75. Zweibaum, A. Differentiation of human colon cancer cells: A new approach to cancer of the colon. *Ann. Gastroenterol. Hepatol.* **1993**, *29*, 257–261; discussion 261–262.
76. Zweibaum, A. Differentiation of human colon cancer cells: A new approach to colon cancer. *Bull. Acad. Natl. Med.* **1993**, *177*, 63–71; discussion 71–63.
77. Grivicich, I.; Regner, A.; da Rocha, A.B.; Kayser, G.B.; Schunemann, D.P.; Grass, L.B.; Alves, P.A.; Henriques, J.A.; Schwartsmann, G. The irinotecan/5-fluorouracil combination induces apoptosis and enhances manganese superoxide dismutase activity in HT-29 human colon carcinoma cells. *Chemotherapy* **2005**, *51*, 93–102.
78. Han, C.; Demetris, A.J.; Michalopoulos, G.K.; Zhan, Q.; Shelhamer, J.H.; Wu, T. PPARgamma ligands inhibit cholangiocarcinoma cell growth through p53–dependent GADD45 and p21 pathway. *Hepatology* **2003**, *38*, 167–177.
79. Nagamine, M.; Okumura, T.; Tanno, S.; Sawamukai, M.; Motomura, W.; Takahashi, N.; Kohgo, Y. PPAR gamma ligand-induced apoptosis through a p53-dependent mechanism in human gastric cancer cells. *Cancer Sci.* **2003**, *94*, 338–343.
80. Takekawa, M.; Saito, H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* **1998**, *95*, 521–530.
81. Smith, M.L.; Chen, I.T.; Zhan, Q.; Bae, I.; Chen, C.Y.; Gilmer, T.M.; Kastan, M.B.; O'Connor, P.M.; Fornace, A.J., Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* **1994**, *266*, 1376–1380.
82. Balakumar, P.; Rose, M.; Ganti, S.S.; Krishan, P.; Singh, M. PPAR dual agonists: are they opening Pandora's Box? *Pharmacol. Res.* **2007**, *56*, 91–98.
83. Basch, E.; Gabardi, S.; Ulbricht, C. Bitter melon (Momordica charantia): A review of efficacy and safety. *Am. J. Health Syst. Pharm.* **2003**, *60*, 356–359.
84. Tenenbaum, A.; Motro, M.; Fisman, E.Z. Dual and pan-peroxisome proliferator-activated receptors (PPAR) co-agonism: The bezafibrate lessons. *Cardiovasc. Diabetol.* **2005**, *4*, doi:10.1186/1475-2840-4-14.

Nutrition and Cancer, 63(2), 227–233

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2011.523506

Routledge
Taylor & Francis Group

Effects of Nobiletin on PhIP-Induced Prostate and Colon Carcinogenesis in F344 Rats

Ming Xi Tang
Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan and Department of Pathology, Luzhou Medical College, Sichuan, China

Kumiko Ogawa and Makoto Asamoto
Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Teera Chewonarin
Department of Biochemistry, Faculty of Medicine, Chiangmai University, Chiangmai, Thailand

Shugo Suzuki
Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Takuji Tanaka
Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

Tomoyuki Shirai
Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

The current study was designed to investigate the effects of nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced prostate and colon carcinogenesis. PhIP was administered to 6-wk-old F344 male rats intragastrically (100 mg/kg) twice a wk for 10 wk. The animals were given 0.05% nobiletin or the basal diet for 50 wk. At the end of the experiment, serum testosterone, estrogen, and leptin did not differ between the 2 groups. The body weights of nobiletin-treated rats were significantly higher than controls ($P < 0.05$), and feeding of nobiletin significantly reduced the relative prostate ($P < 0.05$) and testes ($P < 0.05$) weights as well as the Ki67 labeling index in the normal epithelium in the ventral prostate ($P < 0.01$). The incidence and multiplicity of adenocarcinomas in nobiletin-treated ventral prostate were 50% and 36%, respectively, of controls, but the differences were not statistically significant. However, nobiletin did significantly reduce the total number of colonic aberrant crypt foci (ACF) compared to the control value ($P < 0.05$). Nobiletin, therefore, may have potential for chemoprevention of early changes associated with carcinogenesis in both the prostate and colon.

INTRODUCTION

Dietary habits and lifestyle have been identified as risk factors for prostate and colon cancer development and progression (1,2). Western populations tend to consume large amounts of cooked red meat, from which a number of heterocyclic amines (HCAs), including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), have been identified. PhIP can be metabolized to biologically active metabolites that form DNA adducts (3,4) and is known to be distributed to several tissues in rats (5,6) and humans (7,8). With long-term administration of PhIP experimentally, it was found to induce cancers in the rat prostate, intestine, and mammary gland (9,10). These are common in Western countries and relatively infrequent in Southeast Asian countries (11). Recently, however,

Submitted 28 December 2009; accepted in final form 8 September 2010.

Address correspondence to Kumiko Ogawa, Division of Pathology, National Institute of Health Sciences, Biological Safety Research Center, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Phone: +81-3-3700-9818. Fax: +81-3-3700-1425. E-mail: ogawa93@nihs.go.jp

increasing incidence and mortality have been noted in Asian countries, which may be associated with changes to the Westernized lifestyle, resulting in increased intake of dietary HCAs (12).

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), a polymethoxy-flavonoid extracted from citrus fruits such as oranges, has been found to be a potent chemopreventive agent for cancer development, invasion, and metastasis in a wide range of in vitro (13–16) and in vivo (15–21) experimental studies. In particular, we and others have provided evidence that dietary administration of 0.05% nobiletin in the diet inhibited prostate carcinogenesis in the transgenic rat for adenocarcinoma of the prostate (TRAP) model (15), azoxymethane (AOM)-induced rat colon (19,20), and AOM-induced mouse colon (21) carcinogenesis models.

In the present study, the main objective was to determine whether citrus nobiletin inhibits the induction of rat ventral prostate cancer and/or colon ACF induced by PhIP, one of the most abundant HCAs (22) and candidate for human carcinogen, in F344 rats.

MATERIALS AND METHODS

Animals and Chemicals

Five-week-old male F344 rats were obtained from Charles River Japan (Atsugi, Japan) and housed 3 or 4 to a plastic cage on wood-chip bedding in an air-conditioned specific pathogen-free (SPF) animal room at 22 ± 2°C and 55 ± 5% humidity with a 12-h light/dark cycle. Tap water and food were available ad libitum. All the animal studies were conducted in accordance with established guidelines and protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University School of Medical Sciences. PhIP hydrochloride with a purity >99.9% and nobiletin with a purity >98% were purchased from the Nard Institute, Ltd. (Amagasaki, Hyogo, Japan). The dose of the nobiletin for the present experiment was selected based on the previous studies (15,20), which showed chemopreventive potency but no toxic effects.

