Table 1. Effects of compounds A and B on the development of colonic adenoma and adenocarcinoma

Group no.	Treatment	No. of mice examined	Incidence (%) AD	ADC	Total tumors (AD + ADC)	Multiplicity (no. of tumors/colon) AD	ADC	Total tumors (AD + ADC)
1	AOM[1]/1.5% DSS	28	61	64	71	1.39 ± 1.50[2]	1.96 ± 2.24	3.36 ± 3.34
2	AOM/1.5% DSS/100 ppm GOFA/β-CD	25	40	24[3]	40[4]	0.72 ± 1.06[5]	0.52 ± 1.16[5]	1.24 ± 2.11[6]
3	AOM/1.5% DSS/500 ppm GOFA/β-CD	24	25[7]	13[8]	29[3]	0.33 ± 0.64[6]	0.25 ± 0.74[9]	0.58 ± 1.21[9]
4	AOM/1.5% DSS/100 ppm AUR/β-CD	24	46	46	50	0.96 ± 1.27	1.21 ± 1.61	2.17 ± 2.81
5	AOM/1.5% DSS/500 ppm AUR/β-CD	24	50	25[3]	50	1.00 ± 1.32	0.42 ± 0.83[4]	1.42 ± 2.06[5]
6	AOM	5	0	0	0	0	0	0
7	1.5% DSS	5	0	0	0	0	0	0
8	500 ppm GOFA/β-CD	5	0	0	0	0	0	0
9	500 ppm AUR/β-CD	5	0	0	0	0	0	0
10	Untreated	5	0	0	0	0	0	0

[1]AOM, azoxymethane; DSS, dextran sodium sulfate; GOFA, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid; CD, cyclodextrin; AUR, auraptene: AD, adenoma; ADC, adenocarcinoma. [2]Mean ± SD. [3]Significantly different from the AOM/DSS group (Group 1) by Chi-square test ($p < 0.005$). [4]Significantly different from the AOM/DSS group (Group 1) by Chi-square test ($p < 0.05$). [5]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.01$). [6]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.05$). [7]Significantly different from the AOM/DSS group (Group 1) by Chi-square test ($p < 0.01$). [8]Significantly different from the AOM/DSS group (Group 1) by Fisher's exact probability test ($p = 0.0001$). [9]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.001$).

Table 2. Effects of compounds A and B on colonic inflammation and development of mucosal ulcer and high-grade dysplasia

Group no.	Treatment	No. of mice examined	Inflammation score (incidence, %)	Number of colonic mucosal ulcer/ colon (incidence)	No. of high-grade dysplasia/colon (incidence)
1	AOM[1]/1.5% DSS	28	2.79 ± 0.96[2]	1.29 ± 1.36 (75%)	2.21 ± 1.83 (82%)
2	AOM/1.5% DSS/100 ppm GOFA/β-CD	25	1.52 ± 1.05[3]	0.36 ± 0.64[4] (28%)	0.64 ± 1.19[4] (32%)
3	AOM/1.5% DSS/500 ppm GOFA/β-CD	24	0.75 ± 0.90[3]	0.33 ± 0.56[3] (29%)	0.50 ± 1.14[3] (25%)
4	AOM/1.5% DSS/100 ppm AUR/β-CD	24	1.71 ± 0.69[3]	0.42 ± 0.58[4] (38%)	1.25 ± 1.67 (50%)
5	AOM/1.5% DSS/500 ppm AUR/β-CD	24	1.17 ± 0.87[3]	0.33 ± 0.70[3] (21%)	0.75 ± 1.45[4] (33%)
6	AOM	5	0	0	0
7	1.5% DSS	5	2.20 ± 0.84	2.40 ± 0.89 (80%)	0
8	500 ppm GOFA/β-CD	5	0	0	0
9	500 ppm AUR/β-CD	5	0	0	0
10	Untreated	5	0	0	0

[1]AOM, azoxymethane; DSS, dextran sodium sulfate; GOFA, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid; CD, cyclodextrin. [2]Mean ± SD. [3]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.001$). [4]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.01$).

Other colonic lesions, including colitis with or without mucosal ulcer (Fig. 3*a*) and cryptal dysplastia (Fig. 3*b*), were also observed in the colon of mice in Groups 1–5 and/or 7 (Table 2). With respect to the inflammation score (Table 2) determined on H&E-stained sections at Week 18, the value of Group 1 was the highest among the groups and the scores of Groups 2–5 were significantly smaller in comparison to Group 1 ($p < 0.001$ for each comparison). Similarly, as shown in Table 2, the number of colonic mucosal ulcer per colon of Group 1 was the greatest, and the values of Groups 2–5 were significantly smaller in comparison to Group 1 ($p < 0.01$ or $p < 0.001$). The inflammation score and number of mucosal ulcer of Group 7 were the second among the group. Colonic inflammation in the mice of Groups 6, 8, 9 and 10 was slight, if present, and there were mucosal ulcers in the colon of the mice belonging to these groups.

PCNA- and survivin-labeling index in the colonic adenocarcinomas

The data for the PCNA-, TUNEL- and survivin-positive rates of adenocarcinomas are illustrated in Figure 4. As shown in Figure 4*a*, the mean labeling indices of PCNA of Groups 2

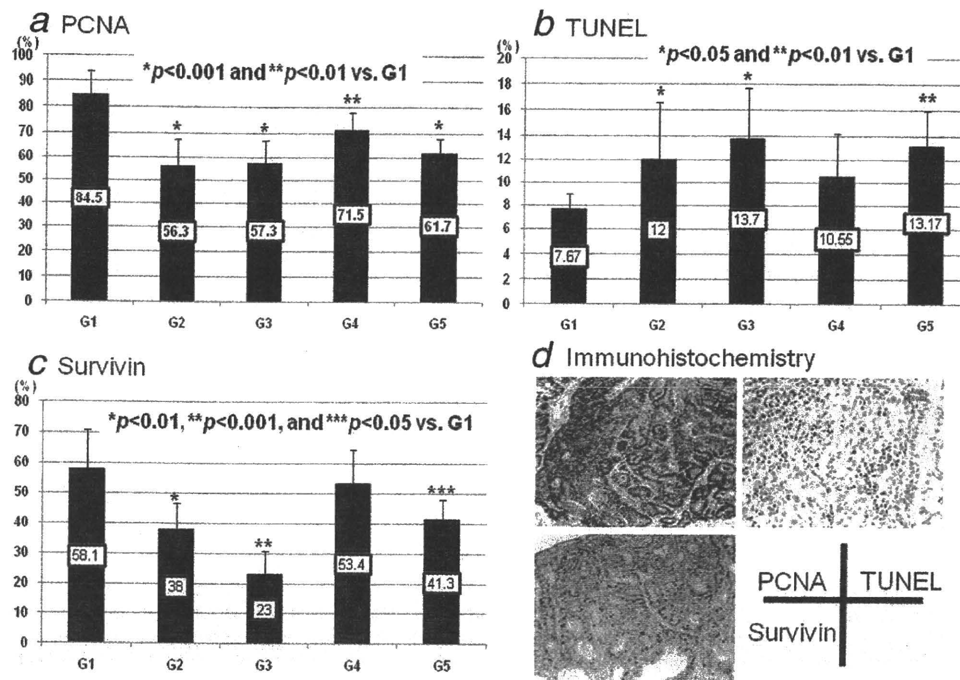

Figure 4. (*a*) The PCNA-labeling index (%, mean ± SD), (*b*) the TUNEL-positive rate (%, mean ± SD) and (*c*) the survivin-positive rate (%, mean ± SD) of colonic adenocarcinomas developed in mice from Groups 1–5. (*d*) The photos show representative PCNA-, TUNEL- and survivin-immunohistochemistry from Group 1.

(56.3 ± 11.2, $p < 0.001$), 3 (57.3 ± 9.6, $p < 0.001$), 4 (71.5 ± 9.4, $p < 0.01$) and 5 (61.7 ± 9.4, $p < 0.001$) were significantly lower in comparison to Group 1 (84.5 ± 9.4) (Fig. 4*d*). The mean TUNEL-positive rates of Groups 2 (12.00 ± 4.56, $p < 0.05$), 3 (13.70 ± 4.04, $p < 0.05$), 4 (10.55 ± 3.62) and 5 (13.17 ± 2.79, $p < 0.01$) were greater in comparison to Group 1 (7.67 ± 1.28) (Fig. 4*d*). With respect to the positive rates of survivin, the values of Groups 2 (38.0 ± 8.5, $p < 0.01$), 3 (23.0 ± 7.6, $p < 0.001$), 4 (53.4 ± 11.3) and 5 (41.3 ± 6.6, $p < 0.05$) were smaller in comparison to Group 1 (58.1 ± 12.6) (Fig. 4*d*).

Scores of NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β immunohistochemistry

The data for the scores of the immunohistochemical expression of these proinflammatory cytokines in colonic adenocarcinomas are illustrated in Figures 5*a*–5*c* and 6*a*–6*c*. Adenocarcinomas and the inflammatory mononuclear cells in the colon positively reacted with the antibodies of the proinflammatory cytokines, such as NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β (Figs. 5*d* and 6*d*). The scores of NF-κB (Fig. 5*a*), Stat3 (Fig. 6*a*), IL-6 (Fig. 6*b*) and IL-1β (Fig. 6*c*) of Groups 2–5 were significantly lower in comparison to Group 1. Similarly, the mean scores of Nrf2 (Fig. 5*b*) and Tnf-α (Fig. 5*c*) of Groups 2, 3 and 5 were significantly smaller in comparison to Group 1. Both values of Group 3 were lower in comparison to Group 1, but the differences were insignificant.

Discussion

The results of our study clearly indicated that the novel prodrugs, GOFA/β-CD and AUR/β-CD, effectively inhibited AOM/DSS-induced colitis-related colonic carcinogenesis without any adverse effects in mice. The effect of GOFA/β-CD was superior in comparison to AUR/β-CD. Dietary feeding with both prodrugs exerted their cancer chemopreventive ability by modulating cell proliferation, inducing apoptosis and suppressing the proinflammatory cytokines (NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β) in adenocarcinomas that developed in the inflamed colon. In turn, the expression of these cytokines may be involved in AOM/DSS-induced colon tumorigenesis. This is the first report showing that prodrugs of GOFA/β-CD and AUR/β-CD exert cancer chemopreventive ability in colitis-related colon carcinogenesis.

