(CRF-1; Oriental Yeast, Tokyo, Japan). Both 1-MT and AOM were purchased from Sigma (St Louis, MO, USA), whereas EGCG was obtained from Mitsui Norin (Tokyo, Japan). For 1-MT and EGCG treatment of rats, 1-MT (0.2%) and EGCG (0.1%) solutions were prepared in tap water and administered to the rats in their drinking water *ad libitum*. Fresh test solutions were prepared three times a week. The concentration of EGCG (0.1%) used in the present study was chosen on the basis of the results of previous chemopreventive studies[19,26] and was within the physiologic range of the daily intake of green tea catechins in humans on a per unit body weight basis.[27]

Experimental procedure

As shown in Figure 1, 60 male F344 rats were quarantined for the first 7 days and then randomized into one of three groups to receive either 0.2% 1-MT, 0.1% EGCG, or no test compounds. One week later, the rats in each group were further grouped to receive subcutaneous injections of AOM (20 mg/kg body weight) or saline (200 μL) once a week for 2 weeks. Rats were given control and test drinking water for 4 weeks, starting 1 week before the first AOM injection. All measurements, including the large bowel excision and the collection of blood samples from the inferior vena cava, were performed from rats that had been killed by CO_2 asphyxiation at Week 4 (9 weeks of age). One-quarter of the excised colons (cecum side) was used for crypt isolation, whereas the remainder was used to determine the number of colonic ACF and BCAC (see below). After the number of ACF had been counted, the colon was rolled like a "Swiss roll"[28] and paraffin-embedded sections were prepared using routine procedures for subsequent histopathologic and immunohistochemical examinations.

Counting colonic ACF and BCAC. The number of ACF and BCAC was determined as described previously.[25,29,30] Briefly, buffered formalin-fixed colons were stained with 0.5% methylene blue solution for 20 s and then placed on microscope slides to count the number of ACF and to determine their size. The number of ACF in the colon was recorded along with the number of crypts in each focus and the data are expressed as the total number of ACF per colon, total number of aberrant crypts (ACs) per colon, number of ACs per focus, and total number of large ACF (i.e. ACF with four or more aberrant crypts) per colon.[29] After the number of ACF had been counted, the rectal mucosa (2.0 cm from the anus) was cut and embedded in paraffin to identify BCAC intramucosal lesions, with 4-μm sections obtained from an *en face* preparation. The number of BCAC on histological sections stained with β-catenin was counted and is expressed as the number of BCAC per cm^2 mucosa.

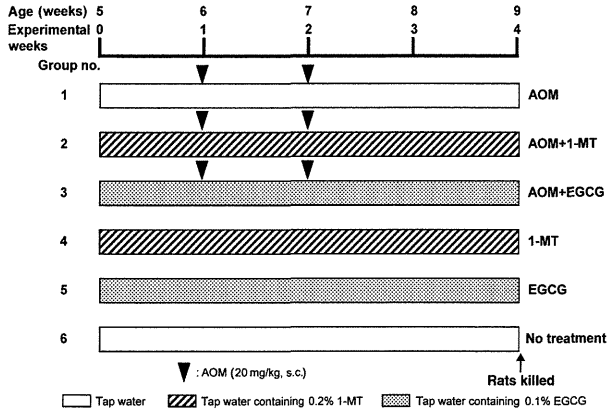

Fig. 1. Experimental protocol. Rats (5 weeks old) were allocated to one of six groups and treated over a period of 4 weeks, as indicated. AOM, azoxymethane; 1-MT, 1-methyltryptophan; EGCG, (−)-epigallocatechin gallate.

Immunohistochemical analysis. After endogenous peroxidase activity had been blocked with H_2O_2, sections were incubated overnight at 4°C with primary antibodies: anti-β-catenin (1:1000; BD Biosciences PharMingen, San Diego, CA, USA), anti-IDO (1:1000; LYFESPAN, Seattle, WA, USA), and anti-L-kynurenine (1:1000; Abnova, Taipei City, Taiwan). Subsequently, sections for the immunohistochemistry of β-catenin and IDO were incubated with biotinylated secondary antibodies against the primary antibodies (DAKO, Carpinteria, CA, USA), followed by incubation with avidin-coupled peroxidase. The sections for L-kynurenine immunohistochemistry were incubated with peroxidase-labeled polymer-conjugated secondary antibodies against the primary antibodies. They were then developed with 3,3′-diaminobenzidine using DAKO Liquid DAB Substrate-Chromogen System (DAKO) and counterstained with hematoxylin.

Crypt isolation. Colonic tissue was washed twice with 1 × Hank's balanced salt solution (HBSS; Sigma) and then incubated with 1 × HBSS containing 30 mM EDTA at 37°C for 15 min. The tissue was dispersed in 1 × HBSS solution by vortexing and separated into epithelial crypts and stromal tissues as described previously.[31]

Quantitative real-time RT-PCR. Total RNA was extracted from isolated epithelial crypts and stromal tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was used for the synthesis of first-strand cDNA. Quantitative real-time RT-PCR was performed using specific primer/probe sets that amplified the *IDO*, *tryptophan 2,3-dioxygenase* (*TDO*), *cyclooxygenase* (*COX*)-*2*, *IFN-γ*, and *GAPDH* genes (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA) and TOYOBO Real-time PCR Master Mix (TOYOBO, Osaka, Japan). Each sample was analyzed on a LightCycler 1.0 (Roche Diagnostics, Mannheim, Germany), as described previously.[32] The expression of each gene was normalized against that of *GAPDH* using the standard curve method.

Determination of IDO activity. Indoleamine 2,3-dioxygenase activity was determined by calculating the ratio of L-kynurenine/L-tryptophan in serum and colonic tissues.[33] Serum samples were deproteinized with 3% perchloric acid. Isolated epithelial crypt and stromal samples were homogenized in 2 μL of 3% perchloric acid per mg tissue. After centrifugation at 4°C and 20 000 *g* for 10 min, aliquots of the supernatant were collected for HPLC determination of L-tryptophan and L-kynurenine concentrations, as described previously.[34]

The enzymatic activity of IDO was also measured using cell-free assays. An aliquot of recombinant human IDO (R&D Systems, Minneapolis, MN, USA) was diluted in 50 mM 2-(*N*-morpholino)ethanesulfonic buffer (pH 6.5). The reaction mixture contained 50 μL enzyme preparation and 50 μL substrate solution, which consisted of 100 mM potassium phosphate buffer (pH 6.5), 50 μM methylene blue, 20 μg catalase, 50 mM ascorbate, 0.4 mM L-tryptophan, and 2000 μM 1-MT or 200 μM EGCG. After incubation of the reaction mixture at 37°C for 1 h, the concentrations of the enzymatic products were measured by HPLC.[35] Enzymatic activity is expressed as the product content per hour.

Statistical analysis. All data are expressed as the mean ± SD. Differences between groups were analyzed by two-way ANOVA and, when statistical significance was found, individual

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differences were evaluated using the Tukey–Kramer multiple comparison test. $P < 0.05$ was considered significant.

Results

General observations. All rats remained healthy and none died during the experimental period. There were no significant differences in the consumption of food (data not shown) and drinking water (Table 1) between the different groups. Body, liver, and relative liver weights, as well as the length of the large bowel, at the end of the study are given in Table 1. The mean body weight of the AOM + 1-MT group was only significantly less than that of the 1-MT group ($P < 0.05$). This decrease may have been due to AOM toxicity, as observed in previous studies,[19,36,37] because 1-MT alone did not reduce body weight in the absence of AOM. Other measurements did not differ significantly among the groups. Histopathologically, there were no findings suggesting toxicity of 1-MT or EGCG in the liver, kidney, or spleen of rats (data not shown).

Effects of 1-MT and EGCG on AOM-induced ACF and BCAC in F344 rats. All rats in the AOM, AOM + 1-MT, and AOM + EGCG groups (i.e. all those treated with AOM) developed ACF and BCAC. In the 1-MT, EGCG, and untreated groups, there were no microscopically observable changes, including ACF or BCAC, in the colon. Compared with the group treated with AOM alone, daily oral administration of 1-MT and EGCG in the drinking water significantly reduced the frequency of ACF ($P < 0.001$ for each comparison). The reduction in the frequency of ACF was significantly greater following EGCG administration than after 1-MT administration ($P < 0.05$). We also noticed a significant reduction in the percentage of large ACF, consisting of four or more aberrant crypts, in the AOM + 1-MT and AOM + EGCG groups compared with the AOM group ($P < 0.001$ for each comparison; Fig. 2a). In addition, the number of BCAC per cm^2 in the AOM + 1-MT and AOM + EGCG groups was significantly less than that in the AOM group ($P < 0.001$ for each comparison; Fig. 2b).

Immunohistochemical analysis of IDO and L-kynurenine in the colonic mucosa. The expression of IDO and L-kynurenine was determined in colonic crypts and preneoplastic lesions (i.e. ACF and BCAC) using immunohistochemical analysis. Compared with colonic crypt cells in untreated control rats, which exhibited only weak positive cytoplasmic staining to IDO, there was a significant increase in IDO staining in the atypical cell cytoplasm of the ACF and BCAC that had developed in AOM-treated rats. Furthermore, L-kynurenine expression, which was very weak in normal crypts of untreated control rats, was slightly increased in the ACF and BCAC of AOM-treated rats (Fig. 3a). Neither EGCG nor 1-MT treatment significantly altered the AOM-induced increases in IDO and L-kynurenine staining (Fig. 3b,c).

Effects of 1-MT and EGCG on IDO and TDO expression in isolated epithelial crypts and stromal cells. In cancer tissues, IDO is overexpressed in both tumor epithelial cells and antigen-presenting cells in the stroma.[38] Tryptophan 2,3-dioxygenase, a hepatic enzyme that catalyses the first step of tryptophan degradation, is also expressed in many tumors.[39] Therefore, after crypt isolation, we determined whether there was increased expression of IDO and TDO in both epithelial crypts and stromal tissues in the colon of AOM-treated rats. As indicated in Figure 4(a,b), quantitative RT-PCR analysis revealed a significant increase in IDO expression in both the crypts and stromal cells of the AOM-treated group compared with the untreated control group ($P < 0.05$ for each comparison). Furthermore, these increases were significantly inhibited by EGCG treatment ($P < 0.05$ for each comparison). Although 1-MT treatment tended to decrease IDO expression in crypts and stromal tissues, the difference failed to reach statistical significance. In the absence of AOM treatment, *IDO* mRNA expression was not affected by the administration of either 1-MT or EGCG. In contrast with IDO, AOM did not induce an increase in *TDO* mRNA expression and neither EGCG nor 1-MT had any effect on TDO expression in crypts and stromal tissues (Fig. 4c,d).

