Lipid metabolism			
NM_007819	*Cyp3a13*	Cytochrome P450, family 3, subfamily a, polypeptide 13	1.20
AK017272	*Lpl*	Lipoprotein lipase	0.74
BC003305	*Lpl*	Lipoprotein lipase	0.69
NM_031884	*Abcg5*	ATP-binding cassette, subfamily G (WHITE), member 5	0.62
NM_024208	*Echdc3*	Enoyl Coenzyme A hydratase domain containing 3	0.24
BI111416	*Echs1*	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	0.19
BC022940	*Acacb*	Acetyl-Coenzyme A carboxylase beta	−0.12
NM_009993	*Cyp1a2*	Cytochrome P450, family 1, subfamily a, polypeptide 2	−0.25
AF127033	*Fasn*	Fatty acid synthase	−0.30
AV027367	*Apoa4*	Apolipoprotein A-IV	−0.31
NM_016741	*Scarb1*	Scavenger receptor class B, member 1	−0.42
NM_009127	*Scd1*	Stearoyl-Coenzyme A desaturase 1	−0.45
BC010769	*Apoa4*	Apolipoprotein A-IV	−0.49
BB224405	*Scarb1*	Scavenger receptor class B, member 1	−0.51
BB138434	*Scarb1*	Scavenger receptor class B, member 1	−0.56
AF047725	*Cyp2c38*	Cytochrome P450, family 2, subfamily c, polypeptide 38	−0.68
BB266455	*Rarb*	Retinoic acid receptor, beta	−0.90
NM_007824	*Cyp7a1*	Cytochrome P450, family 7, subfamily a, polypeptide 1	−1.25
BB667338	*Cyp7a1*	Cytochrome P450, family 7, subfamily a, polypeptide 1	−1.27
AW046066	*Ppard*	Peroxisome proliferator activator receptor delta	−1.51
Apoptosis			
NM_009811	*Casp6*	Caspase 6	0.42
BQ173889	*Ppp3ca*	Protein phosphatase 3, catalytic subunit, alpha isoform	0.25
M60651	*Pik3r1*	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	−0.44
NM_010591	*Jun*	Jun oncogene	−0.51
BB783769	*Xiap*	X-linked inhibitor of apoptosis	−0.58
U21050	*Traf3*	TNF receptor-associated factor 3	−0.61
Oxidative stress			
BM239177	*Mapk14*	Mitogen-activated protein kinase 14	0.78
NM_010442	*Hmox1*	Heme oxygenase (decycling) 1	0.57
NM_013602	*Mt1*	Metallothionein 1	0.55
NM_011435	*Sod3*	Superoxide dismutase 3, extracellular	0.54
AW825835	*Gclc*	Glutamate-cysteine ligase, catalytic subunit	0.31
AV026617	*Fos*	FBJ osteosarcoma oncogene	−1.54

that the hepatic expression levels of several genes associated with lipid biosynthesis and metabolism, apoptosis, and oxidative stress were up- or downregulated in the livers of diosgenin-administered mice (Table 4). Expression of some of these lipid metabolism–associated genes was further confirmed by real-time RT-PCR (Fig. 5). In the diosgenin-treated mice, the expression of lipoprotein lipase, which hydrolyzes triglyceride, was increased 12-fold by the diosgenin treatment. The expression of HMG-CoA synthase 1, HMG-CoA reductase, squalene epoxidase, and *Cyp51*, all of which are involved in the cholesterol biosynthesis pathway, was also upregulated. In contrast, the expression level of *Cyp7a1*, which is associated with a cholesterol-lowering response by the conversion of cholesterol to bile acids, was increased 7.3-fold. These results suggest that diosgenin administration could lead to the

