0.32 ± 0.02, $P < 0.005$) is significantly higher than that of group 1 (AOM alone, 0.24 ± 0.01).

## 4. Discussion

The results described here clearly indicate that dietary administration with EGb and bilobalide, one of the active components of EGb, significantly inhibited AOM-induced ACF formation in male F344 rats. Importantly, rats fed the diets containing EGb or bilobalide showed no adverse effects on food consumption (data was not shown) and growth rate. In the current study, feeding with EGb (group 6) or bilobalide (group 7) alone significantly increased liver weights, but any histological alterations in the liver in these groups were not found. Moreover, the PCNA-labeling indices in liver of rats in all groups were almost similar (data not shown). Therefore, increased liver weight by EGb or bilobalide feeding did not indicate the toxicity of the compounds. These findings suggest that dietary EGb and bilobalide suppress early phase of chemically induced colon carcinogenesis. EGb has already used for a therapeutic in Europe [2–5], EGb and bilobalide may apply for human trial of cancer, although a long-term in vivo experiment confirming that EGb and bilobalide could safely inhibit colonic adenocarcinoma is needed. In addition, other constituents of EGb, flavone glycosides, terpene lactones, and organic acids [33] might contribute the reduction of ACF, since the inhibition rate of ACF development in group 3 (AOM + 500 ppm EGb) is the most remarkable among the groups.

Several explanations for the inhibitory effects of EGb and bilobalide on ACF-formation by AOM are considered. Cell proliferation has long been suspected to play a significant role in the initiation step as well as the promotion/progression of carcinogenesis [34,35]. In the present study, the PCNA-labeling indices of 'normal-appearing' crypts were decreased by dietary administration of EGb or bilobalide. Thus, the inhibitory effect of EGb and bilobalide may be in part due to modification of cell proliferation activity in the cryptal cells.

In rats given EGb and bilobalide, the amounts of total CYP and the activities of GST and QR in liver were elevated. Increased liver weights of rats given EGb or bilobalide alone might be due to alterations in these enzymes. CYP is known to play a prominent role in the biotransformation of carcinogenic xenobiotics. CYP2E1 is one of the enzymes to catalyze the biotransformation of AOM and MAM to a DNA-alkylating species, and the initiation events occur [36,37]. Shinozuka et al. [38] documented that EGb has no effect on the activities of not only CYP2E1 but also CYP1A1 and 1A2 in the liver of rats. Also, Gurley et al. [39] reported that supplementation with Ginkgo biloba for 28 days did not affect serum CYP activity in 6 female healthy volunteers. In contrast their findings, in the current study treatment with EGb or bilobalide significantly increased liver CYP2E1 activity. Also, the treatment significantly elevated liver GST and QR activities. Although further studies to demonstrate the protective roles of EGb and bilobalide against the initiation events caused by AOM are needed, it may be possible that elevation in GST and QR activities overcomes alteration of CYP 2E1 in liver, leading reduction in ACF formation in the current study. Previously, we reported that activities of GST and QR were significantly increased in the liver in EGb- and bilobalide-treated mice [40]. Additionally, treatment with these components elevated glutathione level in the mouse liver [40]. Glutathione acts not only as substrate for glutathione-related enzymes such as GST, but also directly in the destruction of reactive oxygen species [41]. Although we did not examine expression of cyclooxygenase (COX)-2 in this study, ginkgetin, one of the components of EGb down-regulates COX-2 induction in vivo [42]. This effect of EGb or bilobalide might be associated with suppressing incidence of ACF.

Another mechanism of chemoprevention of EGb and bilobalide is due to their antioxidant effect. Oxidative stress is potentially harmful to cells, and is implicated in the etiology and progression of many diseases, including cancer [43]. EGb appears to have a superoxide scavenging effect, a superoxide dismutase (SOD) activity [44] and scavenging properties against proxyl radicals [45]. Bilobalide also increases the activities of the antioxidant enzyme SOD and catalase [46]. Recently, EGb and bilobalide are reported to antagonize the homocysteine effect on inducible nitric oxide synthase (iNOS) expression in macrophages via their antioxidant effect resulting in attenuation of NF-κB activation [47]. EGb inhibits expression of iNOS messenger RNA isolated ischemic-reperfused rat

hearts [48]. Therefore, the observed chemopreventive potential of EGb and bilobalide against early phase of colon carcinogenesis induced by AOM, might be at least partly, via its antioxidant effect.

In conclusion, the findings described here demonstrate for the first time that dietary administration with EGb or bilobalide significantly inhibits the development of AOM-induced colonic ACF. Although the exact mechanisms by which EGb or bilobalide inhibits ACF development remains to be elucidated, it would appear that the modulation of colon tumorigenesis by these components are associated with the alteration of cell proliferation activity and drug metabolizing enzymes' activity.

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# Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-*db/db* mice

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**Epidemiological studies have shown that obesity and diabetes mellitus may be risk factors for colon cancer. However, the underlying mechanisms of how these chronic diseases promote colon carcinogenesis remain unknown. C57BL/KsJ-*db/db* mice have obese and diabetic phenotypes because of disruption of the leptin receptor. The present study was designed to investigate whether development of azoxymethane (AOM)-induced dysplastic and early neoplastic (premalignant) lesions of the colon is modulated in *db/db* mice. Homozygous *db/db* mice, heterozygous *db/+* mice and littermate controls (+/+) were injected with AOM under food restriction (~10.8 kcal/mouse/day) and killed 5 weeks after the carcinogen treatment. Their colons were assessed for premalignant lesions induced by AOM. We found a significant increase in the multiplicity of the total premalignant lesions in *db/db* mice when compared with *db/+* or +/+ mice. Phenotypically, serum leptin and insulin levels in *db/db* mice were significantly higher than those in *db/+* or +/+ mice, whereas the body weights and glucose levels in blood of *db/db*, *db/+* and +/+ mice were comparable. In addition, immunostaining of the leptin receptor and insulin-like growth factor-I receptor showed up-regulation of these protein levels specifically in the lesions. Our data indicate that development of AOM-induced premalignant lesions is enhanced in *db/db* mice with hyperleptinemia and hyperinsulinemia. The results have important implications for further exploration of the possible underlying events that affect the positive association between colon cancer and chronic diseases (obesity and diabetes).**

## Introduction

Numerous epidemiological results suggest that obesity is a risk factor for colon cancer (1,2). In spite of the accumulating evidence in epidemiology, experimental and laboratory studies to confirm the aforementioned proposal and reveal the underlying mechanisms have not been extensively performed so far. This may be because obesity is a complex, heterogeneous and multifactorial syndrome resulting from both genetic susceptibility and environmental factors (3). Besides obesity, it is well known that several factors, including a high fat and low fiber diet (4), low physical activity (5), inflammatory bowel diseases (6) or hereditary disorders such as familial adenomatous polyposis and non-polyposis syndrome (7), increase the risk for development of colorectal cancers. Interestingly, some of the risk factors for colon cancer, such as a high fat diet or decreased physical activity, enhance obesity as well (8,9), suggesting that there might be common biological events or undefined interactive events that affect the positive association between colon cancer and weight gain. Identification and evaluation of such phenomena will be critical in the near future for preventive strategies against both diseases.

C57BL/KsJ-*db/db* mice are genetically altered models with phenotypes of obesity and diabetes mellitus (10). Disruption of the leptin receptor (Ob-R) gene in these mice leads to overexpression of leptin in the adipose tissue and a concomitantly high concentration of leptin in the blood of the mice (11,12). It is widely accepted that leptin functions as a satiety factor through Ob-R, which is mainly expressed in the hypothalamus (12). Because of a deficiency of the leptin-mediated satiety signaling, abnormal dietary habits such as hyperphagia occur in homozygous *db/db* mice, resulting in complex phenotypes. It is now well established that leptin not only interacts with pathways in the central nervous system, but also functions in the peripheral tissues as a mediator of energy expenditure, a permissive factor for puberty and a signal of metabolic status (13). Interestingly, some lines of evidence suggest that leptin in the periphery behaves as a growth factor in lung (14), breast (15) and colonic tissues (16). A plausible role of leptin in tumorigenesis remains undetermined, although there have been several studies suggesting the leptin-related pathway as a possible modulator in neoplastic development (17–19).

In the present study we conducted a short-term assay to examine whether occurrence of azoxymethane (AOM)-induced dysplastic and early neoplastic lesions of colon is modulated in genetically obese *db/db* mice. The main goal of this study was to assess the involvement of obesity-related events such as hyperleptinemia in colon carcinogenesis *in vivo*. To do so, the involvement of dietary factors, including hyperphagia, was kept minimal in this experiment because these may dominate or mask obesity-related interactive factors. To this end, food intake by all the experimental mice was moderately and equally restricted during the study.

## Materials and methods

### *Animals, diets, and carcinogen*

A total of 50 animals (15 male 4-week-old C57BL/KsJ-*db/db* mice, 18 male 4-week-old C57BL/KsJ-*db/+* mice and 17 male 4-week-old C57BL/KsJ-+/+ mice), purchased from Jackson Laboratories (Bar Harbor, ME), were used. The mice were housed in a holding room under controlled conditions of a 12 h

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; EIA, enzyme immunoassay; H&E, hematoxylin and eosin; IGF-I, insulin-like growth factor-I; IGF-IR, insulin-like growth factor-I receptor; MAPK, mitogen-activated protein kinase; Ob-R, leptin receptor.

light/dark cycle, 23 ± 2°C room temperature and 50 ± 10% relative humidity. From 4 weeks of age they were randomly assigned to experimental (10–13 mice of each genotype) and control (5 mice each) groups and were housed in small cages separately to control the food intake of all animals. MF (Oriental Yeast Co., Tokyo, Japan) was used as a basal diet, which consists of 5.3% fat, 21.6% protein, 6.1% minerals, 2.9% fiber and 62.1% carbohydrate and others (~3.6 kcal/g). The major fatty acids present in MF were linoleic acid, oleic acid and palmitic acid. Administration of MF to each mouse was controlled (3 g/mouse/day) whereas water was available *ad libitum* during the experiment. AOM was obtained from Sigma (St Louis, MO).

*Experimental procedures*

Starting at 4 weeks of age, 10 homozygous *db/db* mice, 13 heterozygous *db/+* mice, and 12 littermate controls (+/+) were given s.c. injections of AOM (15 mg/kg body wt) once weekly for 5 weeks to induce dysplastic and early neoplastic lesions of the colon. The remaining five animals of each genotype were injected with 0.2 ml of saline without AOM and served as controls. The food intakes of all the mice were restricted equally by feeding the same amount of MF during the experiment. All the mice were carefully observed daily under the food-restricted conditions. The experiment was terminated 10 weeks after the first injection of AOM (13 weeks of age) and all animals were killed. At autopsy, colons of all the mice were removed, cut open longitudinally and fixed in 10% buffered formalin. After removing the rectal sides (1 cm from the anus), the colons were cut into two portions (distal and proximal) and the distal colons were used in this study. They were embedded in paraffin blocks using an *en face* preparation technique (20) and processed for histopathological examination with hematoxylin and eosin (H&E) staining. Dysplastic and early neoplastic lesions in the colonic mucosa were identified microscopically according to the criteria described by Chang (21) and Risio *et al.* (22). Although we did not evaluate the formation of aberrant crypt foci (ACF) in the unsectioned colons, dysplastic lesions may include dysplastic ACF, but not hyperplastic ones. Epididymal fat tissues were also removed and weighed.