In our study, several proinflammatory cytokines were expressed in the colonic tumors and the inflammatory mononuclear cells infiltrated the tumors both internally and peripherally. As the expression of these cytokines may be involved in tumor growth,[52–54] we evaluated the effects of dietary GOFA/β-CD and AUR/β-CD on their expression in adenocarcinomas developed in Groups 1–5. The treatment with GOFA/β-CD and AUR/β-CD significantly lowered colonic inflammation induced by DSS. Chronic inflammation is involved in oncogenesis in certain tissues, including the large bowel. Therefore, the suppression of chronic inflammation through the modulation of expression of several

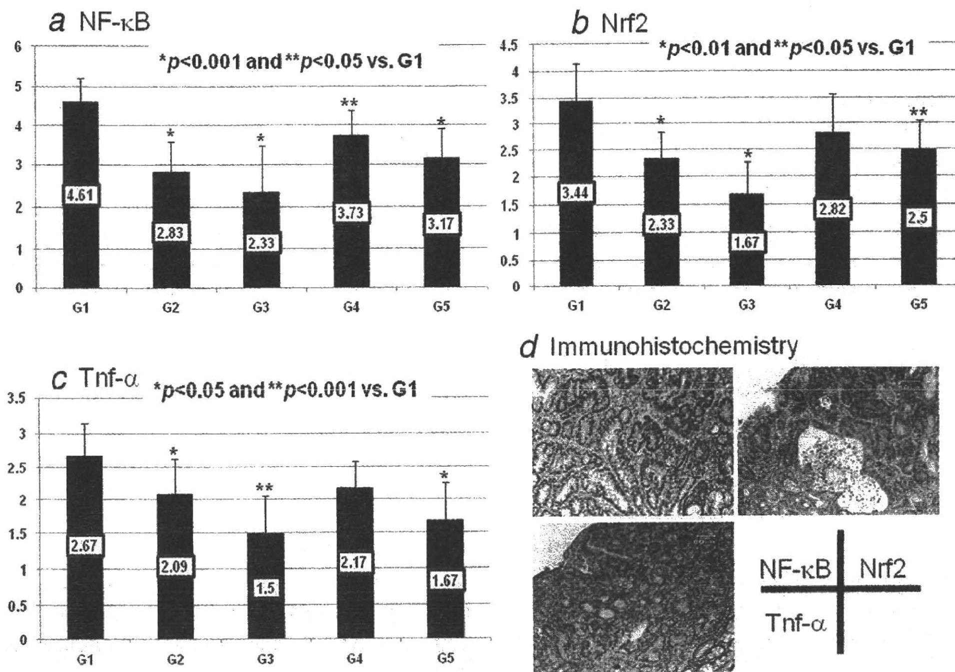

Figure 5. The scores (mean ± SD) of (*a*) NF-κB-, (*b*) Nrf2- and (*c*) Tnf-α-immunoreactivity of colonic adenocarcinomas developed in mice from Groups 1–5. (*d*) The photos show representative NF-κB-, Nrf2- and Tnf-α-immunohistochemistry from Group 1. Note: The adenocarcinoma cells strongly expressed NF-κB, Nrf2 and Tnf-α.

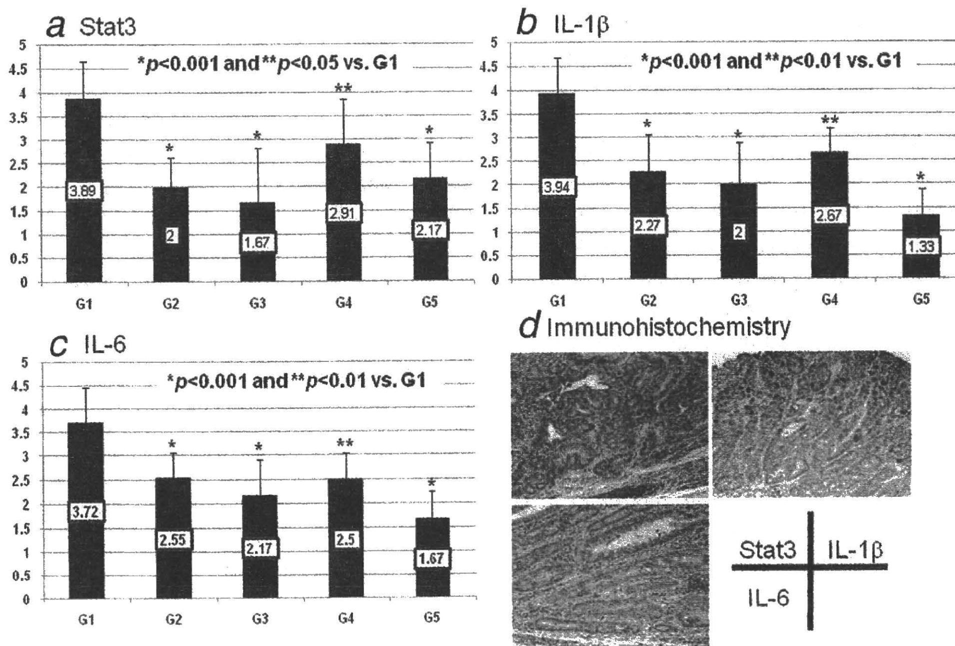

Figure 6. The scores (mean ± SD) of (*a*) Stat3-, (*b*) IL-1β- and (*c*) IL-6-immunoreactivity of colonic adenocarcinomas developed in the mice from Groups 1–5. (*d*) The photos show representative Stat3-, (*b*) IL-1b- and (*c*) IL-6-immunohistochemistry from Group 1. Note: The adenocarcinoma cells strongly expressed Stat3, IL-1β and IL-6.

proinflammatory gene products that mediate several events of carcinogenesis may result in cancer chemoprevention.[55] The modulation of inflammation and expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in the colon results in the suppression of colitis-related colon carcinogenesis of mice.[56] Several molecular targets for the

suppression of inflammation-associated carcinogenesis were proposed.[36] In addition to the highly expressed levels of COX-2 and iNOS of colonic adenocarcinomas in our study (data not shown), the proinflammatory cytokines, such as NF-κB, Tnf-α, Stat3, Nrf2, IL-6 and IL-1β, were strongly expressed in adenocarcinomas that developed in the colon of the mice that received AOM and DSS. Moreover, dietary feeding with GOFA/β-CD and AUR/β-CD suppressed their expressions. The Nrf2-deficient mice are susceptible to DSS-induced colitis.[57] IL-6 and IL-1β are involved in the development of IBD and IBD-related colon cancer.[58,59] These proinflammatory cytokines are thus molecular targets for the chemoprevention of inflammation-related carcinogenesis.[31,37,55,60,61] They are candidate biomarkers of colon tumorigenesis,[62,63] because the expression of NF-κB, Tnf-α and IL-1β is involved in colonic tumorigenesis by affecting proliferation and apoptosis.[64–67] The activation of NF-κB, a transcription factor that is activated by several cytokines released during inflammation and is responsible for many of their proinflammatory effects, was shown to promote the growth of the colon tumors in experimental models.[31,55,60,68] Because of the strong link of NF-κB to different stress signals, including cigarette smoke, NF-κB has been called a "smoke-sensor" of the body.[69] In this context, the findings that a tobacco-specific carcinogen enhances AOM/DSS-induced colon carcinogenesis[70] are of interest. In addition, Stat3 expression is an important factor in colon carcinogenesis, tumor invasion[71] and survival/proliferation of the colonic preneoplastic cells.[72] In addition, the anti-inflammatory potential of melatonin through the suppression of the expression of NF-κB and chemokines (IL-8 and monocyte chemoattractant protein) in a rat colitis model[73,74] is of interest, and it is important to further investigate the cancer chemopreventive ability of this bioactive substance, as was done in our study.

In our study, the treatment with both compounds in the diet significantly lowered colonic inflammation induced by DSS. As chronic inflammation involves tumorigenesis and accelerate carcinogenic steps, the suppression of chronic inflammation through the modulation of the expression of several proinflammatory gene products that mediate a critical role in several events of carcinogenesis may result in the inhibition of cancer development, and it may also serve as cancer chemoprevention.[55] AUR and GOFA possess anti-inflammatory activities.[20,75] In addition, we previously reported on the cancer chemopreventive ability of AUR[22,76] and a prodrug, GOFA (called GAP in the study[23]) of the secondary metabolite of ferulic acid in colitis-associated colon carcinogenesis.[23,76] Several molecular targets for the suppression of inflammation-associated carcinogenesis were proposed.[36] Our recent study demonstrated that the modulation of inflammation and the expression of COX-2, iNOS and other proteins in the colon contribute to the suppression of colitis-related colon carcinogenesis.[10,56]

CDs (cyclic oligomers of glucose) that have the properties of forming inclusion complexes with lipophilic drugs have been widely used in therapy to improve water solubility and bioavailability of drugs. Target tissue bioavailability is an important determinant of these efficacies of chemopreventive agents.[77] In our study, we selected β-CD, which is soluble in water and organic solvents. When compared the chemopreventive efficacy of the inclusion complexes of GOFA and AUR with β-CD in our study to that of previous studies,[22,23] GOFA with β-CD was superior to GOFA[23] and AUR with β-CD was less effective in comparison to AUR.[22] This may be related to the differences between the thermal stability of GOFA/β-CD and AUR/β-CD. A thermogram of the AUR/β-CD complex showed that this coumarin derivative melting endotherm had a substantial reduction in peak area, thus implying that the molecular arrangement of AUR in the solid complex was different from the pure crystal compound.[25,27,45,46] Also, the different effects of these compounds with or without b-CD in the activity of matrix metalloproteinases of inflamed colon.[78]

In conclusion, the novel prodrugs of GOFA/β-CD and AUR/β-CD are effective in inhibiting colon cancer development in a two-stage colitis-related mouse colon carcinogenesis through modulation of inflammation, proliferation and the expression of several proinflammatory cytokines (NF-κB, Tnf-α, Stat3, Nrf2, IL-6 and IL-1β) in the inflamed colon of the mice that received AOM and DSS. Our findings therefore support the development of novel site-specifically delivered prodrugs for colon cancer prevention in the inflamed colon.

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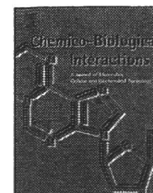

Increased visceral fat mass and insulin signaling in colitis-related colon carcinogenesis model mice

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ABSTRACT

Leptin, a pleiotropic hormone regulating food intake and metabolism, plays an important role in the regulation of inflammation and immunity. We previously demonstrated that serum leptin levels are profoundly increased in mice which received azoxymethane (AOM) and dextran sulfate sodium (DSS) as tumor-initiator and -promoter, respectively, in a colon carcinogenesis model. In this study, we attempted to address underlying mechanism whereby leptin is up-regulated in this rodent model. Five-week-old male ICR mice were given a single intraperitoneal injection of AOM (week 0), followed by 1% DSS in drinking water for 7 days. Thereafter, the weights of visceral fats and the serum concentration of leptin were determined at week 20. Of interest, the relative epididymal fat pad and mesenteric fat weights, together with serum leptin levels in the AOM and/or DSS-treated mice were markedly increased compared to that in untreated mice. In addition, leptin protein production in epididymal fat pad with AOM/DSS-treated mice was 4.7-fold higher than that of control. Further, insulin signaling molecules, such as protein kinase B (Akt), S6, mitogen-activate protein kinase/extracellular signaling-regulated kinase 1/2, and extracellular signaling-regulated kinase 1/2, were concomitantly activated in epididymal fat of AOM/DSS-treated mice. This treatment also increased the serum insulin and IGF-1 levels. Taken together, our results suggest that higher levels of serum insulin and IGF-1 promote the insulin signaling in epididymal fat and thereby increasing serum leptin, which may play an crucial role in, not only obesity-related, but also -independent colon carcinogenesis.

