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Monosodium glutamate-induced diabetic mice are susceptible to azoxymethane-induced colon tumorigenesis

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Obese people and diabetic patients are known to be high risk of colorectal cancer (CRC), suggesting need of a new preclinical animal model, by which to extensively study the diverse mechanisms, therapy and prevention. The present study aimed to determine whether experimental obese and diabetic mice produced by monosodium glutamate (MSG) treatment are susceptible to azoxymethane (AOM)-induced colon tumorigenesis using early biomarkers, aberrant crypts foci (ACF) and β-catenin-accumulated crypts (BCACs), of colorectal carcinogenesis. Male Crj:CD-1 (ICR) newborns were daily given four subcutaneous injections of MSG (2 mg/g body wt) to induce diabetes and obesity. They were then given four intraperitoneal injections of AOM (15 mg/kg body wt) or saline (0.1 ml saline/10 g body wt). Ten weeks after the last injection of AOM, the MSG-AOM mice had a significant increase in the multiplicity of BCAC (13.83 ± 7.44, $P < 0.002$), but not ACF (78.00 ± 11.20), when compare to the Saline-AOM mice (5.45 ± 1.86 of BCAC and 69.27 ± 8.06 of ACF). Serum biochemical profile of the MSG-treated mice with or without AOM showed hyperinsulinemia, hypercholesteremia and hyperglycemia. The mRNA expression of insulin-like growth factor-1 receptor (IGF-1R, $P<0.01$) was increased in the MSG-AOM mice, when compared with the mice given AOM alone. IGF-1R was immunohistochemically expressed in the BCAC, but not ACF, in the AOM-treated mice. Our findings suggest that the MSG mice are highly susceptible to AOM-induced colorectal carcinogenesis, suggesting potential utility of our MSG-AOM mice for further investigation of the possible underlying events that affect the positive association between obese/diabetes and CRC.

Introduction

Epidemiological studies have shown that obesity and diabetes mellitus may be one of the risk factors for colorectal cancer (CRC) development (1–7). At present, hyperinsulinemia (8,9), hypercholesterolemia (10,11), hyperglycemia (9,12) and hyperlipidemia (7) are considered to be the possible risk factors of CRC. In addition, insulin-like growth factor (IGF) pathway is involved in colorectal carcinogenesis (13–16) and the signaling pathway is reported to be a potential target of CRC treatment (17–19) and CRC chemoprevention (20,21). Thus, importance of the growth hormone/IGF-1 axis (22) and IGF/IGF-1 receptor (IGF-1R) axis (15,23,24) is postulated in carcinogenesis in CRC development. In fact, our experimental studies indicated that the IGF/IGF-1R axis is altered during carcinogenesis in colorectum (25,26) and other tissue (27,28) and the axis is a good target for cancer chemoprevention (25–28). However, the underlying mechanisms of how these chronic diseases promote colon carcinogenesis still remain unknown (19). On this context, new research animal models are needed to investigate the diverse aspects of the mechanisms.

We have previously reported that development of AOM-induced precancerous lesions is enhanced in C57BL/KsJ-*db/db* mice with hyperleptinemia and hyperinsulinemia (29). Such an animal model may give important implications for further exploration of the possible underlying events that affect the positive association between CRC and obesity and/or diabetes (30–32). A number of animal models for diabetes and/or obesity have been reported. One such model is produced by injection of monosodium glutamate (MSG). When MSG is applied to Crj:CD-1 (ICR) newborn mice (MSG mice), they develop diabetic condition (hyperinsulinemia, hyperglycemia and hyperplastic islets) without polyphagia (33,34).

It is believed that colorectal carcinogenesis is a representative multistep tumorigenesis with events of genetic alterations. Several small lesions, including aberrant crypt foci (ACF) (35,36), mucin-depleted foci (37) and β-catenin-accumulated crypts (BCACs) (38) are proposed as early-appearing preneoplastic lesions (37). While ACF and mucin-depleted foci are recognized on the surface of cancer-predisposed colons of rodents and human (37), BCAC are identified in colonic mucosa at the early stages of colon carcinogenesis (39). Accumulating evidence suggests that BCAC are independent small dysplastic lesions and/or microadenomas and progressed precancerous lesions (40) in colon carcinogenesis when compared with ACF and mucin-depleted foci (39). These early lesions are widely used for investigating pathobiology of colorectal carcinogenesis (37).

In the current study, new born Crj:CD-1 (ICR) mice were treated with MSG to produce diabetes and obesity and, subsequently, they received a colonic carcinogen, azoxymethane (AOM). Our results indicated that the MSG mice are highly susceptible to AOM-induced colorectal carcinogenesis by counting the number of BCAC, but not ACF, and possible involvement of the IGF/IGF-1R axis in colorectal tumorigenesis of diabetic and obese mice induced by MSG and AOM. Our main goal is to assess the involvement of obesity/diabetes-associated events, such as hyperinsulinemia, in colorectal carcinogenesis *in vivo*.

