Inhibition of intestinal carcinogenesis by a new flavone derivative, chafuroside, in oolong tea

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A new flavone derivative, chafuroside, has been isolated as a strong anti-inflammatory compound from oolong tea leaves, and its structure determined to be (2*R*,3*S*,4*S*,4a*S*,11b*S*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4a,11b-tetrahydro-2*H*,10*H*-pyrano[2′,3′:4,5]furo[3,2-g]chromen-10-one. To assess its potential to inhibit intestinal carcinogenesis, 2.5, 5 and 10 p.p.m. chafuroside was given in the diet to *Apc*-deficient Min mice for 14 weeks from 6 weeks of age. Total numbers of polyps were reduced to 83, 73 and 56% of the control value, respectively. Moreover, dietary administration at 10 and 20 p.p.m. reduced azoxymethane (AOM)-induced colon aberrant crypt foci (ACF) development in rats to 69% of the AOM-treated control value with the higher dose. Chafuroside-associated toxicity was not observed at 2.5–10 p.p.m. in Min mice and 10–20 p.p.m. in AOM-treated rats. These results suggest that chafuroside might be a good chemopreventive agent for colon cancer. (*Cancer Sci* 2006; 97: 248–251)

Colon cancer is one of the most common cancers in developed countries[1] and epidemiological studies have shown that a Western-style diet, high in fat and red meat as well as low in fruits and vegetables, increases the risk.[2,3] Thus, foodstuff is a major focus for research, particularly with regard to identification of effective chemopreventive agents.

Epidemiological evidence suggests that drinking green tea (*Camellia sinensis*) is beneficial for cancer prevention.[4–6] Many animal studies also have shown that tea and its components have anticancer properties.[7,8] The major characteristic constituents of green tea are catechins, including EGCG.[9] In black tea, a large proportion of the catechins are converted into theaflavins and thearubigens through oxidation and polymerization. Another tea, oolong tea, is widely consumed in Asia, especially in China and Japan. The difference among green, black and oolong teas lies in fermentation: green tea is unfermented, black tea is completely fermented, and oolong tea is partially fermented.[7]

Recently, a strong anti-inflammatory compound named chafuroside, (2*R*,3*S*,4*S*,4a*S*,11b*S*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4a,11b-tetrahydro-2*H*,10*H*-pyrano[2′,3′:4,5]furo[3,2-g]chromen-10-one, was isolated from oolong tea leaves with the aid of an inhibition test with DNFB-induced contact hypersensitivity in mice, and its total synthesis reported.[10,11] The compound was presumed to be produced during the partial fermentation process and showed strong anti-inflammatory activity in DNFB and 2,4,6-trinitro-1-chlorobenzene-induced contact hypersensitivity models.[10,11] Moreover, a preliminary study demonstrated the effective dose in the DNFB-induced contact hypersensitivity model to be approximately equal to that of indomethacin, a NSAID, which has been proven to have a cancer-chemopreventive influence.[12]

Although several reports have documented antioxidant, antiallergic and antiobesity activities of oolong tea extracts,[13–15] effects of individual constituents on colon carcinogenesis have hitherto not been described. In the present study, we therefore investigated the impact of chafuroside on intestinal polyp formation in *Apc*-deficient Min mice, an animal model of human familial adenomatous polyposis that develops numerous polyps in the intestinal tract.[16] We also investigated the impact of chafuroside on the formation of AOM-induced aberrant crypt foci, which are putative preneoplastic lesions in the F344 rat colon. In both cases chafuroside reduced the number of lesions, pointing to possible application as a chemopreventive agent for intestinal cancer.

Materials and Methods

Animals and chemicals

Female C57BL/6J-*Apc*$^{Min/+}$ mice (Min mice) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 5 weeks of age and genotyped using a method reported previously.[16] Heterozygotes, as well as wild-type (C57BL/6J) mice, were acclimated to laboratory conditions for 1 week, along with male F344 rats obtained from Charles River Japan (Atsugi, Japan) at 5 weeks of age. Three to five animals were housed per plastic cage, with sterilized softwood chips as bedding, in a barrier-sustained animal room, air-conditioned at 24 ± 2°C and 55% humidity, on a 12:12 h light:dark cycle. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Bodyweights and food consumption were measured weekly. The experiments were conducted according to the 'Guidelines for Animal Experiments in the National Cancer Center' of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

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Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; COX, cyclooxygenase; DNFB, 2,4-dinitrofluorobenzene; EGCG, (–)-epigallocatechin-3-gallate; NSAID, non-steroidal anti-inflammatory drug.

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AOM was purchased from Sigma Chemical Co. (St Louis, MO, USA). Chafuroside was synthesized chemically at the University of Shizuoka (Shizuoka, Japan).[11] Its chemical structure is shown in Fig. 1. The purity of the compound was examined by ^{1}H nuclear magnetic resonance and high-performance liquid chromatography, and showed no concomitant peaks. The compound was pure enough, estimated to be above 99% (melting point of the compound was 229–232°C). Chafuroside concentrations of 2.5, 5, 10 and 20 p.p.m. were mixed into the powdered basal diet AIN-76 A (CLEA Japan, Tokyo, Japan) and confirmed to be stable in the diet under the experimental conditions used in the present study. The doses of chafuroside were selected according to our preliminary study in which chafuroside suppressed intestinal polyp formation in *Apc* gene-deficient mice.

Intestinal polyp formation in Min mice

Female Min mice ($n = 9$–10/group) were fed diets containing 0 (control), 2.5, 5 or 10 p.p.m. chafuroside for 14 weeks from 6 weeks of age. All animals were anesthetized with ether before they were killed. The liver, kidneys and spleen were removed and weighed and the intestinal tract was resected, filled with 10% buffered formalin, and divided into four sections: three segments of small intestine: (1) proximal (4 cm in length from the pylorus ring of the stomach); (2) middle and (3) distal halves of the remainder; and (4) the colon. These segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. Polyp numbers and sizes, and their distributions in the intestine, were determined under a stereoscopic microscope.[17]

AOM-induced ACF development in rats

Male F344 rats, 6 weeks of age, were treated subcutaneously with either AOM in sterile saline at a dose of 15 mg/kg bodyweight or with the saline vehicle, once a week for 2 weeks from 6 weeks of age. From 1 day before the first treatment with AOM, rats were fed control or experimental diets containing chafuroside at 10 or 20 p.p.m. for 4 weeks. At 10 weeks of age, they were killed under ether euthanasia and complete necropsies were carried out. The liver, kidneys and spleen were removed and weighed. The entire colon was resected, filled with 10% buffered formalin, opened longitudinally, and fixed flat between sheets of filter paper in 10% buffered formalin. The colon was then stained with 0.2% methylene blue in saline, and scored under a light microscope for the number of ACF per colon and the mean number of AC per focus.[18]

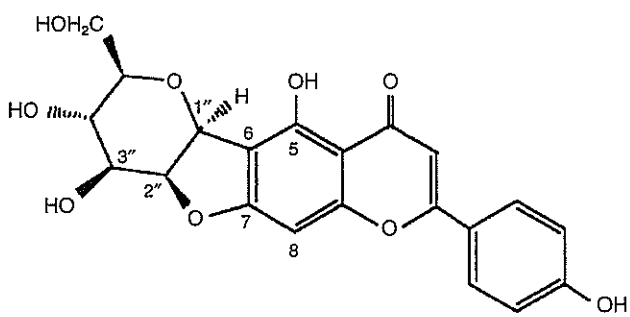

Fig. 1. Structure of chafuroside.

Statistical analysis

The results were expressed as mean ± SD, and statistical analysis was carried out using Dunnett's multiple comparison test. In addition, the linear regression test was also used. Differences were considered to be statistically significant with P-values less than 0.05.

Results

In Min mice, most polyps were located in the small intestine, with a preponderance in the distal parts, and only a few polyps were observed in the colons (Table 1). Treatment with chafuroside at 2.5, 5 and 10 p.p.m. for 14 weeks clearly decreased the total numbers of polyps to 83, 73 and 56% ($P < 0.01$) of the untreated control value, respectively (Table 1). The numbers of polyps in the proximal, middle and distal parts of small intestine in the 10 p.p.m. group were 54, 78 and 46% of the untreated control values, respectively (Table 1). Dose-dependent inhibition was observed in the number of polyps in the proximal ($r = -0.9958$, $P < 0.0005$) and distal parts ($r = -0.9129$, $P < 0.01$) of the small intestine, and in the total number of polyps ($r = -0.9863$, $P < 0.02$). As shown in Fig. 2, administration of chafuroside reduced the number of polyps mainly less than 1.0 mm in diameter. However, the number of polyps measuring ≥1.0 mm in diameter was not affected by chafuroside treatment. Survival rate, general conditions, food consumption and organ weights did not differ among the groups. No significant macroscopic changes were noted in the liver, kidney or spleen. Final body weights in the groups treated with 2.5, 5 and 10 p.p.m. were 103, 105 and 125% of the untreated control value, respectively.