*Leptin and insulin in blood*

At killing, blood samples of saline-treated mice were collected for determination of glucose, leptin and insulin concentrations by enzyme immunoassay (EIA) according to the manufacturer's protocol (R&D systems, Minneapolis, MN).

*Immunohistochemical analysis*

Immunohistochemistry was performed using stain system kits (Dako, Kyoto, Japan; Zymed, South San Francisco, CA). Rabbit polyclonal antibodies against Ob-R (Santa Cruz Biotechnology, Santa Cruz, CA) and insulin-like growth factor-1 receptor (IGF-IR) (Santa Cruz Biotechnology) were applied to the sections according to the manufacturer's protocols. For evaluation of the immunoreactivity cells were considered positive when definite cytoplasmic staining was identified.

*Statistical analysis*

Data were compared by ANOVA and *post hoc* tests. The results were considered statistically significant if the $P$ values were $<0.05$.

## Results

### *General observations*

Food intake of the animals was carefully monitored every day and the total amount of food intake per animal was thought to be the same. The average body weights at the termination of the experiment (Figure 1) were respectively 20.7 ± 4.6 g in AOM-exposed *db/db* mice, 21.7 ± 2.0 g in AOM-exposed *db/+* mice, 19.4 ± 1.6 g in AOM-exposed +/+ mice, 24.3 ± 0.8 g in saline-treated *db/db* mice, 22.9 ± 1.3 g in saline-treated *db/+* mice and 22.6 ± 0.7 g in saline-treated +/+ mice (means ± SD). The body weights of saline-treated +/+ mice were lower than those of saline-treated *db/db* mice ($P < 0.05$) and there was a downward trend in the body weights of AOM-treated mice when compared with the corresponding saline-treated ones. In general, however, the body weights of all mice were controlled favorably. All animals tolerated the AOM injections except three of the *db/db* mice, four of the *db/+* mice and two of the +/+ mice, which died during or soon after the period of AOM exposure, possibly because of a decreased maximum tolerated dose due to the AOM treatment.

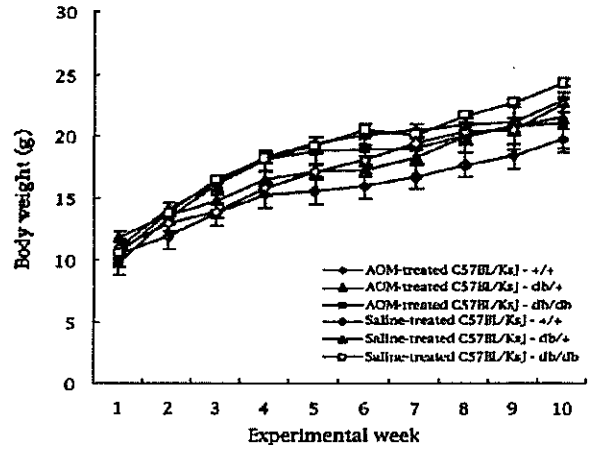


**Fig. 1.** Body weights of the experimental mice (means ± SEM).

**Table I.** Weights of fat tissues and serum glucose, insulin and leptin levels of the experimental mice[a]

| Genotype (no. of mice) | Epididymal fat (g) | Leptin (ng/ml) | Insulin (pg/ml) | Glucose (mg/dl) |
|---|---|---|---|---|
| *db/db* (5) | 1.08 ± 0.11[b,c] | 94.4 ± 14.9[b,c] | 5541.0 ± 4020.1[d,e] | 176.8 ± 20.0 |
| *db/+* (5) | 0.48 ± 0.14[f] | 19.6 ± 5.0 | 389.8 ± 216.0 | 137.4 ± 43.8 |
| +/+ (5) | 0.29 ± 0.03 | 16.9 ± 3.1 | 639.2 ± 523.4 | 172.4 ± 35.0 |

[a]Values are means ± SD.
[b,d,f]Significantly different from +/+ mice ([b]$P < 0.0001$; [d]$P < 0.03$; [f]$P < 0.04$).
[c,e]Significantly different from *db/+* mice ([c]$P < 0.0001$; [e]$P < 0.02$).

### *Concentrations of serum leptin and other factors*

Concentrations of leptin and insulin in the blood of *db/db*, *db/+* and +/+ mice were measured by EIA. As shown in Table I, the levels of both leptin and insulin in *db/db* mice were significantly higher than those in *db/+* or +/+ mice. In contrast, the levels of blood glucose were comparable among these mice. The results indicate that despite the food restriction, hyperleptinemia and hyperinsulinemia occurred in *db/db* mice, but not in *db/+* mice. In support of the results, the epididymal fat weights in *db/db* mice were significantly higher when compared to *db/+* and +/+ mice (Table I) and mRNA transcripts of the leptin gene in the fat tissues were up-regulated specifically in *db/db* mice (data not shown).

### *Dysplastic and early neoplastic lesions*

At killing there were no macroscopic tumors in any colon. On the histological sections with H&E staining a number of dysplastic (Figure 2C) and early neoplastic (Figure 2E) lesions were detected under microscopic examination. As summarized in Table II, AOM treatment induced 29.3 ± 14.1 (mean ± SD) total lesions (dysplasia and early neoplasia) per $cm^2$ of colon in *db/db* mice (Figure 2A), 13.8 ± 3.6 in *db/+* mice and 15.9 ± 9.5 in +/+ mice (Figure 2B). There were 2.1- and 1.8-fold increases in the multiplicity of the total lesions in *db/db* mice when compared to db/+ and +/+ mice, respectively ($P < 0.03$ and $P < 0.04$). In saline-treated

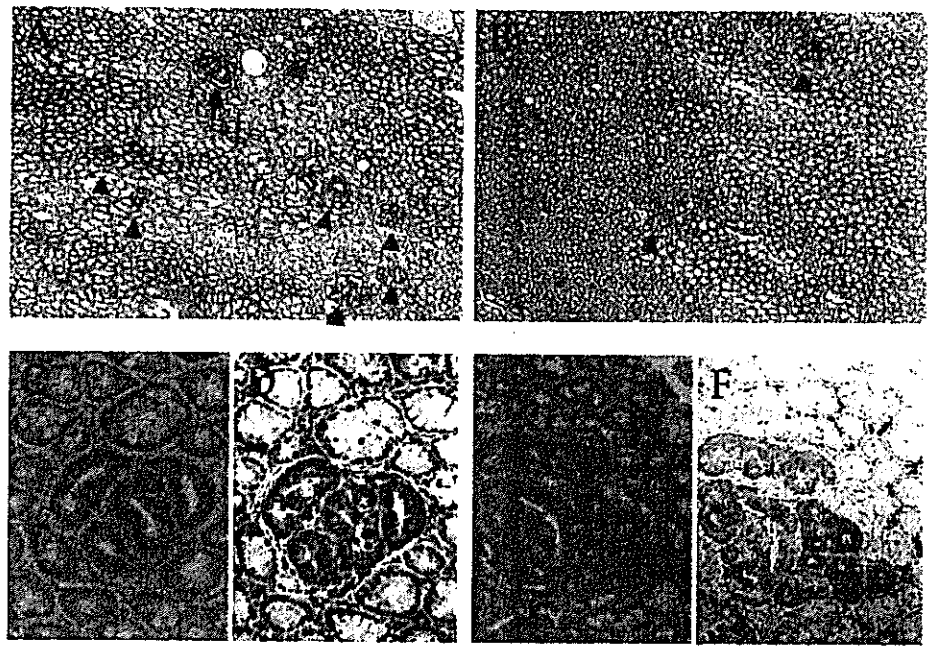

Fig. 2. Histology and immunohistochemistry of Ob-R and IGF-IR in AOM-exposed colonic mucosa. (A) Histology of the colonic mucosa in AOM-exposed *db/db* mice (×20). (B) Histology of the colonic mucosa in AOM-exposed *db/+* mice (×20). Arrowheads (A and B) indicate dysplastic lesions and the arrow (A) points to an early neoplastic lesion. (C) A representative photograph of dysplastic lesions (×200). (D) Up-regulation of Ob-R expression in dysplasia. (E) A representative early neoplastic lesion (×100). (F) IGF-IR expression is up-regulated in early neoplasia. (A)–(C) and (E), H&E staining; (D) and (F), immunohistochemistry of Ob-R and IGF-IR, respectively.

Table II. Multiplicity of dysplastic and early neoplastic lesions of the colon in AOM-treated mice

| Genotype (no. of mice) | Dysplastic | Early neoplastic | Total |
|---|---|---|---|
| *db/db* (7) | 26.7 ± 11.5[a,b,c] | 2.6 ± 2.8 | 29.3 ± 14.1[b,c] |
| *db/+* (9) | 13.1 ± 4.1 | 0.7 ± 1.8 | 13.8 ± 3.6 |
| +/+ (10) | 14.5 ± 9.0 | 1.4 ± 1.3 | 15.9 ± 9.5 |

[a]Means ± SD.
[b]Significantly different from +/+ mice ($P < 0.04$).
[c]Significantly different from *db/+* mice ($P < 0.03$).

mice there were no microscopic lesions in the colonic mucosa. The results indicate that development of AOM-induced premalignant lesions of the colon was enhanced specifically in *db/db* mice.

### *Immunohistochemical analysis of Ob-R and IGF-IR*

Our biochemical analysis showed that hyperleptinemia and hyperinsulinemia occurred in *db/db* mice. To determine the expression levels of the receptors for those hormones, immunohistochemistry using antibodies against Ob-R and IGF-IR was performed. Immunohistochemical expression of both Ob-R and IGF-IR was up-regulated in the cytoplasm of the dysplastic and neoplastic tissues (Figure 2D and F). The results suggest that up-regulation of the expression of these receptors might play a role in colon carcinogenesis and that hyperleptinemia and hyperinsulinemia could affect receptor-mediated signaling in *db/db* mice.

## Discussion

In spite of the recent availability of several animal models for obesity, experimental evidence from these model systems suggesting an interactive mechanism of how obesity promotes colon carcinogenesis is relatively limited. In most of these animal models obesity is induced by dietary factors, such as a high fat diet or hyperphagia. Since the contribution of diet to cancer causation is generally thought to be high (23,24), it is possible that the dominant and complex features of the dietary factors may make it difficult to determine what factor is responsible for the relation between obesity and colon cancer. In this study we moderately restricted the food intake of all animals to equalize the involvement of dietary factors. As a result, the body weights of all the animals examined were controlled favorably, whereas leptin in the blood remained high specifically in *db/db* mice. This enabled us to assess the contribution of possible interactive factors such as hyperleptinemia to colon carcinogenesis. Although it does not suitably reflect human obesity, our model using *db* mice with food restriction is considered to be a novel tool for investigation of colon carcinogenesis in the settings of obesity and diabetes.