Effects of 1-MT and EGCG on IDO activity. We next examined the enzymatic activity of IDO in serum and colon tissues of AOM-treated rats by measuring the concentrations of L-kynurenine and L-tryptophan. The L-kynurenine/L-tryptophan ratios in the serum (Fig. 5a) and stromal cells (Fig. 5c) of the AOM-treated group were significantly higher than in the untreated control group ($P < 0.05$ for each comparison). Treatment of rats with 1-MT and EGCG resulted in a significant decrease in these ratios in AOM-treated rats ($P < 0.05$ for each comparison), suggesting that 1-MT and EGCG significantly inhibit both the systemic (serum) and focal (colonic stromal) AOM-induced increases in IDO activity. In epithelial cells, there were no significant differences in the L-kynurenine/L-tryptophan ratios between the different groups (Fig. 5b). In the absence of AOM treatment, neither 1-MT nor EGCG alone had any effect on the L-kynurenine/L-tryptophan ratios (Fig. 5a–c).

To further investigate whether 1-MT and EGCG directly influence IDO activity, we measured IDO enzyme kinetics (kynurenine production) using recombinant human IDO in a cell-free system. As shown in Figure 6, levels of L-kynurenine produced by IDO were significantly inhibited by 1-MT and EGCG treatment ($P < 0.001$ for each comparison). These findings suggest that both 1-MT and EGCG act directly to inhibit IDO activity.

Effects of 1-MT and EGCG on COX-2 and IFN-γ expression in stromal cells. We next assessed the inhibitory effects of 1-MT and EGCG on COX-2 and IFN-γ expression, because both are regulated by inflammatory cells in the stroma and are implicated in the induction of IDO.[40–43] Using quantitative RT-PCR, we found that the expression of *COX-2* mRNA in stromal tissues was markedly upregulated in the AOM-treated group, but this upregulation was significantly inhibited by

Table 1. General parameters

Treatment	No. rats examined	Drinking water intake (g/day)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	Length of the large bowel (cm)
AOM alone	14	27.3 ± 1.3	203 ± 11	10.2 ± 1.0	5.0 ± 0.5	19.0 ± 1.8
AOM + 0.2% 1-MT	14	25.2 ± 2.3	198 ± 13*	10.1 ± 0.7	5.1 ± 0.4	19.6 ± 1.1
AOM + 0.1% EGCG	14	26.1 ± 2.9	202 ± 8	9.8 ± 0.8	4.9 ± 0.5	19.0 ± 0.9
0.2% 1-MT	6	25.3 ± 4.3	217 ± 12	10.6 ± 0.8	4.9 ± 0.2	20.3 ± 1.6
0.1% EGCG	6	26.5 ± 1.4	212 ± 16	10.2 ± 0.9	4.8 ± 0.6	19.5 ± 1.1
No treatment	6	26.8 ± 0.8	208 ± 9	9.6 ± 0.8	4.6 ± 0.4	19.0 ± 2.1

Data are given as the mean ± SD. *$P < 0.05$ compared with 0.2% 1-methyltryptophan (1-MT) alone. AOM, azoxymethane; EGCG, (–)-epigallocatechin gallate.

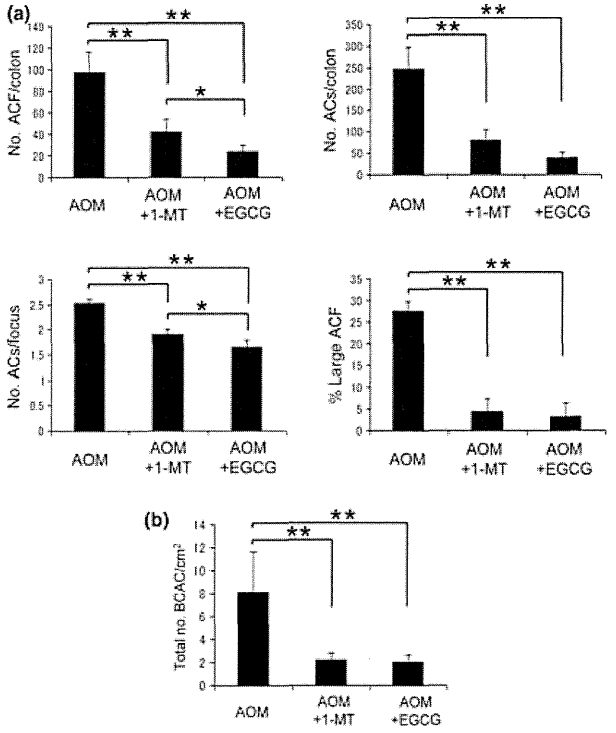

Fig. 2. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on azoxymethane (AOM)-induced formation of aberrant crypt foci (ACF) and β-catenin-accumulated crypts (BCAC). (a) Number of ACF per colon, total number of aberrant crypts (ACs) per colon, the number of ACs in each focus, and the percentage of large ACF (i.e. those with four or more ACs). (b) Number of BCAC per cm^2. Data are the mean ± SD (n = 6). *P < 0.05, **P < 0.001.

EGCG treatment (Fig. 7a). In addition, although AOM increased *IFN-γ* mRNA expression in stromal cells, this increase was not inhibited by EGCG (Fig. 7b). Treatment of rats with 1-MT did not have any significant effect on AOM-induced increases in the expression of *COX-2* or *IFN-γ* mRNA. In the absence of AOM treatment, neither 1-MT nor EGCG alone had any effect on *COX-2* or *IFN-γ* mRNA levels (Fig. 7a,b).

Discussion

The results of the present study suggest that upregulation of IDO is possibly involved in colon carcinogenesis, as evidenced by higher IDO expression (Figs 3a,4a,b) and activity (Fig. 5c) in the colonic mucosa of AOM-treated rats compared with the untreated controls, which did not receive any carcinogen. The results of the present study also provide the first evidence that treatment with the IDO inhibitor 1-MT effectively suppresses the development of colonic preneoplastic lesions (ACF and BCAC) induced by AOM (Fig. 2). This inhibition is considered to be associated with the inhibition of IDO activity, which is increased in AOM-treated rats (Fig. 5a,c), because IDO-mediated immune tolerance plays a critical role in tumor development and progression.[2,3] Therefore, 1-MT may correct IDO-mediated immune escape and thus suppress AOM-induced colorectal carcinogenesis. These results suggest that upregulation of IDO, and most likely subsequent immune tolerance caused by this enzyme, is involved in the early phase of colon carcinogenesis and that targeting IDO may, therefore, be an effective strategy to prevent colorectal carcinogenesis.

The chemopreventive and anti-cancer effects of green tea and EGCG are partially attributed to their anti-oxidative properties, their anti-angiogenic and anti-mutagenic effects, and their anti-inflammatory activities, all of which act in combination to suppress carcinogenesis. Thus, these activities are considered to be the main mechanisms underlying the anti-cancer effects of EGCG.[13,14] Furthermore, earlier studies showed

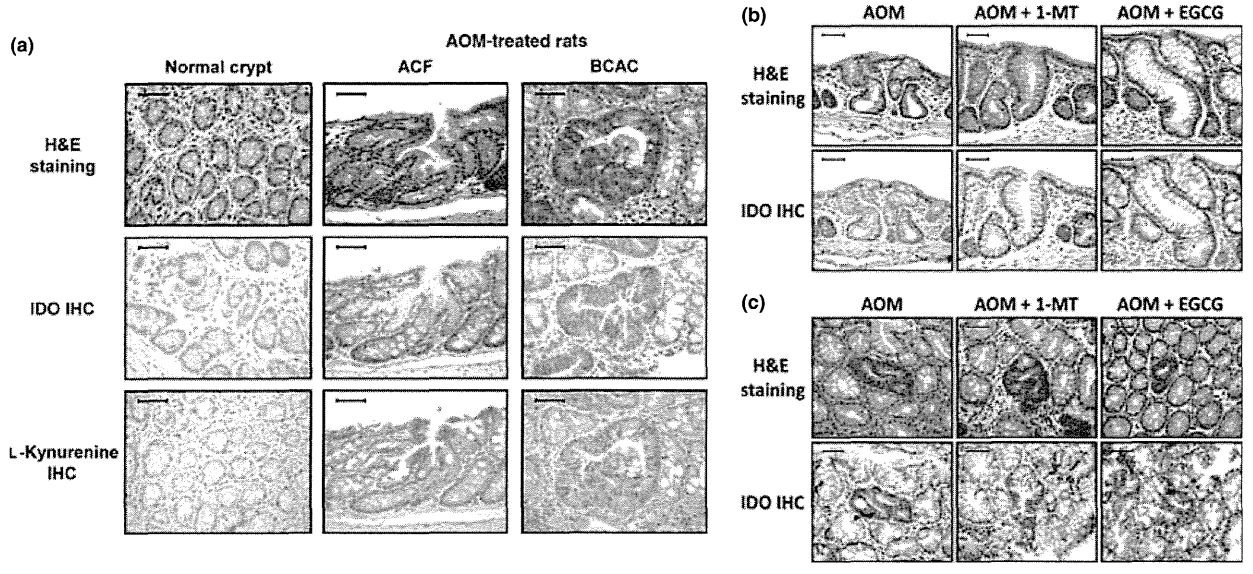

Fig. 3. Immunohistochemical evaluation of the expression of indoleamine 2,3-dioxygenase (IDO) and L-kynurenine in normal crypts from untreated rats and aberrant crypt foci (ACF), and β-catenin-accumulated crypts (BCAC) in the colonic mucosa of rats treated with azoxymethane (AOM). (a) Expression of IDO and L-kynurenine in representative samples of colonic mucosa, as evidenced by H&E staining and immunohistochemistry (IHC). (b,c) Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of IDO in ACF (b) and BCAC (c), as determined by IHC. Scale lines, 50 μm.