Animal Treatment

PhIP suspended in saline was administered to 6-wk-old F344 male rats intragastrically at the dose of 100 mg/kg twice a wk for 10 wk. Animals were then randomized into 2 groups (13 rats/group), to avoid significant difference in the body weights between the two groups before nobiletin administration. Corn oil, either with or without 0.05% nobiletin by weight, was added to the powdered nonpurified standard diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan; http://www.nature.com/jcbfm/journal/v29/n9/extref/jcbfm200980×1.doc) using a ratio of 2 g corn oil for every 98 g basal diet. Both diets were prepared at the same time and fed starting at 16 wk of age and continuing to 66 wk of age. Body weight and food and drinking water consumption were monitored weekly throughout the experiment. At necropsy (66 wk of age), 6 rats/group were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU) solution (100 mg/kg body weight) 1 h before sacrifice to allow investigation of cell proliferation.

Serum and Tissue Collection and Measurement of Testosterone and Estrogen Levels

Under deep pentobarbital anesthesia, blood was collected from the abdominal aorta to evaluate testosterone and estrogen concentrations as well as the serum leptin levels. Testosterone and estrogen levels in serum were analyzed using radioimmunoassays by a commercial laboratory (SRL, Tokyo, Japan).

At sacrifice, tissues were harvested, weighed, and fixed in 10% phosphate-buffered formalin. After fixation for 48 h, the ventral prostate lobes and dorso-lateral lobes containing the urethra and anterior lobes with the seminal vesicles were carefully dissected into individual lobes, and each was weighed. Tissues were routinely processed for embedding in paraffin, and sections were cut and stained with hematoxylin and eosin (H&E).

Colons were also removed, filled with 10% phosphate-buffered formalin for light fixation for 5 min, cut open longitudinally from the cecum to the anus, and then placed between filter papers for further fixation with 10% buffered formalin.

Histopathological Analysis and Immunohistochemistry

In the prostate, we particularly focused on the ventral lobe because PhIP-induced carcinomas develop only in this site (9). We analyzed one entire cross-section of that lobe. As shown in Fig. 1, each acinus was histopathologically classified as normal, or occupied by low- and high-grade prostatic intraepithelial neoplasms (PINs), and adenocarcinomas with cribriform growth pattern as described previously (23,24). The multiplicity of adenocarcinoma was evaluated with the aid of an image analyzer (Image Processor for Analytical Pathology; Sumika Technoservice, Osaka, Japan).

Formalin fixed colons were stained with methylene blue (0.1% in PBS) for counting aberrant crypt foci (ACF) (25) under a stereomicroscope. The number and the size of all foci were assessed separately for 3 portions (ascending, transverse, and descending colon). After observation, visible polyps were removed and embedded individually for histopathological evaluation. Remaining colons were rolled and longitudal strips were embedded in paraffin and stained with H&E. All the sections were used for histopathological evaluation and immunohistochemistry.

Immunohistochemical analysis was performed with a Discovery instrument using DAB Map kits (Ventana Medical Systems, Tucson, AZ), monoclonal mouse anti-BrdU (Dako, Glostrup, Denmark) and polyclonal rabbit anti-Ki67 (NCLKi67p, Novocastra Laboratories, Ltd., Newcastle, UK) antibodies that were applied to heat-pretreated sections at dilutions of 1,000 and 3,000, respectively. Binding was visualized with DAB, followed by light hematoxylin counterstaining.

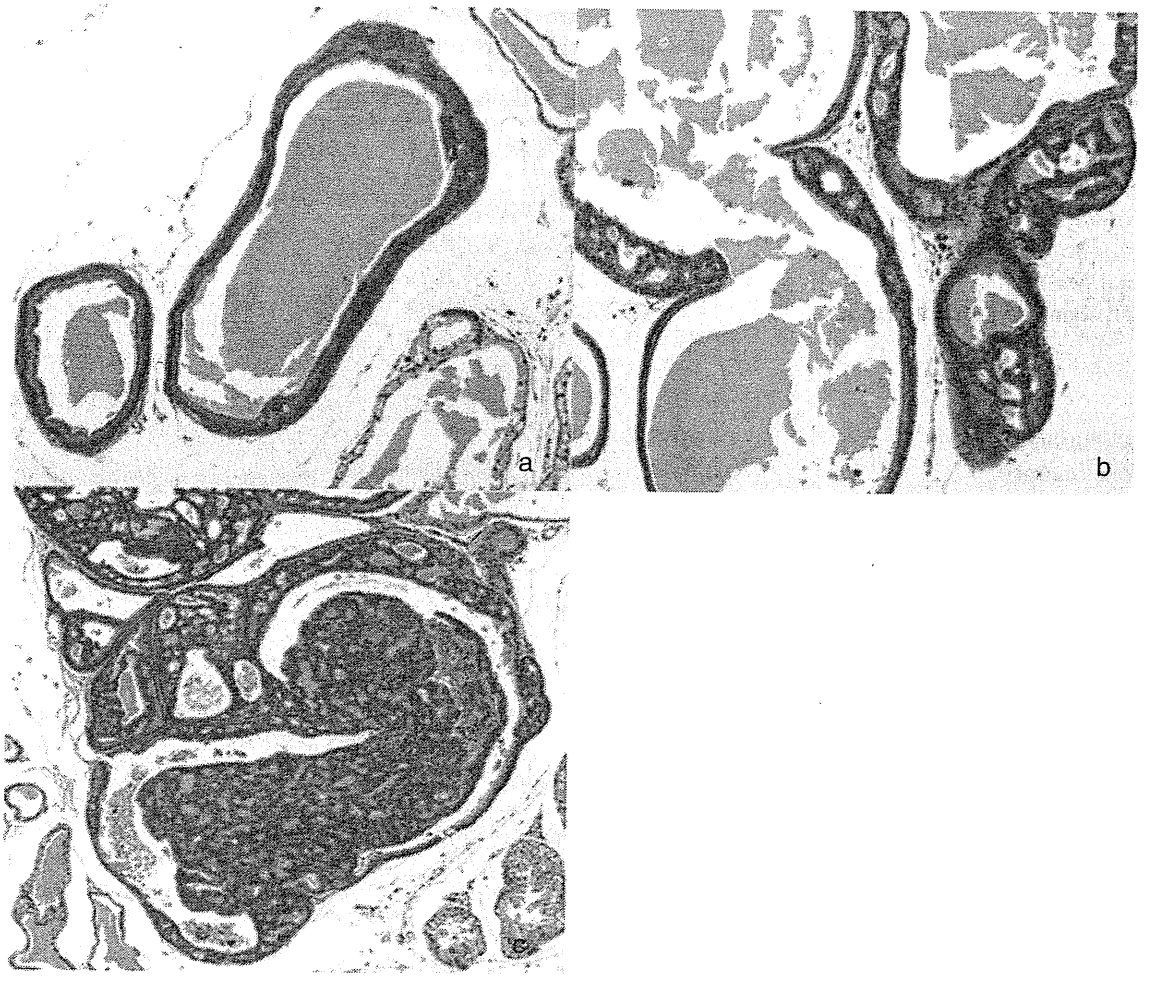


FIG. 1. Representative histological appearance of A: a low-grade prostatic intraepithelial neoplasms (PIN), B: a high-grade PIN, and C: an adenocarcinoma in the ventral prostate.