Our data suggest that development of AOM-induced premalignant lesions of the colon in *db/db* mice with hyperleptinemia was enhanced even under food-restricted conditions. The effect was actually expected and concordant with previous findings that AOM-induced colon carcinogenesis is enhanced in another obese model using Zucker rats (25,26). Concerning the mechanism(s), it is plausible that hyperleptinemia may be critical for this enhancement, because leptin acts as a growth factor in the colon (16) and appears to play a role in tumorigenesis of other organs, such as mammary gland (27). Leptin

exerts its effects through the Ob-R, which has at least five splicing variants (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re) (12). In *db/db* mice a single point mutation in the C-terminal region of the Ob-R gene leads to a splicing abnormality of Ob-Rb (12). Consequently, Ob-Rb, which plays the main role in satiety signaling in the hypothalamus, is specifically deficient in these mice. On the other hand, the other splicing variants, Ob-Ra, Ob-Rc, Ob-Rd and Ob-Re, are preserved in *db/db* mice (12), although the function(s) of these receptors remains undetermined. Interestingly, a recent report showed that Ob-Ra, which is ubiquitously expressed in the periphery, performs signaling transduction (28). Furthermore, mitogen-activated protein kinase (MAPK), a protein kinase which plays a central role in regulating the activity of many nuclear transcriptional factors involved in inflammatory, immune and proliferative responses, has been shown to be one of the signal cascades of leptin mediated through the splicing variants of Ob-R (29). In addition, our immunohistochemical results showed that expression of Ob-R was up-regulated in AOM-induced lesions, suggesting a role in colon carcinogenesis. Taken together, there is a possibility that the enhancement of development of AOM-induced premalignant lesions of the colon in *db/db* mice may be caused by leptin-mediated signaling, such as through MAPK.

This study also showed that insulin in the blood was high specifically in food-restricted *db/db* mice. This result is consistent with previous findings that the expression of insulin is negatively regulated by the leptin pathway (30,31). Unexpectedly, the levels of blood glucose were comparable among the mice, suggesting that resistance to insulin might occur in *db/db* mice under these experimental conditions (32). There is accumulating evidence suggesting that hyperinsulinemia is involved in colon carcinogenesis as well as obesity and diabetes. Several epidemiological studies indicate that diabetic patients with hyperinsulinemia have increased risk for colon cancer (33). Additionally, a previous model assay showed that continuous injections of insulin promote AOM-induced colon carcinogenesis in rats (34). Hence, it seems likely that hyperinsulinemia in *db/db* mice enhanced the development of AOM-induced lesions in the present study. Regarding the mode of action, the current consensus assumes that the insulin-like growth factor-I (IGF-I) pathway plays a role in insulin-related tumor promotion in the colon (35). IGF-I binds to the IGF-IR, activates a signal cascade and triggers cell proliferation in several organs, including colon (36). Insulin at supra-physiological levels also binds to and activates the IGF-IR because of its homology with the insulin receptor (37,38). Furthermore, hyperinsulinemia was shown to indirectly increase bioavailability of IGF-I by regulating levels of IGF-binding proteins (36,39). In addition, our immunohistochemical data showed that IGF-IR expression was up-regulated in AOM-induced dysplastic and early neoplastic tissues of colon. This result was supported by a previous report showing that the IGF-IR is overexpressed in colon cancer in humans (40). Accordingly, it is possible that hyperinsulinemia in *db/db* mice activates the signaling cascades involving the IGF-IR, resulting in a proliferative response.

In conclusion, our data indicate that development of AOM-induced premalignant lesions of the colon was enhanced in *db/db* mice with hyperleptinemia and hyperinsulinemia. However, further studies will be necessary to reveal the specific determinants responsible for the correlation between obesity (and diabetes) and colon carcinogenesis.

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# DIETARY SEED OIL RICH IN CONJUGATED LINOLENIC ACID FROM BITTER MELON INHIBITS AZOXYMETHANE-INDUCED RAT COLON CARCINOGENESIS THROUGH ELEVATION OF COLONIC PPARγ EXPRESSION AND ALTERATION OF LIPID COMPOSITION

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**Our previous short-term experiment demonstrated that seed oil from bitter melon (*Momordica charantia*) (BMO), which is rich in *cis(c)9, trans(t)11, t13*-conjugated linolenic acid (CLN), inhibited the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF). In our study, the possible inhibitory effect of dietary administration of BMO on the development of colonic neoplasms was investigated using an animal colon carcinogenesis model initiated with a colon carcinogen AOM. Male F344 rats were given subcutaneous injections of AOM (20 mg/kg body weight) once a week for 2 weeks to induce colon neoplasms. They also received diets containing 0.01%, 0.1% or 1% BMO for 32 weeks, starting 1 week before the first dosing of AOM. At the termination of the study (32 weeks), AOM induced 83% incidence (15/18 rats) of colonic adenocarcinoma. Dietary supplementation with 0.01% and 0.1% BMO caused significant reduction in the incidence (47% inhibition by 0.01% BMO, $p<0.02$; 40% inhibition by 0.1% BMO, $p<0.05$; and 17% inhibition by 1% BMO) and the multiplicity (64% inhibition by 0.01% BMO, $p<0.005$; 58% inhibition by 0.1% BMO, $p<0.02$; and 48% inhibition by 1% BMO, $p<0.05$) of colonic adenocarcinoma, though a clear dose response was not observed. Such inhibition was associated with the increased content of CLA (c9,t11-18:2) in the lipid composition in colonic mucosa and liver. Also, BMO administration in diet enhanced expression of peroxisome proliferator-activated receptor (PPAR) γ protein in the non-lesional colonic mucosa. These findings suggest that BMO rich in CLN can suppress AOM-induced colon carcinogenesis and the inhibition might be caused, in part, by modification of lipid composition in the colon and liver and/or increased expression of PPARγ protein level in the colon mucosa.**


**Key words:** *conjugated linolenic acid; chemoprevention; colon carcinogenesis; bitter melon; PPARγ*

Colon cancer is the third most malignant neoplasm in the world.[1] It is well known that colorectal cancer is linked to Western lifestyle, which often includes a diet high in fat.[2] In Japan, the incidence of this malignancy, being the third leading cause of cancer death, has been increasing, possibly due to the Westernization of dietary habits, with a rising fat intake. The amount and type of dietary fat consumed are of particular importance for development of this malignancy.[3–6] Epidemiological studies indicate that high intake of fish oil-consumption and fish correlates with a reduced risk of colon cancer.[7,8] Diets containing fish oil are rich in the n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Studies in humans and experimental animals indicate a protective effect of n-3 PUFA (fish, fish oils or EPA ethyl ester), and the mechanism of protection is largely thought to be related to interference with biosynthesis of 2-series prostaglandins (PGs) from arachidonic acid (AA).[9] Our short-term experiment[10] also demonstrated that tuna oil rich in DHA and vitamin $D_3$ inhibits azoxymethane (AOM)-induced rat aberrant crypt foci (ACF), being putative precursor lesions for colonic adenocarcinoma.[11] However, limited studies of α-linolenic acid (α-LN), which is the parent fatty acid of the n-3 family, provide some promising results. Dietary feeding of perilla and flaxseed oils, both rich sources of α-LN, could decrease chemically induced colonic neoplasms and ACF in rat colon carcinogenesis models.[12–14] These results coincide with competitive exclusion of n-6 PUFA from membrane phospholipids and associate reductions in $PGE_2$ concentrations in colonic mucosa.[14] Other fatty acids including n-6 PUFA and their derivatives are also suspected to have possible antitumorigenic property.

Conjugated linoleic acid (CLA) refers collectively to several positional and geometric isomers of linoleic acid (LA) in which the double bonds are in conjugation, typically at positions 9 and 11 or 10 and 12. CLA has been shown to inhibit chemically induced carcinogenesis in various organs, such as mammary glands,[15] skin[16] and forestomach.[17] When compared to studies examining the protective efficacy of CLA on mammary carcinogenesis, evidence for chemoprevention by CLA against colon cancer is less definitive, although gavage with CLA lessened the occurrence of ACF induced by heterocyclic amines.[18,19] On the other hand, the occurrence of other types of conjugated PUFA are present in some seed oils.[20,21] They include conjugated trienoic fatty acids, such as α-eleostearic (*cis(c)9,trans(t)*11,*t*13-CLN), in the seed oil of bitter melon (*Momordica charantia*) oil (BMO), which is an edible plant bitter melon and one of the important food materials in South-East Asia. The cytotoxic effect of *c*9,*t*11,*t*13-CLN isolated from BMO on activity of tumors cells has been indicated in our recent study.[22] In addition, we have reported the protective effect of BMO on the development of ACF with high crypt multiplicity in a short-term *in*

*Abbreviations*: AA, arachidonic acid; AOM, azoxymethane; ACF, aberrant crypt foci; BGO, bitter melon oil; *c,cis*; CLA, conjugated linoleic acid; CLN, conjugated linolenic acid; DHA, docosahexaenoic acid; DMH, 1,2-dimethylhydrazine; DMOX, dimethyloxazoline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; LA, linoleic acid; LN, linolenic acid; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; prostaglandin, PG; sodium dodecyl sulfate, SDS; *t*, *trans*

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*vivo* assay, suggesting a possible inhibitory effect of BMO on colon carcinogenesis.[23]

In our study, chemopreventive ability of BMO on large bowel tumorigenesis was investigated in a long-term *in vivo* assay using a rat colon carcinogenesis model with AOM as a carcinogen. Also, the expression of peroxisome proliferator-activated receptor (PPAR) γ in colonic mucosa and the lipid composition in the liver and colon were estimated to understand the possible mechanisms of modulatory effect of BMO in colon carcinogenesis since fatty acids might be an agonist for PPARs,[24] which suppress colon tumorigenesis.[25–27]

## MATERIAL AND METHODS

### *Animals, chemicals and diets*

A total of 82 male F344 rats, 4 weeks old, obtained from Charles River Japan, Inc. (Kanagawa, Japan), were used. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (3 or 4 rats/cage) with free access to drinking water and a basal diet, AIN-76A,[28] under controlled conditions of humidity (50 ± 10%), lighting (12 hr light/dark cycle) and temperature (23 ± 2°C). AOM was purchased from Sigma Chemical Co. (St. Louis, MO). BMO was extracted from seed of bitter melon according to methods described previously.[23] The fatty acid profile of the total lipids in BMO was generally in harmony with that described in other report,[29] with very high level (60.2%) of 9*c*,11*t*,13*t*-18:3 and a small amount of other CLN isomers, namely, 9*c*,11*t*,13*c*-18:3 (0.6%) and 9*t*,11*t*,13*t*-18:3 (0.3%). These lipids also contained high amount (27.2%) of 18:0 and modest amount of 18:1n-9 (5.9%) and 18:2n-6 (3.8%). Four experimental diets containing various levels of BMO (0%, 0.01%, 0.1% or 1% by weight of diet) based on the AIN-76 formulation were made on the weekly base and stored at −20°C under nitrogen atmosphere in airtight containers for no longer than a week. The composition of the diets is shown in Table I.