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1. Introduction

Leptin, a 16-kDa protein encoded by the *ob* gene, was discovered in 1994 and shown to be a regulator of body weight and energy balance, functioning in the hypothalamus [1]. Although this hormone is produced mainly by WAT, its expression has also been detected in other tissues, including the gastrointestinal tract [2]. In humans, leptin levels are proportional to body mass index and are elevated in obese individuals [3]. Since obesity is known to increase the risk of certain cancers, great effort has been directed to elucidating the possible role of leptin in cancer development [4], and several *in vitro* studies showed that leptin can act as a mitogenic, anti-apoptotic, and tumorigenic factor with various cancer cell lines [5–7]. In addition, data obtained with clinical samples revealed that leptin and its receptor (Ob-R) are expressed in cancer tissues [8]. Thus, the leptin system might promote cancer progression in an autocrine and paracrine manner.

Our previous investigation demonstrated that serum leptin levels were profoundly increased in male ICR mice that received azoxymethane (AOM) and dextran sulfate sodium (DSS) for colitis-related colon carcinogenesis [9]. In addition, a citrus flavonoid, nobiletin, reduced not only those levels, but also colonic tumor development, whereas did not cause significant alterations in serum levels of other adipocytokines (TNF-α, IL-6, and adiponectin) and triglyceride [9]. These findings led us to address and investigate an issue why and how serum leptin level is elevated in the mice treated with AOM and DSS in a colitis-related colon carcinogenesis model.

2. Materials and methods

2.1. Animals and chemicals

Male Crj:CD-1 (ICR) mice (Japan SLC, Shizuoka, Japan) aged 5 weeks were obtained for this study. The animals were handled according to the Guidelines for the Regulation of Animals pro-

Abbreviations: Akt, protein kinase B; AOM, azoxymethane; CD, Crohn's disease; CRC, colorectal cancer; DSS, dextran sulfate sodium; ERK, extracellular signal-regulated protein kinase; IBD, inflammatory bowel disease; IL-6, interleukin-6; mTOR, mammalian target of rapamycin; Ob-R, leptin receptor; TNF-α, tumor necrosis factor-α.

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vided by the Experimentation Committee of Kyoto University and the experimental protocol was approved by the Committee. Upon arrival, all mice were randomized and transferred to plastic cages (5 mice/cage), with free access to drinking water and a pelleted basal diet MF diet (Oriental Yeast, Kyoto, Japan), and controlled conditions of humidity (60 ± 5%), light (12/12 h light/dark cycle) and temperature (24 ± 2 °C). All mice were quarantined for 1 week before starting the experiments.

The colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 (Cat. No. 160110) was purchased from the MP Biochemicals, LLC (Aurora, OH) and used for induction of colitis, after being dissolved in distilled water to a concentration of 1% (w/v).

2.2. Antibodies

Antibodies directed against phospho-protein kinase B (Akt) (Ser483), phospho-S6 (Ser240/244), phospho-mitogen-activate protein kinase/extracellular signaling-regulated kinase (MEK)1/2 (Ser217/221) and phospho-extracellular signaling-regulated kinase (ERK)1/2 (Thr202/Tyr204), as well as horseradish peroxidase (HRP)-conjugated anti-rabbit antibody were obtained from Cell Signaling Technology (Beverly, MA).

2.3. Animal treatment

A total of 80 male ICR mice were divided into four (3 experimental and 1 control) groups. Group 1 served as an untreated control. Mice in groups 2 and 4 were given a single i.p. injection of AOM (10 mg/kg body weight), then starting 1 week after that injection, those in group 4 received 1% DSS in drinking water for 7 days. Mice on group 3 received DSS alone according to the same time schedule. All groups were fed the basal diet throughout the study. At the end of week 2, 5 mice from each group were euthanized, then 15 mice from each group were euthanized after week 20. Euthanasia was performed under ether anesthesia, then blood samples were collected from the abdominal aorta. During the study, body weights of all animals were weighted weekly. At sacrifice, all organs, including adipose tissue, were removed and weighted, and then obtained adipose tissues were frozen using liquid nitrogen, until later use.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the epididymal fat pads using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA with an RNA PCR Kit (AMV). PCR amplification was performed using a thermal cycler (PTC-100TM, MJ Research, Watertown, MA) and conducted with each sense and antisense primer, with *GAPDH* used as the internal standard. The primers for *leptin* were 5′-CCAAAACCCTCATCAAGACC-3′ and 5′-GTCCAACTGTTGAAGAATGTCCC-3′ (Proligo, Kyoto, Japan), which yielded an expected band size of 395 bp, while those for *GAPDH* were 5′-GTGAAGGTCGGA-GTCAACG-3′ and 5′-GGTGAAGACGCCAGTGGACTC-3′ (Proligo), which yielded an expected band size of 300 bp. The primers were used at final concentrations of 0.5 and 0.05 μM, respectively. PCR was performed under the following conditions: 43 cycles at 95 °C for 45 s, 57 °C for 45 s, and 72 °C for 45 s for *leptin*, and 27 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for *GAPDH*. The PCR products were subjected to electrophoresis on 3% agarose gels and stained with SYBR® Gold. The intensity of each band was analyzed using Scion Image.

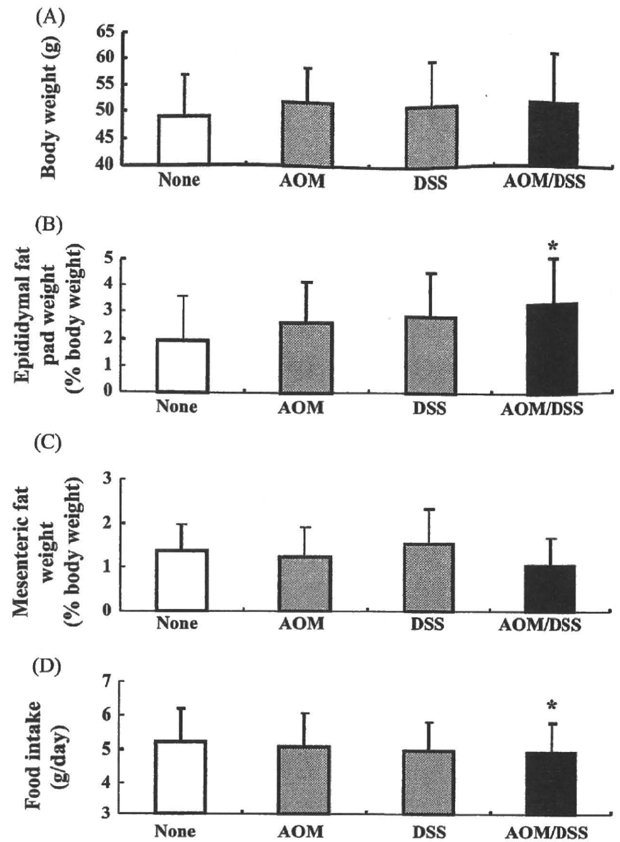

Fig. 1. The mean food intake (A) by the mice during the study, body (B), epididymal fat pad (C), and mesenteric fat (D) weights. The weights were measured at the end of the study.

2.5. Western blotting

To determine the protein expressions of phospho-Akt, phospho-S6, phospho-MEK1/2, and phospho-ERK1/2 in the epididymal fat pads, tissue supernatants were subjected to Western blotting. Frozen adipose tissue was lysed in lysis buffer [10 nM Tris, pH 7.4, 1% sodium dodecyl sulfate (SDS), 1 mM sodium metavanadate (V)], and centrifuged at 3200 × *g* for 5 min. Denatured proteins (40 μg) were separated using SDS-PAGE on a 10% polyacrylamide gel and then transferred to Immobilon-P membranes (Millipore, Billerica, MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h, the membranes were reacted with the corresponding specific primary antibody (1:1000), followed by the corresponding HRP-conjugated secondary antibody (1:1000). The blots were developed using ECL Western blotting detection

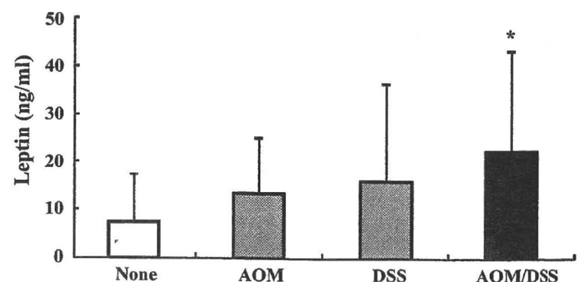

Fig. 2. Serum leptin levels of all groups. The levels were quantified by ELISA, as described in Section 2. Each value is shown as the mean ± SD (n = 15). Statistical analysis was performed using Student's t-test: *P < 0.05 vs. the untreated control (group 1).

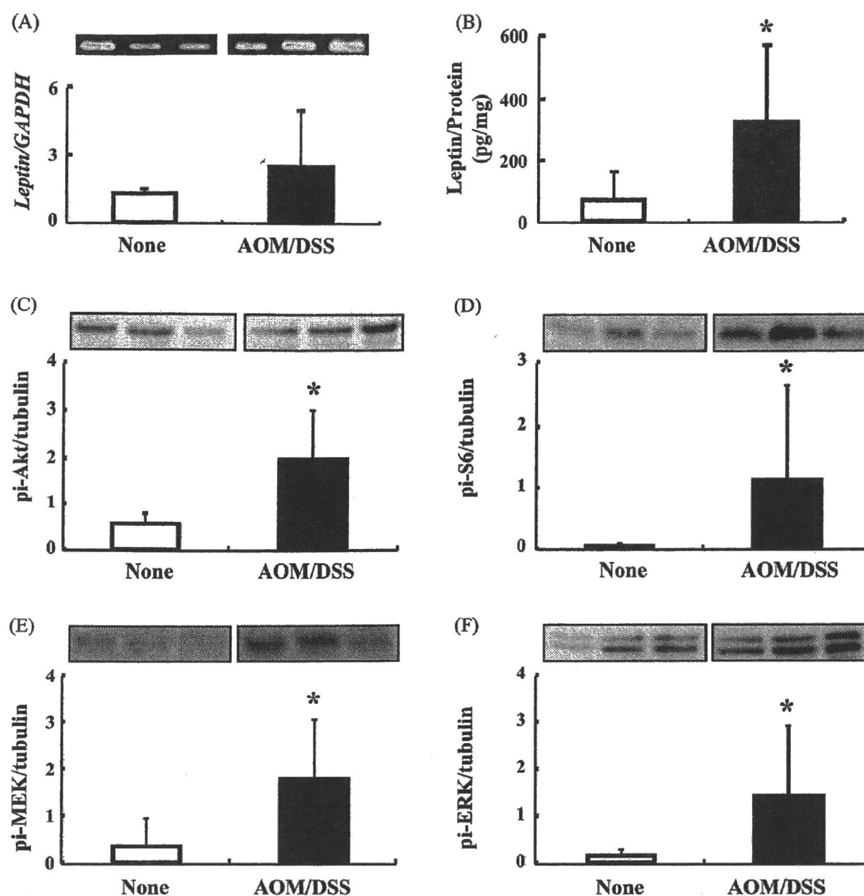

Fig. 3. Leptin production and activation of insulin signaling pathway in epididymal fat pads at the end of the study. (A) Leptin mRNA expression was determined by RT-PCR, as described in Section 2, with the levels corrected by those of GAPDH. Each values is shown as the mean ± SD (n = 5), with 3 representative results presented. (B) Leptin protein amounts were quantified by ELISA, as described in Section 2. Each value is shown as the mean ± SD (n = 10). (C–F) Activation of the insulin signaling pathway was examined by Western blotting, as described in Section 2. Each value is shown as the mean ± SD (n = 10), with 3 representative results presented. Statistical analysis was performed using Student's t-test: *P < 0.05 vs. the untreated control (group 1).

reagents. The intensity of each band was analyzed using Scion Image.