Materials and methods

Animals and chemicals

The pregnant Crj:CD-1 (ICR) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and their newborns were used in the study. MSG was obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan) and AOM from Sigma Chemical Co. (St Louis, MO). Mice used for the experiment were maintained in the well-controlled room with a high-efficiency particulate air filter, a 12 h lighting (7:00–19:00), 25 ± 2°C room temperature and 55 ± 15% humidity. Mice (3–6 mice/cage) were housed in polycarbonate cages measuring W225 × D338 × H140 mm (Japan CLEA, Inc., Tokyo, Japan) with the floor covered with a sheet of roll paper (Japan SLC). MF (Oriental Yeast Co., Ltd, Tokyo, Japan) was used as a basal diet throughout the study. Groundwater that was chlorine-treated and subjected to ultraviolet disinfection was used as drinking water in a bottle. We fully complied with the 'Guidelines Concerning Experimental Animals' issued by the Japanese Association for Laboratory Animal Science and exercised due consideration so as not to cause any ethical problem.

Experimental procedure

The newborns were divided into two groups according to the treatments. The birth date was the beginning of four daily subcutaneous injections of MSG (2 mg/g body wt, MSG mice) and physiological saline (Saline mice). Among these mice, males were subjected to the study. They were divided into four groups at 4 weeks of age: groups 1 (12 males) and 2 (6 males) of the MSG mice received four weekly intraperitoneal injections of AOM (15 mg/kg body wt,

Abbreviations: ACF, aberrant crypts foci; AOM, azoxymethane; BCAC, β-catenin-accumulated crypt; CRC, colorectal cancer; IGF-1R, insulin-like growth factor-1 receptor; MSG, monosodium glutamate.

the MSG-AOM mice) and physiological saline (0.1 ml/10 g body wt, the MSG-Saline mice), respectively. Similarly, two groups of the ICR-Saline mice were given AOM or saline, belonging to groups 3 (11 males, the Saline-AOM mice) and 4 (5 males, the Saline–Saline). At the termination of the experiment (10 weeks after the last injection of AOM and 17 weeks of age of mice), all animals were killed to analyze the number of colonic ACF and BCAC, clinical serum chemistry and mRNA expression of IGF1, IGF2 and IGF-1R in the colonic mucosa.

Counting the numbers of ACF and BCAC

The ACF and BCAC were determined according to the standard procedures described previously (30,31,41). ACF are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia and are larger than adjacent normal crypts (35). BCAC, which have high-frequency mutations in β-*catenin* gene, demonstrate histological dysplasia with a disruption of the cellular morphology (Figure 2A) and an accumulation of this protein (Figure 2B) (39). BCAC do not have a typical ACF-like appearance because the lesion is not recognized on the mucosal surface like ACF and is only identified in the histological sections of 'en face' preparations. Both of these lesions are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive (42–44) and tumor promotion (45) properties. After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water) and then the number of ACF were counted under a light microscope. Thereafter, the distal parts (5 cm from anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area: 0.7 cm^2/colon) was embedded in paraffin and then a total of 20 serial sections (4 μm thick each) per colon were made by an en face preparation (30,31,41). For each case, two serial sections were used to analyze BCAC.

Histopathology and immunohistochemical analyses for β-catenin and IGF-1R

Three serial sections were made from paraffin-embedded tissue blocks. Two sections were subjected to hematoxylin and eosin (H and E) staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. Immunohistochemistry for β-catenin and IGF-1R was performed using the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark), as described previously (30,31). Primary antibodies of anti-β-catenin antibody (1:1000 final dilution) and anti-IGF-1R antibody (1:100 final dilution) obtained from Transduction Laboratories (catalog no. 610154; San Jose, CA) and Santa Cruz Biotechnology, Inc. (sc-7907; Santa Cruz, CA), respectively, were applied on the sections. Negative control sections were immunostained without the primary antibody.

Blood chemistry

At 17 weeks of age, blood samples (0.5–1.0 ml/mouse) were collected for determination of total cholesterol, triglyceride and glucose by a simple measurement device (DRICHEM Fujifilm Medical Co., Ltd, Tokyo). The concentration of blood insulin was measured by an LBIS insulin measuring kit for mice (Shibayagi Co., Ltd, Gunma).

RNA extraction and quantitative real-time reverse transcription–polymerase chain reaction analysis

A quantitative real-time reverse transcription–polymerase chain reaction analysis was carried out in the scraped colonic mucosa of the MSG-AOM mice and the Salin-AOM mice. Total RNA was isolated from the scraped colon mucosa of the mice using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX). The cDNA was synthesized from 0.2 μg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The primers used for the amplification of IGF-1-, IGF-2- and IGF-1R-specific genes were as follows: IGF-1 forward, 5′-CTGGACCAGAGACCCTTTGC-3′ and reverse, 5′-GGACGGGGACTTCTGAGTCTT-3′; IGF-2 forward, 5′-GTGCTGCATCGCTGCTTAC-3′ and reverse, 5′-ACGTCCCTCTCGGACTTGG-3′; and IGF-1R forward, 5′-GTGGGGGCTCGTGTTTCTC-3′ and reverse, 5′-GATCACCGTGCAGTTTTCCA-3′. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the IGF-1, IGF-2 and IGF-1R genes were normalized to the β-actin gene expression level.

Statistical analysis

Measurements are expressed as mean ± SD, and differences if present were compared by one-way analysis of analysis of variance (Tukey–Kramer's multiple comparison's test) or two-tailed unpaired *t*-test. The incidences of intestinal tumors were compared by Fisher's exact probability test. The results were considered statistically significant if the *P* values were <0.05.

