Published OnlineFirst November 24, 2009; DOI: 10.1158/1940-6207.CAPR-09-0061

Dietary Tricin Suppresses Inflammation-Related Colon Carcinogenesis in Male Crj: CD-1 Mice

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Abstract The flavone 4′,5,7-trihydroxy-3′,5′-dimethoxyflavone (tricin) present in rice, oats, barley, and wheat exhibits antigrowth activity in several human cancer cell lines and anti-inflammatory potential. However, the chemopreventive activity has not yet been elucidated in preclinical animal models of colorectal cancer. This study was designed to determine whether dietary tricin exerts inflammation-associated colon carcinogenesis induced by azoxymethane and dextran sulfate sodium in mice. Male Crj: CD-1 mice were initiated with a single i.p. injection of azoxymethane (10 mg/kg body weight) and followed by a 1-week exposure to dextran sulfate sodium (1.5%, *w/v*) in drinking water to induce colonic neoplasms. They were then given the experimental diet containing 50 or 250 ppm tricin. The experiment was terminated at week 18 to determine the chemopreventive efficacy of tricin. In addition, the effects of dietary tricin on the expression of several inflammatory cytokines, including tumor necrosis factor (TNF)-α, were assayed. The development of colonic adenomas and adenocarcinomas was significantly reduced by feeding with 50 and 250 ppm tricin, respectively. Dietary tricin also significantly reduced the proliferation of adenocarcinoma cells as well as the numbers of mitoses/anaphase bridging in adenocarcinoma cells. The dietary administration with tricin significantly inhibited the expression of TNF-α in the nonlesional cypts. Our findings that dietary tricin inhibits inflammation-related mouse colon carcinogenesis by suppressing the expression of TNF-α in the nonlesional cyrpts and the proliferation of adenocarcinomas suggest a potential use of tricin for clinical trials of colorectal cancer chemoprevention.

Cancer mortality rates in the developed countries have increased throughout this century, and has been already the leading cause of death in some Western countries (1, 2). Great advances have been made in the pharmacologic-based treatment of malignant epithelial malignancies. There has also been a marked increase in the understanding of cell and molecular mechanisms underlying a variety of carcinogenic processes (3). However, therapeutic options for advanced neoplastic disease remain limited. This lack of treatment alternatives may be due to the large number of genetic and molecular alterations associated with advanced neoplasms that contribute to the maintenance of neoplastic progression.

The chemopreventive approach to inhibit cancer development and progression is highly attractive. Practical limitations may exist with respect to developing novel and effective chemopreventive agents through the use of appropriate animal models for preclinical evaluation of candidate chemopreventive agents (4). Some herbal and botanical products that contain flavonoids are likely to possess cancer preventive activities (5). A diet rich in fruits and vegetables has long been suggested to correlate with a reduced risk of certain epithelial malignancies, including cancers in the colon, lung, prostate, oral cavity, and breast (5–7). A number of agents have been reported to be candidate *chemo-inhibitors* of cancer development in various tissues, including colon. Among these agents are the flavonoids, a group of phenolic compounds with structural formula of diphenyl-propane and secondary metabolites produced by plants (5, 8, 9).

4′, 5, 7-Trihydroxy-3′, 5′-dimethoxyflavone (tricin; Fig. 1A) is a flavone, a subgroup of the flavonoid group, which is found *in* rice, oats, barley, and wheat (10). Although the physiologic function of tricin in plants is not well defined, the compound is thought to be produced by the plant during times of environmental stress or pathogenic attack (11) and exert potential allelopathic effects (12). Evidence for the biological activity of tricin in rodents has recently been reported. These biological activities

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Received 4/3/09; revised 7/7/09; accepted 7/22/09; published OnlineFirst 11/24/09.

Grant support: Grant-in-Aid for Cancer Research, for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan; Grants-in-Aid grant nos. 18592076 (T. Tanaka), 17015016 (T. Tanaka), and 18880030 (Y. Yasui) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and grant H2008-12 (T. Tanaka) for the Project Research from the High-Technology Center of Kanazawa Medical University.