Western Blot Analysis of Serum for Leptin Levels

Leptin expression is detectable by Western blot analysis only when the total protein concentration of the serum sample is >0.2 mg/ml. After depleting albumin from serum by AlbuSorb (Biotech Support Group, LLC, North Brunswick, NJ) according to the manufacturer's protocol, the serum samples were diluted 2-fold and denatured in the presence of sodium dodecyl sulfate and 2-mercaptoethanol by heating at 100°C for 5 min. A total of 13.9 μg proteins in each sample were electrophoretically transferred to 2 nitrocellulose membranes (Amersham Bioscences Piscataway, NJ). Nonspecific binding on the membranes was minimized with 5% skim milk at room temperature for 1 h, followed by incubation overnight at 4°C in 5% skim milk containing the polyclonal rabbit antileptin antibody (1:200 dilution, A-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing with PBS, the membranes were incubated with secondary antibodies for 1 h. Detection was performed with ECL and Western blotting detection reagents (Amersham Biosciences) and exposure to X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Statistical Analysis

All comparisons of data between the nobiletin-treated group and control rats were performed using the t-test or chi-square test, taking $P < 0.05$ as the level of significance. Multiple comparisons were also performed using Wilks' lambda test.

RESULTS

General Observations

PhIP-pretreatment caused severe suppression of body weight gain, but the mean body weight gain of rats in the nobiletin-treated group was significantly higher than those of the untreated control rats ($P < 0.05$~0.001) during 27 to 66 wk of age (data not shown). At the end of the study, as shown in Table 1, nobiletin feeding resulted in significantly increased body weights ($P < 0.05$) but decreased relative weights (mg/g body weight) of whole prostate ($P < 0.05$) and of the prostate anterior lobe with the seminal vesicles ($P < 0.05$). However, there were no differences in the weights of the ventral and dorsolateral prostates. Of all the prostate lobes, significant decrease of absolute weight

TABLE 1
Final body and relative organ weights, serum concentration, and prostatic lesions

Treatment (No. of Rat)	PhIP + Control Diet (13)	PhIP + Nobiletin (13)
Body weights (g)	373.3 ± 12.0	390.1 ± 19.5*
Relative organ weights (mg/g body weight)		
Whole prostate and SV	5.05 ± 0.82	4.47 ± 0.33*
Ventral prostate	0.19 ± 0.04	0.21 ± 0.04
Dorsolateral prostate[a]	1.65 ± 0.25	1.54 ± 0.07
Anterior prostate and seminal vesicle	3.20 ± 0.59	2.73 ± 0.33*
Testis	8.13 ± 0.23	7.53 ± 0.98*
Liver	25.08 ± 0.97	25.26 ± 0.84
Kidney	5.16 ± 0.20	5.06 ± 0.16
Serum concentration[b]		
Testosterone (ng/ml)	0.67 ± 0.45	0.41 ± 0.23
Estrogen (pg/ml)	5.02 ± 0.45	4.96 ± 0.23
Prostatic lesions		
Incidence of adenocarcinoma (%)	8 (61.5)	4 (30.8)
Multiplicity of adenocarcinoma		
No./mm^2	0.19 ± 0.26	0.06 ± 0.09
% area	1.79 ± 3.09	0.34 ± 0.65
Ratio of prostatic lesion (% acinar)		
Low grade PIN	6.17 ± 3.07	4.03 ± 2.99
High grade PIN	0.39 ± 0.46	0.37 ± 0.50
Adenocarcinoma	0.47 ± 0.55	0.17 ± 0.31

Note: *t*-test. Values are mean ± SD. SV = seminal vesicle; PIN = prostatic intraepithelial neoplasms.
Significantly different from PhIP + control diet group, $^*P < 0.05$.
[a]Dorsolateral prostate containing urogenital organs.
[b]Effective number of control group was 12.

was only observed in the anterior lobe with the seminal vesicles of nobiletin-treated animals (data not shown).

The mean relative weight (mg/g body weight) of the testes in the nobiletin-treated group was significantly lower than the control value ($P < 0.05$) (Table 1), without significant difference of its absolute weight. The average relative (mg/g body weight) liver and kidney weights, serum testosterone and estrogen levels (Table 1), and leptin of the nobiletin-treated group did not differ from those of the controls (data not shown). Food and drinking water consumption also demonstrated no differences (data not shown) between the 2 groups. There were no macroscopic or microscopic alterations in any of the rats related to nobiletin treatment except for the PhIP-target organs (colon and prostate) and testes. Atrophic seminiferous tubules of the unilateral testis were observed in 3 rats given the nobiletin.

Effects of Nobiletin on PhIP-Induced Prostate Carcinogenesis

The dietary administration of nobiletin decreased the incidence and multiplicity of adenocarcinomas in the ventral prostate (Table 1). They were all noninvasive low-grade adenocarcinomas. The incidence of adenocarcinoma in the nobiletin-treated group was 50% (61.5% to 30.8%) of the control value. The multiplicity of adenocarcinoma was evaluated as the number of acini that consisted of adenocarcinoma and the percentage area of the ventral lobe cross-section occupied by adenocarcinoma and was 32% (0.19 to 0.06 acini/mm^2) and 19% (1.79 to 0.34% area) of the control values, respectively. Although significant differences were not evident, the percentage acini involving adenocarcinoma was 36% (0.47 ± 0.55 to 0.17 ± 0.31) of the control value. The incidences of PINs also tended to be decreased. Furthermore, as indicated in Fig. 2, Ki67-labeling indices in the normal epithelium of the nobiletin-treated ventral prostate were significantly decreased ($P < 0.01$) from 1.28 ± 0.49 to 0.89 ± 0.13. There were similar trends in the PINs and adenocarcinomas but without statistical significance.