Results

General observations

As shown in Figure 1, the mean body weight of MSG–Saline mice (group 2) was much greater than Saline–Saline mice (group 4) during the study. The average body weights of the AOM-injected groups belonging to groups 1 (MSG-AOM) and 3 (Saline-AOM) were smaller than that of saline-injected groups, groups 2 (MSG-Saline) and 4 (Saline–Saline), during the study. At the termination of the experiment, the mean body weights of groups 1 and 3 were significantly lower than groups 2 and 4, respectively ($P < 0.001$), as listed in Table I.

The numbers of ACF and BCAC

ACF and BCAC (Figure 2A and B) developed in all the mice belonging to group 1 (the MSG-AOM mice) and 3 (the Saline-AOM mice) but not in the mice of groups 2 (the MSG-Saline) and 4 (the Saline-Salin mice) that did not receive AOM. Table I summarizes the total numbers of ACF and BCAC in all groups. The number of BCAC of group 1 was significantly greater than group 3 ($P < 0.001$), whereas the numbers of ACF developed in groups 1 and 3 were comparable.

Serum levels of glucose, total cholesterol, triglyceride and insulin

The serum concentrations of glucose, total cholesterol, triglyceride and insulin at 17 weeks of age are shown in Table II. MSG treatment significantly elevated all measures regardless of the AOM exposure (group 1 versus group 3, $P < 0.001$; and group 2 versus group 4, $P < 0.001$). However, the AOM administration did not affect all the measurements (group 1 versus group 2; and group 3 versus group 4).

β-Catenin and IGF-IR immunohistochemistry

Immunohistochemical expression of β-catenin revealed the presence of BCACs, where β-catenin was accumulated in the nucleus and/or cytoplasm (Figure 2B). Immunohistochemical expression of IGF-IR in the cytoplasm of BCAC that develop in the MSG-AOM mice was intensive when compared with the surrounding crypts (Figure 2C). Inflammatory cells infiltrated into the surrounding stroma of BCAC also showed positive reaction against IGF-1R.

mRNA expression levels of IGF-1, IGF-2 and IGF-1R in the colonic mucosa

The expression levels of IGF-1, IGF-2 and IGF-1R mRNAs of the colonic mucosa from the MSG-AOM mice (group 1) and the Saline-AOM mice (group 3) were determined. As illustrated in Figure 3, the MSG + AOM treatment mice showed significantly increased mRNA levels of IGF-1 (1. 81-fold increase) and IRF-1R (2.43-fold increase), when compared with the Saline-AOM mice. The increase of IGF-1R was statistically significant ($P < 0.01$). mRNA levels of IGF-2 of the two groups were comparable.

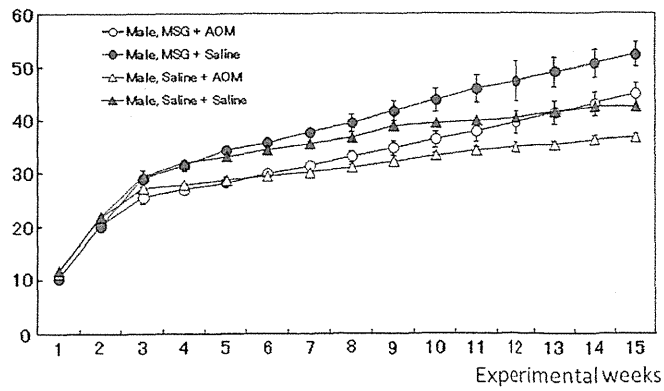

Fig. 1. Body weight gains of all groups during the study.

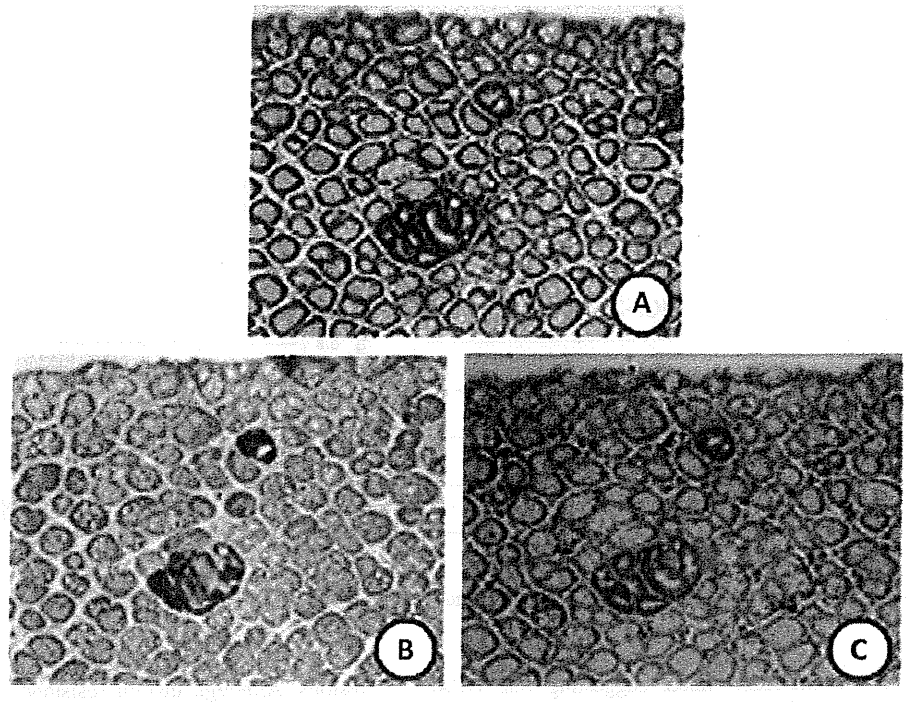

Fig. 2. Histopathology (A) of a BCAC developed in an MSG-AOM mouse. Immunohistochemistry of (B) β-catenin and (C) IGF-1R in the BCAC shows positive reaction of β-catenin in their cell membrane and cytoplasm and IGF-1R in their cytoplasm. Bars = 50 μm. (A) H and E stain, (B) β-catenin immunohistochemistry and (C) IGF-1R immunohistochemistry.