In AOM-treated rats, administration of chafuroside at 10 and 20 p.p.m. in the diet for 4 weeks again did not affect

Table 1. Suppression of intestinal polyp development in Min mice by chafuloside, shown by the number of polyps per mouse

Group (p.p.m)	No. mice	Small intestine			Colon	Total
		Proximal	Middle	Distal		
0	9	17.7 ± 9.8	39.7 ± 11.6	86.8 ± 27.1	0.78 ± 0.67	144.9 ± 37.7
2.5	7	15.3 ± 7.0 (86)	34.4 ± 7.7 (87)	70.0 ± 9.9 (81)	0.86 ± 0.69 (110)	120.6 ± 21.1 (83)
5.0	9	13.0 ± 4.3 (73)	33.4 ± 8.6 (84)	59.2 ± 18.9 (68)*	0.56 ± 0.53 (72)	106.2 ± 26.5 (73)
10.0	8	9.6 ± 3.1 (54)*	31.0 ± 19.9 (78)	40.0 ± 21.8 (46)**	0.75 ± 0.71 (96)	81.4 ± 41.9 (56)**

Data are mean ± SD. Numbers in parentheses are percentages of the control basal diet values. *Significantly different from the basal diet group at $P < 0.05$. **Significantly different from the basal diet group at $P < 0.01$.

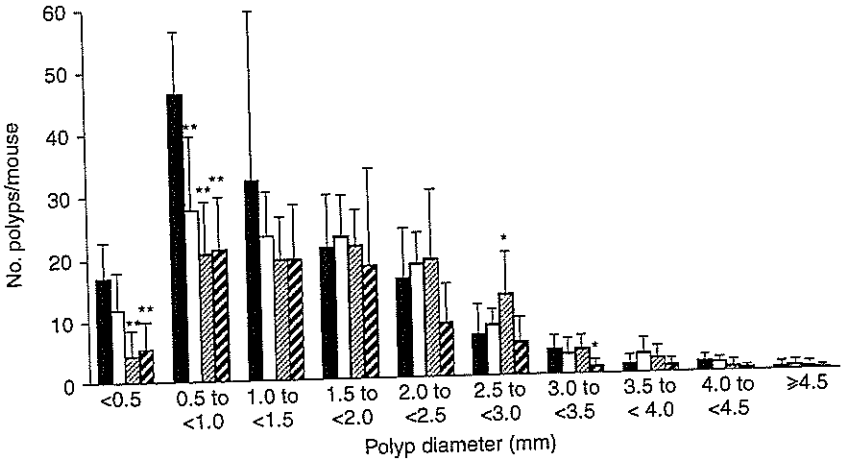

Fig. 2. Effects of chafuroside on the size distribution of intestinal polyps in Min mice. Min mice were fed a basal diet (■) or a diet containing 2.5 (□), 5 (▨) or 10 p.p.m. (▨) chafuroside for 14 weeks. The number of polyps/mouse in each size class is given as a mean value (bars represent SD). *$P < 0.05$, **$P < 0.01$.

general conditions, body weights, food consumption or organ weights. No significant macroscopic changes were observed in the liver, kidney or spleen. ACF were observed in all rats treated with AOM, mainly located in the distal colon. Administration of 10 and 20 p.p.m. chafuroside reduced the total numbers of ACF per colon to 79 ($P < 0.05$) and 69% ($P < 0.01$) of the AOM-treated control value, respectively (Table 2). The total number of AC per colon was also decreased by 16 and 30%, respectively (Table 2). However, treatment with chafuroside did not decrease the mean number of AC per focus (Table 2).

Discussion

In the present study, we obtained clear evidence that a new flavone derivative, chafuroside, suppresses development of intestinal polyps in Min mice and AOM-induced colon ACF in F344 rats at doses of 5–20 p.p.m. in the diet. Although it is a natural compound found in tea leaves, the doses effective in Min mice were much lower than those reported earlier for well-known, naturally occurring and synthesized chemopreventive agents. Indeed, the effective dose to reduce numbers of polyps in Min mice was 10 p.p.m. for chafuroside. This value is lower than with (+)-catechin at 1000 p.p.m.,[19] genistein at 1000 p.p.m.,[20] curcumin at 2000 p.p.m.,[21] and with synthesized aspirin at 250 p.p.m.,[22] piroxicam at 200 p.p.m.[23] and celecoxib at 1500 p.p.m.[24] As treatment with 2.5–10 p.p.m. chafuroside affected only the polyps of smaller size, it might be important to clarify the mechanism by which chafuroside inhibits polyp growth. It has been reported that 100 p.p.m. EGCG is effective for approximately 60% inhibition of AOM-induced ACF formation in F344 rats,[25] and our results thus suggest that chafuroside possesses a strong potential to inhibit development of putative preneoplastic lesions in the colon.

Because there were no signs of chafuroside-induced adverse effects in the present study, long-term consumption for cancer prevention in humans is conceivable. The daily estimated consumption level from a diet containing 10 p.p.m. chafuroside in mice corresponds to approximately 120 mg per day for a 60-kg adult man. The average concentration of chafuroside in a commercially available oolong tea in Japan is almost 55 μg/L (unpublished data). An adult human might reach an effective dose of chafuroside with consumption of more than 100 L of oolong tea. Therefore, taking a chafuroside supplement or drinking concentrated oolong tea may be useful for cancer prevention.

The strong anti-inflammatory and chemopreventive effects are presumably related to the two characteristic moieties of chafuroside: the mannose moiety of which the C1 position provides a C-glycoside linkage with the C6 position of apigenin, and the dihydrofuran moiety, obtained

Table 2. Effects of chafuroside on azoxymethane (AOM)-induced aberrant crypt focus (ACF) formation in F344 rats

Group	No. rats with ACF	Total no. ACF/colon (%)	Total no. AC/colon (%)	Mean no. AC/focus
AOM treatment				
Control diet	9/9	278 ± 51	618 ± 81	2.25 ± 0.18
Chafuroside (10 p.p.m.)	9/9	219 ± 42 (79)*	522 ± 105 (84)	2.40 ± 0.27
Chafuroside (20 p.p.m.)	9/9	192 ± 42 (69)**	435 ± 69 (70)**	2.29 ± 0.30
Saline treatment				
Control diet	0/3	0	0	0
Chafuroside (10 p.p.m.)	0/3	0	0	0
Chafuroside (20 p.p.m.)	0/3	0	0	0

Data are mean ± SD. Numbers in parentheses are percentages of the control basal diet values. *Significantly different from the basal diet group at $P < 0.05$. **Significantly different from the basal diet group at $P < 0.01$. AC, aberrant crypt.

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by ring closure via dehydration between hydroxyl groups at the C7 position of apigenin and at the C2 position of mannose.

According to the mechanism of DNFB-induced contact hypersensitivity, several factors moderated by chafuroside treatment could be assumed. DNFB-induced skin immune responses are divided into a distinct sensitization phase and an early and late elicitation phase. In the sensitization phase, prostaglandin E_2-EP_4 receptor (PGE_2-EP_4) signaling pathways are reported to be involved in antigen uptake into Langerhans cells, and in antigen migration to draining lymph nodes.[26] In addition, other colon tumor-related pathways and mediators, such as ERK1/2 and p38 MAPK signaling pathways, 5-lipoxygenase and inducible nitric oxide synthase, are also reported to be involved in DNFB-induced contact hypersensitivity.[27–29] Thus, chafuroside may exhibit its chemopreventive effects by mediating these inflammatory factors. However, the natural function of chafuroside is not well defined and the anti-inflammatory and chemopreventive mechanisms of this novel flavonoid need to be clarified.

From the present results, we can conclude strong inhibitory effects of chafuroside on intestinal polyp development and ACF formation in *Apc*-deficient mice and AOM-treated rats, respectively, so that chafuroside may be a promising candidate chemopreventive agent for colon cancer.

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ORIGINAL PAPER

Serum triglycerides and colorectal adenoma in a case–control study among cancer screening examinees (Japan)

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Abstract
Objective Most epidemiologic studies have shown serum triglycerides to be associated with colorectal adenoma. However, whether the association can be modified by smoking is unknown. We cross-sectionally investigated the association of serum triglycerides with the risk of adenoma by smoking status.
Methods We identified 782 newly diagnosed adenoma cases from the examinees of a colorectal cancer screening program. All cases were diagnosed by a magnifying colonoscopy with dye spreading. We determined 738 controls without present illness or past history of adenoma from among the examinees. They provided their lifestyle information and fasting blood samples to measure their serum triglycerides. We calculated odds ratios (OR) and 95% confidence intervals (CI) of colorectal adenoma for serum triglycerides.
Results High serum triglycerides were associated with colorectal adenoma (OR 1.5; 95% CI 1.1–2.0 for the highest versus the lowest quartile, P_{trend}, 0.030). A stronger association was observed between three or more adenoma cases and study controls (OR 2.3; 95% CI 1.3–4.2, P_{trend}, < 0.0010). After classifying the study subjects by smoking status, a significant linear risk trend was found in ever-smokers (P_{trend}, 0.0018) but not in never-smokers (P_{trend}, 0.94; $P_{interaction}$, 0.067).
Conclusions Our results suggested that a higher serum triglyceride level may be related to a larger number of adenomas. Adenoma development involving an elevated serum triglyceride level may be modified by smoking.

Keywords Serum triglycerides · Smoking · Colorectal adenoma · Case–control study

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Introduction

Physical inactivity, high body mass index, and high alcohol consumption are convincing or probable risk factors of colorectal cancer [1, 2], and also lead to hypertriglyceridemia [3]. Serum triglycerides may contribute to subsequent development of colorectal neoplasms [4].