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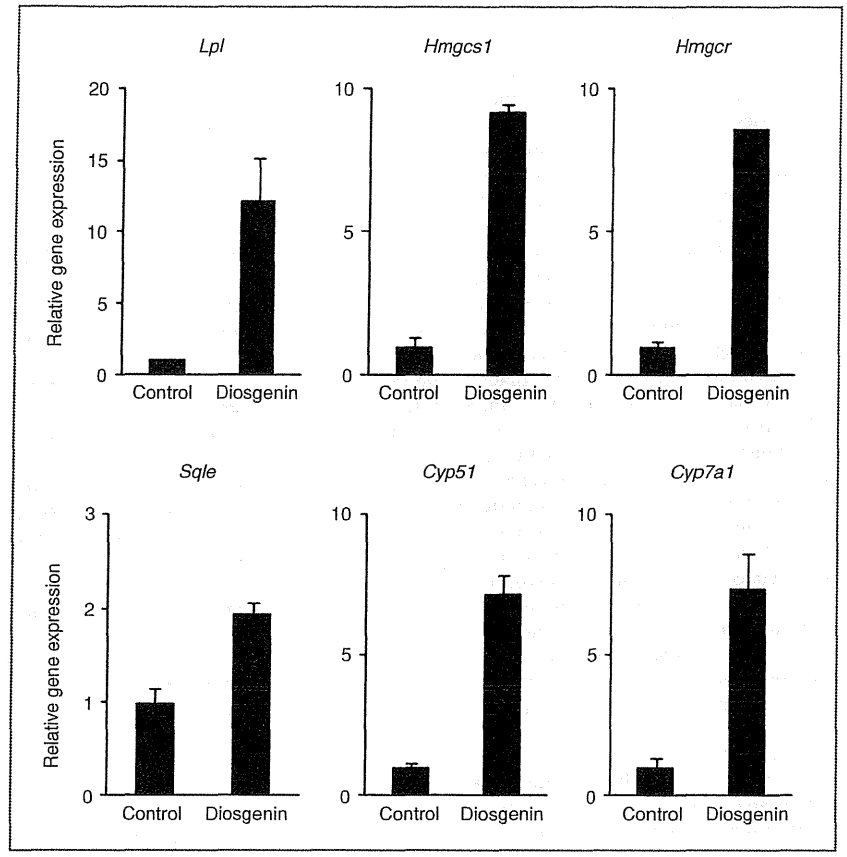

Figure 5. Real-time RT-PCR analyses. Nine-week-old ICR mice were treated with or without 500 ppm diosgenin for 4 weeks. Hepatic RNA was extracted from each mouse, and was subjected to real-time RT-PCR analyses.

improvement of lipid metabolism, which may contribute, at least in part, to decrease serum levels of triglyceride and to chemoprevention in AOM/DSS-induced colon carcinogenesis.

Discussion

In this study, we investigated the effects of orally administered diosgenin or *sanyaku* on mouse colon carcinogenesis induced by AOM/DSS. Our results showed that dietary administration of diosgenin and *sanyaku* significantly inhibited the development of colon cancer induced by AOM/DSS treatment. Raju and colleagues (26) previously reported that 500 to 1,000 ppm of diosgenin and fenugreek seed powder inhibited the formation of colonic precancerous lesions (ACF) in AOM-treated rats, in which the lower dose (500 ppm) of diosgenin was as effective as the higher dose (1,000 ppm) in blocking ACF formation. In this study, we examined the chemopreventive effects of diosgenin and *sanyaku* at doses of 20, 100, and 500 ppm. It was found that diosgenin or *sanyaku* at the lowest concentrations were chemopreventive. However, we could not observe clear dose-dependent responses for anti-inflammatory or anticarcinogenic activity of diosgenin or *sanyaku*. This may be because, even at the lowest concentrations, their effects were saturated. Hence, diosgenin and *sanyaku* may exert chemopreventive effects in humans at low levels, which can be obtained from the human diet.

Several previous studies have shown that the anticancer effects of diosgenin may be attributed to modulation of multiple cell signaling pathways, that is, growth-suppressive effects through cell-cycle arrest and apoptosis induction, modulation of inflammatory processes, and effects on lipid biosynthesis and metabolism pathways (21). Several *in vitro* mechanistic studies have reported that diosgenin induces cell-cycle arrest and apoptosis in several tumor cell lines, including HCT116 or HT29 colon carcinoma cells, where p53 and p21 were upregulated, Bcl-2 was modulated, and caspase-3 was activated (26, 31, 32). In this study, microarray analyses revealed that diosgenin administration altered the expression of pro- and antiapoptotic genes such as caspase-6, protein phosphatase 3, and X-linked inhibitor of apoptosis in mouse liver (Table 4). Taken together, the growth inhibitory effects mediated by cell-cycle arrest