### *Experimental procedure*

After quarantine for 7 days, rats aged 5 weeks were divided into 6 groups as shown in Figure 1. Beginning at 5 weeks of age, all animals were fed each of the 4 different experimental diets. At 6 weeks of age, animals in groups 1 through 4 were *s.c.* injected with AOM (20 mg/kg body weight) once a week for 2 weeks. The rats in groups 1 and 6 were fed the diet containing 5% corn oil. Group 2 was fed the diet containing 0.01% BMO and 4.99% corn oil. Group 3 was given the diet containing 0.1% BMO and 4.9% corn oil. Group 4 and 5 were fed to the diet containing 1% BMO and 4% corn oil. The dose levels of test compound were determined from previous reports.[23] All rats were provided with the experimental diets and tap water *ad libitum*, and weighed weekly. The consumption of experimental diets was also recorded weekly. At the termination of the study (week 32), all of the rats were sacrificed by ether overdose to assess the incidences of neoplastic lesions in all organs including large bowel. At autopsy, all organs, especially the intestine, were carefully inspected grossly, and all abnormal lesions were examined histologically. Colons of 5 rats from each group were randomly selected for measurement of the expression of PPARγ protein and for the lipid analysis in the nonlesional colonic mucosa after resection of tumorous lesions histopathology. Colons of remaining rats were fixed in 10% buffered formalin and processed for histopathological examination by conventional methods using hematoxylin and eosin staining. The liver was excised and weighed, and then the caudate lobe was removed and fixed in 10% buffered formalin for histological examination. Remaining lobes of the liver of all rats were analyzed fatty acid composition. All other tissues were fixed in 10% buffered formalin and histological diagnosis was made. Intestinal neoplasma were diagnosed according to the criteria described by Ward.[30]

TABLE I – PERCENTAGE COMPOSITION OF EXPERIMENTAL DIETS

| Diet ingredients | Control | 0.01% BMO | 0.1% BMO | 1% BMO |
|---|---|---|---|---|
| Casein | 20.0 | 20.0 | 20.0 | 20.0 |
| DL-methionine | 0.3 | 0.3 | 0.3 | 0.3 |
| Corn starch | 15.0 | 15.0 | 15.0 | 15.0 |
| Dextrose | 50.0 | 50.0 | 50.0 | 50.0 |
| Cellulose | 5.0 | 5.0 | 5.0 | 5.0 |
| Corn oil | 5.0 | 4.99 | 4.9 | 4.0 |
| BMO | 0 | 0.01 | 0.1 | 1.0 |
| Mineral mix, AIN-76A | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mix, AIN-76A | 1.0 | 1.0 | 1.0 | 1.0 |
| Choline bitartrate | 0.2 | 0.2 | 0.2 | 0.2 |

### *Western blotting analysis of PPARγ*

Tissue sample were homogenized in CelLyticTM-MT Mammalian Tissue Lysis/Extraction Reagent (Sigma Chemical Co., St. Louis, MO) with a PROTEASE INHIBITOR COCKTAIL (Sigma Chemical Co., St. Louis, MO), and insoluble materials were removed by centrifugation at 4°C. The supernatants were estimated for their protein contents using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin at standard. The solubilized lysates were resolved by sodium dodecyl sulfate (SDS)-PAGE electrophoresis under reducing conditions at a concentration of 50 μg protein of each sample per lane. Detection of PPARγ protein was performed with an anti- PPARγ polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with detection accomplished with an ECL-plus kit (Amersham Bioscience Corp., NJ). Quantitative analysis was performed using Scion Image analysis soft ware (Scion Corp., Frederick, MD).

### *Lipid extraction and analysis*

Lipids in colonic mucosa and liver were extracted with chloroform/methanol (2:1, v/v) as described previously by Folch *et al.*[31] Component peaks were identified by comparison with standard fatty acid methyl ester[32] and quantified by a Shimadzu Chromatopac C-R6A integrator (Shimadzu Seisakusho Co., Ltd., Kyoto, Japan). The identification of CLA and/or CLN isomers was confirmed by using GC-mass spectrometry after conversion of the methyl esters to dimethyloxazoline (DMOX) derivatives.[33] The

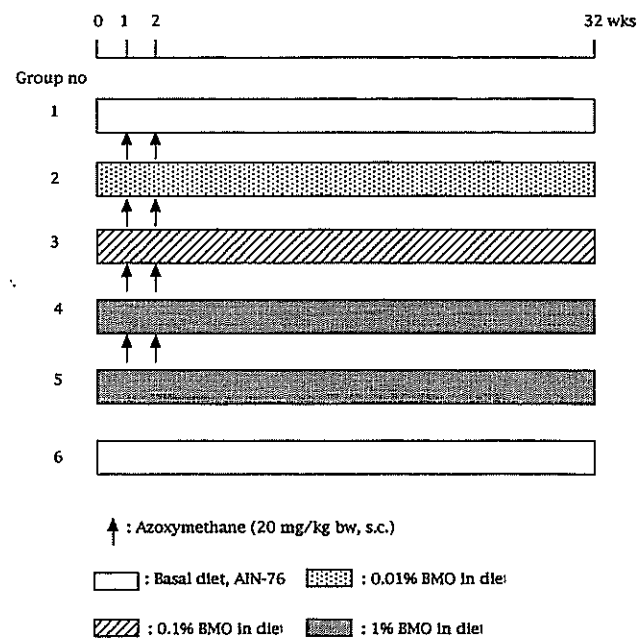


FIGURE 1 – Experimental protocol.

analysis of fatty acid composition was done more than 2 times for each sample and there was no significant difference between results for the same sample. Data are represented as means ± SD.

*Statistical evaluation*

Where applicable, data were analyzed using Student's *t*-test, Welch's *t*-test or Fisher's exact probably test with $p<0.05$ as the criterion of significance.

## RESULTS

*General observation*

During the study, clinical signs of toxicity, low survival and poor condition were not observed in any groups. This was confirmed by histopathological examinations in liver, kidney, spleen, heart and lungs of the rats. Histology of liver revealed no morphological alterations, such as fatty liver. The mean daily intake of diet with or without BMO per rat was between 12.9 and 13.2 g/day/rat. Mean body, liver and % liver weights (g/100 g body weight) in all groups at sacrifice are shown in Table II. There were no significant differences among the groups.

*Incidence and multiplicity of intestinal neoplasms*

Macroscopic observation revealed that most tumors developed in the large intestine and some in the small intestine of rats in groups 1–4. Animals in groups 5 and 6 did not have neoplasms in any organs examined. Colon tumors were sessile or pedunculated tumors and histologically tubular adenoma, adenocarcinoma or signet ring-cell carcinoma, with a higher incidence of adenocarcinoma. The incidence and multiplicity of intestinal tumors are shown in Tables III and IV. The frequencies of large intestinal adenocarcinoma in groups 2 (44%, $p<0.02$) and 3 (50%, $p<0.05$) were significantly smaller than that in group 1 (83%). The incidence of colorectal adenocarcinoma in group 3 (69%) was lower than in group 1, but a significant difference was not present ($p=0.2758$). The incidence of small intestinal adenocarcinoma in groups 2, 3 and 4 did not significantly differ from that in group 1. As presented in Table IV, significant reduction in the multiplicities of colorectal carcinoma (number of carcinomas/rats) in groups 2 ($0.69\pm0.87$, $p<0.005$), 3 ($0.81\pm1.05$, $p<0.02$) and 4 ($1.00\pm0.89$, $p<0.05$) was also found when compared to group 1 ($1.94\pm1.47$).

*Lipid analysis*

The fatty acid profiles of the lipids from liver and colonic mucosa are shown in Tables V and VI, respectively. Although BMO diets contained over 60% of CLN isomer (*c*9,*t*11,*t*13-18:3), any CLN isomer was not detected in both organs of rats fed BMO diets at various doses. On the other hand, the contents of CLA (*c*9,*t*11-18:2) in the liver and colonic mucosa of rats fed BMO were increased in a dose-dependent manner.

*Expression of PPARγ levels in colonic mucosa*

A representative immunoblot analysis of PPARγ expression in colonic mucosa of AOM-treated animals on different dietary regimens is shown in Figure 2. Dietary administration of BMO resulted in enhanced expression of PPARγ protein levels: 1.5-fold increase in groups 2, 1.6-fold elevation in group 3 and 1.9-fold increased in group 4, when compared to rats fed the diet without BMO.

## DISCUSSION

The results described here clearly indicate that dietary administration of BMO rich in *c*9,*t*11,*t*13-CLN significantly inhibits the development of colonic adenocarcinoma induced by AOM in male F344 rats without causing any adverse effects. In addition, significant reduction in the multiplicities of colorectal carcinoma (number of carcinomas/rats) in rats BMO containing diets at all dose levels (0.01%, 0.1% or 1%) was found when compared the AOM alone group. We believe that our results are the first to demonstrate the protective ability of BMO rich in *c*9,*t*11,*t*13-CLN against chemically induced colon carcinogenesis.

In our study, the protective effect of BMO against colon carcinogenesis was not dose dependent. Kimoto *et al.*[34] reported that safflower oil rich in CLA treatment protection in mammary carcinomas, without a clear dose dependence, as found in our study. Dietary CLA between 0.05% and 0.5% was found to produce a dose-dependent inhibition in mammary tumor development,[35] but the inhibitory effect of CLA reached a maximum at about 1%.[36] Thus, there may be the existence of lower threshold of conjugated fatty acids with cancer chemopreventive action.

There are a few studies that investigate the modifying effects of conjugated fatty acids on colon carcinogenesis. Although dietary CLA inhibits cancer development in including mammary gland,[15] skin[16] and forestomach[17] in rodents initiated with a variety of chemical carcinogens, the chemopreventive activity of CLA in the colon is less clear. CLA treatment inhibits the formation of colonic 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-DNA adducts and putative precancerous ACF in colon.[18] Recently, Park *et al.*[37] reported that dietary CLA can inhibit 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis through possibly induction of apoptosis. Cell proliferation is known to play an important role in multistage carcinogenesis with multiple genetic changes.[38] In our previous work, CLN can inhibit the growth of human and mouse cancer cells *in vitro*.[22] Feeding of BMO rich in CLN is reported to increase the number of apoptotic cells and reduce cell proliferation activity.[23] Although we did not estimate cell proliferation activity and apoptotic index in the colonic mucosa and/or neoplasms, it may be possible that inhibitory effect of BMO may be due to, in part, modification of cell proliferation and/or apoptosis induction.