Most epidemiologic studies [5–10] have consistently demonstrated that serum levels of triglycerides are associated with the risk of colorectal adenoma, a precursor lesion of colorectal cancer [11]. Recently, an animal study reported that an age-dependent hyperlipidemic state along with a suppressed lipoprotein lipase, which catalyzes hydrolysis of triglycerides, occurs in *Apc*-deficient mice [12]. The same study group also

showed the improvement of hyperlipidemia and the reduction of intestinal polyp formation by peroxisome proliferator-activated receptor (PPAR) agonists [12] or a lipoprotein lipase inducer without PPAR agonistic activity [13]. Moreover, the lipoprotein lipase inducer simultaneously reduces cyclooxygenase-2 expression levels [13], which are supposed to be involved in colon carcinogenesis [14]. Thus, hypertriglyceridemia is probably associated with colorectal adenoma development in humans as well as in animals.

Moreover, serum triglycerides may promote carcinogen-induced colon tumorigenesis. Some laboratory rats with hypertriglyceridemia such as Zucker obese rats [15], Nagase analbuminemic rats [16], and high-fat diet intake rats [17] are all known to be more sensitive to carcinogen treatments than rats with normal serum lipid levels. After initiating with carcinogen, a clear tumor-promoting effect of triglycerides is observed in these rats. We hypothesized that such a clear effect could be observed in those exposed to a carcinogen such as tobacco smoke. In fact, the International Agency for Research on Cancer announced that an independent effect of smoking may be weak for colorectal cancer [18]. Therefore, colorectal cancer development may need some exposure to promoting factors or environments such as hypertriglyceridemia, after initiating with tobacco smoke.

We examined the association between serum triglycerides and colorectal adenoma, and the different effects between ever- and never-smokers in a case–control study for cancer screening examinees. We conducted a colonoscopic screening with a magnifying instrument and dye spreading to identify adenoma lesions applying the pit-pattern classification. This colonoscopic diagnosis is more efficient and less time-consuming than pathologic diagnosis [19]. Moreover, the validity and reproducibility of this approach have been demonstrated [20, 21].

Subjects and methods

Study subjects

Study subjects were selected from 3,212 colonoscopic screening examinees during February 2004 to February 2005 who participated in the cancer screening program provided by the Research Center for Cancer Prevention and Screening, the National Cancer Center, Japan. These examinees will be annually followed by mail and then reexamined by the same screening process for the following 5 years. Eligible examinees were 2,234 adults, after excluding those out of the age range (less than 50, or 80 or more for men; less than 40, or 80 or more for women); those with a past history of the following diseases and conditions: colorectal adenoma, any cancer, ulcerative colitis, Crohn's disease, familial adenomatous polyposis, carcinoid tumor, or colectomy; those with an unsatisfactory preparation for colonoscopy; those with an incomplete examination; those colonoscopically diagnosed as colorectal cancer. A preparatory magnesium citrate solution, which was both non-absorptive and non-secretion-inducing, was orally administered to each examinee 2 h before screening. No dietary restriction was imposed. Examinees having at least one adenoma were 782 adults (526 men and 256 women) identified by the pit-pattern classification on the magnifying colonoscopy with dye spreading (chromoendoscopy) [19] using a colonoscope (CF-H260AZI; Olympus Medical Systems Corporation, Tokyo, Japan). Of 1,452 examinees not having adenoma, 1,203 (482 men and 721 women) were eligible controls, after excluding those with inflammatory polyps, diverticulitis, submucosal tumor, bowel tuberculosis, or hyperplastic polyps. Since eligible controls were fewer than eligible cases, all 482 men were used for study controls. Of 721 eligible female controls, 256 women were selected using stratified sampling by age and screening periods of female cases. This left 782 adenoma cases and 738 controls. All examinees provided written informed consent. This study was approved by the institutional review board of the National Cancer Center, Tokyo, Japan (G15-01, G16-03).

Questionnaire

Study subjects responded to self-administered questionnaires including demographics, past medical history, family history of cancer, medication, occupation, height, weight, smoking, alcohol consumption, physical activity, working hours, reproductive factors, stress, and dietary habits. Their dietary habits were assessed by intake frequency and relative portions for 145 food items. Various nutrient and food-group intakes were estimated by multiplying the frequency, the relative portions, and the nutrient contents on the Food Composition Table for Japanese foods [22]. This food frequency questionnaire (FFQ) was modified from the FFQ for a population-based prospective study [23, 24] with additional food items. Examinees completed the questionnaire before their screening examinations.

Blood collection and laboratory assays

Study subjects provided their samples of fasting venous blood that were drawn into vacutainer tubes for plasma or serum before any examinations. Blood samplings were mostly conducted one day before colonoscopic screening. In the blood samplings, 74% of examinees were without breakfast, i.e., overnight fasting (about 12 h), while 26% of examinees were without lunch, i.e., approximately 6 h fasting. The blood samples for plasma were divided into four 1-ml aliquots and two buffy-layers. These samples were preserved at –80°C until analysis. The blood samples for serum were used to measure various biomarkers including serum triglycerides. Their serum triglyceride levels were measured using an enzymatic method (Kyowa Medex Co., Ltd., Tokyo, Japan) on a Hitachi 7600 auto-analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

Statistical analysis

Least square means of potential risk factors for colorectal adenoma were calculated by analysis of covariance with adjustment for sex, age, and screening periods using the PROC GLM procedure. Characteristics of cases and controls were compared with the extensions of the Mantel–Haenszel procedure [25] using the PROC FREQ procedure with the CMH option. Odds ratios (OR) of colorectal adenoma for quartile categories of serum triglycerides were calculated using the logistic regression model adjusted for sex, age (less than 50, 50–54, 55–59, 60–64, 65 or more), screening periods (first, second), smoking (0, 1–29, 30–59, 60 or more pack-years), body mass index (lower than 25.0, 25.0–26.9, 27.0–29.9, 30.0 or higher; calculated by measurements at the screening examination), physical activity (METs; quartiles based on controls), alcohol consumption (0, 1–149, 150–299, 300 or more g/week ethanol), family history of colorectal cancer, aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) use, dietary fiber, folate, calcium, vitamin D, and red meat intake (quartiles based on controls; energy-adjusted by the residual method [26]). An adjustment for screening periods has two meanings. One is as a density sampling, which can serve to compare cases and controls like a cohort study. Another is as an indicator of the experience of colonoscopists, because our screening institute was established in February 2004, and all instruments were new for all colonoscopists. Togashi et al. [27] reported that diagnostic accuracy increases with the number of experienced lesions. Serum triglyceride levels were divided into quartiles based on controls' distribution. ORs by the number of colorectal adenomas were assessed by the generalized logit model, i.e., multinomial logistic regression model. Furthermore, we examined whether the association of serum triglycerides was different between never-smokers and ever-smokers, i.e., more than 0 pack-years. The linear trend of ORs was tested using the logarithmic-transformed median serum levels of triglycerides in each category, since the measurements were log-normally distributed. Statistical interaction between serum triglycerides and smoking was assessed based on modeling serum triglycerides as a continuous variable, i.e., median values in each category, with smoking (ever = 1 or never = 0) and a one-degree of freedom test. The p-values for the trend and interaction were evaluated using the two-sided test with 0.05 as the significant level. We used SAS software (version 9.1; SAS institute Inc., Cary, NC) for all statistical analyses.

Results

Adenoma cases were older, more had a family history of colorectal cancer, fewer used aspirin or other NSAIDs, more smoked, had a higher body mass index, and consumed more alcoholic beverages than controls (Table 1). Mean serum triglyceride levels were 113 mg/dl for cases and 101 mg/dl for controls ($p < 0.0010$). Spearman partial rank correlation between serum triglycerides and body mass index in the controls was 0.38, adjusted for sex, age, and screening periods (data not shown in tables). Other potential confounding factors were little correlated with serum triglycerides.

The OR of colorectal adenoma for serum triglycerides was statistically significant in the highest quartile compared to the lowest quartile (OR 1.5; 95% CI 1.1–2.0; Table 2). The linear trend was also statistically significant (P_{trend}, 0.030). The greater the number of adenomas, the higher were the ORs of the highest quartile. The ORs were 1.2 (95% CI 0.85–1.8) for one adenoma, 1.5 (95% CI 0.90–2.6) for two adenomas, and 2.3 (95% CI 1.3–4.2) for three or more adenomas. Medium size (5–9 mm in diameter) adenomas were more strongly associated with serum triglycerides (P_{trend}, 0.011) than smaller (less than 5 mm; P_{trend}, 0.30) or larger ones (10 mm or more; P_{trend}, 0.22). Association of serum triglycerides did not differ among sites of the largest adenomas (data not shown in tables).