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

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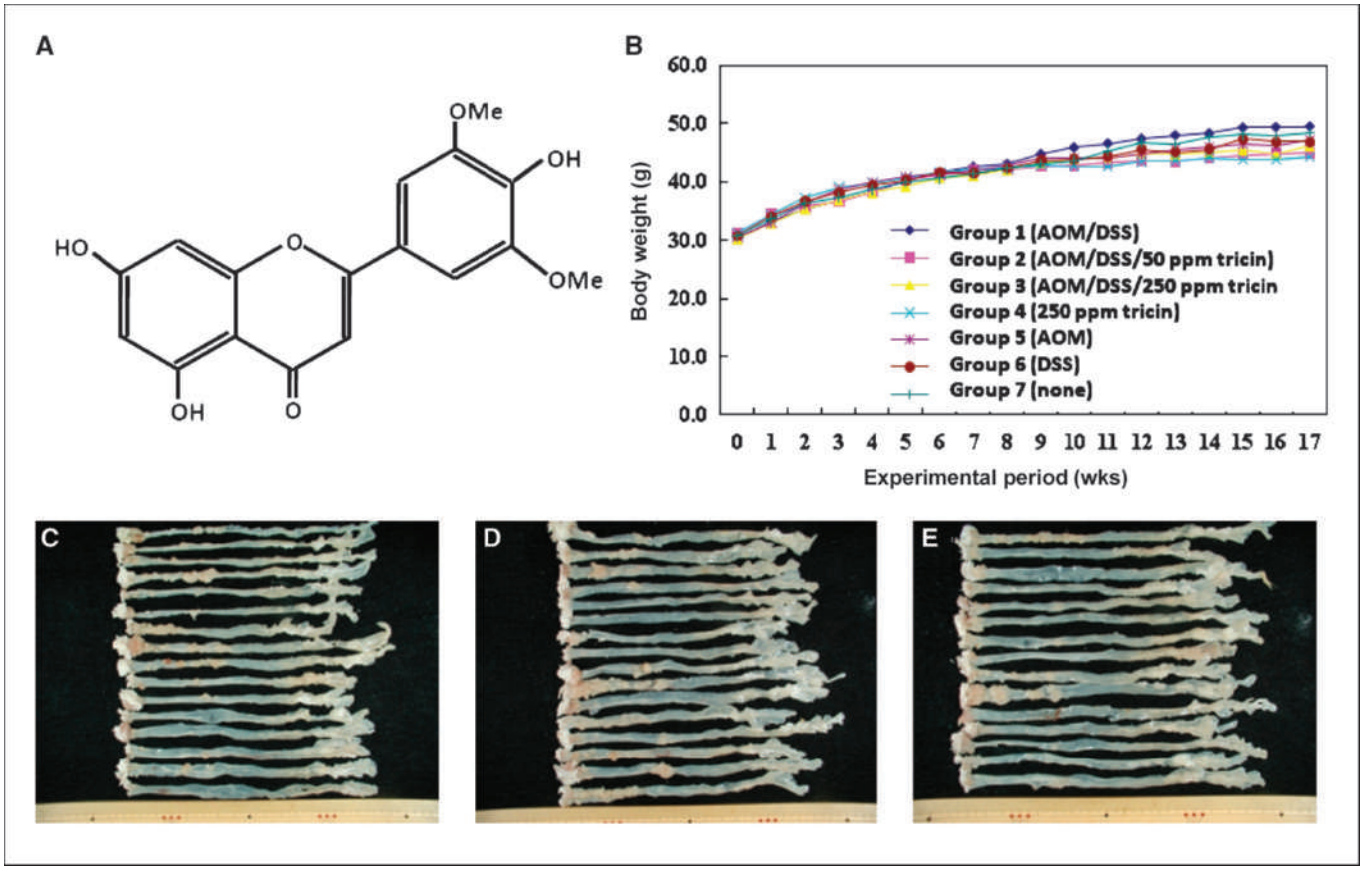

Fig. 1. *A*, the structure of tricin; molecular weight, 330.074. *B*, body weight changes of mice in all groups during the study. Dietary tricin (groups 2, 3, and 5) did not significantly affect the body weight gain. Macroscopic views of the colons from the mice of groups 1 (*C*), 2 (*D*), and 3 (*E*), which received AOM/DSS, AOM/DSS/50 ppm tricin, and AOM/DSS/250 ppm tricin, respectively, at the end of the study (week 18). Although a number of colonic tumors were observed in the mice of group 1, the numbers of the tumors found in groups 2 and 3 were smaller than that in group 1.

include antioxidative (13, 14), antiinflammatory, antiviral (15), and antihistamic (16) activities. These same biological activities have been observed in other promising cancer chemopreventive agents (17–20). The effects of tricin on oncogenesis have been investigated by Gescher et al. Their studies have shown that tricin suppresses the growth of human malignant breast tumor in nude mice (21). Dr. Gescher's group also reported that treatment with tricin-containing extracts from brown rice inhibit the proliferation of human colon and breast cancer cells *in vitro* (22). There are few reports on the effects of dietary tricin on intestinal carcinogenesis. Cai et al. (23) reported that feeding a diet containing 0.2% tricin decreased the size and the number of intestinal adenoma formed in $Apc^{Min/+}$ mice through the inhibition of cyclooxygenase (COX)-2 (23, 24). Dietary tricin did not affect tumor formation in the large bowel (23). Because the concentration of tricin in the mouse intestine is greater than the concentration in the plasma or liver when mice are fed diets containing tricin (25–28), we hypothesized that dietary tricin may affect and possibly inhibit chemically-induced colon carcinogenesis in rodents.

The current study was designed to explore the possible cancer chemopreventive efficacy of tricin. We investigated the effects of dietary tricin on large bowel oncogenesis using an azoxymethane (AOM)/dextran sodium sulfate (DSS)–treated mouse model, which is a useful animal model to study chemoprevention in inflammation-related colon carcinogenesis (29–34). The effects of dietary tricin on the expression of inflammatory enzymes, such as COX-2 (35–37) and inducible nitric oxide synthase (iNOS; refs. 37, 38), and inflammatory cytokines, such as tumor necrosis factor (TNF)-α, (39, 40) NF-κB (17, 40), inhibitor κB (IκB)α, and IκB kinase (IKK)β in the nonlesional colonic mucosa were examined to understand the mechanism(s) by which the compound modify AOM/DSS-induced colon carcinogenesis. In addition, we determined whether dietary tricin affects the chromosomal instability (41) of adenocarcinoma cells by counting the number of anaphase-bridging formations.

