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Animal Models of Carcinogenesis in Inflamed Colorectum: Potential Use in Chemoprevention Study

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Abstract: Inflammation is a risk factor for cancer development in several tissues. In the colorectum, inflammatory bowel disease (ulcerative colitis and Crohn's disease) is a longstanding inflammatory disease with increased risk for colorectal cancer (CRC). Several molecular events involving in chronic inflammatory process contribute to multi-stage carcinogenesis of CRC in the inflamed colon. They include alterations in production of reactive oxygen and nitrogen species, up-regulation of pro-inflammatory cytokines and inflammatory enzymes, and intestinal immune system. In this short review, experimental animal models of inflammation-associated CRC are described. Also, some preclinical data on chemoprevention of inflammation-associated CRC by astaxanthin and a specific inhibitor of nitric oxide synthase using these inflammation-related -CRC models is briefly introduced.

Keywords: Inflammation and cancer, inflammatory bowel disease, colorectal cancer, chemoprevention, animal model.

1. INTRODUCTION

According to a statistical survey published by the Ministry of Health, Labor and Welfare of Japan in 2009, the No. 1 cause of death in Japanese people is malignant neoplasms in 344,000 people, No. 2 is heart diseases in 179,000 people, and No. 3 is cerebrovascular diseases in 121,000 people, with malignant neoplasms occupying the overwhelming top position. That is to say, in the present condition, approximately 30% of the annual mortality total is caused by cancer.

It is considered that lifestyle or life habits have a significant influence on human carcinogenesis [1]. After the World War II, due to changes in lifestyle including dietary habits (high-fat diets), morbidity and mortality due to cancer significantly increased with colorectal cancer (CRC) and lung cancer at the top of the list. Under such present conditions, periodic medical examinations, early detection, and early treatment are strongly emphasized as a means of clinical cancer control [2]. However, is the ultimate cancer control not "prevention of cancer [3]" in the same manner as the control and prevention of other diseases? That is to say, we refer to the control of initiation and promotion of oncogenic pathway [4]. In order to prevent cancer, first improving the environment that increases susceptibility to the onset of cancer, in concrete terms, taking carotenoids-rich vegetables [5, 6], controlling the intake of fats, animal fats in particular, exercising daily, quitting smoking, limiting drinking, etc., may be listed [7]. Another is to actively take tumor suppressing compounds, either natural products or synthetic compounds, in order to stop or retard the processes of initiation and promotion [8, 9]. The latter is referred to as "cancer chemoprevention" that is a concept proposed by Sporn in 1976 [10]. Active cancer prevention by this concept is conducted worldwide from basic researches to clinical studies in different organs [11-19]. However, at present, the actual outcomes are poor when compared to the progress in therapy Fig. (1) [20]. In recent years, along with advancing elucidation of the detailed carcinogenic mechanism, molecular-targeted cancer chemoprevention by chemicals targeting molecules involved in carcinogenesis is also being actively researched [5, 11-13, 21-27].

In this short review, with a focus on CRC, which is on the rise in Asian countries including Japan [28], I would like to introduce the development of an inflammation-related colon carcinogenesis model [29] and some of recent data (Table 1) on preclinical chemoprevention studies using an animal model thereof.

2. DEVELOPMENT OF AN INFLAMMATION-RELATED MOUSE COLON CARCINOGENESIS MODEL

Since the age of Virchow, inflammation has been considered to be involved in the development of cancer [30, 31]. In particular, chronic inflammation is heavily involved in the carcinogenesis of different organs. For example, continued chronic inflammation due to *H. pylori* infection [32] causes chronic atrophic gastritis, leading to the genesis of gastric cancer [33]. That is to say, chronic inflammation due to *H. pylori* infection may exert tumor-promoter effects on gastric carcinogenesis, although its role in chronic inflammation somewhat not fully established. It has been reported that gastric carcinogenesis decreased due to the disinfection or eradication of *H. pylori* in animal experiments [34], with

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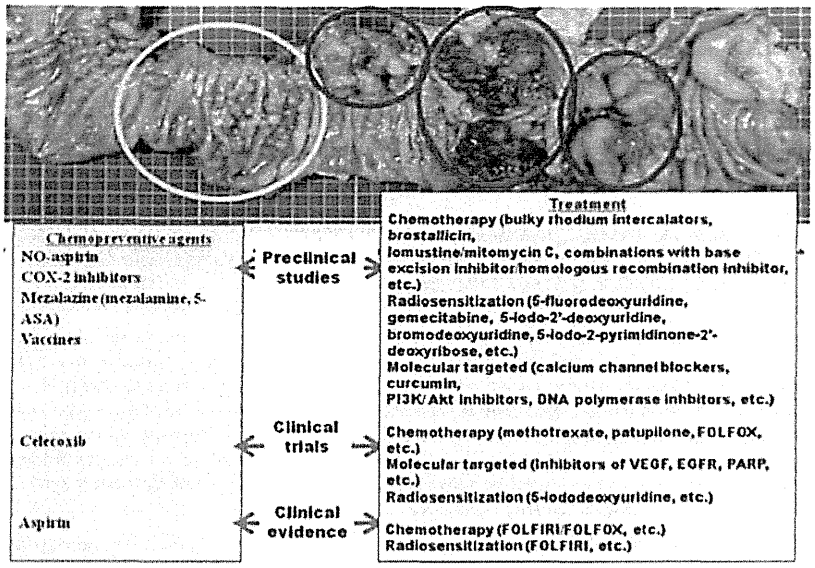