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and/or apoptosis induction possibly contribute to the anticancer activity of diosgenin in AOM/DSS-induced colon carcinogenesis. In addition, although controversial, there are reports that diosgenin can modulate inflammatory processes through the regulation of COX and lipoxygenase activity (33–37). Diosgenin inhibited the activity and expression of COX-2 in human osteosarcoma 1547 cells (33) and also abrogated basal and TNF-induced expression of COX-2 in KBM-5 cells (34), but upregulated COX-2 expression in human erythroleukemia cells (35) and noncancerous human rheumatoid arthritis synoviocytes (36). It has also been reported that diosgenin antagonistically suppressed the inflammatory process in various animal models (38). Diosgenin dose-dependently attenuated subacute intestinal inflammation and normalized bile secretion in indomethacin-induced intestinal inflammation in rats (38). In this study, we showed that oral administration of diosgenin and *sanyaku* markedly reduced the expression levels of inflammatory cytokine genes, including *IL-1β*, *IL-6*, *IL-12b*, and *TNF-α*, which were significantly elevated in the colonic mucosa of mice treated with AOM/DSS (Fig. 4). We also observed that protein levels of COX-2 and iNOS upregulated in the colonic mucosa of AOM/DSS-treated mice were reduced by the administration of diosgenin or *sanyaku* (data not shown). Furthermore, we showed that some genes associated with antioxidative mechanisms, including heme oxygenase-1, superoxide dismutase 3, and glutamate-cysteine ligase, were upregulated in the liver of diosgenin-treated mice (Table 4). These results suggest that anti-inflammatory effects of diosgenin in the intestinal tract may play a role in the prevention of AOM/DSS-induced colon carcinogenesis, because chronic inflammation is an important risk factor for the development of colon cancer (39).

Epidemiologically, a high-fat diet has been associated with an increased risk of colon cancer. Moreover there is a tendency for higher serum triglyceride levels in patients who develop colorectal cancer, as compared with those without colorectal cancer (40). Furthermore, Niho and colleagues (41) have reported that the serum levels of triglyceride in Min mice, an animal model for human familial adenomatous polyps, at the age of 20 weeks were as high as approximately 600 mg/dL, which was approximately 30-fold higher than the levels observed in control mice. They also found that number of intestinal polyps were positively associated with serum levels of triglyceride. The administration of the diethyl benzylphosphonate derivative NO-1886 (a strong inducer of lipoprotein lipase) reduced the serum triglyceride levels to approximately 200 mg/dL and also inhibited intestinal polyp formation in relation to increased lipoprotein lipase activity. It has been recently reported that diosgenin administration resulted in reduced plasma levels of triglyceride and total cholesterol in rodents (23–25), in agreement with our current results showing that dietary diosgenin and *sanyaku* affect lipid metabolism. Higher doses of diosgenin and *sanyaku* tended to reduce serum triglyceride levels, which were elevated in mice treated with AOM/DSS (Table 3). Microarray and real-time RT-PCR analyses showed that administration of diosgenin altered the expression of several genes associated with lipid metabolism in mouse liver. In particular, lipoprotein lipase was significantly upregulated (12-fold) by diosgenin treatment (Fig. 5), which presumably caused a reduction in the levels of serum triglyceride in groups 3, 4, and 7 (Table 3). Similar antihyperlipidemic activities associated with a strong lipoprotein lipase upregulation by diosgenin were also recently reported (42, 43). Thus, our data suggest that the chemopreventive effects of diosgenin on colon carcinogenesis in hyperlipidemic mice may be potentiated by decreasing triglyceride levels *via* upregulation of lipoprotein lipase.

Diosgenin was contained in *sanyaku* at 63.8 ± 1.2 mg/kg dry weight (0.0064%), which was much lower than levels reported in fenugreek (average at 0.54%; ref. 19). It has been reported that when the diosgenin glycoside, dioscin, was orally administered to rats, diosgenin was very poorly absorbed (0.2%; ref. 44). These findings suggest that most ingested diosgenin cannot be absorbed in the stomach and small intestine, and thus enters into the colon, where it exerts chemopreventive effects. On the other hand, we detected other phytosterols in *sanyaku* extracts, including β-sitosterol at a concentration of 0.012%. It has been reported that β-sitosterol supplementation in chow (0.2%) suppressed *N*-methyl-*N*-nitrosourea–induced colon carcinogenesis in rats (45). Therefore, the chemopreventive effects of *sanyaku* may be caused not only by diosgenin but also by other types of phytosterols, such as β-sitosterol present in *sanyaku* and their metabolites.