2.6. Clinical chemistry

The collected blood samples were used for clinical chemistry measurements of leptin (Quantikine Mouse leptin, ELISA/Assay Kit, R&D Systems Inc.), insulin (Insulin measurement kit, Morinaga Institute of Biological Science), and insulin-like growth factor (IGF)-1 (Quantikine Mouse IGF-1, ELISA/Assay Kit, R&D Systems Inc.). The samples were used without dilution for insulin measurements, while they were diluted 20- and 500-fold for leptin and IGF-1.

2.7. Statistical analysis

Data were analyzed using Student's t-test (two-sided) when appropriate, with P < 0.05 considered to indicate significance.

3. Results

3.1. General observations of mice

Food intake was significantly (P < 0.05) lower in group 4 as compared to group 1 (Fig. 1A). The mean body weights of mice in groups 2 through 4 are higher than group 1, though the differences did not reach statistical significance (Fig. 1B). In addition, the AOM and/or DSS treatment led to notable increases in mean relative epididymal fat pad weight (g/100 g body weight), which was most notable in group 4 (AOM and DSS), as the weight was significantly (P < 0.05) increased by 1.7-fold as compared to group 1 (Fig. 1C). However, the mean relative mesenteric fat weight (g/100 g body weight) did not significantly differ among the groups (Fig. 1D).

3.2. Leptin level in the serum and epididymal fat pad

As shown in Fig. 2, the serum concentration of leptin in group 4 was significantly (P < 0.05) elevated by 3.0-fold as compared to group 1, which is consistent with our previous findings [9]. The serum leptin levels in groups 2 and 3 were also increased by 1.8- and 2.2-fold as compared to group 1, respectively, without statistical significance. Since leptin is produced mainly by WAT, we assessed the level of leptin mRNA and protein expression in epididymal fat pads. Leptin mRNA expression in the mice treated with both AOM and DSS (group 4) was increased by 2.0-fold as compared to the untreated control (Fig. 3A), while leptin protein production was also increased by 4.7-fold (P < 0.05) in group 4 mice (Fig. 3B).

3.3. Activation of insulin signaling pathway in epididymal fat pads

Insulin is an important physiological factor that activates mammalian target of rapamycin (mTOR), which is a master regulator of protein synthesis [10], adipose tissue morphogenesis [11], and leptin synthesis/secretion [12] in adipocytes. To determine whether the insulin signaling pathway was activated in the epididymal fat pads of the present ICR mice, we determined the phospho-

rylation status of Akt, S6, MEK1/2, and ERK1/2, all of which are involved in that pathway [13]. As shown in Fig. 3, AOM and DSS treatment substantially increased the phosphorylation of Akt (3.5-fold, $P<0.05$, Fig. 3C), S6 (38-fold, $P<0.05$, Fig. 3D), MEK (5.4-fold, $P<0.05$, Fig. 3E), and ERK (8.8-fold, $P<0.05$, Fig. 3F), respectively, as compared to the untreated control mice.

3.4. Serum levels of insulin and IGF-1

We also assessed the serum levels of insulin and IGF-1 at two time-points, week 2 (just after the cessation of the DSS treatment) and 20 (the termination of the study). As shown in Fig. 4A, serum insulin was elevated in a time-dependent manner by 2.1- and 7.8-fold in groups 1 and 4, respectively. Meanwhile, the level of IGF-1 in group 4 was significantly ($P<0.05$) higher at week 20 than that in group 1, which was in contrast to the findings at week 2 (Fig. 4B).

4. Discussion

Colorectal cancer (CRC) is the most serious complication associated with long standing inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease (CD) [14,15]. Mesenteric WAT is mainly hypertrophic around the intestines in IBD patients, with axial polarity of inflammation the mainstay contributor [16]. To better understand the pathogenesis of IBD-related CRC, one (T.T.) of the present authors and colleagues developed a novel colitis-related CRC model (a two-stage mouse colon carcinogenesis model), which were initiated with AOM and promoted by DSS [17]. In the current study, such sequential treatment significantly increased serum leptin and epididymal fat pad weight, whereas mesenteric fat was not altered (Fig. 1C and D). The individual potencies were less than their combination, suggesting that AOM and DSS act additively to increase the concentration of leptin in serum.

Bendet et al. reported that insulin secretion during a 3-h oral glucose-tolerance test was increased and homeostatic model assessment values were decreased in CD patients [18]. In a later study, Bregenzer et al. found increased insulin resistance and β cell function in CD patients [19]. Furthermore, it should be noted that the concentration of serum C-peptide, a stable indicator of steady-state insulin secretion, was reported to be higher in AOM-treated male rats [20]. Since, it is well known that insulin resistance increases the level of serum IGF-1 [21], the results of this study are consistent with these observations. Furthermore, it is reported that IGF-1 mRNA is markedly up-regulated in the muscularis propria, lamina propria cells and submucosa in inflamed colon [22,23]. Taken together, the increased level of IGF-1 in serum of the present group 4 mice (Fig. 4B) may have been associated with putative insulin resistance and up-regulation of its mRNA in the colon caused by AOM/DSS-induced inflammation with other pathologic conditions, including oxidative stress.

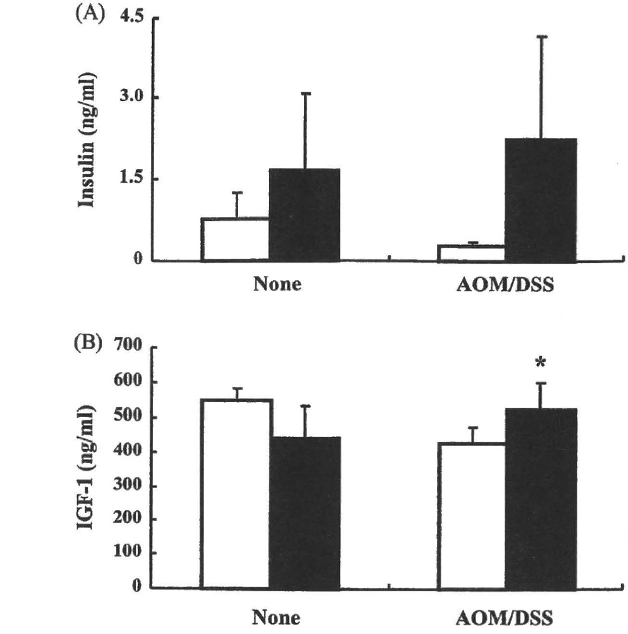

Fig. 4. Levels of serum insulin (A) and IGF-1 (B) after weeks 2 and 20. Serum insulin and IGF-1 levels were quantified by ELISA, as described in Section 2. Open bars; at week 2, closed bars; at week 20. Each value is shown as the mean ± SD ($n=5$ or 15). Statistical analysis was performed using Student's t-test: $^{*}P<0.05$ vs. the untreated control (group 1) for the corresponding time point.

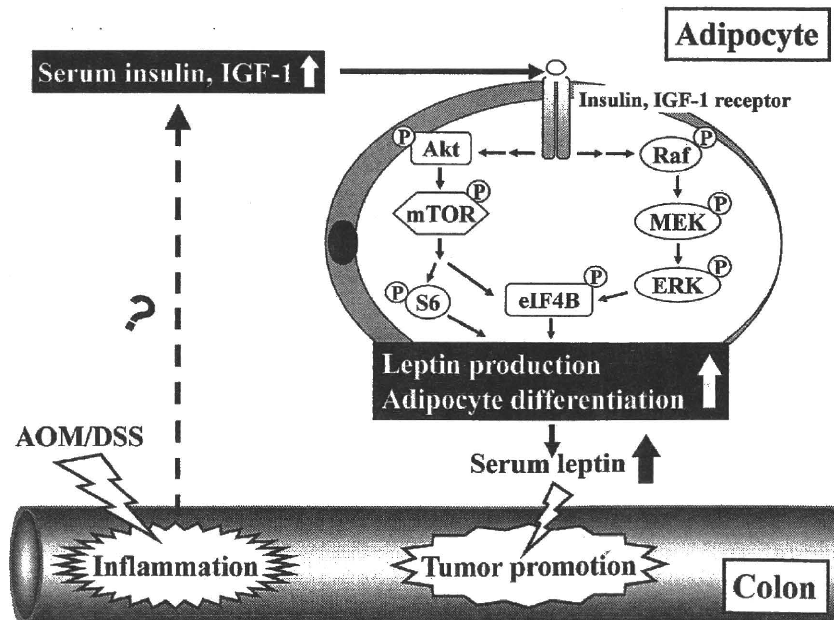

Fig. 5. Proposed schema of events may be involved in the elevation of serum leptin level in AOM and DSS-treated mice. AOM and DSS treatment induces colitis and increases serum insulin/IGF-1 levels by an unknown mechanism. This leads to the activation of the insulin/IGF signaling pathway for adipocyte differentiation and protein translation that induce serum leptin elevation.

Under physiological conditions, serum leptin levels are affected by several factors, and leptin secretion from adipocytes is dominantly dependent on the status of adipocyte signaling molecules. A previous study showed that both insulin and IGF-1 have been reported to be important physiological factors activating mTOR. Activation of this pathway mediates multiple insulin effects, such as stimulation of adipogenesis or lipogenesis [24], and potentiation of leptin secretion. In the present study, the insulin/IGF signaling pathway was consistently activated, and leptin induction and production were promoted in the epididymal fat of mice treated with AOM and DSS (Fig. 3A–F). Thus, it is suggested that these hormones stimulate differentiation and expansion of adipocytes in visceral fat tissue, thereby increasing total amount of epididymal fat tissue. Furthermore, these hormones also accelerate leptin production per one single adipocyte. However, additional studies are necessary to confirm that whether or not these phenomena are specific for epididymal fat pad.

In conclusion, we found that higher levels of serum insulin and IGF-1 promote insulin signaling in epididymal fat, which may lead to increased amounts of visceral fat tissue and a resulting elevation of serum leptin (Fig. 5). These events may play certain critical roles in the development of CRC. However, further studies are warranted for understanding the precise mechanisms underlying AOM and DSS-induced adipose tissue alterations.

Conflict of interest statement

None.