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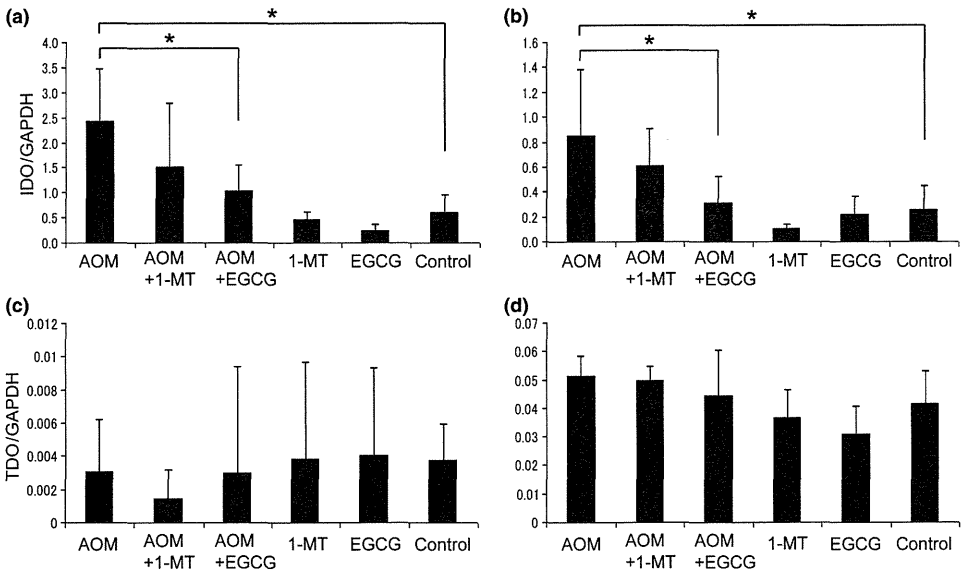

Fig. 4. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of (a,b) indoleamine 2,3-dioxygenase (IDO) and (c,d) tryptophan 2,3-dioxygenase (TDO) in the epithelium (a,c) and stroma (b,d). Total RNA was extracted from epithelial crypts and stromal tissues and IDO and TDO mRNA expression evaluated by quantitative RT-PCR. Expression is normalized against that of GAPDH. AOM, azoxymethane. Data are the mean ± SD (n = 6). *P < 0.05.

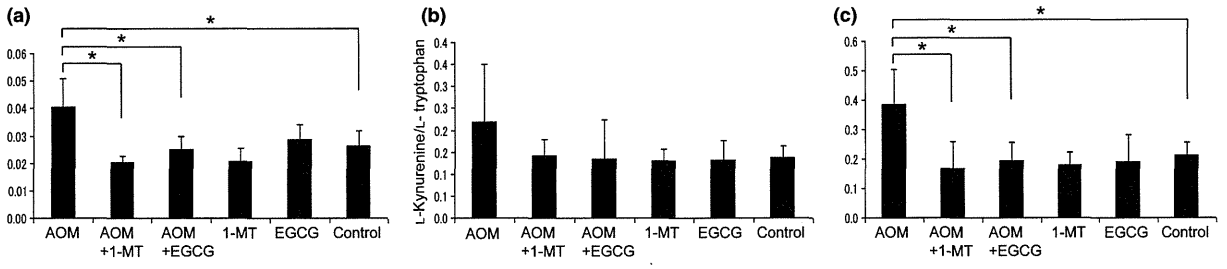

Fig. 5. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on indoleamine 2,3-dioxygenase (IDO) activity in the (a) serum and colonic (b) epithelium and (c) stroma. Functional IDO activity was determined by measuring the concentrations of L-kynurenine and tryptophan using HPLC. The L-kynurenine/L-tryptophan ratio indicates IDO activity. AOM, azoxymethane. Data are the mean ± SD (n = 6). *P < 0.05.

that EGCG suppresses the induction of IDO *in vitro*.[23,24] In the present study, EGCG inhibited the functional enzyme activity of IDO in AOM-treated rats (Fig. 5a,c). In addition, the inhibitory effects of EGCG against AOM-induced increases in *IDO* mRNA expression in the colonic mucosa were greater than those of 1-MT (Fig. 4a,b). This may be associated with the observation that EGCG caused a greater inhibition of the total number of ACF that did 1-MT (Fig. 2a). Therefore, these results suggest that, in addition to the previously reported multiple critical mechanisms of action underlying tumor suppression,[13,14] EGCG may prevent the early phase of colon carcinogenesis, at least in part, by inhibiting the expression and activity of IDO and thus mediating an immune response. The results of a recent study indicating that green tea catechins exert anti-cancer effects by regulating the expression and function of both T and natural killer cells[44] may also strengthen the case for EGCG modulating immune tolerance.

A recent study has revealed the possible roles of toxic tryptophan catabolites produced by IDO in cancer.[45] Of these metabolites, L-kynurenine is considered to play a critical role in the immune escape of malignant cells that occurs within the tumor and its surrounding microenvironment.[4] Conversely, IDO inhibitors can impede the growth of IDO-expressing tumors by reducing the amount of kynurenine present in the microenvironment.[46] Therefore, in addition to inhibiting IDO expression, EGCG has a direct effect in inhibiting IDO enzyme activity (Fig. 6), which may have contributed to its prevention of the development of colonic preneoplastic lesions in the present study.

In the present study, *IDO* mRNA levels in both the epithelium and stroma decreased in rats treated with 1-MT or EGCG; however, the ratio of L-kynurenine/L-tryptophan decreased only in the stroma (Fig. 4a,b,5c). These findings suggest that IDO-induced metabolic conversion of tryptophan to kynurenine occurs mainly in the stroma. For example, in human dendritic cells constitutively expressing IDO protein, the functional activity of this enzyme is tightly regulated and requires additional triggering signals supplied during antigen presentation by $CD4^+$ T cells.[47] Many important immunoregulatory pathways, such as the IFN/JAK/signal transducer and activator of transcription (STAT) pathway and the non-canonical nuclear factor-κB pathway, which are controlled by

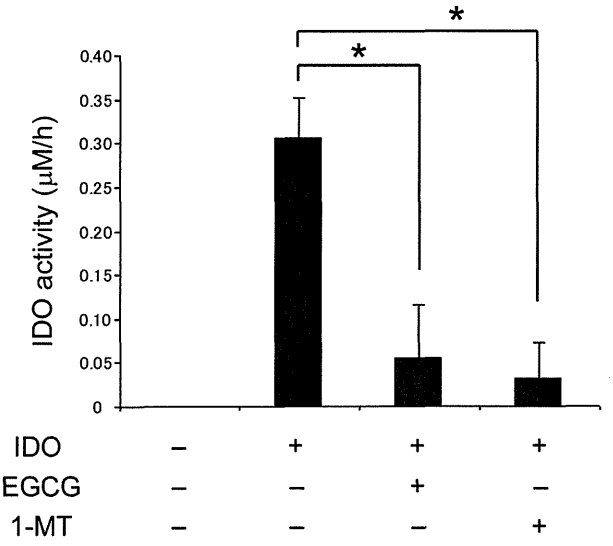

Fig. 6. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on indoleamine 2,3-dioxygenase (IDO) activity in cell-free assays. Functional activity of recombinant human IDO enzyme in response to 1-MT and EGCG was determined by measuring the concentrations of enzymatic products (L-kynurenine) using HPLC. Enzymatic activity is expressed as the product content per hour (μM/h). Data are the mean ± SD. *$P < 0.001$.

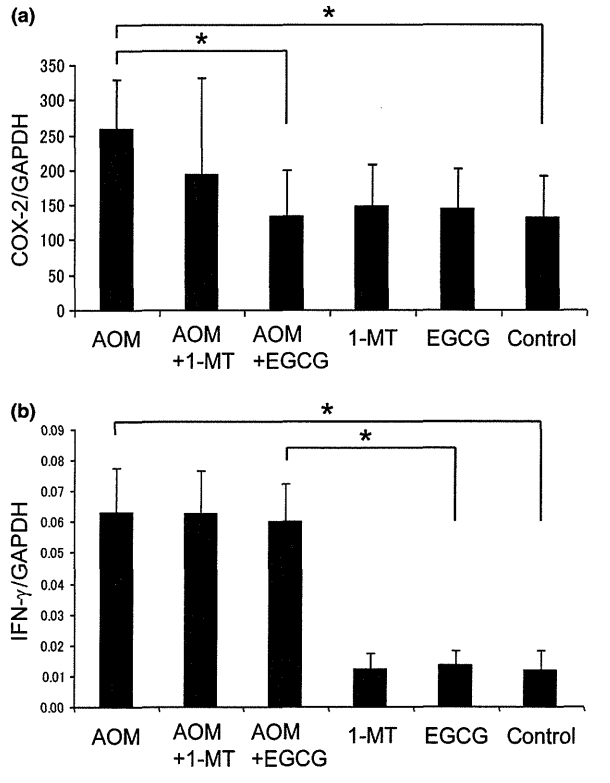

Fig. 7. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of (a) cyclooxygenase (COX)-2 and (b) interferon (IFN)-γ in stromal cells, as determined by quantitative RT-PCR. Expression is normalized against that of GAPDH. AOM, azoxymethane. Data are the mean ± SD ($n = 6$). *$P < 0.05$.

immune cells in the stroma, are related to IDO expression.[38] In addition, several other immune regulatory factors have been implicated as inducers of IDO, including COX-2, which is regarded as one of the most critical inflammatory mediators in the regulation of IDO expression.[42] In the present study, the AOM-induced upregulation of COX-2 in the colonic stroma of rats was significantly inhibited by the administration of EGCG (Fig. 7a). These findings, together with those of Basu *et al.*,[43] who reported a suppressive role of a COX-2 inhibitor against IDO expression in the tumor microenvironment, suggest that EGCG inhibits the expression of IDO, possibly by preventing the induction of COX-2, although further investigations are required to clarify the effects of EGCG. Thus, combination treatment using an IDO inhibitor plus a COX-2 inhibitor may be an effective regimen for the chemoprevention of colorectal cancer because this combination will synergistically inhibit the expression and activity of IDO.

Interferon-γ is also thought to be a major stimulator of IDO,[40,41] and EGCG has been reported to suppress IDO expression by inhibiting STAT-1 activation in response to IFN-γ *in vitro*.[23,24] However, in the present study the expression of IFN-γ in the colonic stroma was not affected by EGCG in the drinking water (Fig. 7b). Other novel mechanisms by which EGCG modulates the expression of IDO may exist; therefore, further studies are needed to clarify the effects of EGCG on the immunoregulatory pathways related to IDO expression.

Aberrant crypt foci have attracted attention as putative precancerous lesions of the colon in experimental models.[48] Numerous molecular abnormalities, including increased expression of K-*ras* and *APC* gene mutations, have been demonstrated in human ACF.[49] In addition, BCAC, which accumulate β-catenin protein in the nucleus and cytoplasm, are regarded as putative precursors to colorectal adenomas.[50] Several rodent studies have shown that both these lesions are useful as biomarkers to evaluate the chemopreventive properties of specific agents.[19,36,37,51] Therefore, our findings, namely that both 1-MT and EGCG markedly inhibit the development of ACF and BCAC, appear to be significant when considering the chemoprevention of colorectal cancer. In particular, a significant reduction of large ACF by 1-MT and EGCG should be emphasized, because large ACF are known to have a strong correlation with the incidence of colonic adenocarcinoma.[20,21]

Finally, it should be mentioned that one limitation of the present study was that the L-kynurenine/L-tryptophan ratio may not directly reflect IDO activity because kynurenine can be metabolized further and TDO can also produce kynurenine from tryptophan.[12] However, in the present study we presumed that TDO exerted little effect on L-kynurenine levels because the expression of TDO was not affected by AOM treatment (Fig. 4c,d). Systemic IDO activity is currently estimated by the serum L-kynurenine/L-tryptophan ratio,[33] as in the present study, because IDO is an intracellular enzyme and circulating IDO concentrations are barely detectable.[3] In fact, a method for analyzing serum IDO protein itself has not been established in experimental animals and there is only one report, published in 2012, of its detection in humans.[52] This limitation needs to be addressed in future studies.