In summary, the present results provide new evidence indicating that diosgenin and *sanyaku* can inhibit colon carcinogenesis in AOM/DSS-induced mice. These effects were potentially caused by the alteration of lipid metabolism (reduced serum triglyceride levels by upregulation of lipoprotein lipase), and the modulation of genes associated with inflammation and multiple signaling pathways. Further studies are required to explore the chemopreventive effects of diosgenin, the Chinese medicine *sanyaku*, and wild yam tuber on colon carcinogenesis in human clinical studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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

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

Introduction

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

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

Materials and methods

Materials

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

Animals, diet and housing conditions

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

Treatments of animals

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

Histological analysis

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

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

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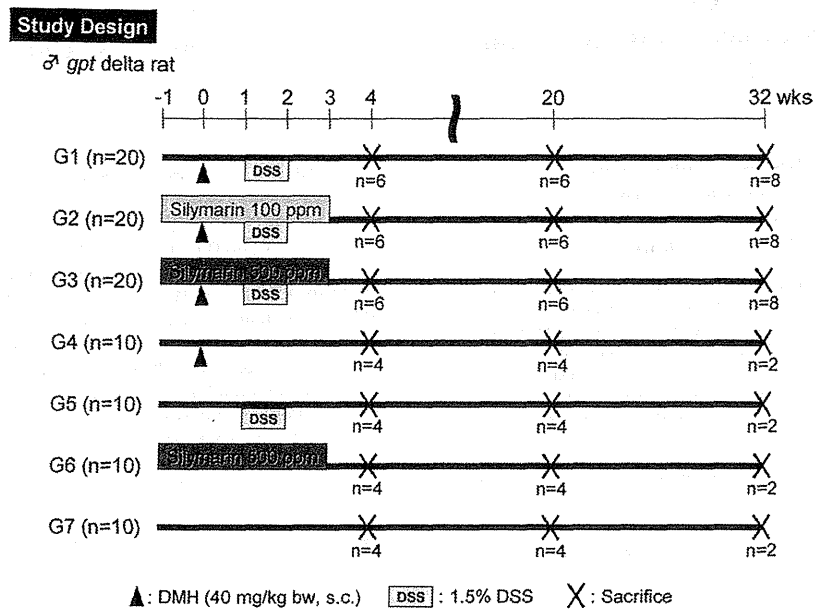

Fig. 1. Experimental protocol. Arrow heads, DMH 40 mg/kg body wt, subcutaneous injection; X, sacrifice.

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

Immunohistochemical procedures

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

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

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

Bacterial reverse mutation test (Ames test)

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

Statistical analysis

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

Results

General conditions of animals

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

Silymarin suppressed preneoplastic lesions

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

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

Gene mutation assay in vivo

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

The colon neoplasms were reduced by silymarin treatment

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

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

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

Discussion

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

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

Table I. Pathological findings in colon (4 weeks)

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

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

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

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

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

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Table III. Incidence of colon tumors in each group