Acknowledgements

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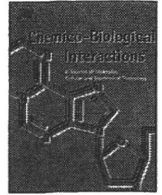

Dietary flavonoids suppress azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-*db*/*db* mice

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ABSTRACT

Obesity is known to be a risk factor for colon carcinogenesis. Although there are several reports on the chemopreventive abilities of dietary flavonoids in chemically induced colon carcinogenesis, those have not been addressed in an obesity-associated carcinogenesis model. In the present study, the effects of 3 flavonoids (chrysin, quercetin and nobiletin) on modulation of the occurrence of putative preneoplastic lesions, aberrant crypt foci (ACF), and β-catenin-accumulated crypts (BCACs) in the development of colon cancer were determined in male *db*/*db* mice with obesity and diabetic phenotypes. Male *db*/*db* mice were given 3 weekly intraperitoneal injections of azoxymethane (AOM) to induce the ACF and BCAC. Each flavonoid (100 ppm), given in the diet throughout the experimental period, significantly reduced the numbers of ACF by 68–91% and BCAC by 64–71%, as well as proliferation activity in the lesions. Clinical chemistry results revealed that the serum levels of leptin and insulin in mice treated with AOM were greater than those in the untreated group. Interestingly, the most pronounced suppression of development of preneoplastic lesions and their proliferation were observed in the quercetin-fed group, in which the serum leptin level was lowered. Furthermore, quercetin-feeding decreased leptin mRNA expression and secretion in differentiated 3T3-L1 mouse adipocytes. These results suggest that the present dietary flavonoids are able to suppress the early phase of colon carcinogenesis in obese mice, partly through inhibition of proliferation activity caused by serum growth factors. Furthermore, they indicate that certain flavonoids may be useful for prevention of colon carcinogenesis in obese humans.

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1. Introduction

Epidemiological studies have shown that obesity is an important cofactor for several types of cancer, including colorectal cancer [1]. Recently, a prospective population based study of about 90,000 subjects conducted by the American Cancer Society confirmed that obesity is directly associated with an increased risk of death from colon cancer [2]. In addition, animal studies have also suggested that obesity enhances tumor development [3], while calorie restriction was reported to inhibit a broad range of spontaneous, transplanted, and chemically induced neoplasms [4]. In fact, the hypothesis that obesity-associated disarrayed metabolic and hormonal plasma parameters, including insulin, triglycerides, glucose and free fatty acids, may promote ACF development and colon cancer has been proposed in the past decade [5]. However, the underlying mechanisms of obesity-related colon carcinogenesis are not fully understood until now.

Leptin, a 16-kDa protein encoded by the *ob* gene, was first documented in 1994 as a regulator of body weight gain and energy balance, with its activities displayed in the hypothalamus [6]. It is well known that serum leptin levels are highly elevated in obese individuals [7] and the protein is mainly secreted by white adipocytes [8]. C57BL/KsJ-*db*/*db* (*db*/*db*) mice are often used as a genetically altered animal model with the genotypes of obesity and diabetes mellitus [9]. In this mouse strain, a mutation in the cytoplasmic domain of the long form of the leptin receptor (Ob-Rb) results in loss of expression of this isoform [10]. In the absence of Ob-Rb, the mice eat excessively and are already obese at 4 weeks of age. Furthermore, they are also demonstrate hyperleptinemia, hyperinsulinemia, hyperglycemia, and hyperlipidemia, as well as increased levels of cholesterol in plasma [11]. The synthesis of leptin in adipocytes, which may be involved in neoplastic processes, is influenced by insulin, tumor necrosis factor-α, glucocorticoids,

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCAC, β-catenin-accumulated crypt; IGF-1, insulin-like growth factor-1; PCNA, proliferating cell nuclear antigen; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor.

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reproductive hormones, and prostaglandins [12]. In addition, leptin can act as a growth factor in colonic epithelial cells [13], while it also modulates the proliferation of colonic cryptal cells [14]. Since leptin might be one of the biological factors involved in the development of colorectal cancer associated with obesity/diabetes, *db/db* mice are quite useful as a model for elucidating the relationship between colon carcinogenesis and obesity/diabetes.

Flavonoids comprise a structurally diverse class of polyphenolic compounds ubiquitously found in plants and produced as a result of plant secondary metabolism [15]. They have several biological effects, such as anti-oxidative and anti-inflammatory activities [16]. We previously reported that chrysin [17], quercetin [18], and nobiletin [19] showed chemopreventive effects toward azoxymethane (AOM)-induced colon carcinogenesis in rats. In addition, administration of green tea polyphenols, including epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, resulted in a significant reduction in body weight gain and body fat accumulation in rodents [20,21]. Furthermore, an *in vitro* study found that certain flavonoids inhibit the growth of 3T3-L1 pre-adipocytes [22]. However, there are known no studies of the effects of flavonoids on obesity-associated carcinogenesis.

In the present study, we first determined the modulatory effects of six different flavonoids; flavone, chrysin, apigenin, luteolin, quercetin, and nobiletin, on leptin secretion from 3T3-L1 cells. Next, we evaluated the effects of dietary chrysin, quercetin, and nobiletin on the occurrence of AOM-induced aberrant crypt foci (ACF) and β-catenin-accumulated crypts (BCACs), putative precursor lesions for colonic adenocarcinoma [23,24], in *db/db* male mice. We also investigated those three flavonoids to determine their effects on clinical chemistry related to the occurrence of colorectal cancer [25]. Since we previously observed high proliferation activities in preneoplastic colonic lesions and non-lesional crypts in *db/db* mice [26], the effects of these flavonoids in regard to proliferation activity in ACF and BCAC were analyzed using an immunohistochemical methods.

2. Materials and methods

2.1. Cell culture

3T3-L1 mouse pre-adipocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum (BS), as well as 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Intracellular lipid accumulation and leptin secretion

3T3-L1 cells (1×10^4/200 μl/well) were seeded into 96-well plates under the growth conditions described above. After reaching confluence, they were incubated for an additional 24 h (designated as day 0). Then, adipocyte differentiation was induced by treatment with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml), components of an Adipogenesis Assay Kit (Chemicon International, Temecula, CA), in DMEM containing 10% FBS for 48 h. The medium was then replaced by DMEM containing 10% fetal bovine serum (FBS) and insulin (5 μg/ml), and changed to fresh medium every 2 days, according to a method previously described by Maeda et al. [27], with some modifications. On day 2, each flavonoid (10, 50, and 100 μM) was dissolved in dimethyl sulfoxide (DMSO), then added to DMEM containing FBS and insulin. The final concentration of DMSO was 0.1% (v/v), which was found to have no effect on cell growth (data not shown). After 12 days, the medium was collected and subjected to ELISA to determine the levels of leptin. The cells were stained with the Oil Red-O component of an Adipogenesis Assay Kit, according to the manufacturer's instructions. Stained oil droplets in 3T3-L1 cells were extracted with dye extraction solution and absorbance of the extracts was measured at 490 nm.

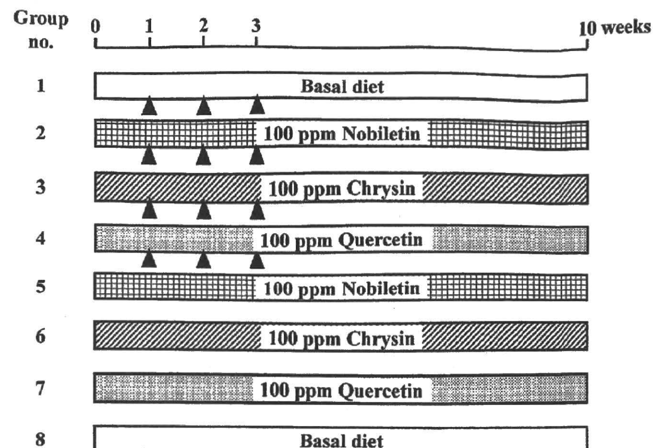


Fig. 1. Experimental protocol for the *in vivo* study. All mice were divided into the following eight experimental and control groups. They were given three weekly intraperitoneal injections of AOM (15 mg/kg body weight, ▲) or saline. Mice in groups 2 through 7 were fed the experimental diets containing each of the flavonoids (100 ppm) for the entire 10-week experimental period. Groups 1 and 8 were given the basal diet without the flavonoids during the study.

2.3. Mice, diet, and carcinogens

Male *db/db* mice were obtained from Jackson Laboratories (Bar Harbor, ME) at the age of 4 weeks and maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. On arrival, all mice were randomized and transferred to plastic cages (2 or 3 mice/cage), and given free access to drinking water and a pelleted basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle), and temperature (23 ± 2 °C). All mice were quarantined for 1 week before starting the experiment. Nobiletin (>98% purity) was obtained from Nard Chemicals (Hyogo, Japan), while other flavonoids were purchased from WAKO Pure Chemicals (Osaka, Japan). Experimental diets were prepared by mixing each flavonoid (100 ppm) separately with powdered CRF-1 every week during the study. Azoxymethane (AOM), a colonic carcinogen, was purchased from Sigma Chemical Co. (St. Louis, MO).

2.4. Experimental procedures

As shown in Fig. 1, all mice were divided into the following eight experimental and control groups: AOM alone (group 1, $n = 9$); AOM + chrysin (group 2, $n = 10$); AOM + quercetin (group 3, $n = 10$); AOM + nobiletin (group 4, $n = 10$); chrysin alone (group 5, $n = 5$); quercetin alone (group 6, $n = 5$); nobiletin alone (group 7, $n = 5$); and untreated (group 8, $n = 5$). All mice were given 3 weekly intraperitoneal injections of AOM (15 mg/kg body weight) or saline, while those in groups 2 through 7 were fed the experimental diets containing the flavonoids (100 ppm) for the entire 10-week experimental period. Groups 1 and 8 were given the basal diet without flavonoids during the study. At week 10, all mice were euthanized after overnight fasting by an intraperitoneal injection of sodium pentobarbital (1 mg/kg body weight). Blood samples were taken from the portal vein before the mice were killed. A complete necropsy was done, and all organs, including the colon, were removed, with the liver, kidneys, pancreas, and epididymal adipose tissue weighted.

2.5. Counting colonic ACF and BCAC

The numbers of ACF and BCAC were determined according to standard procedures described previously [28,29]. Briefly, the colons were cut, placed on filter paper with the mucosal surface up, and fixed in 10% buffered formalin for at least 24 h. The fixed colons were stained with methylene blue (0.5% in distilled water) for 20 s, dipped in distilled water, and placed on microscope slides to count the number and determine the size of ACF. Rectal mucosa (2.0 cm from the anus) was embedded in paraffin to identify intramucosal lesions, considered to be BCAC. A total of 20 serial sections (4 μm thick each) per rectum were prepared using an *en face* method [29]. For each mouse, 2 serial sections were used to analyze the BCAC. The numbers of BCAC in histological sections stained with β-catenin were counted and are expressed as the number of BCAC per cm^2 of mucosa.

2.6. Immunohistochemistry of β-catenin and PCNA

Immunohistochemistry for β-catenin was performed using sections from the distal colon segments with a labeled streptavidin-biotin method (LSAB Kit; Dako, Glostrup, Denmark) and microwave accentuation. Paraffin-embedded sections were heated for 30 min at 65 °C, deparaffinized in xylene, and rehydrated through a graded series of alcohol at room temperature. A 0.05-M Tris–HCl buffer (pH 7.6) was used to prepare the solutions and for washing between the steps. The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4 °C with the primary antibody against β-catenin protein (diluted 1:1000, BD Transduction Laboratories, Lexington, KY). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. Negative-control sections were immunostained without the primary antibody. Immunoreactivity to determine the presence of BCAC was regarded as positive if apparent staining was detected in the cytoplasm and/or nuclei.