Materials and Methods

Chemicals

Tricin (>99% pure confirmed by high performance liquid chromatography) was isolated and prepared from the leaves of *Sasa albo-marginata* (Hououdou Co. Ltd.) by one (M.K.) of the authors (15). In brief, the dried leaves (50 kg) were combined with water (1,000 l) and extracted at 170°C over a period of 3 h. The extracted solution was filtered. The hot water extract of *Sasa albo-marginata* was fractionated successively with ethyl acetate and *n*-butanol. The ethyl acetate fraction (52.0 g) was fractionated using a silica gel 60 (Cica-reagent, 40-50 μm) column (inner diameter 6 × 50 cm, 500 g) and washed with *n*-hexane-ethyl acetate and methanol. This process yielded seven fractions (A-G). Chloroform was added to fraction F (1.50 g) to obtain a chloroform-soluble fraction and an insoluble fraction (solid phase). Tricin (10.0 mg) was recrystallized from the chloroform-soluble fraction as yellow,

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needle-shaped crystals. Finally, a total of 8 g of tricin was prepared from 40,000 kg of the leaves and was used in this study.

AOM was purchased from Sigma-Aldrich. DSS with a molecular weight of 36,000 to 50,000 was obtained from MP Biomedicals, LLC. DSS 1.5% (*w/v*) was prepared shortly before use to induce colitis.

Animals and diets

Five-week-old male Crj: CD-1 (ICR) mice were purchased from Charles River Laboratories, Inc. All animals were housed in plastic cages (three or four mice/cage) and had free access to tap water and a basal diet, Charles River Formula-1 (Oriental Yeast, Co., Ltd.). The animals were kept in an experimental animal room under controlled conditions of humidity (50 ± 10%), light (12/12-h light/dark cycle) and temperature (23 ± 2°C). After 1 wk of quarantine, animals were divided into six experimental groups and one control group. Experimental diets were prepared by mixing tricin in powdered basal diet at two dose levels, 50 and 250 ppm. The highest dose was one eighth of the dose used by Cai et al. (23) because we investigated the potential clinical application of low doses of tricin.

Animal experiment

The experimental and study design were approved by the Committee of Kanazawa Medical University Animal Facility under the Institutional Animal Care guideline. All handling and procedures were carried out in accordance with the appropriate Institutional Animal Care Guidelines.

A total of 95 male ICR mice were divided into six experimental groups and one control group. Mice in groups 1 (n = 20), 2 (n = 20), and 3 (n = 19) were given a single i.p. injection of AOM (10 mg/kg body weight). Beginning 7 d after the AOM injection, they also received 1.5% (*w/v*) DSS in drinking water for 7 d. Beginning 1 wk following the final DSS exposure, the mice in groups 2 were fed an experimental diet containing tricin at the rate of 50 ppm and the mice in group 3 were fed an experimental diet containing 250 ppm tricin. Both groups received the experimental diets for 15 wk. The mice in groups 4 (n = 9) received only the 250 ppm tricin-containing diet. The mice in group 5 (n = 9) received only AOM, and the mice in group 6 (n = 9) received only 1.5% DSS in drinking water. The mice of group 7 (n = 9) served as untreated controls.

At week 8, four mice each from groups 1, 2, and 3 and three mice each from groups 4, 5, 6, and 7 were randomly selected and sacrificed to measure mRNA expression of target inflammatory enzymes and cytokines in the colonic mucosa by quantitative reverse transcription-PCR (RT-PCR). At sacrifice, the large bowel of each animal was removed, the contents (feces) were washed out by physiologic saline, and the length from the ileocecal junction to the anal verge were measured. After the large bowels were cut open longitudinally along the main axis and gently washed with saline, scraped colonic mucosa tissue was dipped into the RNAlater solution (Applied Biosystems/Ambion).

At week 18, all of the remaining animals were euthanized by exsanguinations through the abdominal aorta under diethylether anesthesia and subjected to a complete gross necropsy examination to determine the incidence and multiplicity of tumors in the large bowel. At sacrifice, the large bowel was removed and the length was measured. Each large bowel was cut open longitudinally along the main axis and gently washed with saline, then examined manually to determine the incidence and multiplicity of tumors. The colon was fixed in 10% buffered formalin for at least 24 h. Histopathologic examination was done on H&E-stained sections made from paraffin-embedded blocks. Colonic tumors were diagnosed according to criteria established in a prior study (34). The number and density of mucosal ulcers on H&E-stained sections was also recorded.

Immunohistochemistry of proliferating cell nuclear antigen

Immunohistochemical analysis for the proliferating cell nuclear antigen (PCNA) in the colon with or without tumors was done on 4-μm-thick paraffin-embedded sections by the labeled avidin-biotin-peroxidase complex method using a Vectastain ABC kit (Vector Laboratories), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated with ethanol at room temperature. PBS (pH 7.4; 0.01 mol/L) as used to prepare the solutions and for washes between the preparation steps. Incubations were done in a humidified chamber. The sections were treated for 40 min at room temperature with mouse IgG blocking reagent (Vector Laboratories), and incubated overnight at 4°C with the primary antibody (1:300 dilution; DAKO Japan, Co., Ltd.). The antibody was applied to the sections according to the manufacturer's protocol. Horseradish peroxidase activity was visualized by treatment with H_2O_2 (DAKO Japan, Co., Ltd.) and 3,3′-diaminobenzidine (DAKO Japan) for 5 min. In the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck). For each examination, negative controls were done on serial sections. The numbers of nuclei with positive reactivity for PCNA-immunohistochemistry were counted by two observers (T.O. and T.T.) who were unaware of the treatment groups to which the slides belonged. The positive rates were evaluated in >100 cancer cells each of 15 different areas of the adenocarcinomas and 10 different crypts of the "normal"-appearing colonic mucosa from five mice each from groups 1 to 3 and expressed as percentage (mean ± SD).