In the fatty acid profiles of the lipids from colonic mucosa and liver, we did not detect any CLN isomer in the liver lipids from rat fed the BMO diets, which contained over 60% of CLN isomer (*c*9,*t*11,*t*13-18:3). On the other hand, CLA was found in these lipids and the content of the CLA isomer (*c*9,*t*11-18:2) was significantly greater in rats fed the BMO diets in a dose-dependent manner. This may indicate that part of *c*9,*t*11,*t*13-18:3 in the CLN would be enzymatically converted to *c*9,*t*11-18:2. CLA is known to be a possible chemopreventive agent against ACF formation[18,19] and colon carcinogenesis.[37] Whereas CLA used in published studies[15,17,35,39] contains a mixture of positional and geometrical iso-

TABLE II – BODY, LIVER AND RELATIVE LIVER WEIGHTS

| Group number | Treatment (number of rats examined) | Body weight (g) | Liver weight (g) | Relative liver weight (g/100 g body weight) |
|---|---|---|---|---|
| 1 | AOM (18) | 365 ± 23[1] | 11.9 ± 1.7 | 3.26 ± 0.39 |
| 2 | AOM + 0.01% BMO (16) | 374 ± 20 | 12.7 ± 1.7 | 3.40 ± 0.40 |
| 3 | AOM + 0.1% BMO (16) | 360 ± 18 | 11.8 ± 1.6 | 3.27 ± 0.36 |
| 4 | AOM → 1% BMO (16) | 368 ± 27 | 11.6 ± 1.8 | 3.15 ± 0.37 |
| 5 | 1% BMO (8) | 365 ± 24 | 11.1 ± 1.4 | 3.06 ± 0.29 |
| 6 | None (8) | 376 ± 20 | 12.2 ± 1.2 | 3.24 ± 0.27 |

[1]Mean ± SD.

TABLE III – INCIDENCE OF LARGE BOWEL TUMORS IN EACH GROUP

| Group number | Treatment (number of rats examined) | Number of rats with tumors at | | | | | |
|---|---|---|---|---|---|---|---|
| | | Small intestine | | | Large intestine | | |
| | | Total | AD[1] | ADC | Total | AD | ADC |
| 1 | AOM (18) | 4 (22%) | 0 (0%) | 4 (22%) | 15 (83%) | 7 (39%) | 15 (83%) |
| 2 | AOM + 0.01% BMO (16) | 6 (38%) | 1 (6%) | 5 (31%) | 14 (88%) | 10 (63%) | 7[2] (44%) |
| 3 | AOM + 0.1% BMO (16) | 4 (25%) | 2 (13%) | 3 (19%) | 15 (94%) | 10 (63%) | 8[3] (50%) |
| 4 | AOM + 1% BMO (16) | 3 (19%) | 1 (6%) | 2 (13%) | 14 (88%) | 8 (50%) | 11 (69%) |
| 5 | 1% BMO (8) | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | None (8) | 0 | 0 | 0 | 0 | 0 | 0 |

[1]AD = adenoma; ADC = adenocarcinoma.–[2,3]Significantly different from group 1 by Fisher's exact probability test ([2]$p < 0.02$ and [3]$p < 0.05$).

TABLE IV – MULTIPLICITY OF LARGE BOWEL TUMORS IN EACH GROUP

| Group number | Treatment (number of rats examined) | Multiplicity (number of tumors/rat) of intestinal tumors at | | | | | |
|---|---|---|---|---|---|---|---|
| | | Small intestine | | | Large intestine | | |
| | | Total | AD[1] | ADC | Total | AD | ADC |
| 1 | AOM (18) | 0.28 ± 0.57[2] | 0.00 ± 0.00 | 0.28 ± 0.57 | 2.50 ± 1.79 | 0.56 ± 0.98 | 1.94 ± 1.47 |
| 2 | AOM + 0.01% BMO (16) | 0.38 ± 0.50 | 0.06 ± 0.25 | 0.31 ± 0.48 | 1.56 ± 0.89 | 0.88 ± 0.81 | 0.69 ± 0.87[3] |
| 3 | AOM + 0.1% BMO (16) | 0.31 ± 0.60 | 0.13 ± 0.34 | 0.19 ± 0.40 | 1.56 ± 1.09 | 0.76 ± 0.68 | 0.81 ± 1.05[4] |
| 4 | AOM + 1% BMO (16) | 0.19 ± 0.40 | 0.06 ± 0.25 | 0.13 ± 0.34 | 1.69 ± 0.95 | 0.69 ± 0.79 | 1.00 ± 0.89[5] |
| 5 | 1% BGO (8) | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | None (8) | 0 | 0 | 0 | 0 | 0 | 0 |

[1]AD = adenoma; ADC = adenocarcinoma–[2]Mean ± SD.–[3–5]Significantly different from group 1 by Student's $t$-test ([3]$p < 0.005$, [4]$p < 0.02$ and [5]$p < 0.05$).

TABLE V – EFFECTS OF BMO DIETS ON FATTY ACID COMPOSITION OF LIVER LIPIDS

| Group number | Treatment | Fatty acids (wt%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 16:1n-7 | 18:1n-7 | 18:1n-9 | 18:2n-6 | 18:2(c9,t11) | 20:4n-6 | 22:5n-3 | 22:6n-3 |
| 1 | AOM | 25.4 ± 1.5[1] | 11.5 ± 2.2 | 6.2 ± 0.7 | 5.2 ± 0.3 | 19.5 ± 1.1 | 11.3 ± 0.6 | ND[2] | 12.8 ± 0.9 | 1.9 ± 0.1 | 1.0 ± 0.0 |
| 2 | AOM + 0.01% BMO | 27.9 ± 0.9 | 8.8 ± 0.4 | 7.6 ± 0.6 | 5.4 ± 0.1 | 20.4 ± 1.0 | 11.2 ± 0.4 | 0.04 ± 0.00 | 11.1 ± 0.9 | 1.4 ± 0.1 | 0.9 ± 0.1 |
| 3 | AOM + 0.1% BMO | 24.4 ± 2.4 | 10.1 ± 1.3 | 4.4 ± 2.0 | 5.0 ± 0.6 | 19.9 ± 1.9 | 15.0 ± 2.6 | 0.20 ± 0.02 | 12.6 ± 1.6 | 1.9 ± 0.6 | 1.0 ± 0.1 |
| 4 | AOM + 1% BMO | 23.2 ± 2.5 | 10.4 ± 2.3 | 4.1 ± 1.9 | 4.7 ± 0.4 | 21.5 ± 4.7 | 14.8 ± 3.3 | 1.68 ± 0.30 | 11.7 ± 2.5 | 1.6 ± 0.5 | 0.8 ± 0.2 |
| 5 | 1% BMO | 25.7 ± 0.9 | 10.6 ± 0.8 | 6.4 ± 0.5 | 5.8 ± 0.3 | 20.3 ± 1.1 | 10.7 ± 0.8 | 1.56 ± 0.18 | 12.1 ± 0.7 | 1.3 ± 0.1 | 0.7 ± 0.0 |
| 6 | None | 23.3 ± 0.9 | 11.6 ± 2.7 | 5.2 ± 1.0 | 6.0 ± 0.1 | 19.3 ± 0.8 | 14.5 ± 2.0 | ND | 13.5 ± 1.0 | 1.4 ± 0.2 | 0.8 ± 0.0 |

[1]Mean ± SD.–[2]ND, not detected.

TABLE VI – EFFECTS OF BMO DIETS ON FATTY ACID COMPOSITION OF COLONIC MUCOSA

| Group number | Treatment | Fatty acids (wt%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 16:1n-7 | 18:1n-7 | 18:1n-9 | 18:2n-6 | 18:2(c9,t11) | 20:4n-6 | 22:5n-3 |
| 1 | AOM | 22.8 ± 0.6[1] | 2.9 ± 0.4 | 5.1 ± 0.9 | 5.7 ± 0.3 | 31.4 ± 0.9 | 25.0 ± 1.8 | ND[2] | 1.5 ± 0.5 | 0.0 ± 0.0 |
| 2 | AOM + 0.01% BMO | 22.9 ± 0.7 | 3.5 ± 0.7 | 4.4 ± 0.7 | 6.0 ± 0.3 | 30.5 ± 0.7 | 24.5 ± 1.3 | 0.06 ± 0.03 | 2.3 ± 1.1 | 0.0 ± 0.0 |
| 3 | AOM + 0.1% BMO | 23.2 ± 0.7 | 3.7 ± 0.6 | 4.7 ± 1.0 | 5.5 ± 0.3 | 30.5 ± 0.8 | 24.4 ± 1.4 | 0.40 ± 0.04 | 2.2 ± 0.7 | 0.0 ± 0.0 |
| 4 | AOM + 1% BMO | 22.0 ± 0.6 | 4.6 ± 0.4 | 4.3 ± 1.1 | 5.9 ± 0.3 | 29.3 ± 1.7 | 21.4 ± 1.8 | 3.30 ± 0.23 | 2.8 ± 1.0 | 0.3 ± 0.1 |
| 5 | 1% BMO | 23.3 ± 0.2 | 3.3 ± 0.2 | 5.2 ± 0.3 | 6.5 ± 0.2 | 30.9 ± 0.6 | 21.1 ± 0.9 | 3.66 ± 0.12 | 1.4 ± 0.2 | 0.2 ± 0.0 |
| 6 | None | 22.4 ± 0.6 | 3.6 ± 0.4 | 4.4 ± 0.3 | 6.6 ± 0.3 | 30.0 ± 1.3 | 24.5 ± 0.9 | ND | 2.3 ± 1.0 | 0.0 ± 0.0 |

[1]Mean ± SD.–[2]ND, not detected.

mers, *c*9,*t*11-18:2 isomer is considered to be the active constituent. Therefore, the suppressing effect of BMO on colon carcinogenesis in the current study may be attributed to *c*9,*t*11-18:2 isomer derived from *c*9,*t*11,*t*13-18:3 in the BMO diets. Furthermore, in our study, the contents of linoleic acid (18:2n-6) in the liver lipids of rats fed the BMO containing diets were significantly lower than that of rats fed the diet without BMO. This reduction in the content of linoleic acid may also contribute to the inhibitory effect of BMO on colon carcinogenesis. However, judging from the powerful inhibitory activity of BMO at lower dose levels found in our study, other factors, such as direct action of *c*9,*t*11,*t*13-18:3, should be considered.

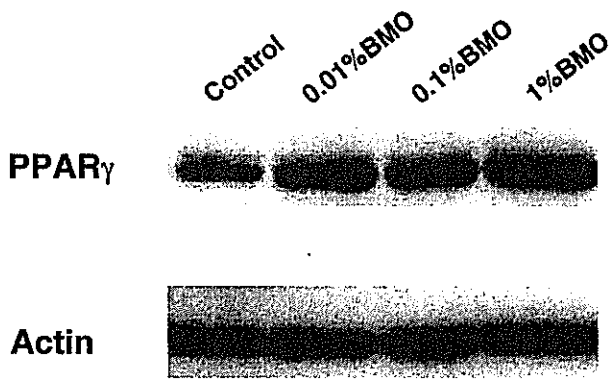


FIGURE 2 – Expression of PPARγ and β-actin proteins analyzed by immunoblot of protein extracts from the colonic mucosa.

Recently, CLA was shown to act a high affinity ligand and activator of PPARα and PPARγ.[40–42] McCarty[41] suggested that part of anticarcinogenic activity of CLA is mediated by PPAγ activation in susceptible tumors. In our study, dietary feeding of BMO enhanced PPARγ expression in nonlesional colonic mucosa. These results are of interest, since we recently demonstrated that synthetic ligands for PPARα and PPARγ effectively inhibit AOM-induced ACF in rats,[26,43] and the findings were confirmed by Osawa *et al.*[27] Thus, it may be possible that feedings with BMO suppresses colon carcinogenesis *via* altering PPARγ expression in colonic mucosa.