For PCNA immunohistochemistry, formalin-fixed, paraffin-embedded distal colon sections were subjected to deparaffinization and dehydration prior to quenching of endogenous peroxidase activity (1.5% H_2O_2 in methanol for 20 min). An antigen-unmasking step was done by placing the slides in a pressure cooker containing 0.01 M sodium citrate (pH 6.0) for 10 min. The sections were incubated for 60 min with the primary mouse anti-rat PCNA monoclonal antibody (Clone PC-10, DakoCytomation) at a dilution of 1:1500 in 10% goat serum. A secondary antibody, biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA), was then applied for 30 min in a 1:500 dilution. Slides were processed with ABC reagent from a Vectastain Elite kit (Vector Laboratories) using DAB as the substrate. Using distal colonic mucosa without lesions from 5 mice in each group, 20 fields were randomly selected from each slide and analyzed at 400× magnification. PCNA-positive cell nuclei were determined in 10 ACF and 10 BCAC each from groups 1 through 4. Cells stained positive for PCNA were scored and expressed as a percentage of total cells in each lesion.

2.7. Clinical chemistry

The collected blood samples were used for clinical chemistry. Leptin (Quantikine Mouse leptin, ELISA/Assay Kit, R&D Systems Inc.), adiponectin (Mouse/Rat adiponectin ELISA kit, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), insulin-like growth factor (IGF)-1 (Quantikine Mouse IGF-1, ELISA/Assay Kit, R&D Systems Inc.), insulin (Insulin measurement kit, Morinaga Institute of Biological Science), triglycerides (Triglyceride E-test, Wako Pure Chemical Industries), cholesterol (Cholesterol E-test, Wako Pure Chemical Industries), and glucose (Glucose CII-test Wako, Wako Pure Chemical Industries) levels were measured. Serum samples without dilution were used for determining insulin, triglycerides, cholesterol, and glucose, while those diluted 100-, 10201-, and 500-fold were used for determining the levels of leptin, adiponectin, and IGF-1, respectively.

2.8. Western blotting

3T3-L1 cells (1×10^5/3 ml/dish) were seeded into 35-mm dishes following treatment with quercetin or nobiletin, then washed twice with PBS and lysed in lysis buffer [10-nM Tris (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1-mM sodium metavanadate (V)], and centrifuged at 3200 × g for 5 min. Denatured proteins (40 μg) were separated using SDS-PAGE on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h, the membranes were reacted with the appropriate specific primary antibody (1:1000), followed by the corresponding HRP-conjugated secondary antibody (1:1000). The blots were developed using ECL Western blotting detection reagents. Antibodies directed against Pi-mitogen-activated protein kinase/extracellular signaling-regulated kinase (MEK)1/2 (Ser217/221), Pi-extracellular signaling-regulated kinase (ERK)1/2 (Thr202/Tyr204), Pi-mammalian target of rapamycin (mTOR) (Ser2448), Pi-S6 (Ser240/244), and Pi-eukaryotic initiation factor 4B (eIF4B) (Ser422), as well as horseradish peroxidase (HRP)-conjugated anti-rabbit antibody, were obtained from Cell Signaling Technology (Beverly, MA).

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells under the same conditions used for Western blotting using TRIzol reagent, according to the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA and an RNA PCR Kit (AMV). PCR amplification was performed using a thermal cycler (PTC-100™, MJ Research, Watertown, MA), and conducted with each sense and antisense primer. The primer sequences and PCR conditions are listed in Table 1. A cyclophilin transcript served as the internal control. PCR products were subjected to electrophoresis through 3% agarose gels and stained with SYBR® Gold.

2.10. Statistical analysis

Where applicable, data were analyzed using a Tukey–Kramer multiple comparison test (GraphPad InStat version 3.05, GraphPad Software, San Diego, CA), Fisher's exact probability test, and Student's *t*-test (two-sided), with $P < 0.05$ as the criterion of significance.

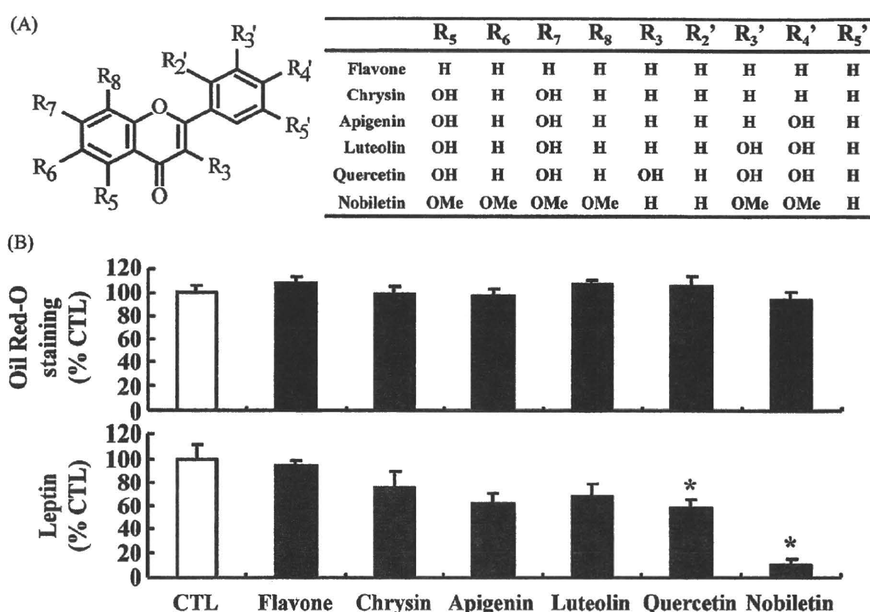
3. Results

3.1. Modulatory effects of flavonoids on Oil Red-O staining and leptin secretion

Adipocyte differentiation was induced by treatment with a mixture of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin in DMEM containing 10% FBS for 48 h, after which differentiated 3T3-L1 adipocytes were separately treated with the six flavonoids (10 μM, Fig. 2A) or the vehicle for 12 days to determine their effects on intracellular lipid accumulation and leptin secretion. Differentiated 3T3-L1 cells were notably loaded with lipid, as detected by Oil Red-O staining, whereas none of the flavonoids had noticeable effects (Fig. 2B). On the other hand, quercetin and nobiletin significantly reduced leptin secretion ($P < 0.01$, Fig. 2B), with the reduction by nobiletin remarkable.

Table 1
List of primer sequences for RT-PCR.

Gene	Primer	Sequence (5′–3′)	Product size (bp)	Cycles	Denaturation Annealing (°C, sec) Extension
Leptin	Forward Reverse	CCA AAA CCC TCA TCA AGA CC GTC CAA CTG TTG AAG AAT GTC CC	395	37	95, 45 57, 45 72, 45
C/EBP α	Forward Reverse	AGG TGC TGG AGT TGA CCA GT CAG CCT AGA GAT CCA GCG AC	238	25	94, 60 54, 60 72, 30
PPAR γ	Forward Reverse	GGT GAA ACT CTG GGA GAT TC CAA CCA TTG GGT CAG CTC TT	268	30	94, 40 58, 40 72, 50
Cyclophilin	Forward Reverse	TTG GGT CGC GTC TCG TTC GA GCC AGG ACC TGT ATG CTT CA	240	20	95, 30 50, 30 72, 60



	R_5	R_6	R_7	R_8	R_3	R_2'	R_3'	R_4'	R_5'
Flavone	H	H	H	H	H	H	H	H	H
Chrysin	OH	H	OH	H	H	H	H	H	H
Apigenin	OH	H	OH	H	H	H	H	OH	H
Luteolin	OH	H	OH	H	H	H	OH	OH	H
Quercetin	OH	H	OH	H	OH	H	OH	OH	H
Nobiletin	OMe	OMe	OMe	OMe	H	H	OMe	OMe	H

Fig. 2. (A) Chemical structures of the flavonoids studied. (B) Modulatory effects of the flavonoids on leptin secretion from 3T3-L1 cells. 3T3-L1 mouse pre-adipocytes were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 h. Differentiated 3T3-L1 cells were treated with DMSO alone or various concentrations of nobiletin for 12 days, then the supernatants were removed for measurements of leptin. The cells were washed twice with PBS and stained with Oil Red-O. Stained cells were viewed under an inverted microscope (Leica Microsystems, Tokyo, Japan) (original magnification 1:200). Leptin secretion was quantified by ELISA. Values are shown as the mean ± SD. Statistical analysis was performed using Student's *t*-test: *$P<0.05$ vs. CTL (DMSO-treated cells).

3.2. General observations of in vivo experiment

We selected 3 of the flavonoids, chrysin, quercetin, and nobiletin, for the *in vivo* experiment based on their chemopreventive efficacy previously shown in colon carcinogenesis models [17–19], together with the present data regarding leptin secretion (Fig. 2B). To investigate the effects of these flavonoids on the early phase of obesity-related carcinogenesis and serum levels of leptin, we performed short-term *in vivo* assays using histological biomarkers, ACF and BCAC, in the *db*/*db* mice. During the study, dietary feeding with the flavonoids did not cause clinical symptoms, including toxicity (data not shown). Body weight gain and food consumption did not markedly differ among the groups except the third week (Fig. 3). Body weight and food consumption of AOM-treated mice (groups 1–4) were slightly low when compared with the control groups (groups 5–8) at the third week. We did not observe significant changes in regard to epididymal fat weight or colon length (Table 2). In contrast, the pancreas weight was significantly increased in group 3 (AOM + quercetin, $P<0.05$) when compared with group 1 (AOM alone).

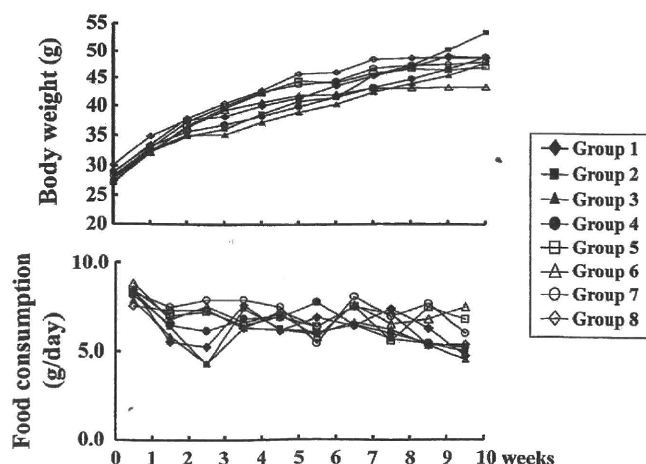


Fig. 3. (A) Body weight and (B) food consumption changes during the experiment period. The amount of food consumption (g/day) are indicated here as means at respective week.

Table 2
General observations in male *db/db* mice.

Group no.	Treatment	Epididymal fat weight (g)	Pancreatic weights (g/100 g body weight)	Length of large bowel (cm)
1	AOM	2.42 ± 0.22	0.530 ± 0.078	11.8 ± 0.4
2	AOM + 100 ppm CHR	2.31 ± 0.28	0.661 ± 0.127	11.9 ± 0.4
3	AOM + 100 ppm QER	2.45 ± 0.31	0.666 ± 0.083[a]	12.1 ± 0.3
4	AOM + 100 ppm NOB	2.47 ± 0.40	0.555 ± 0.013	12.1 ± 0.4
5	100 ppm CHR	2.42 ± 0.35	0.802 ± 0.205[a]	11.6 ± 0.7
6	100 ppm QER	2.63 ± 0.35	0.750 ± 0.209	11.6 ± 0.4
7	100 ppm NOB	2.38 ± 0.36	0.740 ± 0.057[a]	11.9 ± 0.5
8	None	2.35 ± 0.46	0.686 ± 0.162	11.5 ± 1.0

CHR, chrysin; QER, quercetin; NOB, nobiletin. Data are shown as the mean ± SD.
[a] Significantly different in Student's *t*-test, $P < 0.05$ vs. group 1.