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

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

Table IV. Multiplicity of colon tumors in each group

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

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

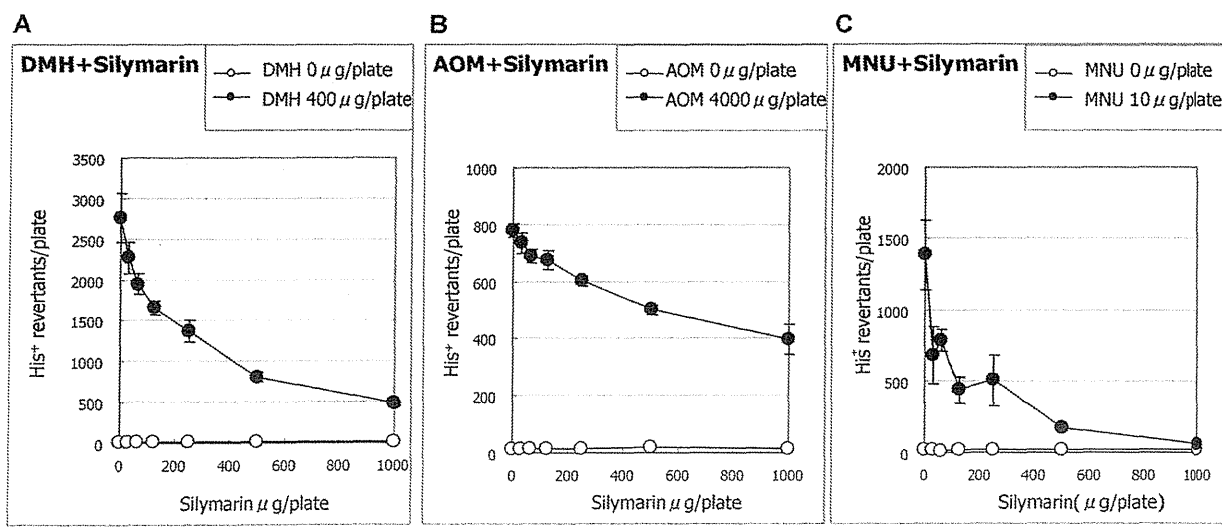

Fig. 2. Antigenotoxic activity of silymarin without S9 mix in *S. typhimurium* strain YG7108 induced by DMH (**A**), AOM (**B**) and MNU (**C**). Filled circle assayed with chemicals, open circle assayed without chemicals.

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

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

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

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

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

Supplementary material

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

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Conflict of Interest Statement: None declared.

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Research Article

Cancer Prevention Research

Selective PGE_2 Suppression Inhibits Colon Carcinogenesis and Modifies Local Mucosal Immunity

Masako Nakanishi[1], Antoine Menoret[2], Takuji Tanaka[3], Shingo Miyamoto[1], David C. Montrose[1], Anthony T. Vella[2], and Daniel W. Rosenberg[1]

Abstract

Prostaglandin E_2 (PGE_2) is a bioactive lipid that mediates a wide range of physiologic effects and plays a central role in inflammation and cancer. PGE_2 is generated from arachidonic acid by the sequential actions of the COX and terminal synthases (PGES). Increased levels of COX-2, with a concomitant elevation of PGE_2, are often found in colorectal cancers (CRC), providing the rationale for the use of COX-2 inhibitors for chemoprevention. Despite their proven efficacy in cancer prevention, however, COX-2 inhibitors exhibit dose-dependent toxicities that are mediated in part by their nonspecific reduction of essential prostanoids, thus limiting their chemopreventive benefit. To achieve enhanced specificity, recent efforts have been directed toward targeting the inducible terminal synthase in the production of PGE_2, microsomal PGES (mPGES-1). In the present study, we show that genetic deletion of *mPGES-1* affords significant protection against carcinogen-induced colon cancer. *mPGES-1* gene deletion results in an about 80% decrease in tumor multiplicity and up to a 90% reduction in tumor load in the distal colon of azoxymethane (AOM)-treated mice. Associated with the striking cancer suppression, we have identified a critical role for PGE_2 in the control of immunoregulatory cell expansion (FoxP3-positive regulatory T cells) within the colon-draining mesenteric lymph nodes, providing a potential mechanism by which suppression of PGE_2 may protect against CRC. These results provide new insights into how PGE_2 controls antitumor immunity. *Cancer Prev Res; 4(8); 1198–208. ©2011 AACR.*

Introduction

Selective inhibition of prostaglandin E_2 (PGE_2) synthesis via pharmacologic targeting of mPGES-1 may provide chemopreventive efficacy while limiting the toxicity that is often associated with long-term use of COX-2 inhibitors (1). mPGES-1 is an inducible terminal synthase with only moderate expression under normal physiologic conditions (2). However, coordinated induction of mPGES-1 and COX-2 is often observed within a variety of cancer types (3). Our laboratory recently reported that genetic deletion of *mPGES-1* in *Apc* mutant mice significantly suppressed intestinal tumorigenesis, a protective effect that occurred in the absence of significant metabolic shunting of the COX-2 product, PGH_2, to other prostaglandins (4).