Effects of Nobiletin on PhIP-Induced Colonic ACF Development

With the stereoscopic observation, PhIP-induced ACF were mostly observed in the transverse colon. The data for ACF are summarized in Table 2. In the transverse colons, nobiletin feeding significantly decreased the number of ACF composed of ≤3 crypts per focus ($P < 0.05$) and the total number of ACF, regardless of size ($P < 0.01$). A significant decrease was also observed in the total number of ACF with >4 crypts per focus

TABLE 2
Quantitative analysis of colonic ACF, adenomas, and BrdU indices

Treatment (No. of Rat)	PhIP + Control Diet (12)	PhIP + Nobiletin (13)
ACF in ascending colon		
<3 crypts	1.1 ± 1.0	1.2 ± 1.4
≥4 crypts	0.8 ± 0.8	0.5 ± 0.7
Total	1.8 ± 1.3	1.7 ± 1.5
ACF in transverse colon		
<3 crypts	6.4 ± 3.5	3.8 ± 1.9*
≥4 crypts	2.5 ± 2.0	1.2 ± 1.0
Total	8.9 ± 4.7	4.9 ± 1.8**
ACF in descending colon		
<3 crypts	2.2 ± 1.5	1.7 ± 2.4
≥4 crypts	1.2 ± 1.0	0.6 ± 0.8
Total	3.3 ± 1.8	2.3 ± 2.5
Total number of ACF		
<3 crypts	9.7 ± 4.4	6.7 ± 4.1
≥4 crypts	4.6 ± 2.8	2.4 ± 1.7*
Total	14.3 ± 6.2	9.1 ± 3.5*
Adenomas (%)	1/12 (8.3)	2/13 (15.4)
BrdU indices in colonic epithelium	11.5 ± 1.4	12.9 ± 1.6

Note: *t*-test. Values are mean ± SD. ACF = aberrant crypt foci.
Significantly different from PhIP + control diet group, $^{*}P < 0.05$. $^{**}P < 0.01$.

($P < 0.05$) and total number of ACF in the whole colons ($P < 0.05$), compared with those in control rats. These significant differences remained when body weight was included as a covariate. Colonic adenomas were found in only 1 control and 2 nobiletin-treated rats. There were no significant differences in the BrdU-labeling indices in nontumorous epithelium of the colon between the control and nobiletin-treated groups (Table 2).

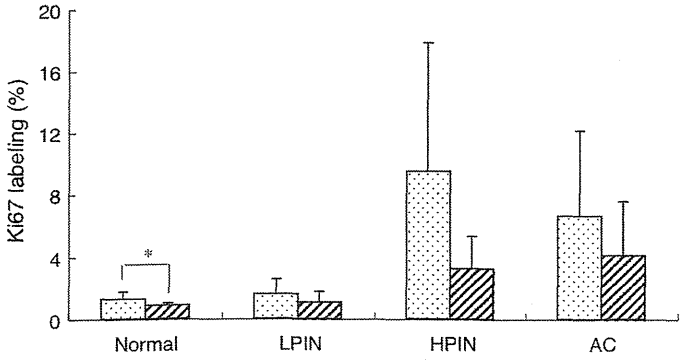


FIG. 2. Mean and SD of the Ki67-labeling indices in the rat prostatic acini of histologically normal epithelium (normal), low-grade prostatic intraepithelial neoplasms (LPIN), high-grade prostatic intraepithelial neoplasms (HPIN), and adenocarcinoma (AC). : PhIP-treated control group. : PhIP + nobiletin-treatment group. *Significantly different from the value in the control group at $P < 0.01$ with the Student's *t*-test.

DISCUSSION

The present experiment demonstrated that 0.05% dietary administration of citrus flavonoid nobiletin inhibited PhIP-induced rat prostate and colon carcinogenesis. This is in agreement with our previous experiment in which nobiletin reduced prostate carcinogenesis in TRAP rats (15). PhIP is the most abundant heterocyclic amine produced during the cooking of meat and fish (22,26), and possesses carcinogenic potential in male rat colon and prostate and female rat mammary gland (9,10). PhIP-DNA adducts have been detected in human mammary glands (7) and prostate (8). Thus, nobiletin might be a good candidate agent for cancer prevention.

In our previous study with TRAP rats, which genetically develop multiple neoplasias in the whole prostate, the number of acini consisting of adenocarcinomas was decreased while that of PIN was increased by nobiletin-treatment (15). Thus, it was suspected that nobiletin suppressed tumor progression from PIN to carcinoma. In the present experiment with PhIP, which induces a lower extent of neoplasia in the prostate, the multiplicity of both adenocarcinoma and PIN, as well as the incidence of adenocarcinoma, tended to decrease. It was thus suggested that nobiletin inhibited not only promotion from PIN to carcinoma but also development of PIN, so that chemopreventive potential against prostate carcinogenesis might extend to early stages.

Regarding colon carcinogenesis, the number of ACF tended to be decreased in both the transverse and descending colon. The number of adenomas observed was too limited for evaluation of chemopreventive effects. Although there has been controversy

as to the significance of ACF as a preneoplastic lesion (27), taken together with the previous report indicating the preventive effect of nobiletin on AOM-induced colon ACF and adenocarcinoma (19,20), the present finding of suppression of ACF formation supports the possibility of chemoprevention.

The inhibitory effect on cell proliferation observed in the nobiletin-treated prostate epithelium is clearly of interest. On the other hand, no effect was observed in nobiletin-treated colonic epithelium. Morley et al. (28) earlier documented evidence that nobiletin significantly suppressed cell proliferation by blockage at the G1 phase in human breast cancer cell lines, MDA-MB-435 and MCF-7, and a human colon cancer cell line, HT-29. In addition, G2/M arrest and apoptosis, with decreased Bcl-2 and increased Bax, were also observed in a nobiletin-treated human lung adenocarcinoma cell line, A549 (16). More recently, it was reported that nobiletin inhibited angiotensin II-induced vascular smooth muscle cell proliferation via inhibition of JNK activation (29).

Hormone dependence of prostate cancer growth is widely accepted and testosterone or its metabolites are the most recognized progression factors. In the present experiment, reduced weight of the testis was observed in 3 rats treated with nobiletin, and the average of serum testosterone in the nobiletin-treated rats was lower than in the control group. In general, serum testosterone levels varied and the standard deviation of these data in this study was also quite high. Moreover, the relative organ weight of the ventral prostate, which is considered to be most tightly regulated by androgen among prostatic lobes (30), was not reduced. Furthermore, absolute weight of testis did not show statistical significance between groups. Although we could not entirely exclude the possibility of antiandrogenic and/or estrogenic activity of nobiletin, we concluded that no major alterations in serum testosterone and estrogen levels were induced by the nobiletin administration.

Antiinflammatory effects of nobiletin due to interference with production of PGE_2 via selective downregulation of the COX-2 gene in human synovial fibroblasts has been demonstrated (31). However, in our study, obvious inflammatory changes were not histologically observed in either the prostate or the colon at the end point, and other measures of inflammation were not measured. Further experiments are clearly needed to clarify the mechanisms of the suppressive effects of nobiletin on carcinogenesis.

In conclusion, these data show that nobiletin tends to inhibit PhIP-induced rat prostate carcinogenesis as well as development of colonic ACF. Although further investigations are needed, a possible mechanism of the chemoprevention effects of nobiletin is the reduction of cell proliferation, at least in the prostate.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labour, and Welfare, Japan; a Grant-in-aid from the Ministry of Health, Labour, and Welfare for the Second Term Comprehensive 10-Year Strategy for Cancer Control, Japan; and a grant from the Society for Promotion of Toxicological Pathology of Nagoya, Japan.