Mitotic index and anaphase bridging index of adenocarcinoma cells

To examine the effects of dietary tricin on chromosomal instability (41) in adenocarcinoma cells, the anaphase bridging index (ABI) was determined on H&E-stained sections. The numbers of mitoses and anaphase bridging were counted in >100 cancer cells from five adenocarcinomas each from groups 1 through 3. The mitotic index (MI; number of mitoses per cancer cells) and ABI (number of anaphases with bridging per mitoses) were expressed as percentages (mean ± SD).

Quantitative RT-PCR

The normal-appearing colonic mucosa of mice from groups 1 through 3 were assayed for mRNA expression of COX-2, iNOS, TNF-α, NF-κB, IκBα, and IKKβ by RT-PCR. RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen Co.). Real-time PCR was done in a LightCycler (Roche Diagnostics Co.) with SYBR Premix Ex Taq (TAKARA BIO, INC.). The expression level of each gene was normalized to the β-actin expression level using the standard curve method. Each assay was done six times and the average was calculated. The primers used for amplifications are listed in Supplementary Table S1.

Statistical analysis

Where applicable, data were analyzed using one-way ANOVA with Tukey-Kramer Multiple Comparisons Test or Bonferroni (GraphPad Instat version 3.05, GraphPad Software) with $P < 0.05$ as the limit for statistical significance. Fisher's Exact Probability test or the χ^2 test were used for comparison of the incidence of lesions between the two groups. Data on mRNA expression (mean ± SEM) were analyzed by Mann-Whitney U test.

Results

General observation

All animals remained healthy throughout the experimental period. Food consumption (grams/day/mouse) did not differ significantly among the groups (data not shown). The body weight gains by mice in all of the seven groups were similar during the study (Fig. 1B). The mean body weight of group 2 (AOM/DSS/50 ppm tricin) was significantly lower than that of group 1 ($P < 0.01$; Supplementary Table S2). The mean colon length of group 1 was significantly shorter than the mean

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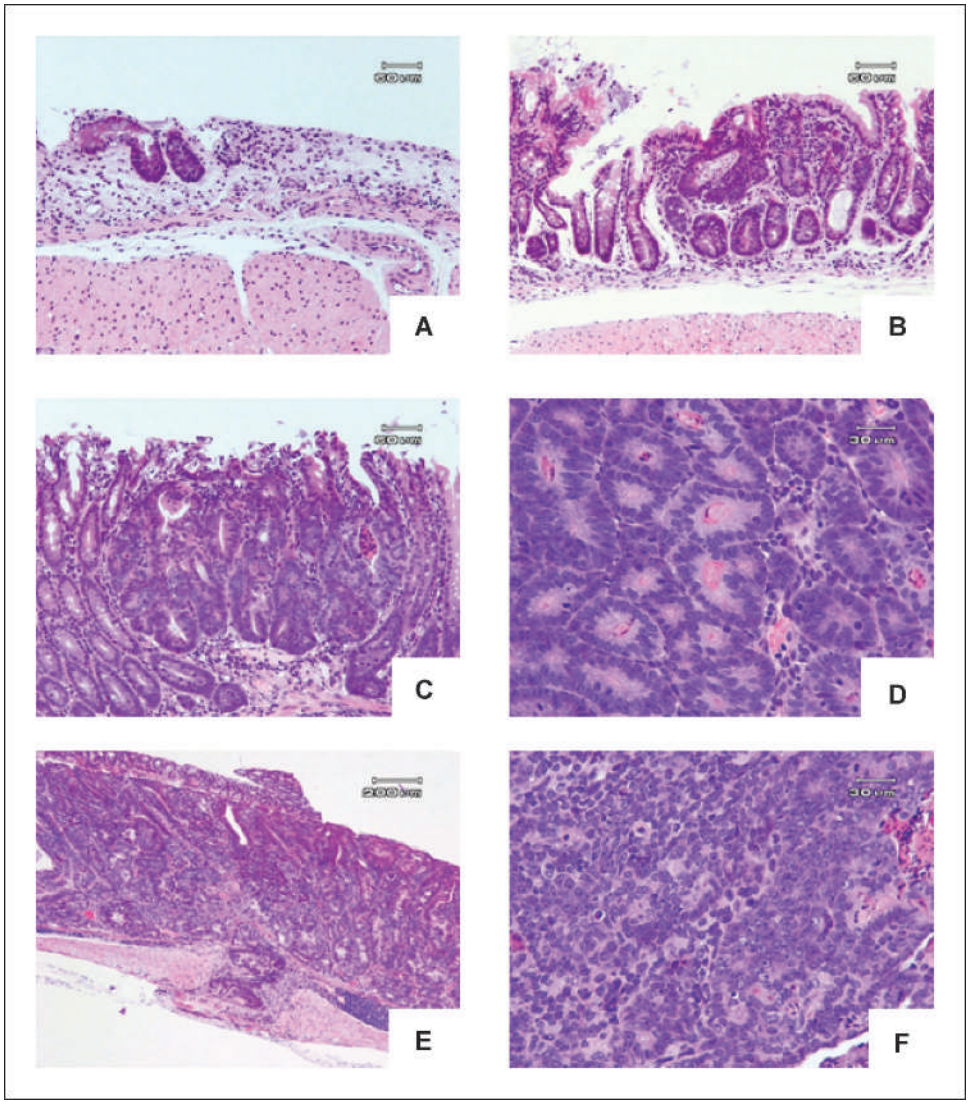

Fig. 2. Representative histopathology of the colonic lesions in group 1 (AOM/DSS). *A,* mucosal ulcer; *B,* dysplastic crypts; *C* and *D,* tubular adenomas; *E* and *F,* moderately differentiated tubular adenocarcinomas.

colon length of group 7 (no treatment; $P < 0.01$; Supplementary Table S2).