Table 1 Characteristics of adenoma cases and controls

	Cases	Controls	*p*
n	782	738	
Men, *n* (%)	526 (67)	482 (65)	0.38[a]
Women, *n* (%)	256 (33)	256 (35)	
First screening period, *n* (%)	322 (41)	328 (44)	0.22[b]
Age, mean, year[c]	60.5	59.7	0.0075
Family history of colorectal cancer, *n* (%)	129 (17)	92 (12)	0.020[d]
Non-steroidal anti-inflammatory drug use, *n* (%)	33 (4.2)	55 (7.5)	0.0022[d]
Smoking, mean, pack-years[d]	15.3	9.86	<0.0010
Body mass index, mean, kg/m^2 [d]	23.0	22.5	<0.0010
Physical activity, mean, METs/day[d]	37.4	36.7	0.15
Alcohol consumption, mean, g/week ethanol[d]	153	122	0.0016
Energy intake, mean, kcal/day[d]	1,955	1,895	0.065
Dietary fiber intake, mean, g/day[e]	13.5	14.0	0.035
Folate intake, mean, μg/day[e]	382	394	0.064
Calcium intake, mean, mg/day[e]	607	620	0.44
Vitamin D intake, mean, μg/day[e]	8.37	7.87	0.15
Red meat intake, mean, g/day[e]	35.6	33.7	0.14
Serum triglycerides, mean, mg/dl[d]	113	101	<0.0010

Note: Least square means ("mean") were calculated by analysis of covariance with adjustment for the following factors. Differences between cases and controls were tested by the extensions of the Mantel–Haenszel procedure with each adjustment

[a] Adjusted for age and screening periods

[b] Adjusted for sex and age

[c] Adjusted for sex and screening periods

[d] Adjusted for sex, age, and screening periods

[e] Adjusted for sex, age, screening periods, and energy intake

Table 2 Odds ratios (OR) and 95% confidence intervals (CI) of colorectal adenoma for serum triglycerides

Range	Serum triglycerides (mg/dl)				P_{trend}
	<68	68–94	95–127	128+	
Median	55	81	109	169	
Controls	176	186	184	183	
1+ adenomas	145	188	192	252	
OR1[a] (95% CI)	1.0 (reference)	1.2 (0.92–1.7)	1.3 (0.93–1.7)	1.7(1.2–2.2)	0.0010
OR2[b] (95% CI)	1.0 (reference)	1.2 (0.88–1.6)	1.1 (0.82–1.5)	1.5(1.1–2.0)	0.030
1 adenoma	95	130	98	123	
OR[b] (95% CI)	1.0 (reference)	1.3 (0.94–1.9)	0.93 (0.65–1.3)	1.2 (0.85–1.8)	0.59
2 adenomas	30	35	60	60	
OR[b] (95% CI)	1.0 (reference)	1.0 (0.58–1.7)	1.6 (0.95–2.6)	1.5(0.90–2.6)	0.058
3+ adenomas	20	23	34	69	
OR[b] (95% CI)	1.0 (reference)	1.0 (0.53–1.9)	1.3 (0.68–2.4)	2.3(1.3–4.2)	<0.0010

[a] Adjusted for sex; age (<50, 50–54, 55–59, 60–64, 65+); and screening periods (first, second)

[b] Adjusted for sex; age (<50, 50–54, 55–59, 60–64, 65+); screening periods (first, second); smoking (0, 1–29, 30–59, 60+ pack-years); body mass index (<25.0, 25.0–26.9, 27.0–29.9, 30.0+); physical activity (quartiles based on controls); alcohol consumption (0, 1–149, 150–299, 300+ g/week ethanol); family history of colorectal cancer; aspirin or other non-steroidal anti-inflammatory drug use; dietary fiber, folate, calcium, vitamin D, and red meat intake (quartiles based on controls; energy-adjusted)

Serum triglyceride levels were associated with colorectal adenoma in ever-smokers, but not in never-smokers (Table 3). A statistically significant OR for the highest quartile was found in ever-smokers (OR 2.0; 95% CI 1.3–3.2), in which the linear trend of OR was evident ($P_{trend,}$ 0.0018). Serum triglyceride levels were clearly associated with three or more adenomas (OR 3.4 for the highest versus the lowest; 95% CI 1.5–7.9; $P_{trend,}$ < 0.0010). In contrast, no elevated OR of adenoma was shown in never-smokers (OR 1.1 for the highest versus the lowest quartile; 95% CI 0.71–1.8), where the statistical interaction between smoking and serum triglycerides was borderline significant ($P_{interaction,}$ 0.067). A similar trend was found when

Table 3 Odds ratios (OR[a]) and 95% confidence intervals (CI) of colorectal adenoma for serum triglycerides stratified by smoking

Range	Serum triglycerides (mg/dl)				P_{trend}
	<68	68–94	95–127	128+	
Median	55	81	109	169	
Never-smokers					
Controls	108	94	103	71	
1+ adenomas	89	101	84	73	
OR (95% CI)	1.0 (reference)	1.3 (0.85–2.0)	0.87 (0.57–1.3)	1.1 (0.71–1.8)	0.94
1 adenoma	66	72	43	37	
OR (95% CI)	1.0 (reference)	1.3 (0.82–2.1)	0.64 (0.39–1.1)	0.88 (0.51–1.5)	0.24
2 adenomas	12	20	30	16	
OR (95% CI)	1.0 (reference)	1.8 (0.80–4.0)	2.3 (1.1–5.0)	1.7 (0.73–4.1)	0.18
3+ adenomas	11	9	11	20	
OR (95% CI)	1.0 (reference)	0.78 (0.29–2.1)	0.64 (0.24–1.7)	1.9 (0.76–4.6)	0.14
Ever-smokers					
Controls	68	92	81	112	
1+ adenomas	56	87	108	179	
OR (95% CI)	1.0 (reference)	1.3 (0.80–2.1)	1.6 (0.99–2.6)	2.0 (1.3–3.2)	0.0018
1 adenoma	29	58	55	86	
OR (95% CI)	1.0 (reference)	1.6 (0.91–2.8)	1.6 (0.87–2.8)	1.9 (1.1–3.3)	0.053
2 adenomas	18	15	30	44	
OR (95% CI)	1.0 (reference)	0.67 (0.30–1.5)	1.3 (0.64–2.7)	1.4 (0.72–2.9)	0.092
3+ adenomas	9	14	23	49	
OR (95% CI)	1.0 (reference)	1.5 (0.58–3.8)	2.2 (0.89–5.4)	3.4 (1.5–7.9)	<0.0010

[a] Adjusted for sex; age (<50, 50–54, 55–59, 60–64, 65+); screening periods (first, second); body mass index (<25.0, 25.0–26.9, 27.0–29.9, 30.0+); physical activity (quartiles based on controls); alcohol consumption (0, 1–149, 150–299, 300+ g/week ethanol); family history of colorectal cancer; aspirin or other non-steroidal anti-inflammatory drug use; dietary fiber, folate, calcium, vitamin D, and red meat intake (quartiles based on controls; energy-adjusted)

classified according to adenoma size (0–4 mm, 5–9 mm, or 10 or more mm in diameter; data not shown in tables).

Since the use of statin and intake of saturated, monounsaturated, or polyunsaturated fatty acid could influence the serum triglyceride levels, we repeatedly performed the same analyses with adjustment for these factors. However, the result did not substantially change compared to that without such adjustment. An analysis was made after deleting subjects taking statin, nonsteroidal anti-inflammatory drugs, or hormone replacement therapy. The association between serum triglycerides and colorectal adenoma was slightly attenuated in the overall analysis but somewhat deattenuated in stratified analysis by smoking. Moreover, we made stratified analyses by all covariates in our multivariate model to control any confounding by these covariates. As a result, we observed a clearer association between serum triglycerides and colorectal adenoma in men than women, or a lower (less than median) than a higher (median or more) red meat intake group. The association did not differ between strata classified according to the other covariates. Since the use of estrogen could reduce the incidence of colon neoplasms, we also adjusted for a history of hormone replacement therapy when separately analyzing female subjects, although it did not substantially influence the association. We also analyzed the association of serum triglycerides and polypoid (0-Ip, 0-Is) or flat/depressed adenoma (0-IIa, 0-IIc; no 0-IIb adenoma in our study subjects) [28]. A clearer association was observed for the risk of polypoid than for flat/depressed adenoma.

Discussion

Our results were consistent with those of previous studies that reported a positive association between serum triglycerides and colorectal adenoma [5–10]. The association was confirmed by dose-dependent relationships between serum levels of triglycerides and the number of adenomas, which is associated with the risk of colorectal cancer [11]. Total colonoscopy was sparsely used in previous epidemiologic studies [8, 10]. Our method of screening by total colonoscopy was more useful than by sigmoidoscopy in reducing the number of misclassifications, since many proximal adenomas could not be detected with sigmoidoscopy [29, 30].

Smoking seemed to play an important role in the association between serum triglycerides and colorectal adenoma. Our results showed that serum triglycerides were associated with colorectal adenoma only in ever-smokers. This suggested that serum triglycerides may be

involved in adenoma formation after DNA damage to colorectal epithelia by carcinogens within tobacco smoke. In short, serum triglycerides may be at work in the promotion phase of carcinogenesis. In fact, *Apc*-deficient mice showed age-dependent hypertriglyceridemia and a number of intestinal polyp formations, which were suppressed by anti-hyperlipidemic medicines [12, 13]. In another animal study, azoxymethane injection of obese rats with hypertriglyceridemia resulted in an increased number of advanced colon aberrant crypt foci, putative precursors of colon cancer [15]. Those animals probably showed such a clear association between triglycerides and intestinal neoplasms due to initiation by a genetic defect or carcinogen. However, the biological or molecular mechanism is unclear so far. Further laboratory and epidemiologic studies are necessary to substantiate this association among smoking, serum triglycerides, and colorectal adenoma.