Table I. Body weights and numbers of ACF and BCAC per colon of mice treated with AOM and/or MSG at the end of study (17 weeks of age)

Group number	Number of mice examined	Treatments		Body weight (g)	Total number of ACFs/colon	Total number of BCACs/colon
		MSG (2 mg/kg body wt)	AOM (15 mg/kg body wt)			
1 (MSG-AOM)	12	+ (4 times daily)	+ (4 times weekly)	44.82 ± 2.22[a,b,c]	78.00 ± 11.20[b]	13.83 ± 7.44[b,c]
2 (MSG-Saline)	6	+ (4 times daily)	− (saline)	52.29 ± 2.32[d]	0	0
3 (Salin-AOM)	11	− (saline)	+ (4 times daily)	36.62 ± 4.15[e]	69.27 ± 8.06[f]	5.45 ± 1.86
4 (Saline–Saline)	5	− (saline)	− (saline)	42.42 ± 0.78	0	0

[a]Mean ± SD.
[b]Significantly different from group 2 ($P < 0.001$).
[c]Significantly different from group 3 ($P < 0.001$).
[d]Significantly different from group 4 ($P < 0.001$).
[e]Significantly different from group 4 ($P < 0.01$).
[f]Significantly different from group 4 ($P < 0.001$).

Table II. Serum levels of total cholesterol, triglycerides, glucose and insulin of mice treated with AOM and/or MSG at the end of study (17 weeks of age)

Group number	Number of mice examined	Treatments		Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Glucose (mg/dl)	Insulin (ng/dl)
		MSG (2 mg/kg body wt)	AOM (15 mg/kg body wt)				
1 (MSG-AOM)	12	+ (4 times daily)	+ (4 times weekly)	167.92 ± 19.96[a,b]	96.25 ± 14.38[b]	196.67 ± 34.09[b]	10.66 ± 1.31[b]
2 (MSG-Saline)	6	+ (4 times daily)	− (saline)	177.50 ± 23.09[c]	93.17 ± 12.64[c]	202.67 ± 15.24[c]	11.18 ± 1.41[c]
3 (Salin-AOM)	11	− (saline)	+ (4 times daily)	107.64 ± 18.65	49.45 ± 13.87	110.36 ± 10.48	0.49 ± 0.14
4 (Saline–Saline)	5	− (saline)	− (saline)	104.60 ± 13.69	45.20 ± 9.98	123.60 ± 11.30	0.50 ± 0.07

[a]Mean ± SD.
[b]Significantly different from group 3 ($P < 0.001$).
[c]Significantly different from group 4 ($P < 0.001$).

Discussion

As expected, the findings described suggest that development of AOM-induced precancerous lesions, BCAC, of the colon in the MSG mice with hyperinsulinemia, hypercholesterolemia, hyperglycemia and hyperlipidemia was increased, when compared with the Salin-AOM mice. Our findings are in accordance with our previous findings that AOM-induced colon carcinogenesis is enhanced in another obese model using C57BL/KsJ-db/db mice (29–31). In the current study, the number of ACF was not different between the

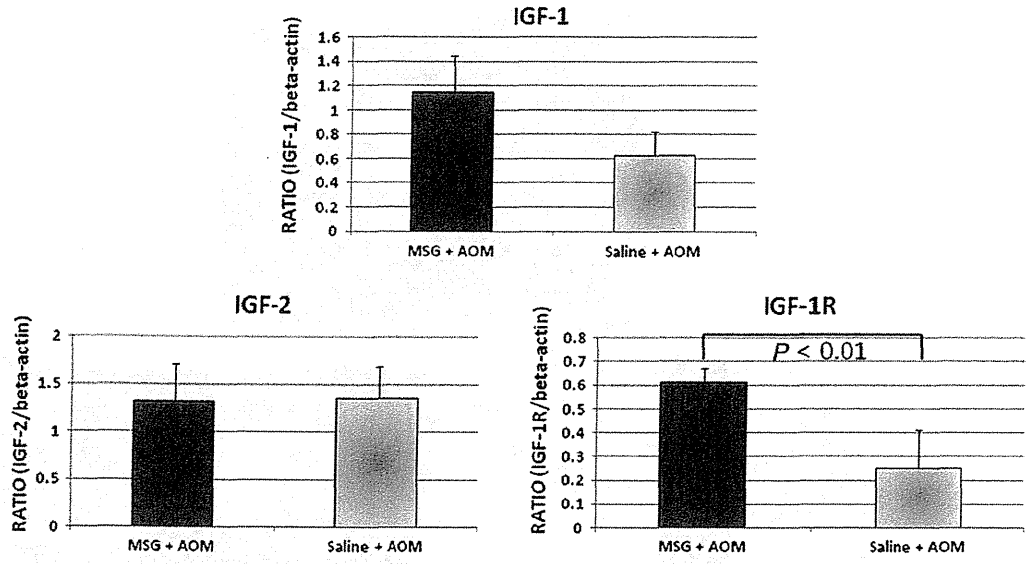

Fig. 3. mRNA expression of IGF-1, IGF-2 and IGF-1R in the colonic mucosa of the MSG-AOM mice and the Saline-AOM mice.