Incidence and multiplicity of colonic lesions

The incidence of macroscopic colonic lesions, including tumors and small ulcerations, were seen in the mice in group 1, 2, 3, and 6 (Fig. 1C-E). All mice in groups 1 through 3, which were treated with AOM/DSS with or without tricin, developed colonic tumors (adenoma and/or adenocarcinoma). The mice of group 4, 5, and 7 did not develop colonic tumors.

Microscopic examinations revealed various pathologic colonic lesions in mice from groups 1, 2, 3, and 6. The lesions included mucosal ulcers (Fig. 2A), dysplastic crypts (Fig. 2B), tubular adenomas (Fig. 2C and D), and tubular adenocarcinomas (Fig. 2E and F). Some of the adenocarcinomas that developed in the group 1 mice invaded the subserosa of the colon (Fig. 2E). Table 1 summarizes the microscopic data on the incidence and multiplicity of colonic lesions. The dietary administration of 50 ppm tricin (group 2) significantly reduced the incidence ($P = 0.0117$) and multiplicity ($P < 0.05$) of adenomas and the number of total tumors (adenoma + adenocarcinoma, $P < 0.05$) when compared with group 1. Feeding with 250 ppm tricin (group 3) also significantly lowered the numbers of adenocarcinomas and total tumors when compared with group 1 ($P < 0.05$ for each comparison). The mean numbers of dysplastic crypts in groups 2 ($P < 0.05$) and 3 ($P < 0.01$) were significantly lower than that of dysplastic crypts in group 1. The mean numbers of mucosal ulcers in group 2 ($P < 0.05$) and 3 ($P < 0.001$) were also significantly smaller than that of group 1.

PCNA labeling indices of the normal-appearing crypts and adenocarcinomas

The data on the proliferative kinetics in the normal-appearing crypts and colonic adenocarcinomas by estimating the PCNA labeling indices are shown in Fig. 3. The dietary administration of tricin significantly lowered the PCNA labeling index of the normal-appearing crypts in group 2 (38 ± 11, $P < 0.05$) and group 3 (36 ± 12, $P < 0.05$) when compared with group 1 (48 ± 11). The PCNA labeling indices for colonic adenocarcinomas in groups 2 (74 ± 6, $P < 0.05$) and 3 (71 ± 4, $P < 0.001$) were significantly lower than in group 1 (80 ± 8).

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Table 1. Incidence and multiplicity of colonic lesions

Group no.	Treatment (no. of mice examined)	Mucosal ulcer	Dysplasia (high grade)	Adenoma	Adenocarcinoma	Total tumors (AD+ADC)
1	AOM/1.5% DSS (16)	100% (2.69 ± 0.95)*	100% (5.00 ± 3.79)	88% (4.19 ± 4.22)	94% (4.63 ± 3.74)	94% (8.81 ± 6.21)
2	AOM/1.5% DSS/50 ppm tricin (16)	94% (1.81 ± 1.11)	80% (2.56 ± 1.79)†,‡	44%§ (1.44 ± 1.79)†,‡	75% (3.19 ± 2.64)	75% (4.63 ± 4.05)†,‡
3	AOM/1.5% DSS/250 ppm tricin (15)	60% (0.87 ± 0.83)‖	73% (1.53 ± 1.13)¶	67% (1.87 ± 1.73)	67% (1.80 ± 2.04)†,‡	80% (3.67 ±3.37)†,‡
4	250 ppm tricin (6)	0%	0%	0%	0%	0%
5	AOM (6)	0%	0%	0%	0%	0%
6	1.5% DSS (6)	33% (0.33 ± 0.52)	0%	0%	0%	0%
7	None (6)	0%	0%	0%	0%	0%

Abbreviations: AD, adenoma; ADC, adenocarcinoma.
*Mean ± SD.
†Significantly different from group 1 by one-way ANOVA, and Tukey-Kramer Multiple Comparisons test.
‡$P < 0.05$.
§Significantly different from group 1 by Fisher's exact probability test ($P = 0.0117$).
‖$P < 0.001$.
¶$P < 0.01$.

The effects of tricin on the MI and ABI

Dietary administration with tricin affected the number of mitosis (Fig. 4A) and anaphase bridging (Fig. 4B) in adenocarcinomas. As illustrated in Fig. 4C, dietary feeding with tricin significantly decreased the MI in group 2 (17.4 ± 0.9, $P < 0.05$) and group 3 (12.7 ± 2.0, $P < 0.001$) compared with group 1 (20.8 ± 2.4). The treatment also lowered the ABI in group 2 (0.50 ± 0.24) and group 3 (0.29 ± 0.10, $P < 0.05$) compared with group 1 (1.10 ± 0.57).