However, serum triglyceride levels are not necessarily associated with colorectal cancer incidence [31, 32] or death [33]. Other factors such as hyperinsulinemia associated with physical inactivity or high body mass index may be needed for further neoplastic development [34, 35]. An elevated insulin level leads to a rise in insulin-like growth factor-I (IGF-I) [35]. IGF-I has potent anti-apoptotic and mitogenic properties in both normal and neoplastic cells. Although serum triglycerides may not be a specific predictor of subsequent risk of colorectal cancer, we might at least consider smokers with high serum triglycerides for colorectal screening and polypectomy as well as risk stratification by age and family history used in the algorithm for colorectal cancer screening [36]. This consideration might contribute to further reduction of the risk of colorectal advanced lesions or deaths [37, 38].

There are several limitations in this study. First, the adenoma cases in our study might include a few false positive cases because the overall accuracy of pit-pattern diagnosis is approximately 90%, whereas our institute data investigating overall accuracy showed more than 95% accurate diagnosis [19]. We could not analyze the association of serum triglycerides by several types of adenoma such as tubular, villous, or serrated adenoma [39] because of a lack of pathologic diagnosis. However, biopsies for all suspicious lesions including adenoma and hyperplastic polyps are unrealistic and time-consuming. Now, magnifying chromoendoscopy is a feasible and efficient method to determine neoplastic lesions such as adenoma. This method is also valid on inter- and intra-observer consistency [20, 21]. Second, serum levels of triglycerides were obtained by single measurements of study subjects. These measurements might show a wider variation due to measurement errors than would the means of multiple measurements. However, a positive association between serum triglycerides and colorectal adenoma would not be due to these diagnostic or measurement errors, which would be random misclassifications occurring in both cases and controls. If we could entirely exclude the misclassification, that positive association would be clearer than the present one. Third, smokers have unhealthy diet habits associated with hypertriglyceridemia in general. We cannot completely rule out a residual confounding with unhealthy diet associated with smoking when we explain the association between hypertriglyceridemia and adenoma only among smokers. Finally, the association between serum triglycerides and colorectal adenoma cannot be conclusively determined to be a causal relationship, since both were assessed cross-sectionally. However, colorectal adenoma could not alter serum triglycerides or dietary habits, which influence serum triglycerides in subjects with colorectal adenoma, since it is an asymptomatic lesion. Therefore, this cross-sectional assessment may be at least useful to infer a causal relationship between serum triglycerides and colorectal adenoma. A Japanese population was originally thought to be different from a Western population in terms of the high prevalence of nonpolypoid (flat and depressed) adenoma. However, such nonpolypoid lesions have now been reported around the world [40]. Therefore, our results can be generalizable not only to the Japanese population but also to other populations including Western ones.

Our results suggest that a higher level of serum triglycerides may be related to a larger number of adenomas. Adenoma development involving an elevated level of serum triglycerides may be modified by smoking.

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Randomized, double-blind, placebo-controlled trial of bovine lactoferrin in patients with chronic hepatitis C

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Several studies have suggested that lactoferrin administration may decrease the serum level of hepatitis C virus (HCV) RNA in patients with chronic hepatitis C. The aim of the present study was to confirm the efficacy of orally administered bovine lactoferrin (bLF) in patients with chronic hepatitis C. The patients with chronic hepatitis C randomly received either oral bLF at a dose of 1.8 g daily for 12 weeks, or an oral placebo. The primary endpoint was the virologic response, defined as a 50% or greater decrease in serum HCV RNA level at 12 weeks compared with the baseline. The secondary endpoint was the biochemical response, which was defined as a 50% or greater decrease in the serum alanine aminotransferase (ALT) level at 12 weeks compared with the baseline. One hundred and ninety-eight of 199 patients were evaluable for efficacy and safety. bLF treatment was well tolerated and no serious toxicities were observed. A virologic response was achieved in 14 of 97 patients (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group. There was no significant difference in virologic response rates between the two groups (−4.4%, 95% confidence interval −14.8, 6.1). In addition, bLF intake did not have any favorable effect on the serum ALT level. The virologic responses were not different between two groups in any subgroup analysis. In conclusion, orally administered bLF does not demonstrate any significant efficacy in patients with chronic hepatitis C. (*Cancer Sci* 2006; 97: 1105–1110)

Hepatitis C virus is a leading cause of chronic liver disease in Japan, and nearly two million people are estimated to be infected.[1] It is well known that HCV infection frequently causes chronic hepatitis, and that chronic hepatitis eventually progresses to liver cirrhosis and HCC approximately 30 years after HCV infection.[2] In Japan, more than 30 000 people die of HCC annually, and approximately 80% of HCC patients are infected with HCV.[3] Therefore, effective anti-HCV therapy is necessary to reduce the number of patients suffering from cirrhosis or HCC. To date, interferon-based therapy is the only effective treatment used clinically for chronic hepatitis C. A sustained complete virologic response (loss of detectable serum HCV RNA) occurs in 15–20% of patients with chronic hepatitis C after interferon therapy.[4] Moreover, recent studies have demonstrated that interferon with ribavirin or peginterferon with ribavirin improves the sustained complete virologic response rate by up to 40–50%.[5,6] However, because more than half of patients do not respond to interferon therapy, and because interferon therapy sometimes induces strong adverse effects, further developments in the treatment of chronic hepatitis C are required.

Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is present mainly in breast milk and other exocrine secretions. Several biological activities of lactoferrin have been demonstrated, including regulation of iron absorption in the intestine and modulation of immunoreactions.[7] Lactoferrin also plays an important role in human innate defense mechanisms against bacteria, fungi and viruses.[8] *In vitro* studies to date have shown that lactoferrin has antiviral effects against human immunodeficiency virus-1 and human cytomegalovirus.[9] Recent experimental studies have suggested that lactoferrin has antiviral effect against HCV.[10–12] Yi *et al.* have reported that lactoferrin binds to HCV envelope proteins *in vitro*.[10] Ikeda *et al.* have reported that lactoferrin prevents HCV infection in cultured human hepatocytes, and suggested that the anti-HCV activity of lactoferrin might be related to its direct binding to viral surfaces.[11,12] In addition, recent clinical studies have demonstrated the potential efficacy of lactoferrin against chronic hepatitis C.[13,14] Tanaka *et al.* reported that 8-week oral administration of bLF at a dose of 1.8 or 3.6 g/day decreased the serum level of HCV RNA markedly in three of four patients with a low pre-treatment HCV RNA level (<100 Kcopy/mL).[13] Iwasa *et al.* administered bLF (3.6 g/day) orally to 15 patients with high viral loads (≥100 KIU/mL), and reported that the mean serum HCV RNA level decreased significantly from 1106 KIU/mL at entry to 612 KIU/mL after 6 months of treatment ($P < 0.01$).[14] Based on these promising findings, we planned to investigate the efficacy of orally administered bLF in patients with chronic hepatitis C. First, we conducted a dose-finding study in 45 patients with chronic hepatitis C.[15] In that study, three dose levels of bLF (1.8, 3.6 and 7.2 g/day) were scheduled, and 15 patients at each dose level received the determined dose of bLF for 8 weeks. bLF treatment was well tolerated up to 7.2 g/day, and no serious adverse events were observed. Although no relationship between bLF dose and efficacy was recognized, a 50% or greater decrease in the serum HCV RNA level was seen in four of 45 patients (8.9%). Furthermore, the HCV RNA level was decreased by 50% or more in eight patients (17.8%) at week 8 after the end of treatment. These results encouraged us to conduct further investigations, and the present randomized

[16]To whom correspondence should be addressed. E-mail: hiueno@ncc.go.jp
Abbreviations: ALT, alanine aminotransferase; bLF, bovine lactoferrin; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL, interleukin; NK, natural killer.

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trial was designed to clarify the anti-HCV activity of bLF in patients with chronic hepatitis C.

Patients and Methods

Patients. Each patient was required to meet the following eligibility criteria: 20–74 years of age; positivity for anti-HCV antibody; an HCV RNA level of 0.5–850 KIU/mL evaluated within 1 month before entry; a sustained elevation of serum ALT level for at least 6 months; a serum ALT level of at least twice the upper normal limit evaluated within 1 month before entry; no evidence of HCC on the basis of ultrasonography or computed tomography carried out within 3 months before entry; and adequate bone marrow function (white blood cell count $\geq 4000/mm^3$, platelet count $\geq 100\,000/mm^3$, and hemoglobin level ≥ 11 g/dL), liver function (total bilirubin level ≥ 2.0 mg/dL, serum albumin level ≥ 3.5 g/dL, and serum aspartate aminotransferase and ALT level ≥ 200 IU/L) and renal function (normal serum creatinine and blood urea nitrogen levels).

The exclusion criteria were: positivity for hepatitis B surface antigen; interferon therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin therapy within 1 month before entry; past or present history of bLF tablet intake; pregnant or lactating females; severe hepatic disease (e.g. autoimmune hepatitis and primary biliary cirrhosis); other serious medical conditions (e.g. gastrointestinal bleeding, active infection, severe pulmonary disease and psychiatric disorders).

Methods. This double-blind, placebo-controlled phase III trial was conducted at 11 centers in Japan. The study was approved by the institutional review board at each center, and all the participants provided written informed consent. Eligible participants were assigned randomly to one of two treatment groups in equal proportions using permutation blocks stratified by centers. A randomization list was drawn up using the SAS random number generator at the data center (Quintiles Transnational Japan K. K. Tokyo, Japan). The treatments consisted of bLF at a dose of 1.8 g/day or a placebo, administered orally twice daily for 12 weeks. In the current study, bLF at 1.8 g/day was selected on the basis of the previous dose-finding study, which indicated that there was no significant relationship between bLF dose (range, 1.8–7.2 g/day) and anti-HCV activity.[15] After the treatment allocation, the data center sent a numbered container of bLF or placebo tablets to a participant. During treatment, combined use of interferon, immunomodulatory therapy, corticosteroid and intravenous glycyrrhizin was prohibited. bLF (450 mg/tablet) and placebo tablets were provided by Morinaga Milk Industries (Tokyo, Japan).

