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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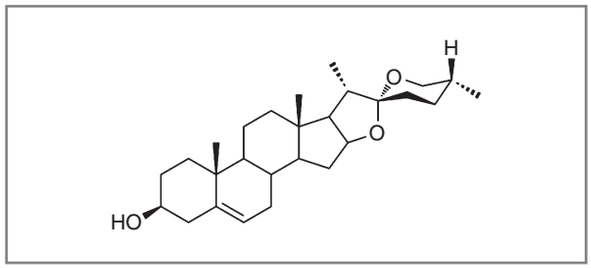

Figure 1. Chemical structure of diosgenin.

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.

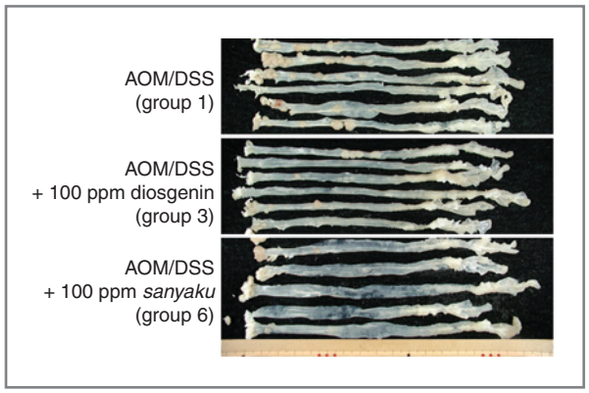

Figure 2. Representative macroscopic view of the colon of mice treated with AOM/DSS (group 1), AOM/DSS + 100 ppm diosgenin (group 3), and AOM/DSS + 100 ppm *sanyaku* (group 6).

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

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

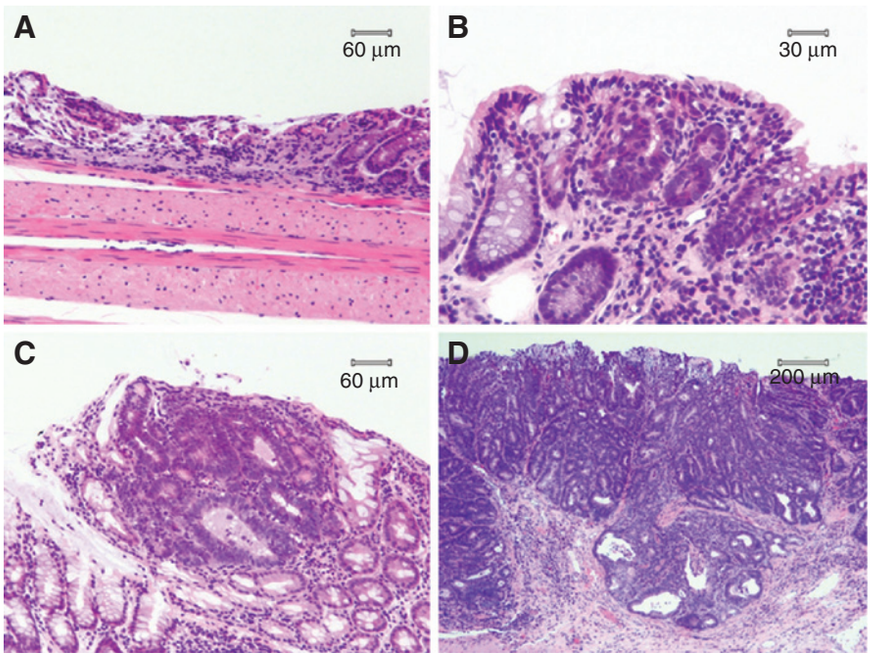

Figure 3. Histopathology of colonic lesions induced by AOM/DSS (group 1). A, mucosal ulcer; B, dysplastic crypts; C, tubular adenoma; and D, invasive ductal adenocarcinoma. Bars in the images represent the distances shown as an indication of magnification.

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]$P < 0.001$).