REFERENCES

1. Syed DN, Khan N, Afaq F, and Mukhtar H: Chemoprevention of prostate cancer through dietary agents: progress and promise. *Cancer Epidemiol Biomarkers Prev* **16,** 2193–2203, 2007.
2. Johnson JJ and Mukhtar H: Curcumin for chemoprevention of colon cancer. *Cancer Lett* **255,** 170–181, 2007.
3. Takayama K, Yamashita K, Wakabayashi K, Sugimura T, and Nagao M: DNA modification by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in rats. *Jpn J Cancer Res* **80,** 1145–1148, 1989.
4. Snyderwine EG, Venugopal M, and Yu M: Mammary gland carcinogenesis by food-derived heterocyclic amines and studies on the mechanisms of carcinogenesis of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Mutat Res* **506–507,** 145–152, 2002.
5. Shirai T, Takahashi S, Cui L, Yamada Y, Tada M, et al.: Use of polyclonal antibodies against carcinogen-DNA adducts in analysis of carcinogenesis. *Toxicol Lett* **102–103,** 441–446, 1998.
6. Takahashi S, Tamano S, Hirose M, Kimoto N, Ikeda Y, et al.: Immunohistochemical demonstration of carcinogen-DNA adducts in tissues of rats given 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): detection in paraffin-embedded sections and tissue distribution. *Cancer Res* **58,** 4307–4313, 1998.
7. Zhu J, Chang P, Bondy ML, Sahin AA, Singletary SE, et al.: Detection of 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine-DNA adducts in normal breast tissues and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev* **12,** 830–837, 2003.
8. Tang D, Liu JJ, Rundle A, Neslund-Dudas C, Savera AT, et al.: Grilled meat consumption and PhIP-DNA adducts in prostate carcinogenesis. *Cancer Epidemiol Biomarkers Prev* **16,** 803–808, 2007.
9. Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, et al.: The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods. *Cancer Res* **57,** 195–198, 1997.
10. Ito N, Hasegawa R, Sano M, Tamano S, Esumi H, et al.: A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-pheny limidazo[4,5-b]pyridine (PhIP). *Carcinogenesis* **12,** 1503–1506, 1991.
11. Parkin DM, Bray FI, and Devesa SS: Cancer burden in the year 2000. The global picture. *Eur J Cancer* **37** (Suppl 8), S4–66, 2001.
12. Sinha R: An epidemiologic approach to studying heterocyclic amines. *Mutat Res* **506–507,** 197–204, 2002.
13. Nishino H, Tokuda H, Satomi Y, Masuda M, Osaka Y, et al.: Cancer prevention by antioxidants. *Biofactors* **22,** 57–61, 2004.
14. Miyata Y, Sato T, Imada K, Dobashi A, Yano M, et al.: A citrus polymethoxyflavonoid, nobiletin, is a novel MEK inhibitor that exhibits antitumor metastasis in human fibrosarcoma HT-1080 cells. *Biochem Biophys Res Commun* **366,** 168–173, 2008.
15. Tang M, Ogawa K, Asamoto M, Hokaiwado N, Seeni A, et al.: Protective effects of citrus nobiletin and auraptene in transgenic rats developing adenocarcinoma of the prostate (TRAP) and human prostate carcinoma cells. *Cancer Sci* **98,** 471–477, 2007.
16. Luo G, Guan X, and Zhou L: Apoptotic effect of citrus fruit extract nobiletin on lung cancer cell line A549 in vitro and in vivo. *Cancer Biol Ther* **7,** 966–973, 2008.
17. Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, et al.: Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res* **60,** 5059–5066, 2000.

18. Minagawa A, Otani Y, Kubota T, Wada N, Furukawa T, et al.: The citrus flavonoid, nobiletin, inhibits peritoneal dissemination of human gastric carcinoma in SCID mice. *Jpn J Cancer Res* **92,** 1322–1328, 2001.
19. Kohno H, Yoshitani S, Tsukio Y, Murakami A, Koshimizu K, et al.: Dietary administration of citrus nobiletin inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Life Sci* **69,** 901–913, 2001.
20. Suzuki R, Kohno H, Murakami A, Koshimizu K, Ohigashi H, et al.: Citrus nobiletin inhibits azoxymethane-induced large bowel carcinogenesis in rats. *Biofactors* **22,** 111–114, 2004.
21. Miyamoto S, Yasui Y, Tanaka T, Ohigashi H, and Murakami A: Suppressive effects of nobiletin on hyperleptinemia and colitis-related colon carcinogenesis in male ICR mice. *Carcinogenesis* **29,** 1057–1063, 2008.
22. Wakabayashi K, Nagao M, Esumi H, and Sugimura T: Food-derived mutagens and carcinogens. *Cancer Res* (Suppl) **52,** 2092S–2098S, 1992.
23. Asamoto M, Hokaiwado N, Cho YM, Takahashi S, Ikeda Y, et al.: Prostate carcinomas developing in transgenic rats with SV40 T antigen expression under probasin promoter control are strictly androgen dependent. *Cancer Res* **61,** 4693–4700, 2001.
24. Kandori H, Suzuki S, Asamoto M, Murasaki T, Mingxi T, et al.: Influence of atrazine administration and reduction of calorie intake on prostate carcinogenesis in probasin/SV40 T antigen transgenic rats. *Cancer Sci* **96,** 221–226, 2005.
25. McLellan EA and Bird RP: Aberrant crypts: potential preneoplastic lesions in the murine colon. *Cancer Res* **48,** 6187–6192, 1988.
26. Sinha R, Rothman N, Brown ED, Salmon CP, Knize MG, et al.: High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Res* **55,** 4516–4519, 1995.
27. Mori H, Yamada Y, Kuno T, and Hirose Y: Aberrant crypt foci and beta-catenin accumulated crypts; significance and roles for colorectal carcinogenesis. *Mutat Res* **566,** 191–208, 2004.
28. Morley KL, Ferguson PJ, and Koropatnick J: Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Lett* **251,** 168–178, 2007.
29. Zhou CH, Wu XH, and Wu YQ: Nobiletin, a dietary phytochemical, inhibits vascular smooth muscle cells proliferation via calcium-mediated c-Jun N-terminal kinases pathway. *Eur J Pharmacol* **615,** 55–60, 2009.
30. Suzuki T, Fujimoto N, Kitamura S, and Ohta S: Quantitative determination of lobe specificity of mRNA expression of androgen-dependent genes in the rat prostate gland. *Endocr J* **54,** 123–132, 2007.
31. Lin N, Sato T, Takayama Y, Mimaki Y, Sashida Y, et al.: Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. *Biochem Pharmacol* **65,** 2065–2071, 2003.