In conclusion, the escape of precancerous cells from the immune system caused by immune tolerance is involved in certain types of carcinogenesis and, therefore, may be an effective target for the implementation of chemoprevention. The results of the present study support the notion that IDO upregulation, which induces immune tolerance, contributes to the early phase of colon carcinogenesis. Furthermore, the present study is the first to provide evidence that the anti-carcinogenic properties of 1-MT and EGCG may be related to inhibition of

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IDO activity, suggesting that targeting IDO and correcting IDO-mediated immune tolerance with EGCG or an IDO inhibitor could be a promising strategy for the prevention of colorectal cancer development in the future. Further experiments using IDO-knockout mice would strengthen the connection between IDO activity and the development of colorectal cancer, and may prove useful in the exploration of IDO inhibitors as chemopreventive agents for colorectal cancer.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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(-)-Epigallocatechin gallate inhibits the expression of indoleamine 2,3-dioxygenase in human colorectal cancer cells

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Abstract. Immune escape, the ability of tumor cells to avoid tumor-specific immune responses, occurs during the development and progression of several types of human malignancies, including colorectal cancer (CRC). Indoleamine 2,3-dioxygenase (IDO), the tryptophan catabolic enzyme, plays a significant role in regulating the immune response and provides tumor cells with a potent tool to evade the immune system. In the present study, we examined the effects of (-)-epigallocatechin gallate (EGCG), the major catechin in green tea, on the inhibition of IDO expression induced by interferon (IFN)-γ in human CRC cells. We found that IFN-γ increased the expression levels of IDO protein and mRNA in HT29 and SW837 CRC cell lines. Treatment of SW837 cells with EGCG significantly decreased IFN-γ-induced expression of IDO protein and mRNA in a dose-dependent manner. Enzymatic activity of IDO, determined by the concentration of L-kynurenine in the culture medium, was also significantly inhibited by EGCG treatment. Phosphorylation of signal transducer and activator of transcription 1 (STAT1) induced by IFN-γ was also significantly inhibited by EGCG. Reporter assays indicated that EGCG inhibited the transcriptional activities of IDO promoters, IFN-stimulated response element and IFN-γ activation sequence, activated by STAT1 phosphorylation. These findings suggest that EGCG may exert antitumor effects on CRC, at least in part, by inhibiting the expression and function of IDO through the suppression of STAT1 activation. EGCG may, thus, serve as a potential agent for antitumor immunotherapy and be useful in the chemoprevention and/or treatment of CRC.

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Abbreviations: CRC, colorectal cancer; EGCG, (-)-epigallocatechin gallate; GAS, interferon-γ activation sequence; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; ISRE, interferon-stimulated response element; JAK, janus kinase; RT-PCR, reverse transcription-polymerase chain reaction; STAT1, signal transducer and activator of transcription 1

Key words: indoleamine 2,3-dioxygenase, (-)-epigallocatechin gallate, colorectal cancer cells, interferon-γ, signal transducer and activator of transcription 1

Introduction

Numerous immune effector cells and molecules recognize and destroy preneoplastic cells (1). The escape of such cells from the immune system is involved in the development and progression of several types of tumors (2). Recent studies have suggested that tryptophan catabolism, via indoleamine 2,3-dioxygenase (IDO), may be a critical mechanism of immune escape (3). IDO, an intracellular enzyme that degrades the essential amino acid tryptophan along the kynurenine pathway, is constitutively expressed by tumor cells and dendritic cells in tumor-draining lymph nodes (4). L-kynurenine and certain other metabolites derived from tryptophan by IDO may inhibit proliferation and induce apoptosis in T cells and natural killer cells (5). IDO overexpression correlates with poor clinical outcomes in patients with several types of malignancies, including colorectal cancer (CRC) (6-8). We have recently demonstrated that increased levels of IDO expression in tumor cells and serum concentration of L-kynurenine, which reflects the enzymatic activity of IDO, are associated with poor prognosis in patients with diffuse large B-cell lymphoma (9-11). Alternatively, 1-methyl-tryptophan, an IDO inhibitor, effectively suppresses chemically induced colorectal carcinogenesis in rats (12). These studies suggest that targeting IDO and regulating tryptophan catabolism may be effective strategies for the treatment of certain types of human malignancies, including CRC (6).

Several immune factors are regarded as mediators in the regulation of IDO expression. Among these factors, interferon (IFN)-γ, which is released by activated T cells and natural killer cells within the tumor microenvironment (13), is considered to be a major inducer of IDO in numerous human

cell types (14). IFN-γ binding to its receptor results in the phosphorylation of janus kinase (JAK), which phosphorylates the downstream protein signal transducer and activator of transcription 1 (STAT1) (13). The IDO promoter contains multiple sequence elements that confer responsiveness to IFN-γ, including the interferon-stimulated response element (ISRE) and the IFN-γ activation sequence (GAS) (14). IFN-γ induces IDO by promoting ISRE and GAS sequence elements through the activation of the JAK/STAT1 signaling pathway (15), indicating that the inhibition of STAT1 phosphorylation and its downstream promoter activity may be effective for the downregulation of IDO (16).

(-)-Epigallocatechin gallate (EGCG), the major biologically active component of green tea, exerts its anticancer and cancer chemopreventive effects in various organs. These effects are partially attributed to their antioxidative, antiangiogenic and antimutagenic effects, as well as their anti-inflammatory activities (17,18). We previously demonstrated that EGCG is able to suppress cell proliferation and induce apoptosis in human CRC cells (19-21). The inhibitory effects of EGCG on inflammation- and obesity-related colon carcinogenesis have also been demonstrated in rodent models (22,23). In addition, recent studies revealed that EGCG treatment suppresses the expression of IDO stimulated by IFN-γ in murine dendritic cells (24) as well as in various human cancer-derived cell lines (25,26). These studies suggest that the inhibitory effect of EGCG on IDO expression may contribute to the chemopreventive and anticancer properties of EGCG; however, whether EGCG is able to inhibit expression of IDO in human CRC cells has not yet been examined. The present study investigated the effects of EGCG on the induction of IDO in human CRC cells stimulated with IFN-γ.

Materials and methods

Chemicals. EGCG was obtained from Mitsui Norin Co. (Tokyo, Japan) and recombinant human IFN-γ was purchased from PeproTech (Rocky Hill, NJ, USA).

Cell lines and cell culture. The Caco2, HCT116, HT29, SW480 and SW837 human CRC cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in DF10 medium containing Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS). Cells were then cultured in a humidified incubator with 5% CO_2 at 37°C. The experimental protocol of this study was approved by the Ethics Committee of Gifu University.

RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Quantitative real-time RT-PCR was performed on 1 μg total RNA using specific primer/probe sets that amplify IDO and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes (TaqMan Gene Expression Assays; Life Technologies) and Toyobo Real-time PCR Master mix (Toyobo, Osaka, Japan). Each sample was analyzed on a LightCycler 1.0 (Roche Diagnostics GmbH, Mannheim, Germany) (27). Gene expression levels were normalized to GAPDH expression levels using a standard curve.

Protein extraction and western blot analysis. Total cellular protein was extracted and equivalent amounts of protein (20 μg/lane) were examined by western blot analysis (21,28). The primary antibody for IDO was purchased from Abcam (Cambridge, UK). The primary antibodies for STAT1, phospho-STAT1 (tyr701) and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). An antibody to GAPDH was used as a loading control.

Determination of L-kynurenine concentration. Culture medium was deproteinized with 2 volumes of 3% perchloric acid. Following centrifugation, the concentration of L-kynurenine in the supernatants was measured by high-performance liquid chromatography (29).

ISRE and GAS reporter assays. Reporter assays were performed (20). ISRE and GAS luciferase reporter plasmids were purchased from Qiagen. Plasmid DNA (0.1 μg) was transfected into SW837 cells ($3x10^4$ cells/6.35 mm diameter dish) using Lipofectin reagent (Life Technologies) in Opti-MEM I medium (Life Technologies). After 22 h, cells were preincubated in the absence or presence of various EGCG concentrations (10, 50 or 100 μM) for 2 h and then stimulated with IFN-γ (10 ng/ml) for 24 h. Cell extracts were prepared and luciferase activity was measured using a luciferase assay system (Promega, Madison, WI, USA). Cells were cotransfected with a CMV-β-galactosidase reporter in all reporter assays and differences in transfection efficacy were corrected by normalizing the luciferase activities to the β-galactosidase activities (20).

Statistical analysis. All data were expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using the Tukey-Kramer multiple comparison test and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of IDO in human CRC cells in the presence and absence of IFN-γ stimulation. We initially examined the expression levels of IDO in the presence and absence of IFN-γ in Caco2, HCT116, HT29, SW480 and SW837 human CRC cells. Quantitative real-time RT-PCR analysis revealed that IDO mRNA was not expressed in any of the CRC cell lines without IFN-γ stimulation. However, when the cell lines were stimulated with 10 ng/ml IFN-γ for 24 h, IDO mRNA levels were significantly increased in all cell lines, particularly SW837 and HT29 cells (Fig. 1A). Furthermore, in accordance with the expression levels of mRNA, the cellular levels of IDO protein in the SW837 and HT29 cells were markedly increased by IFN-γ stimulation (Fig. 1B), indicating that CRC cells may induce IDO expression following IFN-γ stimulation. Based on these results, SW837 cells were selected for the following experiments.