Expressions of inflammatory enzyme and cytokine genes in colonic mucosa

At week 8, we assayed mRNA levels of COX-2, iNOS, TNF-α, NF-κB, IκBα, and IKKβ in the nonlesional colonic mucosa of

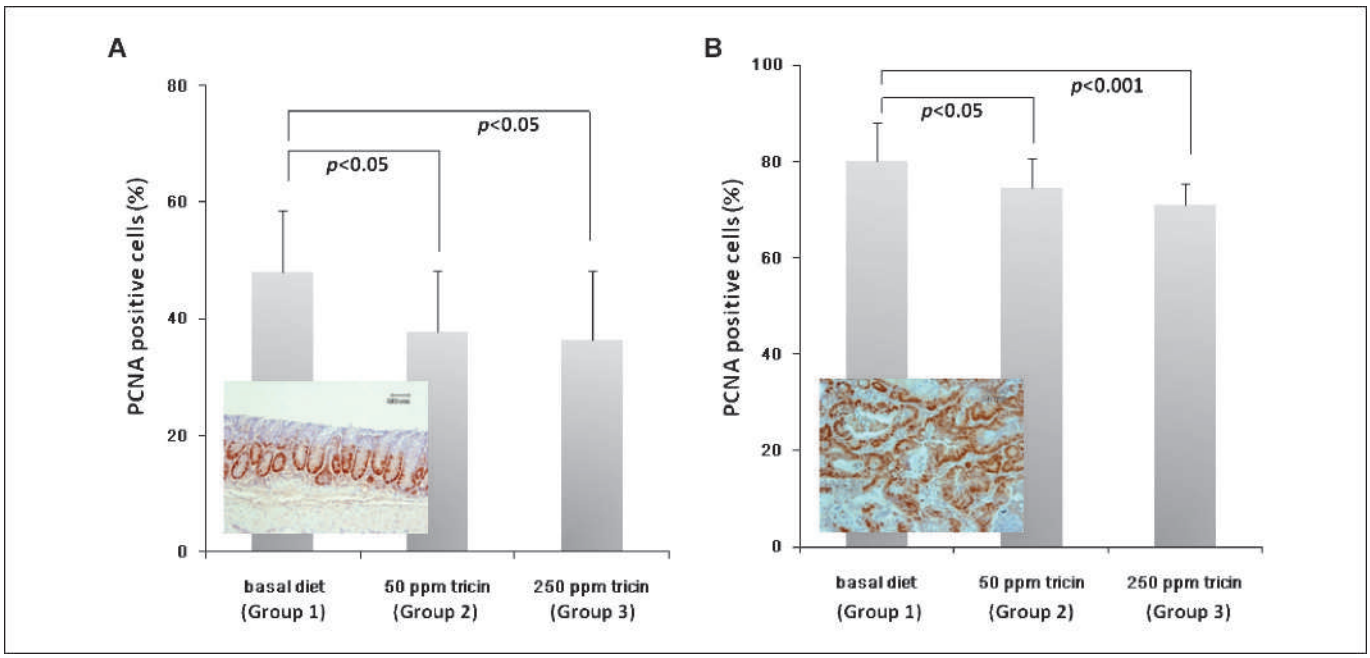

Fig. 3. The PCNA labeling indices of the normal-appearing crypts (*A*) and adenocarcinomas (*B*). Feeding with tricin (groups 2 and 3) significantly lowered the PCNA labeling indices of the normal-appearing crypts ($P < 0.05$ for each comparison) and adenocarcinomas (group 2, $P < 0.05$; and group 3, $P < 0.001$) compared with group 1.

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mice in groups 1 through 3 by semiquantitative real-time RT-PCR (Fig. 5). The TNF-α expression significantly decreased in group 3 compared with group 1 ($P < 0.05$; Fig. 5A). Feeding with tricin did not significantly affect the expression of COX-2 (Fig. 5B), iNOS (Fig. 5C), NF-κB (Fig. 5D), IκBα (Fig. 5E), and IKKβ (Fig. 5F).

Discussion

The results described herein clearly indicate that dietary administration with tricin at two dose levels (50 and 250 ppm) significantly inhibited AOM/DSS-induced colonic tumorigenesis in male ICR mice. The high dose (250 ppm) of tricin significantly inhibited development of adenocarcinomas induced by AOM followed by DSS in mice. The dietary administration with tricin also significantly affected the expression of TNF-α in the colonic mucosa at week 8. The treatment resulted in the reduction of the PCNA labeling index, MI, and ABI in the colonic epithelial malignancies at week 18.

The antitumor and chemoprevention activities of tricin have been reported in both *in vitro* and *in vivo* studies. *In vivo* experiments included transplanted human breast cancer cell lines in nude mice (21). In addition, Cai et al. reported that 0.2% tricin in diet effectively inhibited the number of adenomas in the small intestine of $Apc^{Min/+}$ mice (42). They did not, however, observe inhibition of the development of colonic tumors (42). In the current study, we observed the cancer chemopreventive activity of dietary tricin in carcinogenesis in the inflamed colon. In addition, feeding with tricin lowered the occurrence of mucosal ulcers and preneoplasms (dysplastic crypts).