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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Despite the profound suppression in tumor formation that was observed in this earlier study, the effect of *mPGES-1* deletion in the colon was less apparent due to the propensity of *Apc* mutant mice to develop tumors in the small intestine (5). To address the issue of tissue specificity, we introduced the *mPGES-1* gene deletion onto strain A mice, a line that is highly sensitive to chemical-induced colon cancer (6–8). In the following study, we examined the impact of *mPGES-1* genetic status on colon carcinogenesis induced by azoxymethane (AOM), an organospecific carcinogen that causes multiple adenomas in the distal colon (8). Genetic deletion of *mPGES-1* affords significant protection against AOM-induced colon cancer. Associated with the striking cancer protection, we identified a critical role for PGE_2 in the control of immunoregulatory cell expansion [FoxP3-positive regulatory T cells (Treg)] within the colon-draining mesenteric lymph nodes (MLN), providing a potential mechanism by which inhibition of mPGES-1 and inducible PGE_2 synthesis may protect against colorectal cancers (CRC).

Materials and Methods

Generation of *mPGES-1* deletion on A/J background

Male A/J mice were purchased from The Jackson Laboratory and crossed with female *mPGES-1* knockout (KO) mice (C57BL/6; ref. 9). *mPGES-1* heterozygous mice were backcrossed onto A/J mice for 9 additional generations

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(N10). N10 heterozygous mice were intercrossed to generate A/J:*mPGES-1* KO mice. Genotyping was carried out by tail biopsy. Mice were maintained in a temperature-controlled, light-cycled room and allowed free access to drinking water and standard diet (LM-485; Harlan Teklad).

AOM treatment

Six-week-old wild-type (WT) and KO mice were injected intraperitoneally with AOM (10 mg/kg of body weight; Sigma-Aldrich) or vehicle control (0.9% NaCl) once a week for a total of 6 weeks. Twenty weeks after the last injection, mice were sacrificed and blood, spleen, MLNs, and colon were harvested for further analysis. Colons were flushed immediately with ice-cold PBS and excised longitudinally. Specimens were fixed flat in 10% neutral buffered formalin for 4 hours and stored in 70% ethanol. Animal experiments were conducted after approval by the Animal Care Committee (ACC/IACUS) at the University of Connecticut Health Center.

Quantification of lesions

Whole-mount colons were stained with 0.2% methylene blue and the number and size of aberrant crypt foci (ACF) and tumors were scored under a dissecting microscope. Colon tumor load per mouse was determined using tumor diameter to calculate the spherical tumor volume ($V = 4/3\ \pi\ r^3$). The amount of ulcerated tissue was determined as the percentage across the entire length of colon ($n = 10$ per group).

Immunohistochemistry

Colons were paraffin embedded and sectioned at 5-μm thickness. Sections were treated with 1% to 3% hydrogen peroxide, blocked, and incubated with anti-APC (1:800; Millipore), anti-PCNA (1:150; Novocastra Laboratories Ltd.), anti-PECAM1 (1:500; Santa Cruz Biotechnologies, Inc.), anti-β-catenin (1:2,000; Sigma-Aldrich), anti-cleaved caspase-3 (1:200; Cell Signaling Technology Inc.), anti-cyclinD1 (1:20; Novus Biologicals), anti-mPGES-1 (1:5,000; Abnova), and anti-Ki-67 (1:50; Dako North America, Inc.). Sections were incubated with biotinylated secondary antibody, followed by ABC reagent (Vector Laboratories Inc.). Signal was detected using 3,3′-diaminobenzidine (DAB) solution (Vector Laboratories). Tissues were counterstained with hematoxylin.

Immunofluorescence microscopy

Following antigen retrieval, sections were blocked and incubated with anti-mPGES-1 (1:5,000; Abnova) and then incubated with secondary antibody conjugated with Cy5 (1:500; Millipore). Nuclei were stained with Sytox Orange (1:10,000; Invitrogen). Staining was visualized by confocal microscopy using a Zeiss LSM 510/Confocor II and images were analyzed by LSM image browser software.