Research Article

Cancer Prevention Research

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

Noriyuki Miyoshi[1], Tomoki Nagasawa[1], Ryota Mabuchi[1], Yumiko Yasui[2], Keiji Wakabayashi[1], Takuji Tanaka[3,4], and Hiroshi Ohshima[1]

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,

Authors' Affiliations: [1]Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, and Global Center of Excellence Program, University of Shizuoka, Shizuoka; [2]Department of Physiology and Nutrition, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido; [3]Tohkai Cytopathology Institute: Cancer Research and Prevention, Gifu; and [4]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

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

Corresponding Author: Noriyuki Miyoshi, Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan. Phone: 81-54-264-5536; Fax: 81-54-264-5530. E-mail: miyoshin@u-shizuoka-ken.ac.jp

doi: 10.1158/1940-6207.CAPR-10-0279

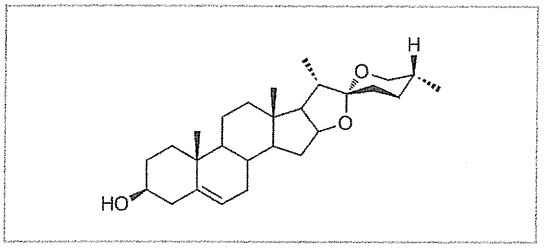


Figure 1. Chemical structure of diosgenin.

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.

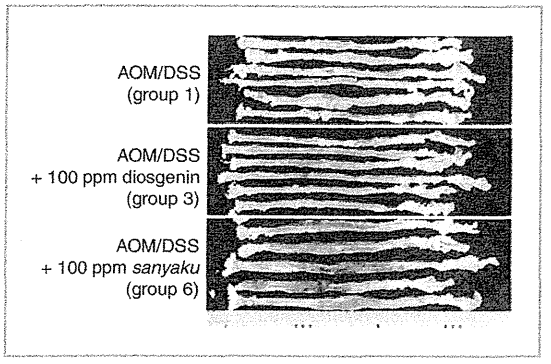


Figure 2. Representative macroscopic view of the colon of mice treated with AOM/DSS (group 1), AOM/DSS + 100 ppm diosgenin (group 3), and AOM/DSS + 100 ppm *sanyaku* (group 6).

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

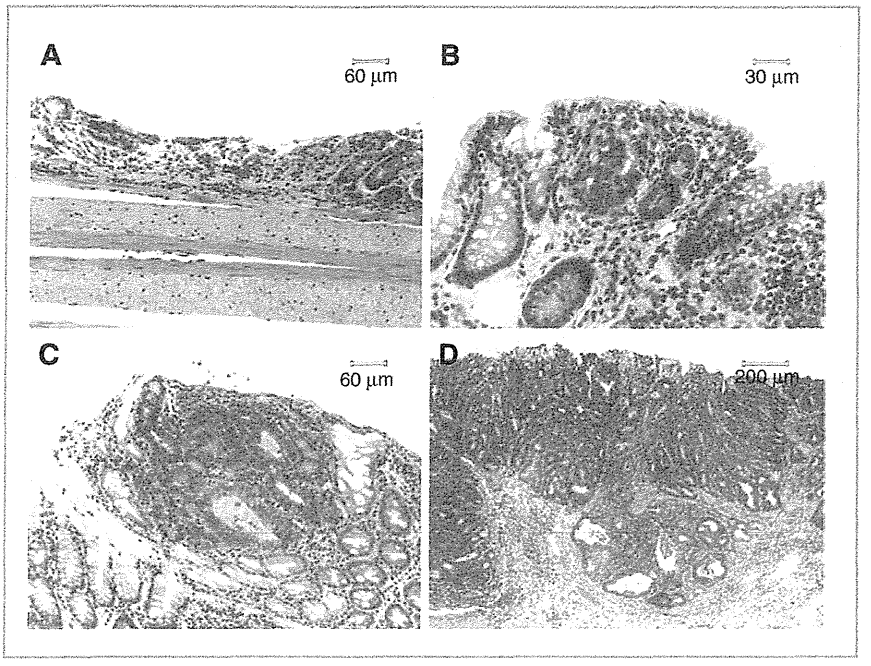


Figure 3. Histopathology of colonic lesions induced by AOM/DSS (group 1). A, mucosal ulcer; B, dysplastic crypts; C, tubular adenoma; and D, invasive ductal adenocarcinoma. Bars in the images represent the distances shown as an indication of magnification.

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]$P < 0.001$).

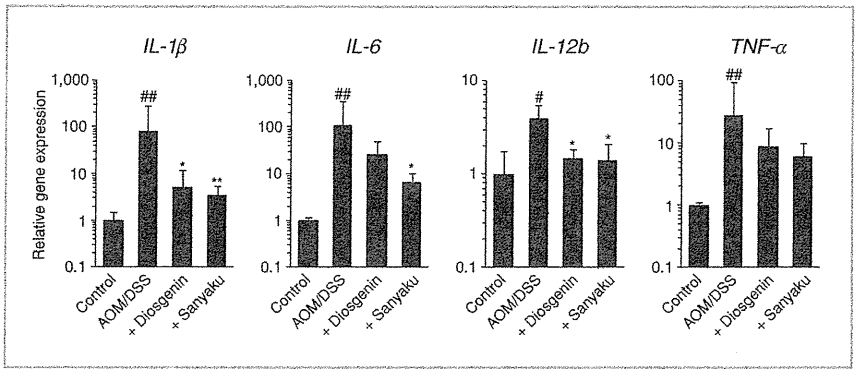


Figure 4. Effects of diosgenin on AOM/DSS-induced inflammatory cytokine gene expression in colonic mucosa. Total RNA was extracted from scraped colonic mucosa of mice treated with AOM/DSS, AOM/DSS followed by 20 ppm diosgenin or *sanyaku* for 3 weeks, and control as described in the Materials and Methods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