The antioxidant activity of *c*9,*t*11,*t*13-18:3 is another possible explanation for inhibitory effect of colon carcinogenesis by feeding of BMO diet. Ha *et al.*[17] demonstrated that CLA may act by antioxidant mechanisms cytotoxicity. In addition, Dhar *et al.*[44] reported that *c*9,*t*11,*t*13-18:3 from BMO acts as an antioxidant. In compounds with more than 2 conjugated double bond, conjugation increases the rate of oxidation. Thus, in the *in vivo* study, conjugated trienoic fatty acids are also likely to be more rapidly oxidized than linoleates by picking up more free radicals, thereby eliminating or reducing the formation of hydroperoxides. Although we did not determine these parameters, it may be possible that in our study BMO reduces the formation of hydroperoxides by lowering the generation of free radicals and peroxidation of PUFA occurring in cell membrane and other lipids. Indeed, BMO treatment could effectively inhibit colitis-related colon carcinogenesis, where production of free radicals increases (manuscript in preparation).

In our study, estimated CLN intake in rats treated with 0.01%, 0.1% and 1% BMO was 2.12, 21.5 and 212.4 mg/kg body weight per day, respectively. Whereas CLA used in animal studies usually contained a mixture of position and geometrical isomers, *c*9,*t*11-18:2 is considered to be the most active constituent.[15,17,39] As shown in our study, only the *c*9,*t*11-18:2 isomer was accumulated in the colonic mucosa and liver when rats were fed BMO containing diets. The finding that even at the 0.1% dose level of BMO, which is greater than the average CLA consumption (approximately 1g/person /day) in the United States,[45] BMO feeding exerted cancer chemopreventive activity may suggest that BMO, a good dietary resource of CLA, is one of promising cancer preventive substances against colon cancer development.

In conclusion, the results of our study suggest that dietary BMO in rich CLN has a beneficial effect on chemically induced rat colon carcinogenesis, providing an effective dietary chemopreventive approach to disease management. Our findings also suggest that BMO could be applied to preclinical studies of the prevention of colon cancer.

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# Pomegranate seed oil rich in conjugated linolenic acid suppresses chemically induced colon carcinogenesis in rats

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**Pomegranate (*Punica granatum* L.) seed oil (PGO) contains more than 70% *cis*(*c*)9,*trans*(*t*)11,*c*13-18:3 as conjugated linolenic acids (CLN). Our previous short-term experiment demonstrated that seed oil from bitter melon (*Momordica charantia*) (BMO), which is rich in *c*9,*t*11,*t*13-CLN, inhibited the occurrence of colonic aberrant crypt foci (ACF) induced by azoxymethane (AOM). In this study, we investigated the effect of dietary PGO on the development of AOM-induced colonic malignancies and compared it with that of conjugated linoleic acid (CLA). To induce colonic tumors, 6-week old male F344 rats were given subcutaneous injections of AOM (20 mg/kg body weight) once a week for 2 weeks. One week before the AOM treatment they were started on diet containing 0.01%, 0.1%, or 1% PGO or 1% CLA for 32 weeks. Upon termination of the bioassay (32 weeks) colon tumors were evaluated histopathologically. AOM exposure produced colonic adenocarcinoma with an incidence of 81% and multiplicity of 1.88±1.54 at week 32. Administration of PGO in the diet significantly inhibited the incidence (AOM+0.01% PGO, 44%, $P<0.05$; AOM+0.1% PGO, 38%, $P<0.01$; AOM+1% PGO, 56%) and the multiplicity (AOM+0.01% PGO, 0.56±0.73, $P<0.01$; AOM+0.1% PGO, 0.50±0.73, $P<0.005$; AOM+1% PGO, 0.88±0.96, $P<0.05$) of colonic adenocarcinomas, although a clear dose-response relationship was not observed at these dose levels. CLA feeding also slightly, but not significantly, reduced the incidence and multiplicity of colonic adenocarcinomas. The inhibition of colonic tumors by PGO was associated with an increased content of CLA (*c*9,*t*11-18:2) in the lipid fraction of colonic mucosa and liver. Also, administration of PGO in the diet elevated expression of peroxisome proliferator-activated receptor (PPAR) γ protein in the non-tumor mucosa. These results suggest that PGO rich in *c*9,*t*11,*c*13-CLN can suppress AOM-induced colon carcinogenesis, and the inhibition is associated in part with the increased content of CLA in the colon and liver and/or increased expression of PPARγ protein in the colon mucosa. (Cancer Sci 2004; 95: 481–486)**

Colon cancer is one of the leading causes of cancer deaths in Western countries. Globally, more than 875,000 men and women were afflicted with this cancer in 1996 and more than 510,000 died in the same year.[1] Diet, especially fat intake, has been regarded as the most important nutritional influence on colon cancer development. Colorectal cancer development is known to be linked to Western lifestyle, which often includes a diet high in fat.[2] The amount and type of dietary fat consumed are of particular importance for development of this type of malignancy.[3–6] Epidemiological investigations indicate that high intake of fish and fish oil rich in $n-3$ polyunsaturated fatty acids (PUFAs) correlates with a reduced risk of colorectal malignancy.[7,8] Laboratory animal model studies indicate that the $n-3$ PUFAs are protective, whereas the $n-6$ PUFAs promote colon carcinogenesis.[9] Our previous study also demonstrated that tuna oil, rich in docosahexaenoic acid and vitamin $D_3$, inhibits azoxymethane (AOM)-induced aberrant crypt foci (ACF),[10] the putative precursor lesions for colonic adenocarcinoma.[11] In this connection, it is noteworthy that conjugated linoleic acid (CLA), a mixture of positional and geometric isomers of linoleic acid (LA), found mainly in milk fat and dairy products[12] inhibits chemically induced tumorigenesis, particularly in rat mammary bioassays,[13] mouse skin carcinogenesis,[14] and mouse forestomach neoplasia.[15] Compared with investigations on the protective efficacy of CLA in mammary carcinogenesis, evidence for chemoprevention by CLA in colon tumorigenesis is less definitive. CLA did not reduce tumorigenesis in $Apc^{Min/+}$ mouse.[16] However, oral administration of CLA inhibited the occurrence of chemically induced ACF.[17–19] The significance of various types of CLA as modulators of pathological processes encouraged us to explore new sources of naturally occurring CLA as chemopreventive agents against colon carcinogenesis.[20] On the other hand, other types of conjugated PUFA are present in some seed oils.[21,22] These include pomegranate seed oil (PGO) from pomegranate seeds (*Punica granatum* L.); PGO contains punicic acid, another form of conjugated linolenic acid (CLN). Studies from our laboratory and elsewhere have demonstrated the cytotoxic effect of *c*9,*t*11,*c*13-CLN isolated from PGO and tung oil on a variety of human cancer cell lines, including colon cancer cells.[23,24] In addition, we have reported the protective effect of *c*9,*t*11,*t*13-CLN against the aggressive development of putative precursor lesions for colonic adenocarcinoma, ACF (with high crypt multiplicity), in a short-term *in vivo* assay.[25] These findings suggest a possible inhibitory effect of CLN on colon carcinogenesis.

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of nuclear receptors and ligand-responsive transcription factors that participate in many processes important for cell and tissue homeostasis.[26] To date, three different isotypes have been described in various species, i.e., PPARα, PPARδ (also called PPARβ), and PPARγ, each exhibiting distinct patterns of tissue distribution and ligand specificity. PPARα regulates numerous aspects of fatty acid catabolism, whereas PPARγ controls adipocyte differentiation, systemic glucose levels, and lipid homeostasis.[27,28] The activity of the PPARs can also be modulated by PUFAs, including LA, linolenic acid, arachidonic acid, and certain eicosanoids (prostaglandin J).[29,30] Houseknecht *et al.* have reported that CLA activates the PPARγ transcription factor *in vitro* in CV-1 cells transfected with a PPARγ expression vector.[31] Several studies indicate that synthetic PPARγ ligands inhibit proliferation and induce differentiation in colon cancer cell lines.[32,33] We have shown that synthetic ligands for PPARγ prevent the development of carcinogen-induced preneoplastic ACF in the colon of rats.[34,35] Our recent investigation has confirmed these findings: a synthetic

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PPARγ ligand effectively inhibits AOM-induced colon carcinogenesis in rats (Hirose, Y., et al., manuscript in preparation).

The present study was designed as an initial step to evaluate the inhibitory activity of PGO against colon carcinogenesis in male F344 rats. Also, the expression of PPARγ in colonic mucosa and the lipid composition of hepatic and colon tissues were determined to understand the possible mechanisms through which PGO suppresses colon tumorigenesis, since PPARs might be activated by fatty acids.[36]

## Materials and Methods

**Animals, chemicals, and diets.** We used a total of 104 male F344/Ducrj rats, obtained from Charles River Japan, Inc. (Kanagawa), at the age of 4 weeks. The rats were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (3 or 4 rats/cage) with free access to drinking water and a basal diet, AIN-76A,[37] under controlled conditions of relative humidity (50±10%), lighting (12-h light/dark cycle) and temperature (23±2°C). AOM was purchased from Sigma Chemical Co. (St. Louis, MO). PGO was extracted from the seeds of pomegranate according to the methods described previously.[25] The fatty acid profile of the total lipids in PGO was similar to that reported.[38] The composition of CLN isomers was as follows: *c*9,*t*11,*c*13-18:3 (75.3%), *c*9,*t*11,*t*13-18:3 (4.2%), *t*9,*t*11,*c*13-18:3 (1.6%), and *t*9,*t*11,*t*13-18:3 (0.5%). CLA was obtained from Rinoru Oil Mills Co., Ltd., Tokyo. CLA contained *c*9,*t*11-18:2 (30.9%) and *t*10,*c*12-18:2 (33.5%). All experimental diets containing various levels of PGO (0%, 0.01%, 0.1%, or 1% by weight of diet) and CLA (1% by weight of diet) were prepared weekly in our laboratory and stored at −20°C under a nitrogen atmosphere in airtight containers for no longer than a week. PGO and CLA were added to the experimental diets at the expense of corn oil. The rats were fed fresh diet every day and the peroxide value of the lipids in the fresh diets was less than 3.0 meq/kg lipid.