We can point several mechanisms by which tricin may suppress AOM/DSS-induced colon carcinogenesis in this study. Our findings that dietary tricin lowered the PCNA labeling index, MI, and ABI of colonic adenocarcinomas may suggest an antigrowth effect of tricin on colonic malignancy. The findings are in agreement with the reports by Cai et al. (21) that showed tricin or tricin-containing extracts of brown rice inhibited the growth of the colon and mammary cells *in vitro* and *in vivo* (22). In addition, the results that dietary tricin lowered the ABI of adenocarcinoma cells suggest that tricin affects the chromosomal instability of cancer cells and possibly their telomerase activity (41). Tricin may exert chemopreventive activity through inhibition of COX-1 and 2 enzymes and prostaglandin E_2 production in human colon cancer cell lines (HT-29 and HCA-7) and the small intestine of $Apc^{Min/+}$ mice (23, 24). Unexpectedly, dietary tricin did not significantly alter the expression of COX-2 or iNOS at week 8. The suppression of NF-κB–signaling pathway by dietary administration with tricin was insignificant. However,

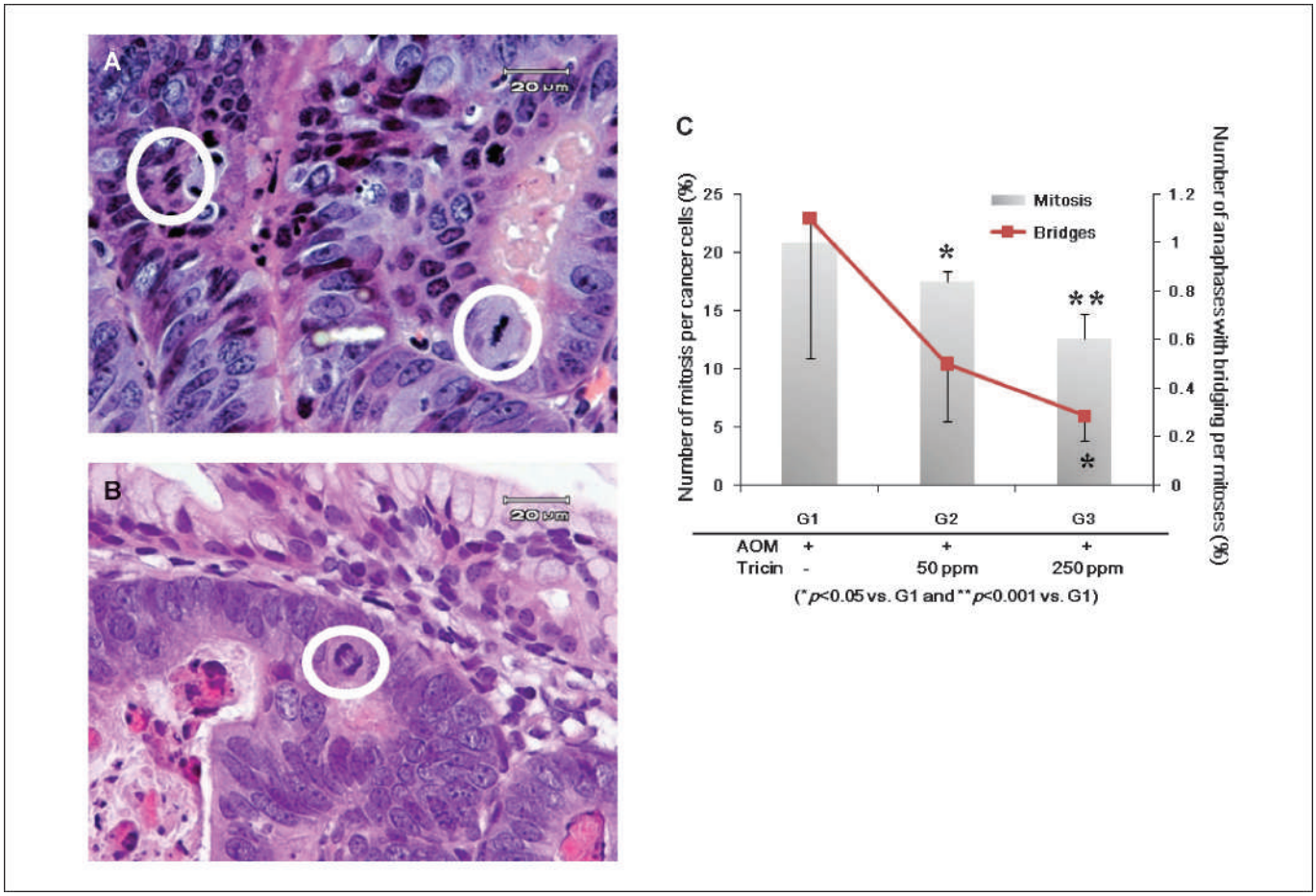

Fig. 4. The effects of dietary tricin on the MI and ABI. *A,* representative mitotic figures (*left circle,* anaphase; *right circle,* metaphase) in an adenocarcinoma, (*B*) representative anaphase bridging (*circle*) in an adenocarcinoma, and (*C*) MI (*columns*) and ABI (*lines*). Dietary administration of tricin significantly reduced the MI (50 ppm tricin, $P < 0.05$; and 250 ppm tricin, $P < 0.001$) and ABI (250 ppm tricin, $P < 0.05$). *G1,* group1; *G2,* group2; and *G3,* group 3.