3.3. *Frequency of preneoplastic lesions (ACF and BCAC) and PCNA-labeling index*

Histological examinations revealed no pathological lesions in any organs except the colon. Table 3 summarizes data for colonic ACF and BCAC formation. All mice in groups 1 through 4, which received AOM with or without a flavonoid, developed ACF (Fig. 4A). In groups 5 through 8, there were no microscopically observable changes, including ACF and BCAC, in our examinations of colonic morphology. The mean number (±SD) of ACF per colon in group 1 was 25.6 ± 8.9. Dietary administration of chrysin, quercetin, and nobiletin significantly reduced ACF incidence by 91%, 89%, and 68%, respectively ($P < 0.001$ versus group 1), while we also saw a significant reduction (85–100% inhibition, $P < 0.001$) in the numbers of large ACF containing four or more aberrant crypts, which are known to be well-correlated with the incidence of colonic adenocarcinoma [30–32], when compared with group 1 (12.5 ± 9.7). Also, large ACF did not develop in the colons of mice in group 3 (AOM + quercetin). As shown in Table 3, the numbers of BCAC (Fig. 4B) per cm^2 in groups 2 (65% inhibition, $P < 0.001$), 3 (71% inhibition, $P < 0.001$), and 4 (64% inhibition, $P < 0.001$) were significantly fewer than that in group 1 (12.5 ± 9.7).

As summarized in Table 3, the mean PCNA-labeling indices of BCAC were greater than those of ACF (Fig. 4C and D) in groups 1 through 4. ACF indices in the mice that received dietary flavonoids (28% reduction by chrysin, $P < 0.05$; 30% reduction by quercetin, $P < 0.001$; and 20% reduction by nobiletin, $P < 0.05$) were significantly smaller than that of mice that received AOM alone (group 1, 39.1 ± 5.2). Also, feeding with chrysin (26% reduction, $P < 0.001$), quercetin (41% reduction, $P < 0.001$), and nobiletin (19% reduction, $P < 0.001$) significantly lowered index for BCAC when compared with group 1 (48.8 ± 6.0).

3.4. *Serum levels of leptin, adiponectin, IGF-1, insulin, triglyceride, cholesterol, and glucose*

Serum profile data are listed in Table 4. The serum concentration of leptin in group 1 was significantly greater (67% increase) than that in group 8 (untreated, $P < 0.05$), while dietary administration of quercetin significantly decreased the serum leptin level by 31% ($P < 0.05$) when compared with group 1. Chrysin feeding also decreased the serum leptin level (11% decrease), though it was not significant. Dietary nobiletin did not have an effect on the level of leptin in serum. The serum level of adiponectin in

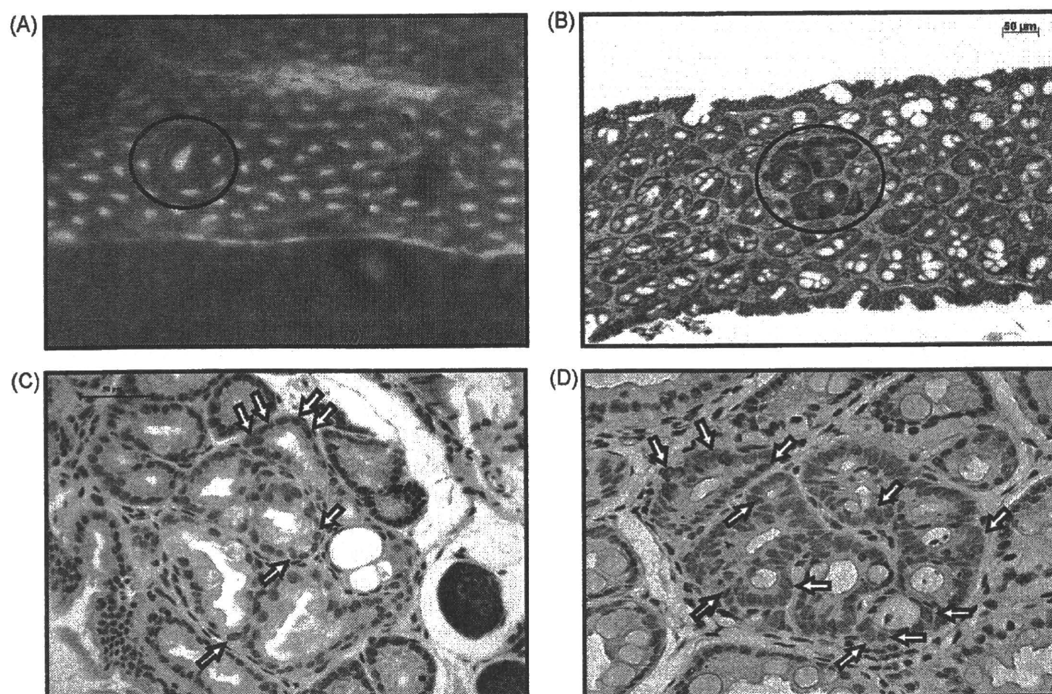


Fig. 4. Representative morphology of preneoplastic lesions (ACF and BCAC) and immunohistochemistry of β-catenin- and PCNA-immunohistochemistry of the lesions in the *db/db* mice that received AOM (group 1). (A) ACF (circled) was observed on the methylene-blue-stained colonic mucosa; (B) BCAC (circled) was detected by β-catenin immunohistochemistry. Arrows in (C) ACF and (D) BCAC are PCNA-labeled nuclei.

Table 3
Inhibitory effects of flavonoids on the development of AOM-induced preneoplastic lesion and PCNA-labeling index.

Group no.	Treatment	Total no. of ACF/colon	Total no. of ACF containing 4 or more ACs/colon	Total no. of BCAC/cm²	PCNA-labeling index (%)	
					ACF	BCAC
1	AOM	25.6 ± 8.9	4.6 ± 2.4	12.5 ± 9.7	39.1 ± 5.2	48.8 ± 6.0
2	AOM + 100 ppm CHR	2.3 ± 2.2[a]	0.1 ± 0.3[a]	4.4 ± 3.1[b]	30.5 ± 6.2[b]	35.9 ± 6.3[c]
3	AOM + 100 ppm QER	2.8 ± 2.0[a]	0	3.6 ± 2.6[c]	27.5 ± 6.3[a]	28.7 ± 9.2[a]
4	AOM + 100 ppm NOB	8.3 ± 4.9[a]	0.7 ± 1.3[a]	4.5 ± 3.2[b]	31.4 ± 6.2[b]	39.5 ± 6.9[b]
5	100 ppm CHR	0	0	0	0	0
6	100 ppm QER	0	0	0	0	0
7	100 ppm NOB	0	0	0	0	0
8	None	0	0	0	0	0

ACF, aberrant crypt foci; AC, aberrant crypts; BCAC, β-catenin-accumulated crypt; PCNA, proliferating cell nuclear antigen; CHR, chrysin; QER, quercetin; NOB, nobiletin. Data are shown as the mean ± SD.

[a] Significantly different in one-way ANOVA with Bonferroni correction test, $P < 0.001$ vs. group 1.
[b] Significantly different in one-way ANOVA with Bonferroni correction test, $P < 0.05$ vs. group 1.
[c] Significantly different in one-way ANOVA with Bonferroni correction test, $P < 0.01$ vs. group 1.

Table 4
Serum profiles in each groups of male *db/db* mice.

Group no.	Treatment	Leptin (ng/ml)	Adiponectin (μg/ml)	IGF-1 (ng/ml)	Insulin (ng/ml)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)
1	AOM	181.4 ± 15.6[a]	8.0 ± 0.3[a]	467.5 ± 93.3	4.2 ± 2.6	204.0 ± 27.9	176.4 ± 13.0	791.6 ± 101.4
2	AOM + 100 ppm CHR	160.9 ± 39.5	8.1 ± 0.2	397.3 ± 61.8	4.6 ± 1.9	183.1 ± 37.2	169.5 ± 13.8	843.9 ± 78.4
3	AOM + 100 ppm QER	125.4 ± 19.3[b]	7.8 ± 0.7	434.2 ± 53.2	2.8 ± 0.8	227.4 ± 44.2	175.8 ± 21.2	882.0 ± 20.6
4	AOM + 100 ppm NOB	179.2 ± 44.3	8.5 ± 0.8	412.2 ± 49.8	5.4 ± 2.2	248.8 ± 66.7	187.0 ± 21.0	817.4 ± 59.4
5	100 ppm CHR	102.3 ± 51.0	7.0 ± 0.8	538.2 ± 175.8	1.5 ± 1.0	277.3 ± 94.7	151.9 ± 36.2	1013.4 ± 79.0
6	100 ppm QER	100.8 ± 44.9	6.5 ± 0.3	495.8 ± 95.4	2.0 ± 1.3	250.4 ± 61.8	171.5 ± 28.3	966.8 ± 94.0
7	100 ppm NOB	102.6 ± 35.9	6.9 ± 0.7	528.1 ± 114.0	2.7 ± 2.9	243.7 ± 17.5	168.0 ± 28.8	886.7 ± 102.5
8	None	108.8 ± 36.8	6.4 ± 0.3	473.0 ± 35.7	2.8 ± 1.7	275.0 ± 15.9	179.0 ± 24.8	1068.2 ± 27.2

IGF-1, insulin-like growth factor-1; CHR, chrysin; QER, quercetin; NOB, nobiletin. Data are shown as the mean ± SD.

[a] Significantly different in Student's *t*-test, $P < 0.05$ vs. group 8.
[b] Significantly different in Student's *t*-test, $P < 0.05$ vs. group 1.

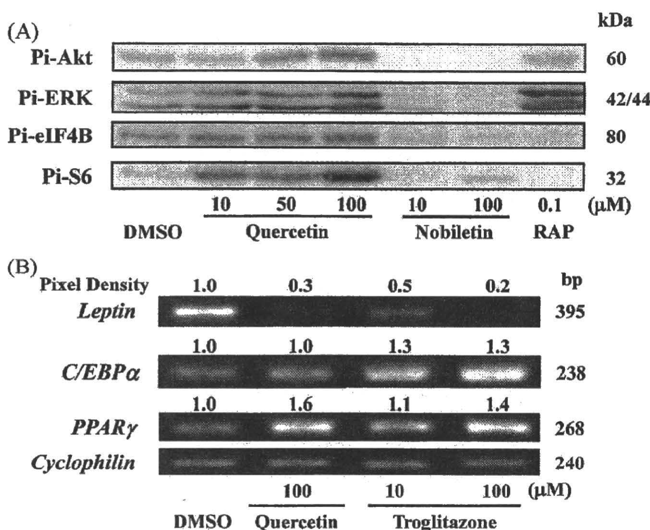


Fig. 5. (A) Modulatory effects of quercetin, nobiletin, and rapamycin on the Akt/mTOR signaling pathway in differentiated 3T3-L1 cells. (B) Modulatory effects of quercetin and troglitazone on the expression of transcription factors in differentiated 3T3-L1 cells. The relative density of the bands normalized to cyclophilin is indicated. 3T3-L1 mouse pre-adipocytes (1×10^5 cells in 35 mm dish) were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 h. Differentiated 3T3-L1 cells were treated with DMSO alone, quercetin, nobiletin, rapamycin, or troglitazone for 12 days. The cells were washed twice with PBS and analyzed using Western blotting and RT-PCR methods. Rap, rapamycin.

group 1 was significantly higher than that in group 8 ($P < 0.05$). However, dietary administration with the flavonoids (groups 2 through 4) did not have any effects on serum adiponectin levels. The serum level of IGF-1 in group 1 was comparable to that in group 8. Dietary flavonoids (groups 2 through 4) decreased the level, though the differences were not significant. Treatment with the different flavonoids did not have a significant effect on the increase of insulin in serum caused by AOM administration. There were no marked differences in regard to the levels of triglyceride, cholesterol, and glucose among the groups.