Effects of EGCG on the expression levels of IDO in SW837 CRC cells. We next examined the effects of EGCG on the

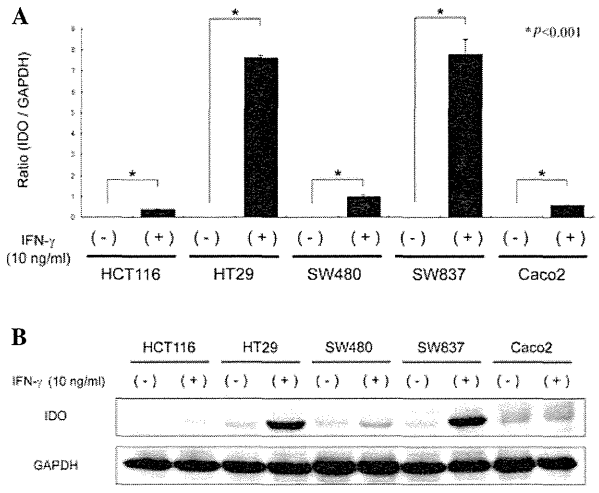

Figure 1. Expression of IDO in human CRC cell lines. Caco2, HCT116, HT29, SW480 and SW837 cells were treated with or without IFN-γ (10 ng/ml) for 24 h. (A) Total RNA was extracted from each cell line and IDO mRNA expression was measured by quantitative real-time RT-PCR. Gene expression levels were normalized to GAPDH. Columns and bars indicate the mean and SD. (B) IDO protein expression levels were detected by western blot analysis. Total cellular protein was extracted and equivalent amounts of protein were examined. Equal protein loading was confirmed by the detection of GAPDH. Repeated western blots gave similar results. IDO, indoleamine 2,3-dioxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.

expression of IDO mRNA and protein in SW837 cells. As demonstrated in Fig. 2A, expression levels of IDO mRNA induced by IFN-γ ($P<0.001$) were significantly suppressed when the cells were pretreated with 50 and 100 μM EGCG for 2 h prior to IFN-γ stimulation for 24 h ($P<0.001$ for each comparison). Similarly, increasing levels of EGCG treatment, particularly 100 μM, caused a marked decrease in the levels of IDO protein induced by IFN-γ in SW837 cells (Fig. 2B). These findings suggest that EGCG inhibits the expression of IDO mRNA and protein levels.

Effects of EGCG on the enzymatic activity of IDO induced by IFN-γ in SW837 cells. We examined whether EGCG inhibits the enzymatic activity of IDO induced by IFN-γ in SW837 cells. IDO activity was determined by measuring the concentration of L-kynurenine in culture medium (29). As demonstrated in Fig. 2C, the L-kynurenine concentration was markedly increased in the culture medium of IFN-γ-stimulated SW837 cells compared to that of the untreated cells ($P<0.001$). This increase was significantly inhibited by treatment with 100 μM EGCG ($P<0.001$), suggesting that, in addition to the expression levels, EGCG also suppresses the enzymatic activity of IFN-γ-induced IDO in CRC cells.

Effects of EGCG on the phosphorylation of STAT1 protein in SW837 cells. To determine the molecular mechanisms involved in the EGCG suppression of IDO expression in CRC cells, we examined whether EGCG inhibits STAT1 protein phosphorylation as the JAK/STAT1 pathway is mediated by IFN-γ to induce IDO expression (14). Western blot analysis revealed that stimulation with IFN-γ for 30 min markedly increased the expression of phosphorylated STAT1 protein in SW837 cells. However, this induction was significantly decreased by EGCG treatment in a dose-dependent manner (Fig. 3A), suggesting that at least part of the EGCG inhibition of the IFN-γ-induced IDO expression was due to the suppressed activation of STAT1.

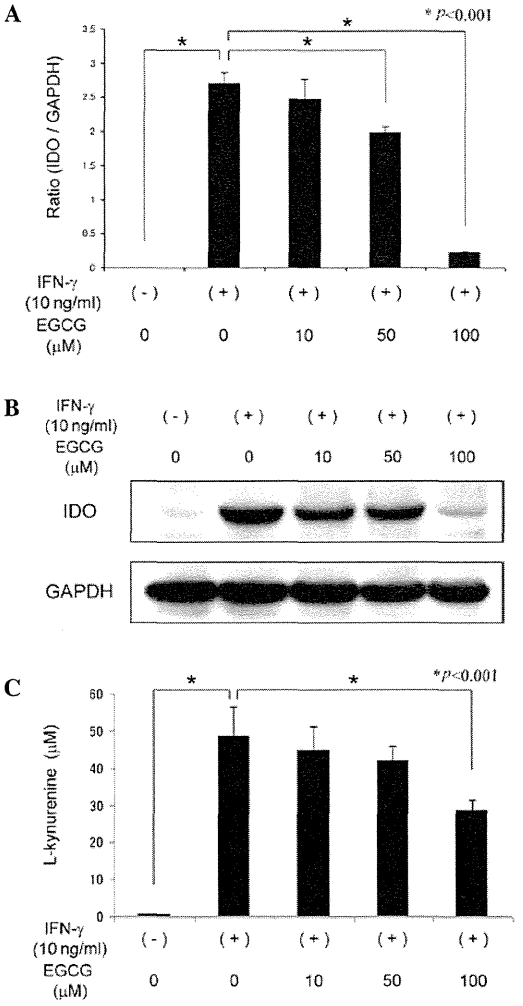

Figure 2. Effects of EGCG on the expression levels and enzymatic activity of IDO in SW837 cells. Cells were pretreated with various concentrations (0, 10, 50 or 100 μM) of EGCG for 2 h and were then stimulated with IFN-γ for 24 h. (A) Total RNA was extracted and the IDO mRNA expression levels were measured by quantitative real-time RT-PCR. (B) Total cellular protein was extracted and the levels of IDO protein expression were determined by western blot analysis. (C) Functional IDO enzymatic activity was determined by measuring the concentrations of L-kynurenine in culture medium under the same conditions. Columns and bars indicate the mean and SD. Repeated western blots gave similar results. IDO, indoleamine 2,3-dioxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; EGCG, (-)-epigallocatechin gallate; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.

Effects of EGCG on the transcriptional activity of IDO promoters in SW837 cells. To elucidate whether transcription

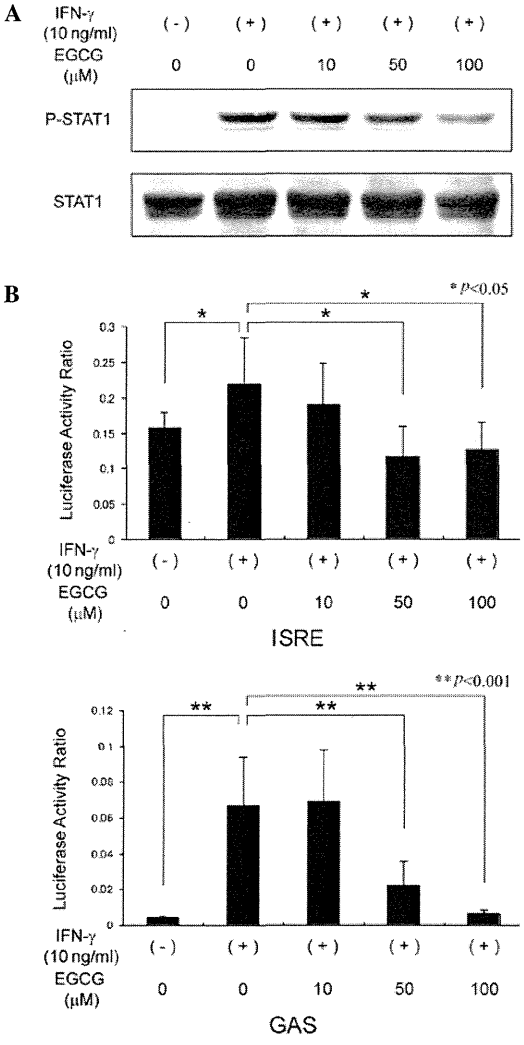

Figure 3. Effects of EGCG on the phosphorylation of STAT1 protein and transcriptional activity of IDO promoter in SW837 cells. (A) Cells were pretreated with various concentrations of EGCG for 2 h and were then stimulated with IFN-γ for 30 min. Total cellular protein was extracted and the expression of phosphorylated STAT1 protein was determined by western blot analysis. (B) Transient transfection reporter assays using ISRE and GAS luciferase reporter plasmids were performed using cell extracts pretreated with various concentrations of EGCG for 2 h and then stimulated with IFN-γ for 24 h. Columns and bars indicate the mean and SD. IFN, interferon; EGCG, (-)-epigallocatechin gallate; STAT1 signal transducer and activator of transcription 1; ISRE, interferon-stimulated response element; GAS, IFN-γ activation sequence; IDO, indoleamine 2,3-dioxygenase; SD, standard deviation.

of IDO is inhibited by EGCG, we next examined the effects of EGCG on the transcriptional activity of ISRE and GAS, two sequence elements of the IDO promoter, which are activated by the binding of STAT1 and associated with IFN-γ-induced IDO gene transcription (15). Transient transfection luciferase reporter assays demonstrated that IFN-γ stimulation upregulated the transcriptional activity of ISRE ($P<0.05$) and GAS ($P<0.001$) in SW837 cells. However, treatment with 50 and 100 μM EGCG significantly inhibited the promoter activities of ISRE ($P<0.05$) and GAS ($P<0.001$) induced by IFN-γ (Fig. 3B). These results provide evidence that the inhibitory effects of EGCG on IDO expression occur at the gene transcription level.

Discussion

The IDO-mediated escape of cancer cells from the immune system plays a critical role in tumor development and progression. Therefore, inhibition of inappropriate IDO activity in tumors may attenuate the ability of malignant cells to evade immune surveillance and promote their clearance (2). Several preclinical studies have demonstrated that IDO inhibitors, including 1-methyl-tryptophan, have a therapeutic effect in rodent cancer models (30,31). The results of this study in human CRC cells provide the first evidence that a naturally occurring compound, EGCG, effectively inhibits the IFN-γ-induced expression and activity of IDO (Fig. 2). These findings suggest that EGCG may exert its chemopreventive and anticancer properties, at least in part, by improving potential antitumor immune responses. These findings are significant in the context of chemoprevention and therapy using EGCG since clinical trials have demonstrated that high expression levels of IDO in cancer tissues are associated with disease progression and poor overall survival rates of patients with CRC (7,8).

Efficient EGCG inhibition of IFN-γ-induced IDO expression in CRC cells may be explained by the inhibition of STAT1 phosphorylation as IFN-γ/JAK/STAT1 signaling plays a critical role in IDO induction (14). Tyrosine phosphorylation of STAT1 by IFN-γ-induced reactions promotes the binding of STAT1 to two regulatory elements of the IDO gene promoter region, ISRE and GAS (14,15). In the present study, STAT1 phosphorylation (Fig. 3A) and transcriptional activities of ISRE and GAS (Fig. 3B) induced by IFN-γ stimulation were markedly suppressed by EGCG treatment. Our findings suggest that EGCG may prevent IFN-γ-induced expression of IDO at the gene transcription level through suppressing STAT1 activation. These results are consistent with previous studies demonstrating that EGCG efficiently inhibited IFN-γ-elicited phosphorylation and/or DNA-binding activity of STAT1 in several types of human cancer-derived cell lines (25,26). In addition to cancer cells, EGCG also inhibits IDO expression induced by IFN-γ in dendritic cells through the inhibition of STAT1 phosphorylation (24). Therefore, these studies (24-26), together with our findings, indicate that IFN-γ/JAK/STAT1 signaling is a critical target of EGCG in the suppression of IDO induction.