In the current study, we tested the hypothesis that oral administration of bLF would: (1) reduce the serum HCV RNA level; and (2) reduce the serum ALT level in patients with chronic hepatitis C. In addition, we investigated the influence of orally administered bLF on systemic immune response in a small group of participants. The participants were evaluated every 4 weeks as outpatients until 4 weeks after completion of treatment. Serum HCV RNA level and serum ALT level were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after treatment. Serum HCV RNA level was determined by reverse transcription–polymerase chain reaction using the Amplicor-HCV monitor V 2.0 kit with a sensitivity of 0.5 KIU/mL (Roche Diagnostics, Tokyo, Japan). Anti-HCV antibody was determined by chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Tokyo, Japan). HCV serotyping was carried out as described previously.[16] HCV serotype 1 corresponds to genotypes 1a and 1b of the Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.[17] Serum concentration of IL-18 was measured in participants at two institutions (National Cancer Center Hospital and Osaka Red Cross Hospital), and the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes was measured in participants at the National Cancer Center Hospital. IL-18 and all lymphocytes were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after completion of treatment. Serum concentration of IL-18 was assayed with a human IL-18 enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan). Lymphocyte surface phenotypes of CD4, CD8, CD16 and CD56 were determined by flow cytometry.

Adverse events were graded for severity according to the Japan Society for Cancer Therapy criteria,[18] which are similar to the National Cancer Institute Common Toxicity criteria. During treatment, participants were asked to record in a daily journal both compliance and any adverse events they experienced.

Assessment of efficacy and statistical analysis. Analyses were carried out on an intention to treat basis. The primary endpoint was a virologic response. In the current study, we defined a virologic response as a 50% or greater decrease in the serum HCV RNA level at 12 weeks compared with the baseline. Secondary endpoints were a biochemical response, as were changes in serum HCV RNA level and serum ALT level. If the serum ALT level at 12 weeks showed both a ≥50% decrease compared with the baseline and was ≤ twice the upper normal limit, we considered it a biochemical response. Response rate was calculated as the number of responders divided by the total number in each group. Participants whose HCV RNA (or ALT) data at 12 weeks were missing were included only in the denominator. Change in HCV RNA level (or ALT level) was calculated as the logarithm of the HCV RNA level (or ALT level) at 12 weeks minus the logarithm of these at the baseline. Differences in the virologic or biochemical response rates between two groups were analyzed using a test for the difference between two proportions. Differences in the change in HCV RNA level or ALT level between two groups were analyzed using a test for the difference between two means. In addition to the above planned analyses, subgroup analyses for virologic response were carried out based on pretreatment variables including age, serum HCV RNA level and HCV serotype. In a small group of participants, change in the serum concentration of IL-18 and changes in the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes during the study period were investigated. Analyses were carried out using JMP4.0 and PC SAS Release v.8.02 (SAS Institute Japan Ltd, Tokyo, Japan). All P-values are two-tailed, and differences at $P < 0.05$ were regarded as statistically significant.

We estimated that a total of 250 participants would be the maximum to enroll for a 2-year enrollment period. Subsequent power analysis revealed that 125 participants per group would have 75% power to detect a 10% difference in the virologic response rate (15 vs 5%) at the 5% level of significance. An interim analysis by the independent data monitoring committee was planned after the first 125 participants had been enrolled. All trial personnel and participants were blinded to treatment assignment for the duration of the trial. Only the trial statistician and the independent data monitoring committee saw unblinded data. In the interim analysis of the primary endpoint, the O'Brien–Fleming method was used.[19]

Results

Patients. Enrollment began at seven institutions in April 2001. Because 250 participants were not enrolled for the 2 years planned originally, we extended the registration period for one more year and increased the number of participating institutions from seven to 11. An interim analysis was carried out in March 2004 with the data from the first 125 participants. Because the results of the interim analysis indicated that it was highly unlikely that a significant difference in treatment efficacy between

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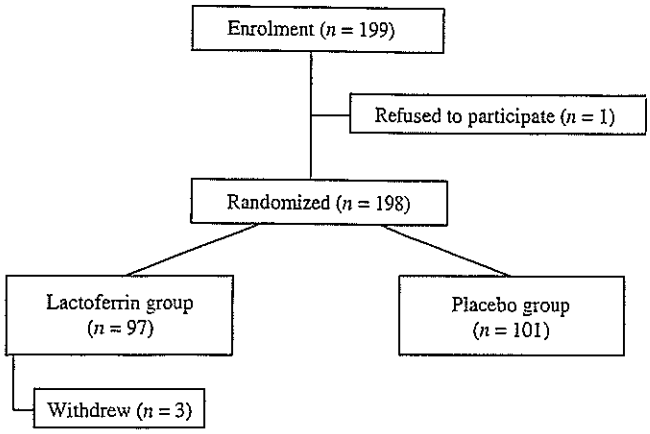

Fig. 1. Flow diagram of participant enrolment.

Table 1. Baseline characteristics of the patients

Characteristic	Bovine lactoferrin	Placebo
No. patients	97	101
Age (years)†	61 (29–74)	58 (31–74)
Sex (male/female)	53/44	55/46
History of interferon therapy	25	29
ALT level (IU/L)†	91 (41–340)	98 (27–250)
HCV RNA level (KIU/mL)†	378 (8.8–960)	452 (8.0–1560)
HCV serotype (1/2/ND)	78/17/1	76/22/3

†Median (range). ALT, alanine aminotransferase; HCV, hepatitis C virus; ND, not determined.

the two groups would be observed with the planned full enrollment of 250 participants, the data monitoring committee recommended discontinuation of further enrollment. Therefore, enrollment was stopped on 31 March 2004, at which point 199 participants had been enrolled. Because one patient refused to participate in the study before randomization, efficacy and safety were analyzed in the remaining 198 participants (97 bLF and 101 placebo) (Fig. 1). Although three participants in the bLF group discontinued treatment for reasons other than an adverse event, the remaining 195 participants completed the scheduled 12 weeks of treatment. The baseline characteristics of the 198 participants are shown in Table 1. There was no significant difference between the bLF and placebo groups regarding the pretreatment characteristics including age, sex, serum ALT level and serum HCV RNA level.

Virologic efficacy. Virologic response, the primary endpoint, was assessed in all 198 participants who received at least one dose of treatment. Virologic response was observed in 14 of 97 participants (14.4%) in the bLF group, and in 19 of 101 (18.8%) in the placebo group (Table 2). No complete virologic response (loss of detectable serum HCV RNA) was seen in either of the groups. There was no significant difference in the virologic response rate with bLF treatment in comparison with the placebo (−4.4%, 95% CI −14.8, 6.1). Change in the HCV RNA level at 12 weeks compared with the baseline was assessed in 190 participants (93 bLF group, 97 placebo group), excluding eight participants for whom HCV RNA data at 12 weeks were lacking. The change in the mean logarithm of the HCV RNA level was −0.09 in the bLF group and −0.09 in the placebo group, indicating no significant difference between the groups ($P = 1.00$).

Biochemical efficacy. Biochemical response was assessed in 198 participants. Biochemical response was seen in six of 97 participants (6.2%) in the bLF group, and in four of 101 participants (4.0%) in the placebo group (Table 2). No significant difference in the biochemical response rate was seen between the groups (2.2%, 95% CI −3.9, 8.3). Change in the serum AST level was assessed in 192 participants (93 bLF group, 99 placebo group), excluding six participants for whom ALT data at 12 weeks were lacking. The change in the mean logarithm of the ALT level was −0.085 in the bLF group and −0.080 in the placebo group, indicating no significant difference ($P = 0.93$).

Subgroup analysis. The rates of virologic response with respect to pretreatment variables are presented in Table 3. Among participants with a low HCV RNA level (<100 KIU/mL), the virologic response rate was 29.4% in the bLF group and 15.4% in the placebo group, indicating no significant difference between the groups (14.0%, 95% CI −15.2, 43.2). The virologic responses were also not different between two groups in other subgroup analyses such as age, sex and HCV serotype.

Analysis of IL-18 and lymphocytes. The serum concentration of IL-18 was measured in 73 participants enrolled at the National Cancer Center Hospital and Osaka Red Cross Hospital (36 bLF, 37 placebo). Figure 2 shows the changes in the mean IL-18 levels in the bLF group and placebo group. The mean IL-18 levels in the bLF and placebo groups were 293.9 pg/mL and 309.9 pg/dL at the baseline and 280.7 pg/mL and 291.5 pg/mL at 12 weeks, respectively. The corresponding changes in the mean IL-18 level at 12 weeks were −14.5 pg/mL and −15.9 pg/mL, respectively, indicating no significant difference between the groups ($P = 0.91$). Similarly, there were no significant differences between the groups at any other points during the study period. The percentage of lymphocyte was measured in 46 participants at the National Cancer Center Hospital (bLF 23, placebo 23), and the results are shown in Fig. 3. The percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes remained almost unchanged throughout the study in both groups, and the differences between them were not significant.