MSG-AOM mice and Saline-AOM mice, suggesting that BCAC rather than ACF has potential to progress to malignancies (39,42). This may be explained by the differences of pathobiological characteristics between two lesions: BCAC is dysplastic and microadenomas and ACF is consisted of hyperplastic and dysplastic lesions.

ACF have attracted attention as putative precancerous lesions in the colon in both experimental models and in humans (36). A number of molecular abnormalities, including increased expression of K-*ras* and *APC* gene mutations, are demonstrated in human ACF (46–48). BCAC, which accumulate β-catenin protein in the nucleus and cytoplasm, are also regarded as putative precursors to colorectal adenomas (37). Several rodent studies have shown that both of these lesions are useful as biomarkers to evaluate the chemopreventive properties of specific agents (49,50). In human colorectum, increased plasma IGF-1 levels are associated with the number of dysplastic ACF (51), suggesting that IGF-1 may be a promoter of the growth of dysplastic ACF and an independent risk factor of CRC. It may be possible that the number of dysplastic ACF, but not hyperplastic ACF, may be increased in the MSG-AOM mice, although we did not analyze two types of ACF.

Neonatal injections of MSG to mice or rats cause hyperthalamic damage (52,53), and as a consequence, these animals present several neuroendocrine and metabolic alterations, which lead central obesity, type 2 diabetes, insulin resistance, hyperinsulinemia, hypertriglyceridemia and hyperlipidemia (33). These abnormalities are risks for the development of CRC (9). The pancreatic islets in the mice subcutaneously injected MSG in neonatal period are hyperplastic up to 54 weeks of age (33). Therefore, our model described here may be suitable to study the pathobiology of diabetes- and obesity-associated colorectal carcinogenesis.

In this study, blood insulin level of the MSG-Saline mice (group 2) was the highest among the groups. There is accumulating evidence suggesting that hyperinsulinemia is involved in colon carcinogenesis, obesity and diabetes (3,8,9,12,54,55). Several epidemiological studies indicate that type 2 diabetic patients with hyperinsulinemia increases risk for CRC (3,12). Additionally, continuous injections of insulin promote AOM-induced colon carcinogenesis in rats (56,57). Hence, it seems likely that hyperinsulinemia in the MSG-AOM mice enhanced the development of AOM-induced lesions in the present study. Hyperglycemia and hypercholesterolemia observed in the MSG-AOM mice also contribute the development of BCAC and ACF in the colorectum because these conditions are positively associated with CRC occurrence (9,11,12). Hyperinsulinemia, hyperglycemia and hypercholesterolemia may singly or synergistically promote the development of preneoplastic and neoplastic colonic lesions. Although insulin resistance in colorectal carcinogenesis of obese people and/or type 2 diabetic patients is reported (7,55,58,59), we did not investigate presence or absence of insulin resistance in this study, Corpet *et al.* (60) reported that diet that increase some indirect insulin resistance markers does not promote colon carcinogenesis in female rats when ACF are used as a biomarker.

Regarding the mode of action, the current consensus assumes that the IGF-1 pathway plays a role in insulin-related tumor promotion in the colon (20,61). IGF-1 binds to the IGF-1R, activates a signal cascade and triggers cell proliferation in several tissues, including colon (62). Insulin at supra-physiological levels also binds to and activates the IGF-1R because of its homology with the insulin receptor (62). Furthermore, hyperinsulinemia was shown to indirectly increase bioavailability of IGF-1 by regulating levels of IGF-binding proteins (63). In this study, IGF-1R immunohistochemical expression of IGF-1 was strongly positive in the cytoplasm of BCAC developed in the MSG-AOM mice. Indeed, overexpression of IGF-1R was also reported in human CRC (64). Accordingly, it may be possible that hyperinsulinemia in MSG-AOM mice activates the signaling cascades involving the IGF-IR, resulting in a proliferative response (19,59,61). Another interesting findings regarding IGF-1R immunohistochemistry is that inflammatory cells infiltrated around BCAC were positively reacted against the IGF-1R antibody. Significance of the findings is not known, but similar findings have been reported in human Crohn's disease (65). IGF-1 and IGF2 are potentially relevant mediators in the chronic inflammation (27) and mediate the majority of their biological action through IGF-1R (66). Thus, our findings on the IGF-1R immunohistochemical positivity in inflammatory cells suggest that inflammation in the microenvironment of precancerous lesions for CRC may contribute to the growth of the lesions (67,68).

In conclusion, our data indicate that the MSG-AOM mice with hyperinsulinemia, hypercholesterolemia, hyperglycemia and hypercholesterolemia are highly susceptible to colorectal carcinogenesis and the MSG-AOM mouse model could be useful for investigating the mechanisms of obesity/diabetes-associated events involving in colorectal carcinogenesis and the therapeutic and chemopreventive strategies of CRC in obese people and/or type 2 diabetic patients.