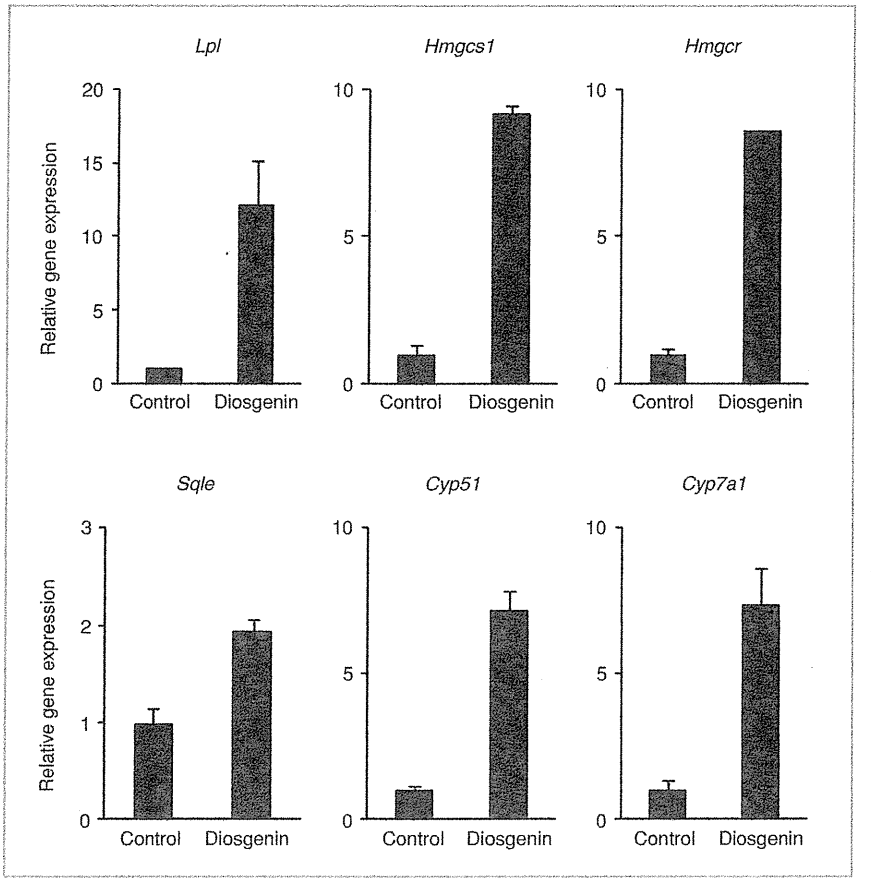


Figure 5. Real-time RT-PCR analyses. Nine-week-old ICR mice were treated with or without 500 ppm diosgenin for 4 weeks. Hepatic RNA was extracted from each mouse, and was subjected to real-time RT-PCR analyses.

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-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.

Grant Support

This study was supported by Grants-in-Aid (21680052 to N. Miyoshi and 21300280 to H. Ohshima) from the Ministry of Education, Science, Culture, and Sport; Grants-in-Aid for Cancer Research (19-19 and 21-1-2) from the Ministry of Health, Labour, and Welfare, Japan; and the Global COE program at the University of Shizuoka.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

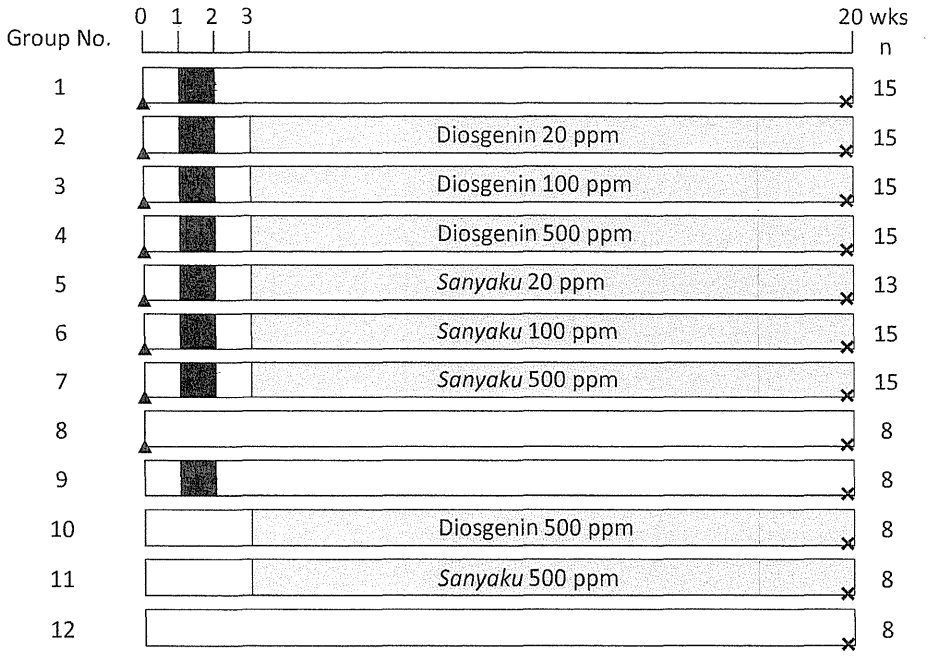
Received October 13, 2010; revised January 26, 2011; accepted February 18, 2011; published OnlineFirst March 2, 2011.