**Experimental procedure.** After quarantine for 7 days, male F344 rats, 5 weeks of age were assigned to one of eight groups. At the time, and throughout the assay, all rats were fed the control and experimental diets containing PGO or CLA. At 6 weeks of age, the rats in groups 1 through 5, designated for carcinogen treatment, were subcutaneously injected with AOM (20 mg/kg body weight) once a week for 2 weeks and those scheduled to receive vehicle treatment received equal volumes of normal saline. The unmodified diet was the control AIN-76A diet containing 5% corn oil. Group 2 was fed this AIN-76A diet containing 0.01% PGO. Group 3 was given the modified diet containing 0.1% PGO and 4.9% corn oil. Groups 4 and 6 were fed the modified diet containing 1% PGO (4% corn oil). Groups 5 and 7 were fed the modified diet containing 1% CLA (4% corn oil). All rats were provided with experimental diet and tap water *ad libitum*, and weighed weekly. The food intake was also recorded weekly. At the termination of the study (week 32), all rats were sacrificed using an overdose of ether. At autopsy, all organs, especially the intestine, were carefully examined grossly, and all abnormal lesions were examined histologically. Colons of five rats from each group were randomly selected for measurement of the expression of PPARγ protein and for lipid analysis in the non-lesional colonic mucosa. Colons of the remaining rats were fixed in 10% buffered formalin and processed for histopathological examination by conventional methods using hematoxylin and eosin staining. Intestinal neoplasms were diagnosed according to the criteria described by Ward.[39] The liver was excised and weighed, then the caudate lobe was removed and fixed in 10% buffered formalin for histological examination. Remaining lobes of the liver of all rats were analyzed for fatty acid composition. All other tissues were fixed in 10% buffered formalin and submitted to histological examination.

**Western blotting analysis of PPARγ.** Colon samples were homogenized in CelLyticTM-MT Mammalian Tissue Lysis/Extraction Reagent (Sigma Chemical Co.) with a protease inhibitor cocktail (Sigma Chemical Co.), and insoluble materials were removed by centrifugation at 4°C. The supernatants were used to determine protein contents using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard. The solubilized lysates were resolved by sodium dodecyl sulfate-PAGE electrophoresis under reducing conditions at a concentration of 50 μg protein of sample per lane. Detection of PPARγ protein was performed with an anti-PPARγ polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) utilizing an ECL-plus kit (Amersham Bioscience Corp., NJ). Quantitative analysis was performed using Scion Image analysis software (Scion Corp., Frederick, MD).

**Lipid extraction and analysis.** Lipids in colonic mucosa and liver were extracted with chloroform/methanol (2:1, v/v) as described previously by Folch *et al.*[40] Component peaks were identified by comparison with standard fatty acid methyl ester [41] and quantified by a Shimadzu Chromatopac C-R6A integrator (Shimadzu Seisakusho Co., Ltd., Kyoto). The identification of CLA and/or CLN isomers was confirmed by using GC-Mass spectrometry after conversion of the methyl esters to dimethyloxazoline derivatives.[42] The analysis of fatty acid composition was done in duplicate for each sample. Data are represented as means±SD.

**Statistical evaluation.** Where applicable, data were analyzed using Student's *t* test, Welch's *t* test or Fisher's exact probability test with $P<0.05$ as the criterion of significance.

## Results

**General observations.** Experimental diet containing different levels of PGO and CLA did not produce any observable toxicity or any gross change in any organ examined. This was confirmed by histopathological examinations in liver, kidney, spleen, heart, and lungs of the rats. Histology revealed no morphological evidence of fatty liver. The mean daily intake of diet with or without PGO was between 13.1 and 13.5 g/day/rat. Mean weights of body and liver (g/100 g body weight) in all groups at sacrifice are shown in Table 1. There were no significant differences among the groups.

**Incidence and multiplicity of intestinal neoplasms.** Macroscopic observation revealed that most tumors developed in the large intestine and some in the small intestine of rats in groups 1–5. Rats treated with saline and fed either control or experimental diet showed no evidence of tumor formation in any organ examined. Colon tumors were sessile or pedunculated and histologically tubular adenomas, adenocarcinomas, or signet ring-cell carcinomas with a preponderance of adenocarcinomas. The incidence (% animals with intestinal tumors) and multiplicity (number of tumors/rat) of intestinal tumors are summarized in Tables 2 and 3, respectively. Administration of 0.01% and 0.1% PGO in the diet significantly suppressed the incidence (44% in the AOM+0.01% PGO group, $P<0.05$ and 38% in the AOM+0.1% PGO group, $P<0.01$) and multiplicity (0.56±0.73 in the AOM+0.01% PGO group, $P<0.01$ and 0.50±0.73 in the AOM+0.1% PGO group, $P<0.005$) of adenocarcinomas in the colon, when compared the AOM alone group (81% incidence and 1.88±1.54 multiplicity). Whereas 1% PGO in the diet had no significant effect on the incidence of adenocarcinomas, it significantly inhibited the multiplicity of adenocarcinomas in the colon (0.88±0.96 multiplicity, $P<0.05$). Another important observation is that 1% CLA in the diet had no effect on the incidence and multiplicity of colon carcinomas.

**Table 1. Body, liver and relative liver weights**

| Group no. | Treatment (no. of rats examined) | Body weight (g) | Liver weight (g) | Relative liver weight (g/100 g body weight) |
|---|---|---|---|---|
| 1 | AOM (16) | 366±21[1)] | 12.0±1.6 | 3.28±0.37 |
| 2 | AOM+0.01% PGO (16) | 375±17 | 11.8±1.4 | 3.13±0.27 |
| 3 | AOM+0.1% PGO (16) | 362±30 | 11.5±1.1 | 3.17±0.25 |
| 4 | AOM+1% PGO (16) | 369±26 | 11.4±1.5 | 3.10±0.35 |
| 5 | AOM+1% CLA (16) | 368±26 | 11.4±1.4 | 3.11±0.57 |
| 6 | 1% PGO (8) | 377±28 | 11.8±1.1 | 3.13±0.33 |
| 7 | 1% CLA (8) | 380±10 | 12.7±1.3 | 3.34±0.35 |
| 8 | None (8) | 377±19 | 12.2±1.1 | 3.24±0.25 |

*1)* Mean±SD.

**Table 2. Incidence of intestinal tumors in each group**

| Group no. | Treatment (no. of rats examined) | No. of rats with intestinal tumors at: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Entire intestine | | | Small intestine | | | Large intestine | | |
| | | Total | AD[1)] | ADC | Total | AD | ADC | Total | AD | ADC |
| 1 | AOM[2)] (16) | 13 (81%) | 7 (44%) | 13 (81%) | 4 (25%) | 0 (0%) | 4 (25%) | 13 (81%) | 7 (44%) | 13 (81%) |
| 2 | AOM+ 0.01% PGO (16) | 12 (75%) | 8 (50%) | 9 (56%) | 6 (38%) | 2 (13%) | 4 (25%) | 10 (63%) | 7 (44%) | 7[3)] (44%) |
| 3 | AOM+ 0.1% PGO (16) | 12 (75%) | 10 (63%) | 9 (56%) | 7 (44%) | 3 (19%) | 4 (25%) | 10 (63%) | 9 (56%) | 6[4)] (38%) |
| 4 | AOM+ 1% PGO (16) | 14 (88%) | 8 (50%) | 11 (69%) | 7 (44%) | 2 (13%) | 5 (31%) | 12 (75%) | 7 (44%) | 9 (56%) |
| 5 | AOM+ 1% CLA (16) | 14 (88%) | 5 (31%) | 11 (69%) | 4 (25%) | 0 (0%) | 4 (25%) | 12 (75%) | 5 (31%) | 8 (50%) |
| 6 | 1% PGO (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 1% CLA (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | None (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*1)* AD, adenoma; ADC, adenocarcinoma.
*2)* AOM, azoxymethane.
*3, 4)* Significantly different from group 1 by Fisher's exact probability test ([3)]$P<0.05$ and [4)]$P<0.01$).

**Table 3. Multiplicity of intestinal tumors in each group**

| Group no. | Treatment (no. of rats examined) | Multiplicity (no. of tumors/rat) of intestinal tumors at: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Entire intestine | | | Small intestine | | | Large intestine | | |
| | | Total | AD[1)] | ADC | Total | AD | ADC | Total | AD | ADC |
| 1 | AOM (16) | 2.81 ±2.01[3)] | 0.63 ±1.02 | 2.19 ±1.72 | 0.31 ±0.60 | 0.00 ±0.00 | 0.31 ±0.60 | 2.50 ±1.90 | 0.63 ±1.02 | 1.88 ±1.54 |
| 2 | AOM+ 0.01% PGO (16) | 1.50[4)] ±1.21 | 0.69 ±0.70 | 0.80[4)] ±0.83 | 0.38 ±0.50 | 0.13 ±0.34 | 0.25 ±0.45 | 1.13[4)] ±1.26 | 0.56 ±0.73 | 0.56[5)] ±0.73 |
| 3 | AOM+ 0.1% PGO (16) | 1.75 ±1.57 | 1.00 ±1.03 | 0.75[5)] ±0.86 | 0.44 ±0.51 | 0.19 ±0.40 | 0.25 ±0.45 | 1.31[4)] ±1.35 | 0.81 ±0.83 | 0.50[6)] ±0.73 |
| 4 | AOM+ 1% PGO (16) | 2.00 ±1.16 | 0.81 ±0.98 | 1.19 ±1.05 | 0.38 ±0.50 | 0.13 ±0.34 | 0.31 ±0.48 | 1.56 ±1.15 | 0.69 ±0.87 | 0.88[4)] ±0.96 |
| 5 | AOM+ 1% CLA (16) | 1.63 ±1.02 | 0.38 ±0.62 | 1.25 ±1.06 | 0.25 ±0.45 | 0.00 ±0.00 | 0.25 ±0.45 | 1.38 ±1.09 | 0.38 ±0.62 | 1.00 ±1.10 |
| 6 | 1% PGO (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 1% CLA (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | None (8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*1)* AD, adenoma; ADC, adenocarcinoma.
*2)* AOM, azoxymethane.
*3)* Mean±SD.
*4–6)* Significantly different from group 1 by Student's *t* test or Welch's *t* test ([4)]$P<0.05$, [5)]$P<0.01$, and [6)]$P<0.005$).

**Lipid analysis.** The fatty acid profiles of the lipids from liver and colonic mucosa are shown in Tables 4 and 5, respectively. Although PGO diets contained over 70% of CLN isomer *c*9,*t*11,*c*13-18:3, this isomer was not detected in the liver and colon of rats fed PGO diets at various doses. On the other hand, the contents of CLA (*c*9,*t*11-18:2) in the liver and colonic mucosa of rats fed PGO were elevated in a dose-dependent manner. Rats receiving the diet containing CLA showed *c*9,*t*11-18:2 and *t*10,*c*12-18:2 in both the liver and colonic mucosa.