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Suppression of azoxymethane-induced colonic preneoplastic lesions in rats by 1-methyltryptophan, an inhibitor of indoleamine 2,3-dioxygenase

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The escape of preneoplastic cells from the immune system, which is caused by immune tolerance, occurs during the development of several types of tumors. Indoleamine 2,3-dioxygenase (IDO) plays a critical role in the induction of immune tolerance. In the present study we investigated the effects of 1-methyltryptophan (1-MT), an IDO inhibitor, and (–)-epigallocatechin gallate (EGCG), the major catechin in green tea, on the development of azoxymethane (AOM)-induced colonic preneoplastic lesions by focusing on the inhibition of IDO. To induce colonic premalignant lesions, male F344 rats were injected with AOM (20 mg/kg body weight, s.c.) once a week for 2 weeks. They also received 0.2% 1-MT or 0.1% EGCG in their drinking water for 4 weeks, starting 1 week before the first dose of AOM. Both 1-MT and EGCG significantly decreased the total number of aberrant crypt foci and β-catenin-accumulated crypts, which overexpressed IDO protein. Treatment with EGCG decreased IDO mRNA expression in both the colonic epithelium and stroma of rats induced by AOM. The AOM-induced increase in *cyclooxygenase-2* mRNA expression in the colonic stroma was significantly decreased by EGCG. Furthermore, AOM-induced increases in IDO activity in the serum and stroma were significantly inhibited by 1-MT and EGCG. Inhibition of IDO activity by 1-MT and EGCG was also observed in cell-free assays. These findings suggest that upregulation of IDO activity is observed in the early stages of colon carcinogenesis and that the use of IDO inhibitors, such as 1-MT and EGCG, which suppress the occurrence of colonic preneoplastic lesions, could be a novel strategy for the chemoprevention of colon cancer. (*Cancer Sci* 2012; 103: 951–958)

The immune system recognizes preneoplastic cells and, in most cases, eliminates these cells before they expand into clinically detectable tumors. Therefore, the escape of precancerous cells from the immune system, which is closely associated with immune tolerance, is involved in the development of several types of tumors.[1] Recent studies have suggested that indoleamine 2,3-dioxygenase (IDO) plays a crucial role in the induction of immune tolerance.[2] Indoleamine 2,3-dioxygenase is an intracellular enzyme that catalyses the first and rate-limiting steps in the catabolism of the essential amino acid tryptophan along the kynurenine pathway.[3] In the tumor microenvironment, increased IDO activity inhibits the proliferation of T and natural killer cells and induces apoptosis through tryptophan depletion and the production of toxic tryptophan catabolites.[4] Overexpression of IDO has been shown to be correlated with poor clinical outcome in patients with ovarian carcinoma, endometrial carcinoma, and colorectal carcinoma.[5–7] We have recently reported that, in diffuse large B-cell lymphoma, IDO expression in tumor cells and serum concentrations of L-kynurenine, which reflect IDO activity, are useful indicators of a poor prognosis.[8,9] Several preclinical studies using rodent cancer models have demonstrated that IDO inhibitors, such as 1-methyltryptophan (1-MT), are therapeutically beneficial, especially when combined with different types of cytotoxic chemotherapeutic agents.[10,11] These reports suggest that targeting IDO, and therefore regulating tryptophan catabolism, may be an effective strategy for the treatment of certain types of human malignancies.[12] However, the possibility of cancer chemoprevention by inhibiting IDO expression and/or activity has not been considered.

(−)-Epigallocatechin gallate (EGCG), one of the major catechins in green tea, is the most biologically active component of green tea. It has been shown to exert its cancer chemopreventive and anti-carcinogenic effects in various organs, including the colon.[13,14] Previously, we demonstrated that EGCG can inhibit the growth of and induce apoptosis in human colorectal cancer cells.[15–17] The inhibitory effects of EGCG on both inflammation- and obesity-related colon carcinogenesis have also been demonstrated.[18,19] In addition, green tea polyphenols in the drinking water have been shown to inhibit the development of putative preneoplastic lesions called aberrant crypt foci (ACF) in rats treated with azoxymethane (AOM), which induces ACF.[20–22]

Recently, it was been reported that EGCG administration suppresses the expression of IDO in interferon (IFN)-γ-stimulated murine dendritic cells[23] and human oral cancer cell lines.[24] Although the mechanisms underlying the role of IDO in carcinogenesis have not yet been clarified, we hypothesized that the inhibitory effect of EGCG on IDO expression may contribute to the anti-carcinogenic properties of EGCG. To confirm our hypothesis, we examined the effects of 1-MT and EGCG on the development in the colon of AOM-induced preneoplastic lesions, namely ACF[21] and β-catenin-accumulated crypts (BCAC),[25] in male F344 rats by focusing on the inhibition of IDO expression and activity.

Materials and Methods

Animals, chemicals, and diets. Male F344 rats, aged 4 weeks (Charles River Japan, Tokyo, Japan), were maintained at the Gifu University Animal Facility according to Institutional Animal Care Guidelines. All rats were housed in plastic cages with free access to drinking water and a pelleted basal diet

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(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.