References

1. Stewart BW, Kleihues SP, editors. World cancer report. Lyon: IARC Press; 2003.
2. Bhalla V, Bhalla MA. Risk factors for colorectal cancer. Oncology News 2009;4:84–5.
3. Herbey II, Ivankova NV, Katkoori VR, Mamaeva OA.Colorectal cancer and hypercholesterolemia. Exp Oncol 2005;27:166–78.
4. Giovannucci E, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. Gastroenterology 2007;132:2208–25.
5. Yamada K, Araki S, Tamura M, Sakai I, Takahashi Y, Kashihara H, et al. Relation of serum total cholesterol, serum triglycerides and fasting plasma glucose to colorectal carcinoma in situ. Int J Epidemiol 1998;27:794–8.
6. Greenwald P, Milner JA, Anderson DE, McDonald SS. Micronutrients in cancer chemoprevention. Cancer Metastasis Rev 2002; 21:217–30.
7. Gescher AJ, Sharma RA, Steward WP. Cancer chemoprevention by dietary constituents: a tale of failure and promise. Lancet Oncol 2001;2:371–9.
8. Wanasundera JPD, Ravindran G. Effects of cooking on the nutrient and antinutrient contents of yam tubers (Dioscorea alata and Dioscorea esculenta). Food Chemistry 1992;45:247–50.
9. Wanasundera JP, Ravindran G. Nutritional assessment of yam (Dioscorea alata) tubers. Plant Foods Hum Nutr 1994;46:33–9.
10. Lape IM, Treche S. Nutritional quality of yam (Dioscorea dumetorum and D rotundata) flours for growing rats. J Sci Food and Agric 1994;66:447–55.
11. Agbor-Egbe T, Trèche S. Evaluation of the chemical composition of Cameroonian yam germplasm. J Food Compost Anal 1995;8: 274–83.
12. Hikino H, Konno C, Takahashi M, Murakami M, Kato Y, Karikura M, et al. Isolation and hypoglycemic activity of dioscorans A, B, C, D, E, and F; glycans of Dioscorea japonica rhizophors. Planta Med 1986;52:168–71.
13. He S, Zhan T, Wang S. Study on chemistry and antioxidation activity of water soluble polysaccharides of Rhizoma Dioscoreae oppositae. J China Pharm Univ 1994;25:373–5.
14. Hu K, Dong A, Yao X, Kobayashi H, Iwasaki S. Three spirostanol glycosides from rhizomes of Dioscorea collettii var hypoglauca. Planta Med 1996;62:573–5.
15. Hu K, Dong A, Yao X, Kobayashi H, Iwasaki S. Four furostanol glycosides from rhizomes of Dioscorea collettii var hypoglauca. Planta Med 1997;63:161–5.
16. Yang DJ, Lu TJ, Hwang LS. Isolation and identification of steroidal saponins in Taiwanese yam cultivar (Dioscorea pseudojaponica Yamamoto). J Agric Food Chem 2003;51:6438–44.
17. Yang DJ, Lu TJ, Hwang LS. Simultaneous determination of furostanol and spirostanol glycosides in Taiwanese yam (Dioscorea spp.) cultivars by high performance liquid chromatography. J Food Drug Anal 2003;11:271–6.
18. Chen Y, Wu Y. Progress in research and manufacturing of steroidal sapogenins in China. J Herbs Spices Med Plants 1994;2: 59–70.
19. Taylor WG, Elder JL, Chang PR, Richards KW. Microdetermination of diosgenin from fenugreek (Trigonella foenum-graecum) seeds. J Agric Food Chem 2000;48:5206–10.
20. Final report of the amended safety assessment of Dioscorea Villosa (Wild Yam) root extract. Int J Toxicol 2004;23:49–54.
21. Raju J, Mehta R. Cancer chemopreventive and therapeutic effects of diosgenin, a food saponin. Nutr Cancer 2009;61:27–35.
22. McAnuff MA, Harding WW, Omoruyi FO, Jacobs H, Morrison EY, Asemota HN, et al. Hypoglycemic effects of steroidal sapogenins isolated from Jamaican bitter yam, Dioscorea polygonoides. Food Chem Toxicol 2005;43:1667–72.
23. Juarez-Oropeza MA, Diaz-Zagoya JC, Rabinowitz JL. In vivo and in vitro studies of hypocholesterolemic effects of diosgenin in rats. Int J Biochem 1987;19:679–83.
24. Son IS, Kim JH, Sohn HY, Son KH, Kim JS, Kwon CS, et al. Antioxidative and hypolipidemic effects of diosgenin, a steroidal saponin of yam (Dioscorea spp.), on high-cholesterol fed rats. Biosci Biotechnol Biochem 2007;71:3063–71.
25. Kwon CS, Sohn HY, Kim SH, Kim JH, Son KH, Lee JS, et al. Antiobesity effect of Dioscorea nipponica Makino with lipase-inhibitory activity in rodents. Biosci Biotechnol Biochem 2003;67: 1451–6.
26. Raju J, Patlolla JM, Swamy MV, Rao CV. Diosgenin, a steroid saponin of Trigonella foenum graecum (Fenugreek), inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon cancer cells. Cancer Epidemiol Biomarkers Prev 2004;13:1392–8.
27. Taylor WG, Zaman MS, Mir Z, Mir PS, Acharya SN, Mears GJ, et al. Analysis of steroidal sapogenins from amber fenugreek (Trigonella foenum-graecum) by capillary gas chromatography and combined gas chromatography-mass spectrometry. J Agric Food Chem 1997;45:753–9.
28. Shah VKG, Dunstan H, Taylor W. An efficient diethyl ether-based soxhlet protocol to quantify faecal sterols from catchment waters. J Chromatogr A 2006;1108:111–5.
29. Giovannucci E. Metabolic syndrome, hyperinsulinemia, and colon cancer. Am J Clin Nutr 2007;86:836–42.
30. Pais R, Silaghi H, Silaghi AC, Rusu ML, Dumitrascu DL. Metabolic syndrome and risk of subsequent colorectal cancer. World J Gastroenterol 2009;15:5141–8.
31. Corbiere C, Liagre B, Bianchi A, Bordji K, Dauça M, Netter P, et al. Different contribution of apoptosis to the antiproliferative effects of diosgenin and other plant steroids, hecogenin and tigogenin, on human 1547 osteosarcoma cells. Int J Oncol 2003;22:899–905.
32. Raju J, Bird RP. Diosgenin, a naturally occurring steroid [corrected] saponin suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression and induces apoptosis in HCT-116 human colon carcinoma cells. Cancer Lett 2007;255:194–204.
33. Moalic S, Liagre B, Corbière C, Bianchi A, Dauça M, Bordji K, et al. A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells. FEBS Lett 2001;506: 225–30.
34. Shishodia S, Aggarwal BB. Diosgenin inhibits osteoclastogenesis, invasion, and proliferation through the downregulation of Akt, I kappa B kinase activation and NF-kappa B-regulated gene expression. Oncogene 2006;25:1463–73.
35. Leger DY, Liagre B, Corbière C, Cook-Moreau J, Beneytout JL. Diosgenin induces cell cycle arrest and apoptosis in HEL cells with increase in intracellular calcium level, activation of cPLA2 and COX-2 overexpression. Int J Oncol 2004;25:555–62.
36. Liagre B, Vergne-Salle P, Corbiere C, Charissoux JL, Beneytout JL. Diosgenin, a plant steroid, induces apoptosis in human rheumatoid arthritis synoviocytes with cyclooxygenase-2 overexpression. Arthritis Res Ther 2004;6:373–83.
37. Nappez C, Liagre B, BenLiagre B, Vergne-Salle P, Corbiere C, Charissoux JL, et al. Diosgenin, a plant steroid, induces apoptosis in human rheumatoid arthritis synoviocytes with cyclooxygenase-2 overexpression. Arthritis Res Ther 2004;6:R373–83.
38. Yamada T, Hoshino M, Hayakawa T, Ohhara H, Yamada H, Nakazawa T, et al. Dietary diosgenin attenuates subacute intestinal inflammation associated with indomethacin in rats. Am J Physiol 1997;273:355–64.
39. Dinarello CA. The paradox of pro-inflammatory cytokines in cancer. Cancer Metastasis Rev 2006;25:307–13.
40. Mutoh M, Akasu T, Takahashi M, Niho N, Yoshida T, Sugimura T, et al. Possible involvement of hyperlipidemia in increasing risk of colorectal tumor development in human familial adenomatous polyposis. Jpn J Clin Oncol 2006;36:166–71.
41. Niho N, Mutoh M, Takahashi M, Tsutsumi K, Sugimura T, Wakabayashi K, et al. Concurrent suppression of hyperlipidemia and intestinal

polyp formation by NO-1886, increasing lipoprotein lipase activity in Min mice. Proc Natl Acad Sci U S A 2005;102:2970–4.
42. Gong G, Qin Y, Huang W, Zhou S, Wu X, Yang X, et al. Protective effects of diosgenin in the hyperlipidemic rat model and in human vascular endothelial cells against hydrogen peroxide-induced apoptosis. Chem Biol Interact 2010;184:366–75
43. Uemura T, Goto T, Kang MS, Mizoguchi N, Hirai S, Lee JY, et al. Diosgenin, the main aglycon of fenugreek, inhibits LXRα activity in HepG2 cells and decreases plasma and hepatic triglycerides in obese diabetic mice. J Nutr 2011;141:17–23.
44. Li K, Tang Y, Fawcett JP, Gu J, Zhong D. Characterization of the pharmacokinetics of dioscin in rat. Steroids 2005;70: 525–30.
45. Raicht RF, Cohen BI, Fazzini EP, Sarwal AN, Takahashi M. Protective effect of plant sterols against chemically induced colon tumors in rats. Cancer Res 1980;40:403–5.

Supplemental figure 1



Supplemental figure 1 Experimental protocol used in this study.
▲: AOM (10 mg/kg i.p.); ■: 1.5% DSS in drinking water; □: Basal diet and tap water;
□ : Diosgenin or *sanyaku*; ×: Sacrifice.