Safety. Safety was assessed in 198 participants who received at least one dose of bLF or placebo during the study. The bLF treatment was well tolerated, and no serious complications occurred during the treatment. Although minor adverse events including neutropenia, γ-GTP elevation and hyperglycemia were observed in participants treated with bLF, their frequency and intensity did not differ from those in the placebo group. HCC was detected in one participant in the bLF group and in one participant in the placebo group during the study period.

Discussion

The present study was carried out to confirm the anti-HCV activity of orally administered bLF in patients with chronic hepatitis C. A virologic response (a 50% or greater decrease in the serum level of HCV RNA at 12 weeks compared with the baseline) was observed in 14 of 97 participants (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group, the difference between the groups being non-significant. The virologic responses were not different between two groups in any subgroup analysis. Furthermore, bLF intake did not have any favorable effect on the serum ALT level. On the basis of these results, we concluded that orally administered bLF did not have any efficacy, including anti-HCV activity, in patients with chronic hepatitis C.

The virologic response rate of 14.4% observed in the bLF group was somewhat higher than that reported in the previous dose-finding study,[15] in which four of 45 patients (8.9%) showed a virologic response at the end of bLF treatment. Nevertheless, the current study failed to demonstrate any anti-HCV activity of bLF, because a similar virologic response rate to that in the bLF group was seen in the placebo group. Having designed this randomized study, we assumed that a virologic

Table 2. Virologic and biochemical efficacy

Characteristic	Bovine Lactoferrin	Placebo	Difference (95% CI)	*P*-value
Virologic efficacy				
Response rate (%)	14.4	18.8	−4.4 (−14.8, 6.1)	
Change in HCV RNA level†	−0.09	−0.09		1.00
Biochemical efficacy				
Response rate (%)	6.2	4.0	2.2 (−3.9, 8.3)	
Change in ALT level†	−0.085	−0.080		0.93

†Mean logarithm. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

Table 3. Virologic response rate as a function of baseline variables

Variable	Bovine lactoferrin (*n* = 97)		Placebo (*n* = 101)		Difference	
	Response/total	%	Response/total	%	%	95% CI
Age						
<65 years	12/62	19.4	14/77	18.2	1.2	−11.9, 14.2
≥65 years	2/35	5.7	5/24	20.8	−15.1	−33.1, 2.9
Sex						
Male	10/53	18.9	10/55	18.2	0.7	−14.0, 15.3
Female	4/44	9.1	9/46	19.6	−10.5	−24.7, 3.8
ALT level						
<100 IU/L	6/57	10.5	7/51	13.7	−3.2	−15.6, 9.2
≥100 IU/L	8/40	20.0	12/50	24.0	−4.0	−21.1, 13.1
HCV RNA level						
<100 KIU/mL	5/17	29.4	2/13	15.4	14.0	−15.2, 43.2
≥100 KIU/mL	9/80	11.3	17/88	19.3	−8.0	−18.8, 2.7
HCV serotype†						
1	11/78	14.1	16/76	21.1	−7.0	−18.9, 5.0
2	3/18	16.7	2/22	9.1	7.6	−31.4, 28.6

†Hepatitis C virus serotype was not measured in four patients. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

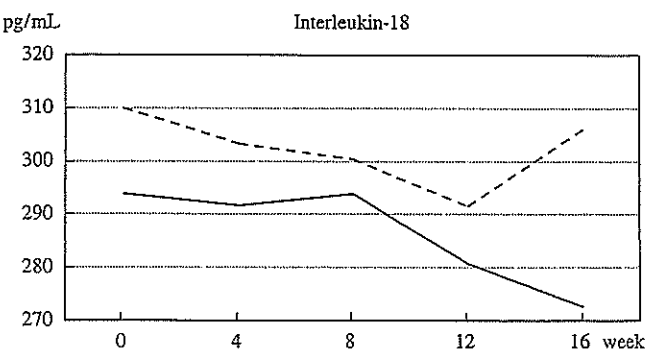

Fig. 2. Changes in the mean serum concentration of interleukin-18 in the bovine lactoferrin group (straight line, *n* = 36) and the placebo group (dotted line, *n* = 37).

response rate of around 5% would be seen in the placebo group due to spontaneous remission of viral activity. However, contrary to our expectation, 19 of 101 participants (18.8%) in the placebo group showed a ≥50% decrease in the HCV RNA level at 12 weeks, indicating that our assumption was inappropriate. Our results suggested that in order to assess the reduction of the HCV RNA level, periodic evaluation would be necessary to exclude the influence of spontaneous fluctuation of HCV RNA.

Several experimental studies have suggested that lactoferrin has some activity against HCV. Yi *et al.*[10] reported that lactoferrin binds to the HCV E1 and E2 envelope proteins *in vitro*, and Ikeda *et al.*[11,12] reported that lactoferrin prevents HCV infection in cultured human hepatocytes. They suggested that the anti-HCV activity of lactoferrin might be due to a neutralizing efficacy, in which the administered lactoferrin became bound directly to the HCV virion, thus inhibiting adsorption of the HCV-lactoferrin complex into human hepatocytes. Therefore, intravenous administration of lactoferrin might improve the viremic state in patients with chronic hepatitis C. However, for practical application, administration of lactoferrin directly into blood does not seem to be a suitable approach because lactoferrin is a large glycoprotein molecule (80 kDa) that may cause allergic reactions. Therefore, oral administration of bLF was selected for the present study, even though the metabolism and mechanism of ingested lactoferrin are yet to be clarified. As to absorption, it has been reported that intact lactoferrin and its fragments are present in the urine of human milk-fed preterm infants.[20] However, in adult rats, lactoferrin and its fragments are not detectable in portal blood after bLF ingestion,[21] and in adult humans, the serum lactoferrin level does not increase after oral administration of recombinant human lactoferrin.[22] However, several studies have suggested that orally administered lactoferrin might enhance immune responses via cytokine production.[23,24] It has been reported that oral administration of bLF to mice enhances the production of IL-18 and interferon-γ in the mucosa of the small intestine, and increases the number of $CD4^+$, $CD8^+$ and NK cells in the small-intestinal epithelium.[25,26] Varadhachary *et al.* reported that oral administration of recombinant human lactoferrin to mice stimulates IL-18 production from gut enterocytes, and augments the NK activity of spleen cells and production of blood $CD8^+$ cells.[27] Furthermore, a recent clinical study has demonstrated that oral administration of bLF (0.6 g/day) for 3 months in 36 patients with chronic hepatitis C increased the serum IL-18 level significantly compared with the

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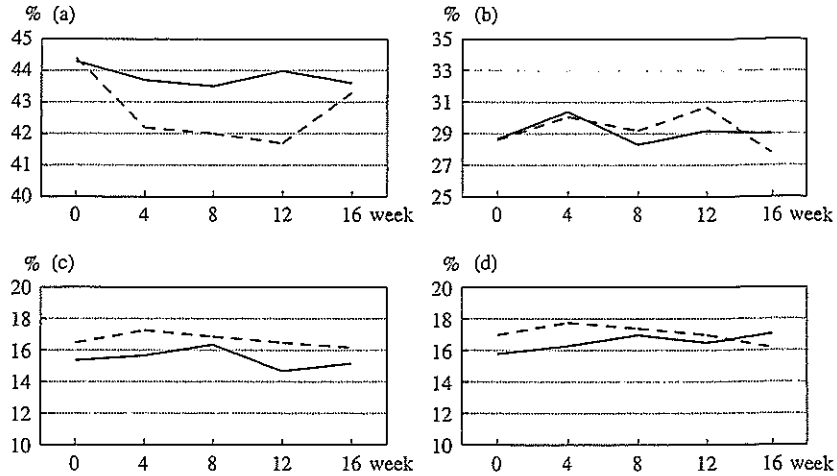

Fig. 3. Changes in the mean percentages of (a) $CD4^+$, (b) $CD8^+$, (c) $CD16^+$ and (d) $CD56^+$ peripheral blood lymphocytes in the bovine lactoferrin group (straight line, $n = 23$) and the placebo group (dotted line, $n = 23$).

baseline.[28] However, our study found no evidence that oral administration of bLF influences the serum concentration of IL-18 or the percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ lymphocytes. Further investigations are required to clarify the peripheral and systemic effects of orally administered lactoferrin. In addition, as many *in vitro* studies have suggested that lactoferrin has direct binding neutralizing efficacy against HCV,[29–31] further investigations are needed to devise a means of delivering lactoferrin or its fragment into the bloodstream safely and effectively.

Recently, several studies have investigated the value of adding lactoferrin to interferon therapy for chronic hepatitis C. Hirashima *et al.* randomly assigned 21 patients with chronic hepatitis C to either a consensus interferon plus oral lactoferrin (3.0 g/day) group or a consensus interferon monotherapy group.[32] Three of 10 patients in the consensus interferon plus lactoferrin group showed a sustained complete virologic response, as did four of 11 patients in the consensus interferon group, indicating no statistically significant difference between the groups. Ishibashi *et al.* conducted a randomized controlled trial to investigate the efficacy of interferon α-2b and ribavirin plus oral lactoferrin (0.6 g/day) compared with interferon α-2b and ribavirin plus placebo in 36 patients with chronic hepatitis C.[33] A sustained complete virologic response was seen in six of 18 patients in the lactoferrin group and in five of 18 patients in the placebo group, there being no statistically significant difference between the groups ($P = 0.7$). Although the numbers of patients recruited in the two randomized trials were small, these results suggested that the additional value of oral lactoferrin combined with interferon therapy would be negative for the treatment of chronic hepatitis C.