Flow cytometry

RBC-depleted spleens and MLN cells were resuspended with staining buffer (balanced salt solution, 3% FBS and 0.1% sodium azide), followed by blocking solution containing normal mouse serum, anti-Fc receptor supernatant from the 2.4 G2 hybridoma (10), and human γ-globulin. Cells were incubated with labeled primary antibodies (CD4, CD8, CD11b, Gr-1 from eBioscience; Ki-67 from BD Biosciences) and analyzed by flow cytometry as described before (11). Intracellular staining of FoxP3 was conducted according to manufacturer's protocol (eBioscience).

In vitro lymphocytes stimulation

Spleens and MLNs were harvested from 20- to 25-week-old untreated female A/J mice, and 1×10^6 cells were stimulated with phorbol-12-myristate-13-acetate (PMA; 50 ng/mL; Sigma) and ionomycin (1 μg/mL; Sigma) for 4 hours in the presence of Brefeldin A (10 μg/mL; Sigma). Intracellular staining was conducted for interleukin (IL) 10, IFN-γ, and Foxp3 (eBioscience,) as described earlier ($n = 6$ per group).

Histologic evaluation

Colon histology was evaluated on Swiss-rolled hematoxylin and eosin (H&E) sections by a board-certified pathologist (T.T.). Colonic crypt dysplasia was diagnosed according to established criteria (12), and colon tumors were further diagnosed using histopathologic descriptions (13).

PGE_2 and serum cytokine analyses

Serum was prepared from blood and samples were purified using a PGE_2 affinity column (Cayman Chemical). PGE_2 concentrations were determined by ELISA (Cayman Chemical; $n = 13$ per group). Serum cytokine levels were determined using a Bio-Plex Pro assay (Bio-Rad Laboratories, Inc.; $n = 5$ per group).

Measurement of the proliferative zone

Quantification of the proliferative zone was assessed on proliferating cell nuclear antigen (PCNA)-stained tissues, randomly selecting 25 crypts per mouse for analysis ($n = 5$ per group). The ratio of positive cells to total cells within the crypt was calculated for each colon, and an average of the ratios were compared between WT and *mPGES-1* KO mice.

Statistical analyses

Statistical analyses of tumor size and multiplicity, as well as a comparison of PGE_2 levels and the frequency of immune cells using fluorescence-activated cell-sorting (FACS) analysis, were conducted by Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

mPGES-1 deletion suppresses colon carcinogenesis

mPGES-1 KO mice and WT littermates were injected with AOM and colons were harvested for analysis 20 weeks after the last injection. In response to AOM treatment, WT mice