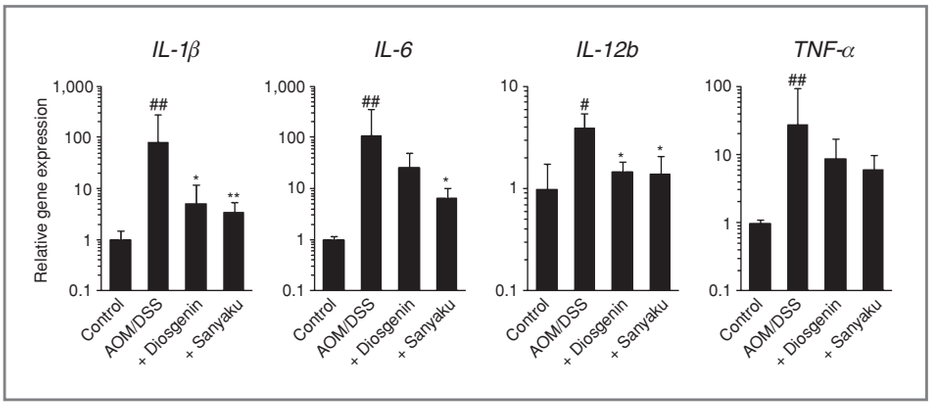

Figure 4. Effects of diosgenin on AOM/DSS-induced inflammatory cytokine gene expression in colonic mucosa. Total RNA was extracted from scraped colonic mucosa of mice treated with AOM/DSS, AOM/DSS followed by 20 ppm diosgenin or *sanyaku* for 3 weeks, and control as described in the Materials and Methods 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.

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

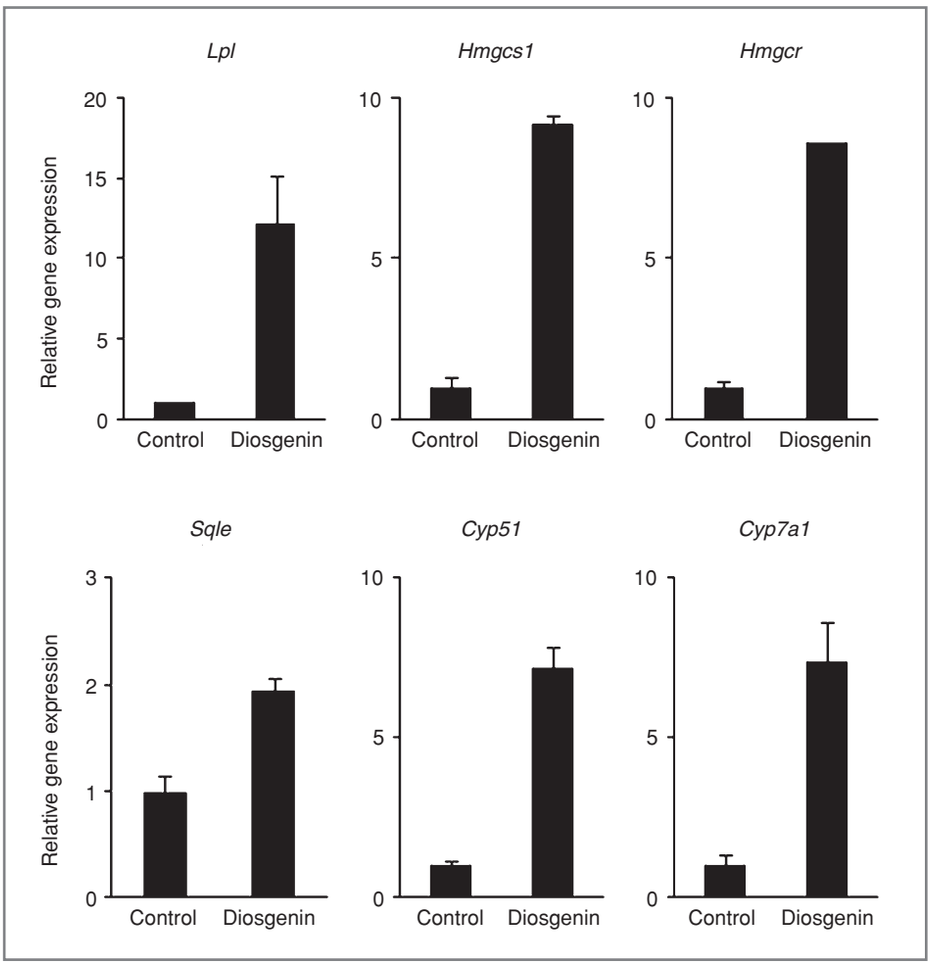

Figure 5. Real-time RT-PCR analyses. Nine-week-old ICR mice were treated with or without 500 ppm diosgenin for 4 weeks. Hepatic RNA was extracted from each mouse, and was subjected to real-time RT-PCR analyses.

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

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-1*β, *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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