In the present study, the expression levels of IDO mRNA were significantly increased by IFN-γ stimulation in all CRC cell lines (Fig. 1A). Additionally, the concentration of L-kynurenine, which reflects the enzymatic activity of IDO in culture medium was also increased by SW837 CRC cells that overexpress IDO (Fig. 2C). These findings may be associated with the immune escape of malignant cells that occurs within the tumor and its surrounding microenvironment since tryptophan depletion and production of toxic tryptophan catabolites resulting from IDO activity may inhibit T cell and natural killer cell proliferation (5). The serum kynurenine/tryptophan ratio is significantly increased, while the serum tryptophan level is significantly decreased, in CRC patients when compared to control noncancerous patients (32). Conversely, IDO inhibitors can impede the growth of IDO-expressing tumors through

the reduction of kynurenine in the microenvironment and enhancement of T cell functions (33). Therefore, the effects of EGCG on the suppression of L-kynurenine levels increased by IFN-γ-stimulated human CRC cells (Fig. 2C) may also contribute to the inhibition of growth of IDO-expressing CRC cells and improve immune tolerance caused by IDO.

In this study, we demonstrate that EGCG downregulates the expression and enzymatic activity of IFN-γ-induced IDO in CRC cells through the inhibition of STAT1 activation. Our findings suggest the possibility that EGCG may exert its anticancer and chemopreventive effects by inhibiting the expression and function of IDO. A recent experiment in rats has demonstrated that upregulation of IDO activity is possibly involved in colon carcinogenesis, and EGCG treatment effectively suppresses the development of chemically induced colonic preneoplastic lesions by inhibiting the expression levels and enzymatic activity of IDO (12). In conclusion, targeting IDO and improving IDO-mediated immune escape of premalignant and cancer cells with EGCG may be an effective strategy for the prevention and treatment of CRC.

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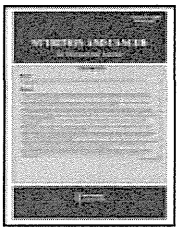

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Preventive Effects of Curcumin on the Development of Azoxymethane-Induced Colonic Preneoplastic Lesions in Male C57BL/KsJ-*db/db* Obese Mice

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Obesity-related metabolic abnormalities include a state of chronic inflammation and adipocytokine imbalance, which increase the risk of colon cancer. Curcumin, a component of turmeric, exerts both cancer preventive and antiinflammatory properties. Curcumin is also expected to have the ability to reverse obesity-related metabolic derangements. The present study examined the effects of curcumin on the development of azoxymethane (AOM)-induced colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice. Feeding with a diet containing 0.2% and 2.0% curcumin caused a significant reduction in the total number of colonic premalignant lesions compared with basal diet-fed mice. The expression levels of tumor necrosis factor-α, interleukin-6, and cyclooxygenase-2 (COX-2) mRNAs on the colonic mucosa of AOM-treated mice were significantly decreased by curcumin administration. Dietary feeding with curcumin markedly activated AMP-activated kinase, decreased the expression of COX-2 protein, and inhibited nuclear factor-κB activity on the colonic mucosa of AOM-treated mice. Curcumin also increased the serum levels of adiponectin while conversely decreasing the serum levels of leptin and the weights of fat. In conclusion, curcumin inhibits the development of colonic premalignant lesions in an obesity-related colorectal carcinogenesis model, at least in part, by attenuating chronic inflammation and improving adipocytokine imbalance. Curcumin may be useful in the chemoprevention of colorectal carcinogenesis in obese individuals.

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INTRODUCTION

Colorectal cancer (CRC) is a serious global health care problem. There is increasing evidence that obesity and its related metabolic abnormalities are associated with colorectal carcinogenesis. Several biological mechanisms linking obesity to the development of CRC have been demonstrated, including the emergence of insulin resistance and alterations in the insulin-like growth factor (IGF)/IGF-1 receptor axis (1,2). Imbalance of adipocytokines (such as decreased adiponectin levels and increased leptin levels) and a state of chronic inflammation, both of which are highly correlated with the presence of excess adipose tissue, also play roles in obesity-related colorectal carcinogenesis (1,2). For instance, there is a close correlation between lower levels of serum adiponectin and the development of colorectal tumors (3,4). Increased leptin levels exert tumor-promoting effects in obesity- and inflammation-related CRC (5–9). Tumor necrosis factor (TNF)-α, a central mediator of chronic inflammatory diseases, has markedly increased expression levels in adipose tissue, and its dysregulation is associated with stimulation of tumor promotion and progression of carcinogenesis (10,11). These reports suggest that targeting adipocytokine imbalance and chronic inflammation may provide effective strategies for preventing the development of obesity-related CRC (6–8).

Curcumin, a yellow-colored pigment derived from turmeric (Curcuma longa), has been shown to exert numerous pharmacological effects, including antiinflammatory and chemopreventive properties (12–14). In rodent models, dietary curcumin was found to inhibit chemically induced colorectal carcinogenesis (see the Chemopreventive Database at http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html). Curcumin also inhibited cell growth and induced apoptosis in human CRC-derived cells

by suppressing the expression of cyclooxygenase-2 (COX-2) (15,16), which is one of the most critical targets of CRC chemoprevention (17). Curcumin showed benefits in the treatment of ulcerative colitis in a randomized clinical trial (18), and it was also found to attenuate chronic experimental colitis in rats by reducing the expression of TNF-α and COX-2 on the colonic mucosa (19). Therefore, curcumin may be capable of exerting chemopreventive and antiinflammatory effects on the colon through regulation of TNF-α and COX-2 expression.

In addition, recent studies have revealed that curcumin has the potential to improve obesity-related chronic inflammatory conditions and metabolic derangements. In rodent models of obesity and diabetes, administration of curcumin significantly ameliorates diabetes, increases adiponectin production by adipose tissue, and decreases serum levels of TNF-α and interleukin (IL)-6 (20,21). Therefore, curcumin may be a promising agent for the prevention of obesity-related pathogenesis including CRC development. In the present study, we investigated the effects of curcumin on the development of colonic premalignant lesions, aberrant crypt foci (ACF), and β-catenin accumulated crypts (BCAC) (22,23) in an animal model for obesity-related colorectal carcinogenesis (5–8), which was produced by administrating the colonic carcinogen azoxymethane (AOM) to obese and diabetic C57BL/KsJ-*db/db* (*db/db*) mice.

MATERIALS AND METHODS

Animals and Chemicals

Four-week-old male homozygous *db/db* mice were obtained from Japan SLC (Shizuoka, Japan) and were maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. Curcumin and AOM were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Procedure

A total of 38 male *db/db* mice were divided into the following 5 experimental and control groups: untreated (Group 1, $n = 5$); 2% curcumin alone (Group 2, $n = 5$); AOM alone (Group 3, $n = 10$); AOM plus 0.2% curcumin (Group 4, $n = 9$); and AOM plus 2% curcumin (Group 5, $n = 9$). The curcumin concentrations (0.2% and 2%) were chosen based on the following 2 reasons: 1) the doses of dietary curcumin in the most published rodent experiments were between 0.05 and 2% from the Chemopreventive Database; and 2) the dose level of 0.2% curcumin in diet, which is equivalent to 300mg/kg/day (24), is relevant to the dose used in clinical trials. At 5 wk of age, all mice were given 4 weekly subcutaneous injections of saline (Groups 1 and 2) or AOM (15 mg/kg body weight; Groups 3, 4, and 5). The mice in Groups 1 and 3 were fed the basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), throughout the experiment (for 11 wk). Group 2 was fed the basal diet containing 2% curcumin throughout the experiment. Groups 4 and 5 were given the basal diet containing 0.2% and 2% curcumin, respectively, for 7 wk, starting 1 wk after the last injection of AOM. At the termination of the study (16 wk of age), all mice were sacrificed for analysis of ACF and BCAC. The animal experiment was approved by the Committee of the Institutional Animal Experiments of Gifu University.

Identification and Counting of ACF and BCAC

The numbers of ACF and BCAC were determined according to standard procedures (6–8). After fixing flat in 10% buffered formalin for 24 h, the colons were stained with methylene blue (0.5% in distilled water) to count the number of ACF. The distal parts of the colon (2 cm from anus; mean area, 0.7 cm^2/colon) were then cut and embedded in paraffin, and a total of 20 serial sections (each 4 μm thick) per mouse were created by an en face preparation to identify BCAC intramucosal lesions (6–8). For each case, 2 serial sections were used to analyze BCAC.

Histopathology and Immunohistochemical Analyses for β-Catenin and Nuclear Factor-κB

Two serial sections, prepared from the paraffin-embedded tissue blocks, were subjected to hematoxylin and eosin staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC (6–8). In addition, phospho-nuclear factor-κB (NF-κB) p65 immunohistochemistry was performed in vertical histological sections of colonic mucosa to estimate NF-κB activity in the crypt cells and mucosal interstitial cells (25). Immunohistochemistry for β-catenin and phospho-NF-κB p65 was performed using the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark). Anti-β-catenin antibody (1:1000 final dilution) was obtained from BD Transduction Laboratories (San Jose, CA). Antiphospho-NF-κB p65 antibody (Ser276; 1:50 final dilution) was from Cell Signaling Technology (Danvers, MA). In the phospho-NF-κB p65-immunostained sections, the cells with intensely stained nuclei were considered to be active in NF-κB. Cells with active NF-κB in both the colonic epithelium and interstitium were counted and expressed as a percentage of the total number of cells in the tissues. A positive cell index (%) was determined by counting at least 500 cells in the colonic epithelium and 300 cells in the interstitium of each mouse.

Protein Extraction and Western Blot Analysis

Total proteins were extracted from the scraped mucosa from the remaining colon portions of the AOM-treated mice (Groups 3 to 5), and equivalent amounts of proteins (20 μg/lane) were examined by a Western blot analysis (6–8). The primary antibodies for AMP-activated kinase (AMPK), phosphorylated form of AMPK, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (6–8). An antibody against GAPDH served as a loading control.

RNA Extraction and Quantitative Real-Time Reverse Transcription-PCR analysis

Total RNA was isolated from the scraped colonic mucosa of the AOM-treated mice using the RNAqueous-4PCR kit

(Ambion Applied Biosystems, Austin, TX). The cDNA was synthesized from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, San Diego, CA). A quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify the TNF-α, IL-6, COX-2, and GAPDH genes, as previously described (8). Real-time RT-PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN) with the SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the TNF-α, IL-6, and COX-2 genes were normalized to the GAPDH gene expression levels.