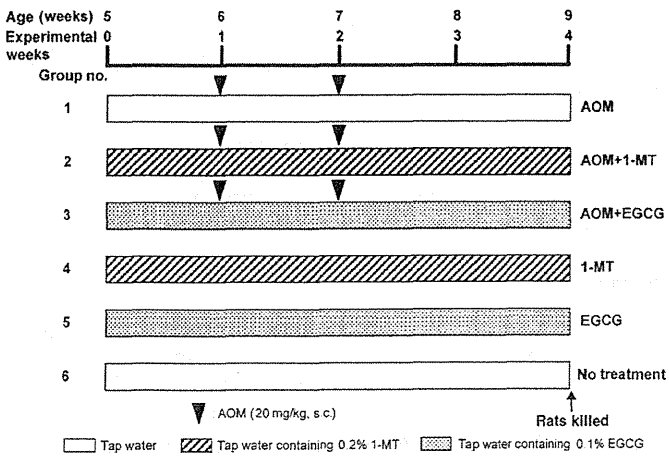

Fig. 1. Experimental protocol. Rats (5 weeks old) were allocated to one of six groups and treated over a period of 4 weeks, as indicated. AOM, azoxymethane; 1-MT, 1-methyltryptophan; EGCG, (−)-epigallocatechin gallate.

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.

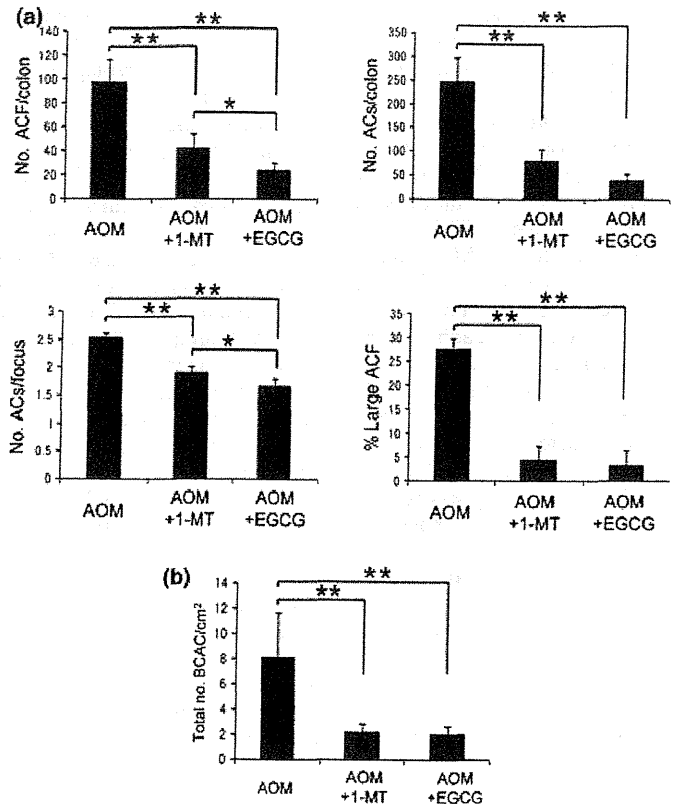

Fig. 2. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on azoxymethane (AOM)-induced formation of aberrant crypt foci (ACF) and β-catenin-accumulated crypts (BCAC). (a) Number of ACF per colon, total number of aberrant crypts (ACs) per colon, the number of ACs in each focus, 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