In summary, oral administration of bLF at a dose of 1.8 g/day for 12 weeks showed an acceptable safety profile in patients with chronic hepatitis C. However, there was no significant difference in the virologic responses between patients who received oral bLF and those receiving placebo. In addition, bLF intake did not have any favorable effect on the serum ALT level. These findings do not support the practical use of oral bLF in patients with chronic hepatitis C.

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RESEARCH COMMUNICATION

Suppression of Azoxymethane-induced Colonic Premalignant Lesion Formation by Coenzyme Q10 in Rats

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Abstract

Reactive oxygen species cause damage to proteins, lipids and DNA. Coenzyme Q10 (CoQ10) is a compound with mitochondrial bioenergetic functions. The reduced form of CoQ10 shows antioxidant activity. In the present study, effects of CoQ10 on development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and mucin-depleted foci (MDF) in F344 male rats were investigated. To induce ACF and MDF, 6-week old rats were given two weekly subcutaneous injections of AOM (15 mg/kg body weight) and also received a control diet or experimental diets containing CoQ10 (200 or 500 ppm) for 4 weeks, starting one day before the first dose of AOM. At 10 weeks of age, all animals were sacrificed and their colons were evaluated for numbers and sizes of ACF and MDF. Administration of 200 and 500 ppm CoQ10 resulted in reduction of ACF numbers, to 77% and 68% of the carcinogen control value, respectively. The percentages of ACF consisting of more than 4 crypts in these groups were also significantly lower than in the controls. Treatment with 500 ppm CoQ10 furthermore decreased the number of sialomucin-producing ACF and MDF per colon to 42% and 38% of the carcinogen control value without CoQ10, respectively. These results suggest that CoQ10 may be an effective chemopreventive agent against colon carcinogenesis.

Key Words: Azoxymethane - aberrant crypt foci - CoQ 10 - colon - chemoprevention

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Introduction

Colon cancer is one of the leading causes of cancer deaths in both men and women in Western countries (Jemal et al., 2003). In Japan, its incidence has been increasing, and it is now the third leading cause of cancer death. Primary cancer prevention, including chemoprevention, is therefore important. Several epidemiological studies have suggested that high consumption of fruit and vegetables, especially those containing high amounts of antioxidants, may protect people from colon cancer (Terry et al., 2001).

An increasing amount of experimental and epidemiological evidence implicates reactive oxygen species (ROS) in the pathogenesis of cancer. ROS, which increase in inflammation and in exposure to exogenous stimuli, including smoking, can cause DNA damage, oxidize lipids and proteins, and alter signal transcription pathways that enhance cancer risk. Antioxidative agents (e.g., vitamin E, vitamin C, N-acetylcysteine and other phytochemicals) that scavenge ROS can act as cancer preventive agents (Borek, 2004; Khanzode et al., 2004).

Coenzyme Q10 (CoQ10) is a well-known electron transporter in complexes I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase), and III (ubiquinone-cytochrome c oxidoreductase) of the mitochondrial respiratory chain (Lenaz et al., 1993; Lenaz et al., 1998), which is synthesized endogenously in humans and is found in virtually all aerobic organisms (Tran et al., 2001). CoQ10 is also taken in through food intake. In addition to its role as an electron carrier in the mitochondrial electron transport chain, the immunostimulating action of CoQ10 has been reported (Folkers et al., 1982, 1993). The reduced form of CoQ10 also acts as an antioxidant (Frei et al., 1990; Overvad et al., 1999).

Aberrant crypt foci (ACF) are generally considered as putative preneoplastic lesions for colon cancer in both rodents (Bird, 1987; McLellan and Bird,1988) and humans (Pretlow, 1991). However, most are hyperplastic and relatively few ACF present in the colon actually develop into tumors. Therefore, it is important to identify subgroups of lesions that may be more predictive of eventual tumorigenesis. Ochiai et al. have reported that dysplastic ACF can be detected by adding a simple decolorization process with 70% methanol after conventional 0.2%

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methylene blue staining (2005). Additional markers of colon cancer risk have been suggested, based on their correlation with tumor formation and/or histological changes reflecting DNA mutations. These include ß-catenin accumulated crypts (BCAC) (Yamada et al., 2000; Mori et al., 2005), flat dysplastic ACF (Paulsen et al., 2005), sialomucin-producing ACF (Jenab et al., 2001) and mucin-depleted foci (MDF) (Caderni et al., 2003). ACF producing sialomucins have a higher rate of cell proliferation, higher degree of dysplasia, and greater distortion of the luminal opening than ACF producing sulfomucins (Jenab et al., 2001; Caderni et al., 1995; Uchida et al., 1997). Most MDF are histologically dysplastic and feature accumulation of β-catenin. In order to examine the chemopreventive activity of CoQ10 in colon carcinogenesis, in the present study, we investigated effects of CoQ10 on the development of azoxymethane (AOM)-induced sialomucin-producing ACF and MDF in addition to classical ACF.

Materials and Methods

Animals and diets

Male F344 rats, 5 weeks of age, were purchased from Charles River Japan (Atsugi Japan) and quarantined for 1 week before being randomized into six groups. Three animals each were housed in a plastic cage. The animal room was controlled at 23±2°C, 50±10% humidity, and a 12-h light/dark cycle. Powdered AIN-76A (Bio-Serv., Frenchtown, NJ) was used as the basal diet during the experiment. CoQ10 (ubiquinone) was produced in Kaneka Corporation (Osaka, Japan). Water and basal diet or experimental diets, with addition and thorough mixing of CoQ10 at the indicated concentrations, prepared every week, were given ad libitum. Food consumption and body weights were measured weekly. CoQ10 was confirmed to be stable for at least 4 weeks at the room temperature when added to the basal diet.

Experimental protocol

A total of 36 male F344 rats were divided into six groups (Table 1); three or nine rats each in saline or AOM treated groups, respectively. At 6 weeks of age, all rats except those intended for vehicle treatment were given s.c. injections of AOM (Nard Institute, Ltd., Amagasaki, Japan) at a dose rate of 15 mg/kg body weight once a week for 2 weeks. The controls received equal volumes of normal saline (5 ml/kg body weight). Starting 1 day before the first dose of AOM, rats were fed on a control diet or experimental diets containing 200 or 500 ppm CoQ10 throughout the experiment. At 10 weeks of age, the rats were sacrificed under ether anesthesia to assess the occurrence of colonic lesions. The experimental protocols were approved by the Institutional Ethics Review Committee for Animal Experimentation.

Table 1. Effects of CoQ10 on Body Weights and Food Intake of Rats

Treatment	No. of rats	Body weight (g)[1]	Mean food consumption (g/rat/day)[1]
AOM	9	222 ± 7	12.5 ± 1.6
AOM + 200 ppm CoQ10	9	222 ± 11	12.7 ± 1.6
AOM + 500 ppm CoQ10	9	228 ± 8	13.2 ± 1.6
Saline	3	226 ± 10	12.9 ± 1.4
Saline + 200 ppm CoQ10	3	233 ± 9	13.2 ± 1.2
Saline + 500 ppm CoQ10	3	230 ± 7	12.7 ± 1.4

[1]Data are means ± SD

Determination of ACF and mucin production

All colons were removed, flushed with saline, slit open longitudinally from the cecum to the anus, placed between filter papers and fixed in 10% neutral buffered formalin for 24 h. They were then stained with 0.2% methylene blue in saline and placed, mucosal side up, on a microscope slide and observed under a microscope. ACF were recorded according to standard procedures used routinely in our laboratory (Kawamori et al., 1995). After ACF determination, methylene blue-stained colons were processed for high-iron diamine Alcian blue (HID-AB) staining of mucin as described previously (Caderni et al., 1995). The HID-AB method stains sulphomucins dark brown, while light or dark blue staining predominantly

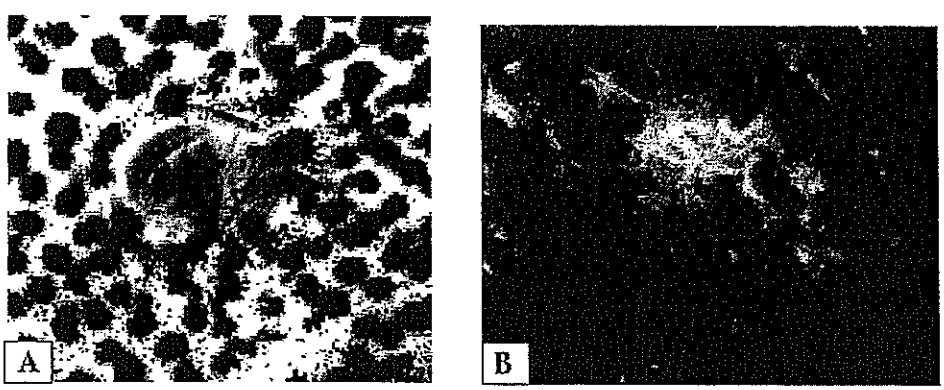

Figure. 1. Lesions identified in the colon of AOM-treated rats by HID-AB staining. (A) Appearance of a sialomucin-producing ACF (original magnification, X40). (B) Appearance of an MDF (original magnification, X40)