Clinical Chemistry

The blood samples, which were collected at the time of sacrifice after 6 h of fasting, were used for chemical analyses. The serum concentrations of adiponectin and leptin (Shibayagi, Gunma, Japan) were determined by using an enzyme immunoassay according to the manufacturer's protocol.

Statistical Analyses

The results are presented as the mean ± SD and were analyzed using the GraphPad Instat software program, v. 3.05 (GraphPad Software, San Diego, CA) for Macintosh. The differences among the groups were analyzed by 1-way analysis of variance (ANOVA) or by 2-way ANOVA if required. When ANOVA showed a statistically significant effect ($P < 0.05$), comparison of each experimental group with the control group was performed using the Tukey-Kramer multiple comparisons test. The differences were considered statistically significant when the 2-tailed P-value was less than 0.05.

RESULTS

General Observations

During the study, dietary feeding with curcumin did not cause clinical symptoms or evidence of toxicity (data not shown). The mean body weights of the AOM-injected groups (Group 3: 40.9 ± 5.5 g, Group 4: 35.0 ± 2.6 g, and Group 5: 39.1 ± 6.1 g) were lower than that of the saline-injected group (Group 1: 54.6 ± 8.8 g) at the termination of the experiment ($P < 0.01$ for each comparison). This might be caused by the toxicity of AOM, which has been observed in previous experiments (6–8). No significant differences were observed in the mean weights of the liver and kidney among the groups (data not shown). Histopathological examination also revealed that there were no alterations visible in the liver and kidney tissues. This provides further evidence of the lack of toxicity of curcumin with respect to the liver and kidney of the mice in Groups 3 to 5 (data not shown).

Effects of Curcumin on AOM-Induced ACF and BCAC Formations in *db/db* Mice

Table 1 summarizes the total numbers of ACF and BCAC in each group. ACF and BCAC were observed to develop in the colon of all mice that received AOM (Groups 3 to 5), but not in the control groups (Groups 1 and 2). The mean number of ACF in the AOM alone group (Group 3) was 134.0 ± 24.5 and dietary feeding with 0.2% (Group 4) and 2% (Group 5) curcumin significantly reduced the incidence of ACF by 27% ($P < 0.05$) and 43% ($P < 0.01$), respectively. Moreover, the number of BCAC in Group 5 was significantly lower than that in Group 3 (76% inhibition, $P < 0.01$).

Effects of Curcumin on the Expression Levels of TNF-α, IL-6, and COX-2 mRNAs in the Colonic Mucosa of AOM-Injected *db/db* Mice

Quantitative real-time RT-PCR analysis indicated that feeding with 2% of curcumin significantly decreased the expression levels of TNF-α (Fig. 1A, $P < 0.05$) and COX-2 (Fig. 1C, $P < 0.05$) mRNAs in the colonic mucosa of AOM-injected mice relative to those of the curcumin-untreated control mice. In addition, the expression levels of IL-6 mRNA were apparently decreased to a greater extent by administration of both doses of curcumin (Fig. 1B, $P < 0.01$).

Effects of Curcumin on the Activation of AMPK and the Expression of COX-2 Proteins in the Colonic Mucosa of AOM-Injected *db/db* Mice

A Western blot analysis indicated that administration of both concentrations of curcumin caused significant phosphorylation

TABLE 1
Effects of curcumin on AOM-induced ACF and BCAC formation in the colon of male *db/db* mice

Group no.	Treatment	No. of mice	Length of colon (cm)	Total no. ACFs/colon	Total no. BCACs/cm^2
1	Saline	5	11.1 ± 0.8[a]	0	0
2	Saline + 2% curcumin	5	10.5 ± 0.2	0	0
3	AOM alone	10	10.6 ± 0.8	134.0 ± 24.5	3.4 ± 1.8
4	AOM + 0.2% curcumin	9	9.6 ± 1.6	98.2 ± 36.6[b]	1.5 ± 1.1
5	AOM + 2% curcumin	9	10.4 ± 0.4	76.4 ± 13.6[c]	0.8 ± 1.4[c]

[a]Mean ± SD.
[b]Significantly different from Group 3 ($P < 0.05$).
[c]Significantly different from Group 3 ($P < 0.01$).

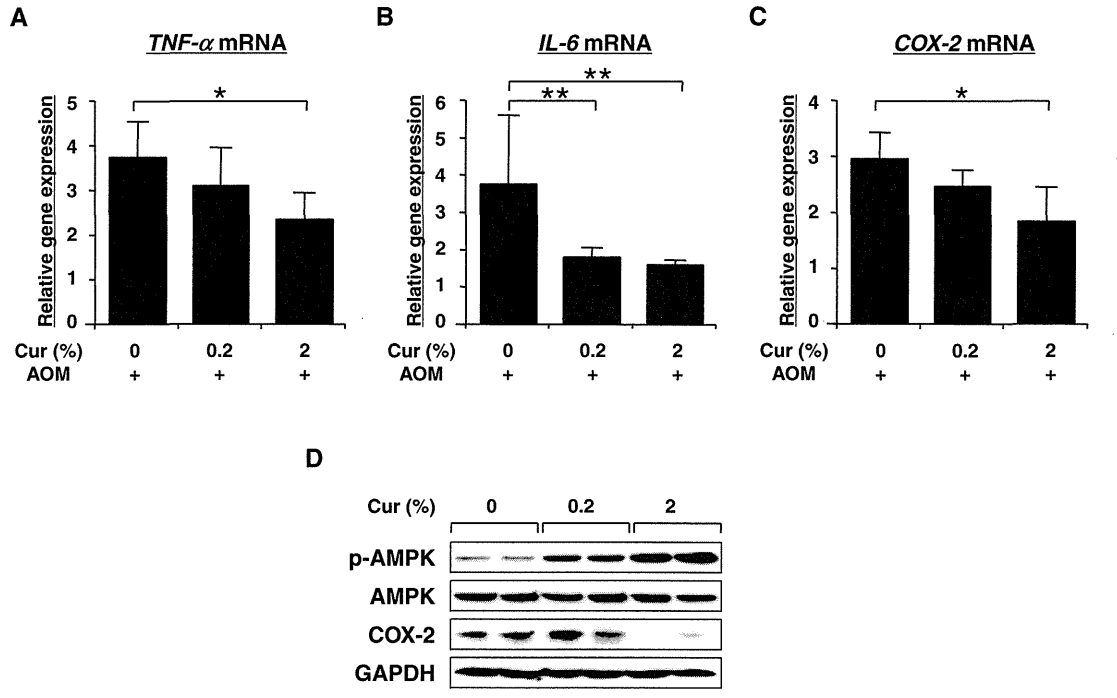

FIG. 1. Effect of curcumin (Cur) on the levels of tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), and cyclooxygenase-2 (COX-2) (C) mRNAs and on the activation of AMP-activated kinase (AMPK) and expression of COX-2 proteins (D) in the colonic mucosa of azoxymethane (AOM)-treated *db/db* mice. cDNA was synthesized from the colonic mucosa and real-time RT-PCR was performed using TNF-α (A), IL-6 (B), and COX-2 (C) specific primers. The expression levels of these genes were normalized to the level of the GAPDH gene. Each experiment was done in triplicate and the average was subsequently calculated. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to mice not fed with curcumin. Total proteins were extracted from the scraped colonic mucosa and equivalent amounts of proteins were examined by a Western blot analysis using phosphorylated AMPK (p-AMPK), AMPK, and COX-2 specific antibodies (D). Two lanes represent protein samples from 2 different mice in each group (Groups 3 to 5). An antibody to GAPDH served as a loading control.

(activation) of AMPK protein in the colonic mucosa of AOM-treated mice. In addition, there was an apparent decrease in the expression levels of COX-2 proteins in the colonic mucosa of mice after feeding the diet containing 2% curcumin (Fig. 1D).

Effects of Curcumin on the NF-κB Activity in the Colonic Mucosa of AOM-Injected *db/db* Mice

Curcumin inhibits the activation of NF-κB and this is one of the critical mechanisms for the antiinflammatory effects of this agent (12,13). Therefore, the effects of curcumin on NF-κB activity were examined in the colonic mucosa of AOM-injected mice. As shown in Fig. 2, the indices of phospho-NF-κB p65-positive cells in both the colonic epithelium and interstitium of curcumin-supplemented mice were significantly smaller than those of the curcumin-untreated mice ($P < 0.01$, for each comparison), thus indicating that curcumin supplementation significantly inhibits the NF-κB activity in the colonic mucosa, mainly crypt and inflammatory cells, of the AOM-treated *db/db* mice.

Effects of Curcumin on the Serum Levels of Adiponectin and Leptin and on the Relative Weights of White Adipose Tissue in *db/db* Mice

The serum concentrations of adiponectin in the 2% curcumin-treated groups were significantly higher than those of the groups that did not receive curcumin ($P < 0.01$ and $P < 0.05$), regardless of AOM-injection (Fig. 3A). In AOM-injected groups, administration of both doses of curcumin showed a significant decrease in the serum levels of leptin ($P < 0.05$ for each comparison) when compared to mice that did not receive curcumin (Fig. 3B). In addition, administration of curcumin also significantly reduced the relative weights of white adipose tissue (periorchis and retroperitoneum) compared with the untreated groups ($P < 0.05$ and $P < 0.01$), regardless of AOM-treatment (Fig. 3C).