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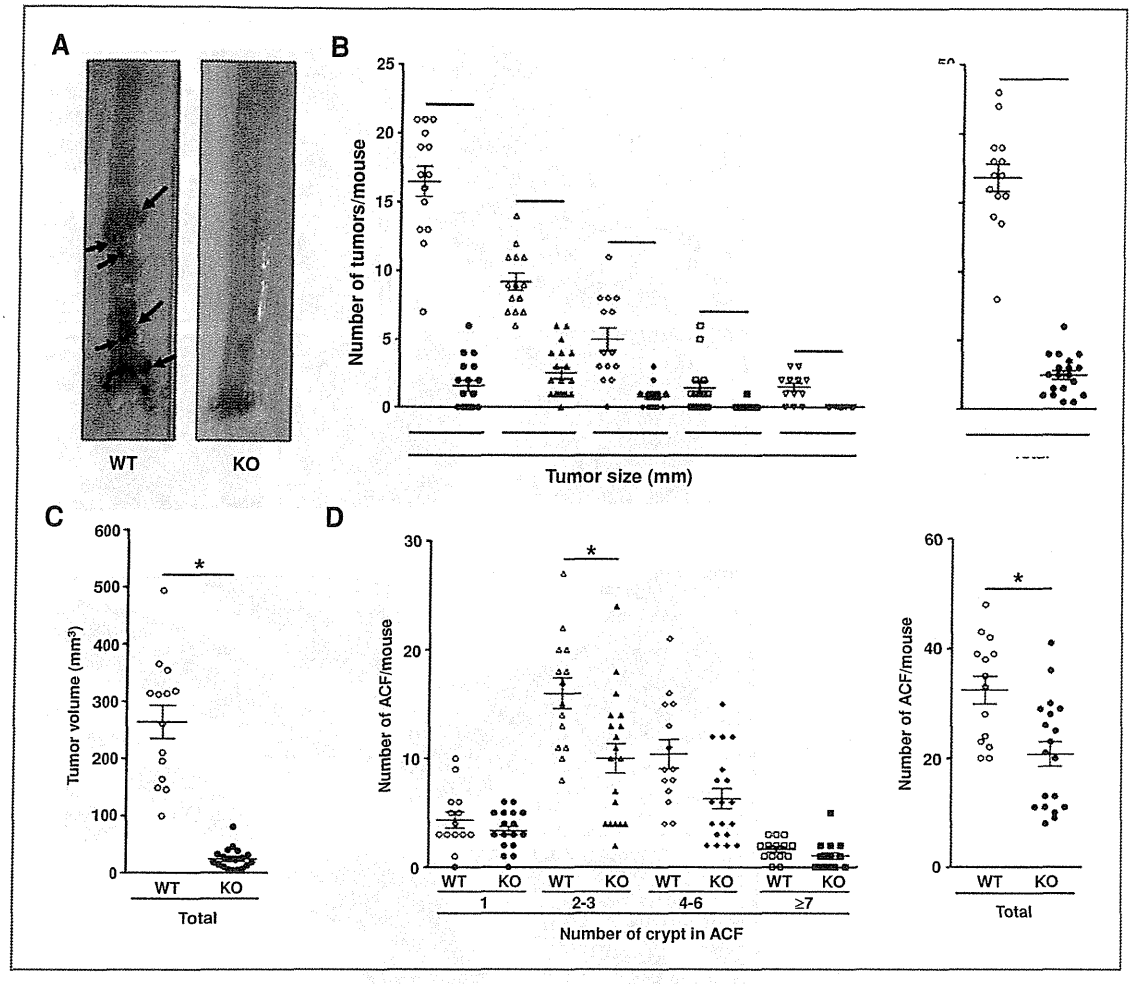

Figure 1. Genetic deletion of *mPGES-1* suppresses AOM-induced colon cancer. A, representative whole-mount colons showing numerous large, well-vascularized tumors in the WT mice (arrows). B, size distribution and total number of tumors per colon rounded to the nearest whole number, as well as total tumor volume (mm^3). C, size distribution and total number of ACF. Each data point represents an individual mouse. Bars indicate means ± SEM. *, $P < 0.05$ compared with WT mice.

developed multiple, large, and highly vascularized tumors, primarily confined to the distal colon (Fig. 1A, arrows). In *mPGES-1* KO mice, however, only several colons had macroscopically visible tumors (Fig. 1A). Tumor enumeration revealed a remarkable suppression (up to 85%) in *mPGES-1* KO mice (33.6 ± 2.0 vs. 5.0 ± 0.7 in WT and KO, respectively; $P < 0.0001$; Fig. 1B), whereas tumor load was reduced by up to 90% (264.0 ± 29.0 vs. 24.3 ± 4.3 in WT and KO, respectively; $P < 0.0001$; Fig. 1C). Despite the virtually complete protection against tumor formation, the total number of ACF, a preneoplastic lesion common to the distal colon, was reduced by less than 40% (32.4 ± 2.5 vs. 20.7 ± 2.3 in WT and KO, respectively; $P < 0.002$; Fig. 1D). The most effective protection occurred in ACF of intermediate size, including those with 2 to 3 (16.0 ± 1.4 vs. 10.0 ± 1.4 in WT and KO, respectively; $P < 0.005$) and 4 to 6 crypts per focus (10.4 ± 1.3 vs. 6.3 ± 0.9 in WT and KO, respectively; $P < 0.01$).

In WT mice, analysis of tumor histology identified primarily 2 forms of large adenomas, characterized by either a pedunculated or flat morphology (Fig. 2, WT). The tubular adenomas often exhibited an elongated structure with a stalk (Fig. 2, arrow). In the *mPGES-1* KO mice, however, only 1 of 19 mice developed a large adenoma (>3 mm), which had either a flat or a slightly raised morphology (Fig. 2, KO). Microadenomas were found in the colons from either genotype, although carcinomas *in situ* were limited to the WT mice (Fig. 2, WT). The

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