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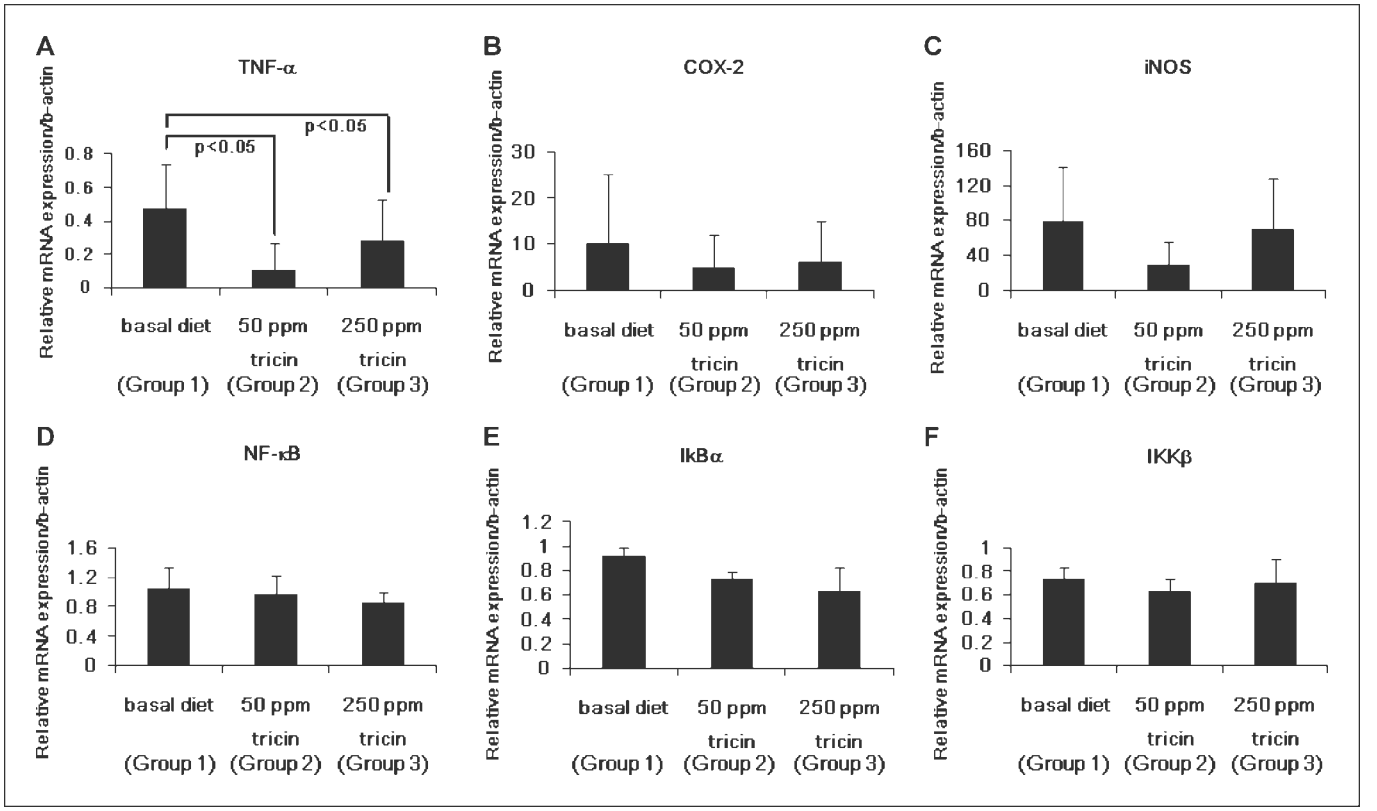

Fig. 5. The expression of (*A*) TNF-α (*B*) COX-2, (*C*) iNOS, (*D*) NF-κB, (*E*) IκBα, and (*F*) IKKβ in the normal-appearing colonic mucosa of groups 1 to 3 that were assessed by semiquantitative real-time RT-PCR. The expression of TNF-α was significantly inhibited by feeding with tricin (groups 2 and 3, $P < 0.05$ for each comparison). Feeding with tricin lowered the expression of COX-2, iNOS, and the NF-κB signaling pathway, but the reduction did not reach statistical significance. The expression was normalized to β-actin mRNA expression. Samples were analyzed in triplicate. *Columns*, mean of three independent experiments; *bars*, SEM; $n = 12$, Statistical analysis was done by the Mann-Whitney *U* test.

we observed that dietary tricin significantly inhibited the expression of TNF-α in the nonlesions colonic mucosa. Such effects are of interest because TNF-α acts as a master switch to establish an intricate link between inflammation and cancer (39, 40).

In conclusion, the dietary administration with tricin effectively suppressed AOM/DSS-induced colon carcinogenesis by suppressing the expression of TNF-α in the early phase and MI and ABI in the later phase. The effects of tricin on TNF-α expression are also important in the chemopreventive activity of tricin in inflammation-associated colorectal carcinogenesis. The safety of tricin was reported by Verschoyle et al. (43). A natural flavonoid tricin is present in edible plants, including rice, oats, barley, and wheat (10). In the current study, we isolated tricin from the dried leaves of *Sasa albo-marginata* that contain a large amount (0.2 ppm) of tricin than rice (Oryza sativa L.; 0.066 ppm). Tricin is thus a candidate for clinical use for fighting colorectal cancer development in patients without colitis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

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Cancer Prev Res 2009;2:1031-1038. Published OnlineFirst November 24, 2009.