**Expression of PPARγ levels in colonic mucosa.** A representative immunoblot analysis of PPARγ expression in colonic mucosa of AOM-treated rats on different dietary regimens is shown in

**Table 4. Effects of PGO-containing CLN diets on fatty acid composition of liver lipids**

| Group no. | Treatment | Fatty acids (wt %) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1*n*−9 | 18:2*n*−6 | 18:2(*c*9,*t*11) | 18:2(*c*9,*t*11) | 20:4*n*−6 |
| 1 | AOM | 26.0±0.8[1)] | 10.4±0.2 | 19.6±0.9 | 11.4±0.5 | ND | ND | 12.8±0.7 |
| 2 | AOM+0.01% PGO | 26.3±0.7 | 8.6±0.1 | 20.9±0.7 | 12.5±0.6 | 0.06±0.05 | ND | 11.1±0.5 |
| 3 | AOM+0.1% PGO | 25.2±1.0 | 10.5±1.7 | 18.0±3.1 | 16.9±4.9 | 0.24±0.02 | ND | 12.5±1.3 |
| 4 | AOM+1% PGO | 26.4±0.7 | 9.0±1.3 | 20.9±1.6 | 12.4±1.2 | 2.54±0.28 | ND | 11.0±1.6 |
| 5 | AOM+1% CLA | 25.5±1.2 | 11.5±0.3 | 18.1±0.8 | 10.8±0.4 | 1.09±0.04 | 0.30±0.04 | 13.2±0.3 |
| 6 | 1% PGO | 23.0±1.9 | 12.4±1.1 | 18.4±3.0 | 10.9±1.4 | 1.94±0.13 | ND | 15.1±2.5 |
| 7 | 1% CLA | 23.8±0.3 | 12.5±0.3 | 17.6±0.0 | 12.0±1.0 | 1.18±0.08 | 0.30±0.02 | 14.5±0.3 |
| 8 | None | 22.9±0.5 | 10.4±0.9 | 19.4±0.7 | 14.6±1.6 | ND | ND | 13.5±0.8 |

1) Mean±SD.

**Table 5. Effects of PGO-containing CLN diets on fatty acid composition of colonic mucosa lipids**

| Group no. | Treatment | Fatty acids (wt %) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1*n*−9 | 18:2*n*−6 | 18:2(*c*9,*t*11) | 18:2(*c*9,*t*11) | 20:4*n*−6 |
| 1 | AOM | 22.6±0.4[1)] | 2.8±0.2 | 31.4±0.8 | 26.0±0.8 | ND | ND | 1.3±0.3 |
| 2 | AOM+0.01% PGO | 22.4±0.5 | 3.2±0.4 | 30.3±0.3 | 24.8±1.1 | 0.11±0.02 | ND | 2.2±0.4 |
| 3 | AOM+0.1% PGO | 22.2±1.3 | 4.7±2.3 | 27.6±3.6 | 23.3±1.9 | 0.38±0.12 | ND | 4.0±3.0 |
| 4 | AOM+1% PGO | 22.8±0.7 | 4.1±0.9 | 28.9±1.9 | 20.6±0.9 | 4.10±0.23 | ND | 2.9±1.0 |
| 5 | AOM+1% CLA | 24.6±0.6 | 3.5±0.7 | 29.0±1.1 | 21.1±1.4 | 2.46±0.17 | 0.95±0.13 | 1.8±0.8 |
| 6 | 1% PGO | 23.3±0.6 | 2.8±0.4 | 30.0±0.8 | 21.6±0.8 | 4.44±0.13 | ND | 1.3±0.7 |
| 7 | 1% CLA | 23.5±0.9 | 4.2±0.9 | 27.7±1.8 | 20.1±1.6 | 2.18±0.28 | 0.89±0.16 | 3.6±1.1 |
| 8 | None | 22.4±0.6 | 3.4±0.5 | 30.0±1.2 | 24.6±1.6 | ND | ND | 2.4±1.0 |

1) Mean±SD.

Fig. 1. Dietary administration of PGO resulted in enhanced expression of PPARγ protein as compared to the control, namely, a 2.4±0.8-fold increase in group 2, a 2.3±0.8-fold elevation in group 3, and a 1.8±0.7-fold increase in group 4. Feeding of CLA also increased the expression of PPARγ protein (2.1±0.6-fold) as compared to the control diet.

## Discussion

The results described here clearly indicate that dietary administration of PGO, which is rich in *c*9,*t*11,*c*13-CLN, significantly inhibits the development of AOM-induced colonic adenocarcinomas in male F344 rats without causing any adverse effects. In addition, there was a significant reduction of the multiplicity of carcinomas in the colon of rats fed PGO at any dose level (0.01%, 0.1%, or 1%) as compared with the AOM-induced rats. Previous studies have shown that CLA does not alter CYP expression.[43,44] However, it is possible that PGO may affect liver CYPs, including CYP2E1, which activates AOM, since dietary CLN suppressed AOM-induced ACF when fed during AOM exposure.[25] The results described here suggest that dietary feeding of PGO suppressed progression of these lesions through adenoma to malignant neoplasm in the post-initiation phase. We believe that our results are the first to demonstrate the chemopreventive efficacy of PGO, which is rich in *c*9,*t*11,*c*13-CLN, against chemically induced colon carcinogenesis.

Recent evidence suggests that pharmacological activation of PPARγ may prevent cancer.[45] PPARγ might exert its anticancer effect through the modification of cell growth[32] and the regulation of cyclooxygenase-2.[46] The highly potent and specific PPARγ ligand GW7845 significantly reduces rat mammary carcinogenesis.[47] We and others have also demonstrated that synthetic ligands for PPARα and PPARγ effectively inhibit AOM-induced ACF in rats.[34,35,48] CLA was shown to act as a high-affinity ligand and activator of PPARα and PPARγ.[31,49,50] McCarty[50] suggested that a part of the anticarcinogenic activity of CLA is mediated by PPARγ activation in susceptible tumors.

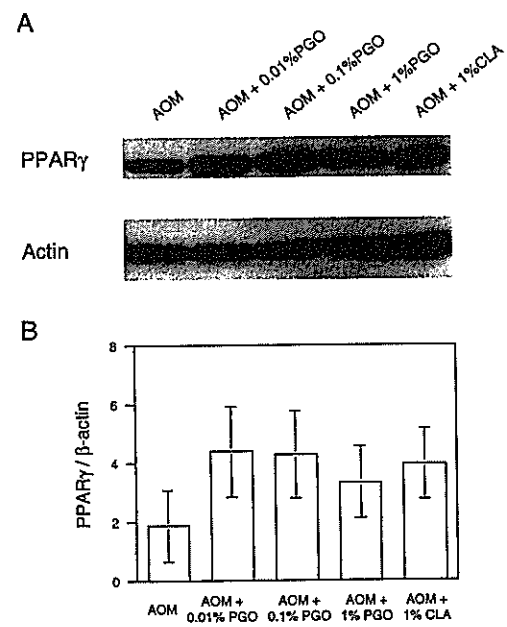


**Fig. 1.** Expression of PPARγ in colonic mucosa. (A) PPARγ and β-actin proteins analyzed by immunoblotting of protein extracts from the colonic mucosa. (B) Quantitative analysis using Scion Image analysis software

In the current study, dietary administration of PGO enhanced PPARγ expression in non-lesional colonic mucosa of rats, and thus, induction of PPARγ activity may account for the diminished colon tumor incidence and multiplicity that we observed.

We did not detect any CLN isomer in the lipids extracted from the livers of rats fed the PGO diets, although the PGO contained over 70% of the *c*9,*t*11,*c*13-CLN isomer. On the other hand, CLA was found in the extracted lipids: the CLA

isomer (*c*9,*t*11-18:2) was significantly and dose-dependently higher in extracts from rats fed the PGO diets. These findings are in accordance with other reports.[51,52] This may indicate that a part of *c*9,*t*11,*c*13-18:3 in the PGO diets was enzymatically converted to *c*9,*t*11-18:2. Therefore, the suppressing effect of PGO on colon carcinogenesis in the current study may be attributed to the *c*9,*t*11-18:2 isomer derived from *c*9,*t*11,*c*13-18:3 in the PGO diets. However, feeding 1% PGO enhanced the accumulation of *c*9,*t*11-CLA, the active component of the CLA isomer. On the other hand, the suppressive effect against colon carcinogenesis of PGO at the 1% level was weaker than that of lower dose administration (0.01% and 0.1%). Ip *et al.*[53] found that dietary CLA at between 0.05% and 0.5% produced a dose-dependent inhibition of mammary tumor development, but the inhibitory effect of CLA reached a maximum at about 1%.[54] Thus, there may exist an optimal dose of conjugated fatty acids to exert an optimal cancer chemopreventive action.

The present study also demonstrates that dietary feeding of CLA enhanced PPARγ expression in non-lesional colonic mucosa. However, the protective effect of CLA against colon carcinogenesis was relatively weaker than that of PGO. The differences in effect on PPARγ expression are possibly due to the differences of positional and geometrical isomers between CLA and PGO. In the current study, CLA contained 30.9% *c*9,*t*11-CLA and 33.5% *t*10,*c*12-CLA. Feeding of CLA enhanced the accumulation of *c*9,*t*11-CLA and *t*10,*c*12-CLA in colonic mucosa and liver. Recently, Brown *et al.*[55] suggested that *c*9,*t*11-CLA is an agonist, and *t*10,*c*12-CLA an antagonist of PPARγ. Therefore, it is likely that, when given as an isomeric mixture, they may largely negate each other's effects.

A possible contribution of antioxidant activity to the inhibition of colon carcinogenesis by PGO cannot be justified without evidence indicating that oxidative stress is involved in AOM-induced colon carcinogenesis. Ha *et al.*[15] suggested that the inhibitory effects of CLA intubation on benzo(*a*)pyrene-induced mouse forestomach tumorigenesis might be due to the antioxidative property of CLA. Interestingly, the *c*9,*t*11-CLA isomer was incorporated into forestomach phospholipids after CLA intubation in their study. In addition, Dhar *et al.*[56] reported that *c*9,*t*11,*t*13-18:3 from bitter melon acts as an antioxidant. These compounds have more than two conjugated double bonds, and conjugation is known to increase the rate of oxidation. Conjugated trienoic fatty acids may be more rapidly oxidized than LA. After oxidation, conjugated trienoic fatty acids may form more hydroperoxides than LA. Similarly, docosahexaenoic acid, eicosapentaenoic acid and α-LA are more readily oxidized to hydroperoxides than LA.[57] Although we did not determine these parameters, it is possible that, in this study, PGO reduces the formation of hydroperoxides by diminishing the generation of free radicals and the peroxidation of PUFAs in cell membranes of cryptal cells initiated with AOM. Additional in-depth studies of the mechanisms of action of PGO as a colon tumor inhibitor are warranted to provide a clearer understanding of its effects.

In the present study, the estimated daily CLN intakes in rats given diets containing 0.01%, 0.1%, and 1% PGO were 2.63, 27.9, and 273 mg/kg body weight respectively. Though the CLA used in animal feeding studies contains a mixture of positional and geometrical isomers, *c*9,*t*11-18:2 is considered to be the most active constituent.[13,15,58] In this current study, only the *c*9,*t*11-18:2 isomer accumulated in the colonic mucosa and liver when rats were fed PGO. The finding that PGO exerted cancer chemopreventive activity even at the 0.1% dose level suggests that PGO has promise as a naturally occurring preventive agent against colon cancer development.

In conclusion, this study has explored for the first time whether PGO, a food component, exhibits chemopreventive efficacy against experimental colon carcinogenesis. Our findings suggest that dietary PGO rich in CLN has a preventive effect on chemically induced rat colon carcinogenesis. The efficacy of a chemopreventive agent may depend on the timing of administration. Thus, further experiments on the chemopreventive ability of PGO are needed, to establish whether pre-initiation or post-initiation administration is preferable to inhibit tumor development. Such a study is under way in our laboratory.

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