DISCUSSION

Obesity-related physiological alterations, such as a state of chronic inflammation, are known to influence the risk of CRC development (1,2). This is the first study to report evidence

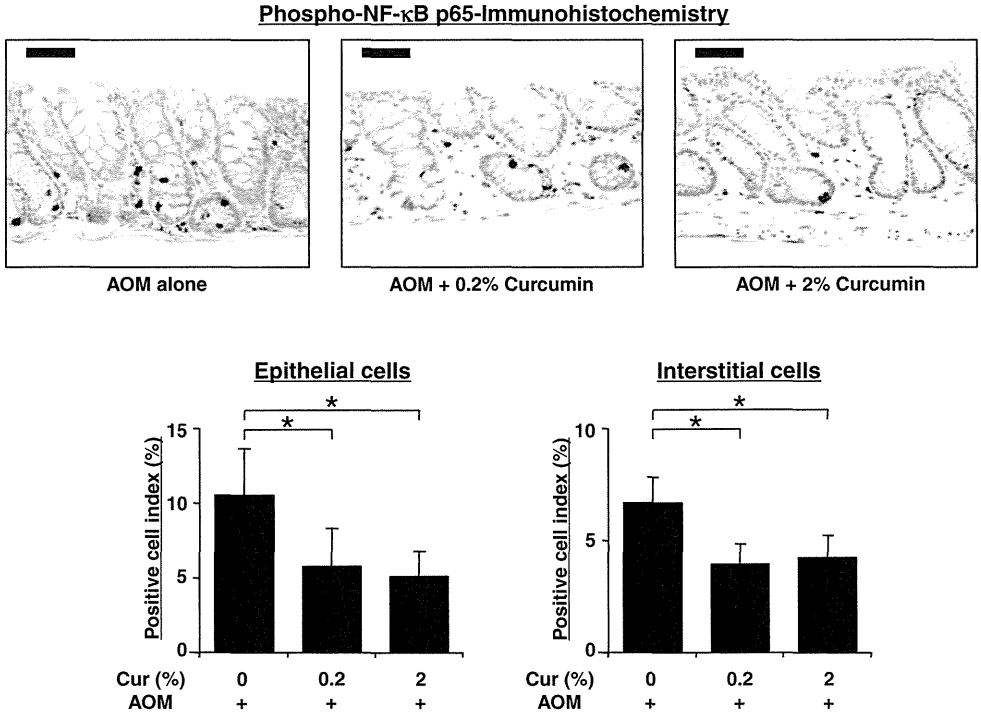

FIG. 2. Effects of curcumin (Cur) on the nuclear factor (NF)-κB activity in the colonic mucosa of azoxymethane (AOM)-treated *db/db* mice. Sections of the colon were stained with antiphospho-NF-κB p65 antibody. Representative photographs from each group are shown in the upper panels. The positive cell indices, which were determined by counting the phospho-NF-κB p65-positive cells in both the colonic epithelium and interstitial inflammatory cells, are shown in the lower panels. Bars: 50 μm. $^*P < 0.01$ compared to mice not fed with curcumin.

of curcumin effectively inhibiting the development of putative precursor lesions (ACF and BCAC) of colonic adenocarcinoma in the obese and diabetic *db/db* mice (Table 1). These suppressive effects are most likely associated with the decrease in expression of proinflammatory cytokines TNF-α and IL-6 in the colonic mucosa of AOM-treated *db/db* mice (Figs. 1A and 1B), although other anticancer mechanisms, such as modulation of cell proliferation and apoptosis, could be considered (12–14). The expression levels of COX-2, which represents an early response to proinflammatory mediators and a critical target for CRC chemoprevention (17), are also inhibited in the colonic mucosa of mice fed with curcumin (Figs. 1C and 1D). In addition, curcumin supplementation inhibited NF-κB activity, which is involved in regulation of COX-2 expression (26), in the colonic mucosa of AOM-treated *db/db* mice (Fig. 2). These findings are consistent with those of a previous study indicating that curcumin inhibits the induction of COX-2 by TNF-α stimulus via the inhibition of NF-κB activation in human colon epithelial cells (27). Curcumin suppresses the growth of CRC-derived cells by inhibiting the expression of COX-2 (16). Curcumin also inhibits the secretion of TNF-α and IL-6 from monocytes cultured under high-glucose conditions, and lowers the serum levels of TNF-α and IL-6 in diabetic rats (21). Therefore, overexpression of inflammatory mediators, especially TNF-α, IL-6, and COX-2, which are relevant to excessive storage of lipid, might represent critical targets of curcumin to prevent the development of obesity-related CRC.

The present study also demonstrated the first evidence that administration of curcumin markedly enhances AMPK activation in the colonic mucosa of obese and diabetic mice (Fig. 1D). This finding is significant because AMPK, which functions as a major metabolic switch for maintenance of energy homeostasis, controls processes relevant to tumor development and therefore may be a promising target for cancer chemoprevention (28). For instance, the antidiabetic drug metformin, a pharmacological AMPK activator, exerts growth inhibitory effects on colon cancer xenografts as well as on human CRC cells (29,30). Pitavastatin, which is used to treat hyperlipidemia, inhibits development of AOM-induced BCAC in *db/db* mice by activating AMPK and decreasing the expression levels of TNF-α, IL-6, and COX-2 mRNAs in the colonic mucosa (8). Curcumin also inhibits proliferation and induces apoptosis in human CRC cells

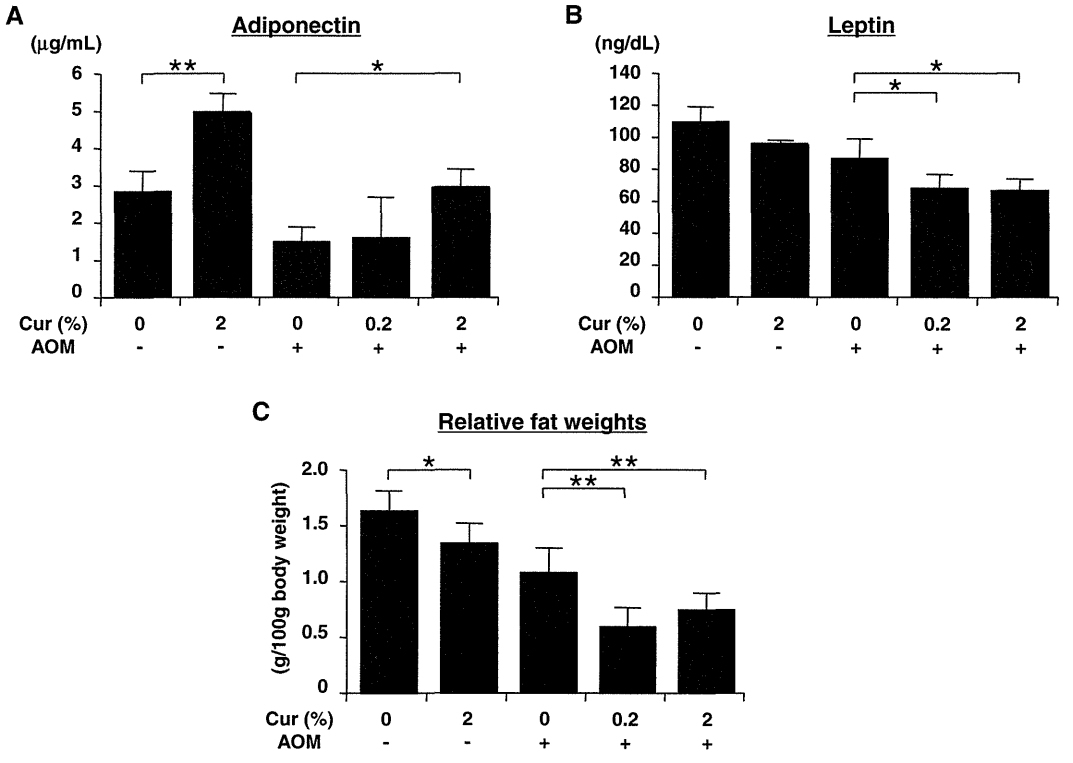

FIG. 3. Effect of curcumin (Cur) on the serum levels of adiponectin and leptin and on the relative weights of fat in experimental mice. The serum concentrations of adiponectin (A) and leptin (B) were measured by using an enzyme immunoassay. At sacrifice, white adipose tissue of the periorchis and retroperitoneum was excised and the relative weights (g/100 g body weight) were calculated (C). Values are expressed as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to mice not fed with curcumin. AOM indicates azoxymethane.

by activating AMPK and decreasing COX-2 expression (15). These reports (8,15,29,30), together with our present findings (Table 1 and Fig. 1D), suggest that curcumin may prevent the development of obesity-related colorectal carcinogenesis, at least in part, through the activation of AMPK.

Dysregulation of adipocytokines is also involved in the link between obesity and colorectal carcinogenesis (1,2). In particular, a low level of serum adiponectin, which raises the risk of colorectal tumorigenesis (3,4), is associated with obesity-based chronic inflammation because adiponectin exerts antiinflammatory effects by downregulating the production of TNF-α and IL-6 (31). Adiponectin inhibits the growth of CRC cells through the activation of AMPK and suppresses colonic epithelial proliferation in mice that are fed a high-fat diet (32,33). On the other hand, a higher level of serum leptin, which is proportional to the amount of body fat stored, plays a role in enhancing the development of obesity- and inflammation-related CRC (5–9). Leptin also increases cell growth and promotes cell mobility and invasion in CRC-derived cells (34,35). These reports suggest that targeting the imbalance of adiponectin and leptin might be an effective strategy for preventing obesity-related CRC. Indeed, improvement of adipocytokine imbalance by certain agents may contribute to the inhibition of obesity-related tumorigenesis in the colorectum (6–8). Therefore, our findings that dietary curcumin significantly increased the levels of adiponectin as well as decreased the levels of leptin in the serum of *db/db* mice, which might be caused by the reduction in white adipose tissue (Fig. 3), is important to explain the chemopreventive effects of this agent on obesity-related colorectal carcinogenesis. Similarly, it was also reported that curcumin administration ameliorates diabetes and increases the expression of adiponectin both in serum and adipose tissue of leptin-deficient *ob/ob* mice (20). Therefore, in addition to the improvement of metabolic abnormalities, curcumin has the potential to suppress obesity-related colorectal carcinogenesis by affecting the serum levels of adiponectin and leptin.

The limitation of present study is that the dose of curcumin in Group 5 (2%) is relatively high when considering the use of this

agent in clinical practice. However, our histopathologic examination revealed no alterations in the vital organs of the mice that received the diet containing 2% curcumin. Our ongoing study on the safety and chemopreventive ability of different doses of curcumin may show dose-response efficacy in inhibiting colorectal tumorigenesis of mice, as suggested for ACF-inhibition in this study (see Group 4 in Table 1). Moreover, a recent clinical trial has shown a significant result indicating that a daily oral dose of 3.6 g curcumin, which is well tolerated at patients with advanced CRC (36), achieves pharmacologically efficacious levels in the colorectum with negligible distribution of this agent outside the gut (37). This finding suggests that regions of the gastrointestinal tract, such as the colorectum, may be more appropriate targets for curcumin to exert its cancer chemopreventive effects because such organs are directly exposed to high concentrations of this agent.

It should be emphasized again that obesity and obesity-related metabolic abnormalities represent serious global health care problems, and CRC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis, which includes chronic inflammation and adipocytokine imbalance, might be a promising strategy for treating obese individuals who are at an increased risk of developing CRC. Curcumin, which has been shown to exert various chemopreventive and anticancer properties (12–14), appears to be a potentially effective candidate for this purpose because this phytochemical can attenuate inflammation while improving the imbalance of adipocytokines and can, furthermore, prevent the development of colonic precancerous lesions in obese and diabetic *db/db* mice.

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