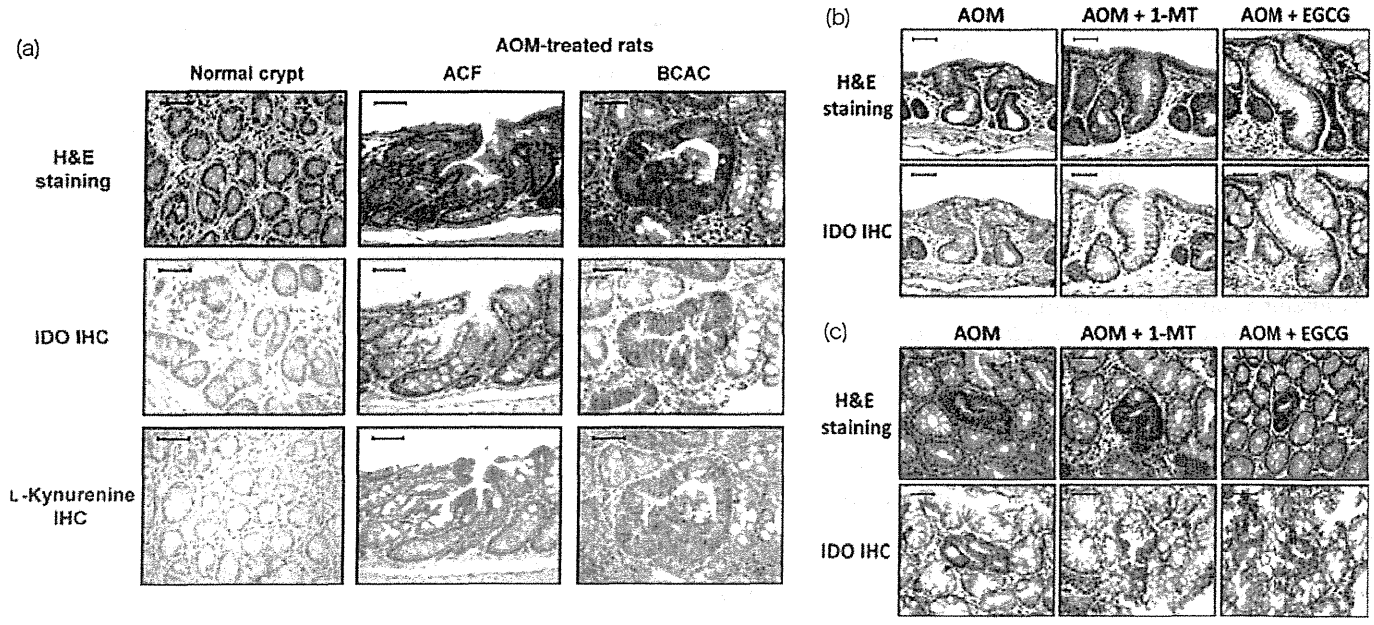

Fig. 3. Immunohistochemical evaluation of the expression of indoleamine 2,3-dioxygenase (IDO) and L-kynurenine in normal crypts from untreated rats and aberrant crypt foci (ACF), and β-catenin-accumulated crypts (BCAC) in the colonic mucosa of rats treated with azoxymethane (AOM). (a) Expression of IDO 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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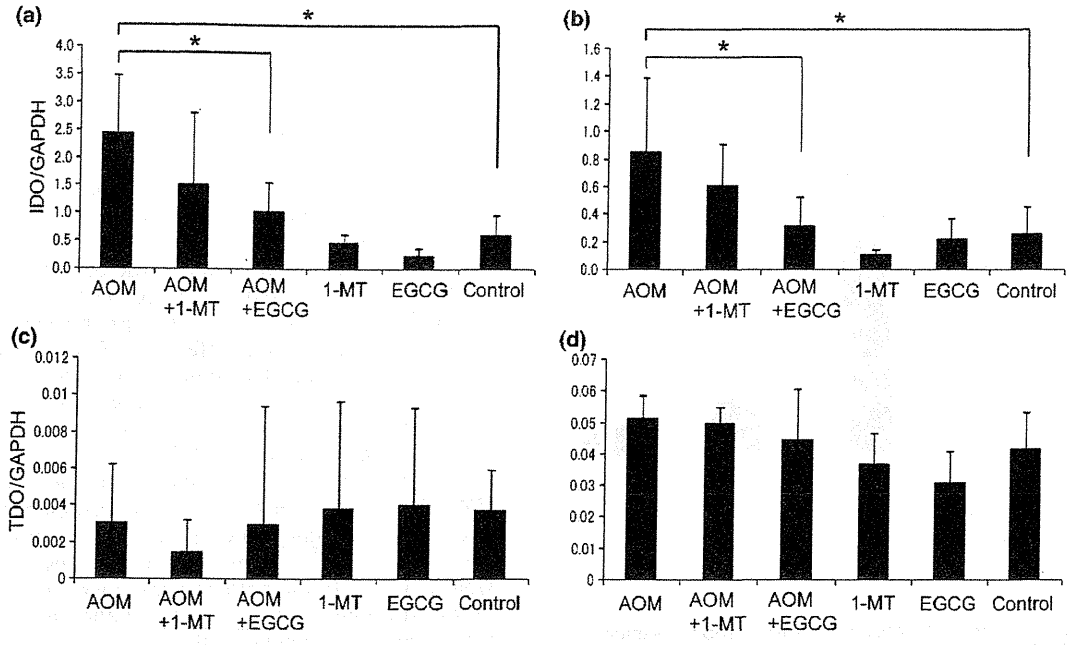

Fig. 4. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on the expression of (a,b) indoleamine 2,3-dioxygenase (IDO) and (c,d) tryptophan 2,3-dioxygenase (TDO) in the epithelium (a,c) and stroma (b,d). Total RNA was extracted from epithelial crypts and stromal tissues and 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.

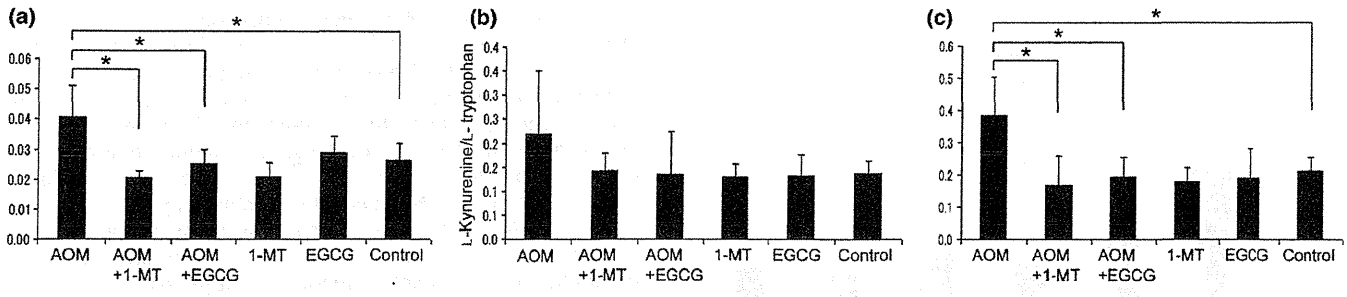

Fig. 5. Effects of 1-methyltryptophan (1-MT) and (−)-epigallocatechin gallate (EGCG) on indoleamine 2,3-dioxygenase (IDO) activity in the (a) serum and colonic (b) epithelium and (c) stroma. Functional IDO activity was determined by measuring the concentrations of L-kynurenine and tryptophan using HPLC. 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