3.5. Quercetin inhibition of leptin mRNA expression

The Akt/mTOR signaling pathway, including eIF4B, is considered to play a crucial role as a regulator of adipogenesis [33] and leptin secretion [34]. Our previous study indicated that nobiletin decreased the phosphorylation state of eIF4B partly through inactivation of MEK/ERK [35]. Therefore, we investigated the effects of quercetin on the mTOR signaling pathway, because it exhibited a profound suppressive effect on leptin production *in vivo*. Unexpectedly, the phosphorylation state of Akt, ERK, eIF4B, and S6 was increased in quercetin-treated cells, while nobiletin abolished the increase, as previously reported [35] (Fig. 5A). The differing effects obtained by treatments with quercetin and nobiletin led us to examine whether quercetin has an effect on the expression of *leptin* mRNA. It is well known that CCAAT/enhancer binding protein (C/EBP)α is an important transcription factor of leptin. Peroxisome proliferator-activated receptor (PPAR)γ plays an important role in adipocyte differentiation, though several PPARγ agonists, including thiazolidinediones, were shown to repress leptin gene expression in adipocytes [36]. In the present study, quercetin and troglitazone significantly reduced leptin mRNA expression, while they did not

reduce the level of C/EBPα expression (Fig. 5B). Of interest, inverse correlations for mRNA expression between leptin and PPARγ were observed for quercetin and troglitazone (Fig. 5B).

4. Discussion

Our results clearly indicate that dietary administration of the flavonoids chrysin, quercetin, and nobiletin leads to suppression of the development of precursor lesions (ACF and BCAC) induced by AOM in obese mice, in part by reducing the proliferation activity of the lesions. The order of chemopreventive ability in the present findings was quercetin > chrysin > nobiletin, which is consistent with our previous reports [17,26]. Interestingly, the tested flavonoids, particularly quercetin, lowered the levels of growth factors in serum, especially leptin.

The high susceptibility of *db/db* mice to colon carcinogenesis might be related to high proliferation activities of normal crypts and pre-neoplasms. Obesity itself along with high levels of serum cholesterol, triglycerides, glucose, insulin, and leptin have been suggested to explain that elevated susceptibility [26]. Recently, leptin was reported to act as a mitogenic factor in cultured human colon cancer cells [37] and mouse colon carcinogenesis [35]. Hyperinsulinemia has also been hypothesized to be an underlying factor linking obesity, type 2 diabetes mellitus, and colon tumorigenesis [38]. As for the mechanism of action, insulin resistance is associated with hyperinsulinemia and increased levels of growth factors including IGF-1, which may promote colon carcinogenesis through their effects on colonic cryptal cell kinetics [39]. In this context, a recent report showing that leptin interacts with IGFs to promote the survival and expansion of *APC* deficient colonic epithelial cells, but not of those expressing wild-type *APC*, is interesting [40].

In the present study, the tested dietary flavonoids did not have effects on body weight gain, epididymal fat pad weight, or food intake. However, quercetin markedly lowered serum leptin and insulin concentrations, which were elevated by injection of AOM. Importantly, treatment with each flavonoid alone (groups 5 through 7) did not have an influence on the level of leptin as compared with the control group (Table 4). However, feeding with the flavonoids decreased the serum levels of IGF-1. Although, it is not clear how each of the flavonoids in this study reduces the serum concentrations of these growth factors, such reduction may lead to suppression of proliferation activity in preneoplastic lesions. Quercetin modulates several signal transduction pathways involving MEK/ERK, which are associated with proliferation of several types of cancer cells [41], while nobiletin inhibits the proliferation of a variety of human cancer cell lines, partly due to induction of G1 cell cycle arrest [42]. In addition, chrysin induces G2/M cell-cycle arrest in human colon carcinoma SW480 cells [43]. Together with our findings, it is suggested that the reduction of proliferation in preneoplastic lesions (ACF and BCAC) caused by each flavonoid is related to induction of cell-cycle arrest in aberrant cells.

Leptin release is influenced by the amount of *leptin* mRNA expression in adipocytes. That release is regulated by not only the mTOR signaling pathway, but also *leptin* mRNA transcription, which is activated during adipocyte differentiation. C/EBPα, which belongs to the C/EBP family of transcription factors, plays a central role in the control of energy homeostasis and is expressed during the terminal phase of differentiation immediately prior to the expression of many adipose-specific genes [44]. The PPAR family of proteins also plays an important role in adipocyte differentiation [45]. Taken together, logical candidate transactivators of the leptin promoter include C/EBPα and PPARγ. In fact, the proximal promoter of the *leptin* gene contains a functional C/EBP-binding site, which mediates activation of the leptin promoter by co-transfected C/EBPα in 3T3-L1 pre-adipocytes. However, it is surprising that PPARγ agonists, *e.g.*, thiazolidinediones, were found to down-regulate leptin mRNA levels [36]. Furthermore, since the putative PPARγ response element in the leptin promoter is not involved in negative regulation, it has been hypothesized that PPARγ functionally antagonizes C/EBPα to decrease transcription in response to thiazolidinediones [46].

In the present study, quercetin significantly reduced the mRNA expression of leptin, but not that of C/EBPα, while it up-regulated PPARγ mRNA expression, as did troglitazone (Fig. 5B). Consistent with our findings, Fang et al. recently reported that quercetin inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes by acting as a potential agonist of PPARγ [47]. Furthermore, their competitive ligand-binding assay confirmed that quercetin competes with rosiglitazone in the same binding pocket site as PPARγ. Thus, it is likely that quercetin affects leptin secretion from white adipose tissue in *db/db* mice by acting as a PPARγ agonist. We previously observed that nobiletin suppresses hyperleptinemia in ICR mice given AOM and dextran sulfate sodium [35]. However, no effects of nobiletin were found in the present *db/db* mice that received AOM (Table 4). Since nobiletin suppresses leptin secretion partly by repression of the insulin signaling pathway in 3T3-L1 cells, the differences between the biochemical effects induced by quercetin and nobiletin may explain why the former and not the latter suppresses serum leptin levels in AOM-treated *db/db* mice.

In summary, the present results provide additional evidence that certain dietary flavonoids are potent to inhibit the early phase of colon carcinogenesis in genetically altered obese mice, partly through reduction of proliferation. Those effects were also shown to be related to lowered serum levels of leptin, insulin, and IGF-1. This study investigated the effects of selected flavonoids on colonic pre-malignancy by focusing on lowered levels of serum growth factors, thus additional studies of the exact mechanisms are needed for development of prevention and treatment strategies for obesity-related colonic malignancies.

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BioFactors

The modifying effects of green tea polyphenols on acute colitis and inflammation-associated colon carcinogenesis in male ICR mice

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Abstract.

Reactive oxygen species (ROS) have been implicated as mediators of intestinal inflammation and carcinogenesis. Although green tea polyphenols (GTP) have anticancer property as antioxidants they also generate ROS *in vitro*. In this study, we investigated the modifying effects of GTP on dextran sulfate sodium (DSS)-induced acute colitis and on 1,2-dimethylhydrazine (DMH) and DSS-induced colon carcinogenesis in male ICR mice. At sacrifice after 6 days, the colon shortening induced by 2% DSS was unchanged by 0.1% and 0.25% GTP, but increased by 0.5% and 1% GTP-containing diet. The expression of interleukin-1β and macrophage-migration inhibitory factor in the DSS + 0.1% GTP group were lower than the DSS alone group, whereas the expression levels were increased in the DSS + 0.5% GTP and DSS + 1% GTP groups when compared with the DSS alone group. In a subsequent experiment to determine the effects of 0.01–1% GTP on inflammation-associated colon carcinogenesis induced by DMH/DSS, 0.5 and 1% doses of GTP failed to prevent the development of multiple colon tumors, rather, they tended to increase it. Our results thus indicate that the modifying effects of GTP on DSS-induced acute colitis and DMH/DSS-induced colon carcinogenesis depends upon its dosage and the expression of proinflammatory cytokines.

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Keywords: green tea polyphenols, colitis, colon carcinogenesis, cytokines

1. Introduction

Green tea is a popular beverage in Asian countries and it has recently become popular in Europe and North America [1]. Green tea polyphenols (GTP) have been reported to be potent antioxidants and prevent oxidative stress-related disease, due to their abilities to scavenge reactive oxygen species (ROS) and chelate metal ions, which promote ROS generation [2]. GTP has been shown to prevent inflammation and carcinogenesis in different tissues of rodents, and several mechanisms have been postulated for this activity [3–6]. However, to date, GTP and (−)-epigallocatechin-3-gallate (EGCG), the major constituent in tea catechins, have also emerged as pro-oxidants at least in *in vitro* systems. For instance, EGCG and GTP generated H_2O_2 in cell-free and cell culture systems [7,8]. EGCG induced DNA damage by oxidative stress generation [9], and induced cyclooxygenase-2 and tumor necrosis factor (TNF)-α expression in macrophages [10,11]. In our previous study, EGCG enhanced promatrix metalloproteinase-7 production via spontaneous superoxide generation in HT-29 human colon cancer cells [12].

Colorectal cancer (CRC) is the second leading cause of death from cancer in Western countries including North America [13]. In humans, inflammatory bowel disease (IBD), including chronic ulcerative colitis (UC) and Crohn's disease, predisposes to the development of CRC [14]. Indeed a nongenetic etiology for IBD has become of primary importance in CRC, together with the hereditary syndromes of familial adenomatous polyposis and hereditary nonpolyposis CRC. Previous studies showed that inducible nitric oxide synthase was expressed in epithelial cells and inflammatory cells at the site of carcinogenesis in humans and animal models. Indeed, Reinecker et al. found nitrate and oxidative DNA lesion products in inflamed colonic mucosa of rodents and IBD patients [15,16]. The effects of GTP on colon

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Abbreviations: ACF, aberrant crypt foci; CRC, colorectal cancer; DMH, 1,2-dimethylhydrazine; DSS, dextran sulfate sodium; EGCG, (−)-epigallocatechin-3-gallate; ELISA, enzyme-linked immunosorbent assay; GTP, green tea polyphenols; H&E, hematoxylin & eosin; IBD, inflammatory bowel disease; IL, interleukin; MIF, macrophage-migration inhibitory factor; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

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