Fig. (1). CRCs (circles in red) with multiple polyps (tubular adenomas, a circle in blue) in a UC patient (upper panel). Progress in chemoprevention and therapy (lower panel). Akt, protein kinase B; 5-ASA, 5-aminosalicylic acid; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; FOLFIRI, 5-fluorouracil, leucovorin and irinotecan; FOLFOX, leucovorin, 5-fluorouracil, and oxaliplatin; *NO, nitric oxide*; PARP, poly-ADP ribose polymerase; PI3K, phosphatidylinositide-3-kinase; VEGF, vascular endothelial growth factor.

clinical reports of delaying the time until carcinogenesis [33]. Other environmental factors contribute to inflammation (cancer) in humans include chewing tobacco/betel quid (oral cancer), smoking (lung cancer), asbestos (malignant mesothelioma), Schistosomiasis (bladder cancer), Opisthorchis sinensis (cholangiocarcinoma), hepatitis B and C viruses (liver cell cancer), humanpapilloma virus (uterine cervical cancer), and herpes simplex (Kaposi's sarcoma) [31].

Regarding colitis and CRC, patients suffering from ulcerative colitis (UC) and Crohn's disease (CD), which are refractory inflammatory bowel diseases (IBD) of undetermined etiology, have an exceedingly high risk of CRC, with the risk thereof being proportional to the duration of the disease [35-38]. As the colitis animal model resembling human UC, dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS) are frequently used as colitis-inducing agents in rodents and genetically-modified mice [38, 39]. In particular, as the colon carcinogenesis model with a background of inflammation, a method of repeated (cycle) administration of DSS, which is a promoter class (without genotoxicity) carcinogen to mice and rats is being used [38]. However, a long period of time is required for inducing colorectal tumors with this model. There are carcinogens that are able to produce colorectal neoplasms in rodents. They include azoxymethane (AOM), 2-amino-1-methyl-6-phenyli midazo[4,5-*b*]pyridine (PhIP), 1,2-dimethylhydrazine (DM H), which are colon-specific carcinogens, to mice and rats [40, 41]. Research on carcinogenesis and the suppression of carcinogenesis with precancerous lesions and tumors occurring in the colon of these animals is being widely conducted. To date, many candidate compounds for suppressing colon carcinogenesis have been reported [1, 9, 14, 15, 42]. In contrast to rats, mice need the complex methods to induce colorectal tumors. Administration of the carcinogen(s) is required about 10 times when compared to rats, and a long period (over 40 weeks) is required to induce the colorectal tumors Fig. (**2A**) [40, 41]. Accordingly, we have tried to produce a two-stage colorectal carcinogenesis model with AOM and DSS in mice with a background of inflammation that allows early induction of CRC with a high frequency and multiplicity [24, 29, 40, 41, 43] Fig. (**2B**, TANAKA model using ICR mice). Treatment with AOM and DSS resulted in a number of colorectal tumors development in either sex of ICR mice: susceptibility to AOM/DSS-induced colorectal carcinogenesis was similar between males and females. We have also analyzed alterations of genes' [44] and proteins' [45] expression during this AOM/DSS model. In this inflammation-related colon carcinogenesis mice model, mice are initiated with a single intraperitoneal injection of AOM at the start of the experiment as the tumor-initiator and starting a week after this injection, 2% DSS in drinking water was administered for a week as the tumor-promoter; thereby, it was possible to induce multiple colorectal tumors at a high frequency within 20 weeks [29, 46-48] Fig. (**2B**). The development of colorectal adenoma (AD) and adenocarcinoma (ADC) was observed at 3 and 4 weeks after the administration of AOM, respectively Fig. (**3**) [47]. Dysplastic crypts (DYS, Fig. **3**), which are preneoplastic lesions for colitis-related colorectal ADC, were also observed at week 2. In this model, mice received a single administration of AOM (10 mg/kg body weight) alone did not induce colorectal tumors 17–20 weeks after AOM exposure [29, 46-48]. AOM treatment after DSS exposure did not produce any colonic neoplasms in male ICR mice and AOM exposure during DSS treatment resulted

Table 1. Compounds tested for their effects on inflammation-associated colorectal carcinogenesis model

Chemicals (Doses and Treatment Method)	Mice or Rats* (Dose of DSS)	Effects	Ref. Nos. or PMID Nos. (Year)
Nimesulide (400 ppm in diet)	Mice (2% DSS)	Inhibition	[57]
Troglitazone (500 ppm in diet)	Mice (2% DSS)	Inhibition	[57]
Bezafibrate (500 ppm in diet)	Mice (2% DSS)	Inhibition	[57]
Auraptene (100, 500 ppm in diet)	Mice (1% DSS)	Inhibition	PMID: 16395701 (2006)
Collinin (100, 500 ppm in diet)	Mice (1% DSS)	Inhibition	PMID: 16395701 (2006)
Pitavastatin (1, 10 ppm in diet)	Mice (2% DSS)	Inhibition	[60]
Nobiletin (100 ppm in diet)	Mice (1% DSS)	Inhibition	PMID: 18375960 (2008)
(-)-epigallocatechin gallate (100, 1000 ppm in drinking water)	Mice (2% DSS)	Inhibition	PMID: 21479417 (2008)
Polyphenon E	Mice (2% DSS)	Inhibition	PMID: 21479417 (2008)
Ursodeoxycholoc acid (800, 4000 ppm in diet)	Mice (1% DSS)	Inhibition	PMID: 17438113 (2007)
Sulfasalazine (500 ppm in diet)	Mice (1% DSS)	Inhibition	PMID: 17438113 (2007)
ONO-1714 (a specific inducible nitric oxide synthase inhibitor, 50, 100 ppm in diet)	$Apc^{Min/+}$ mice (1% DSS)	Inhibition	[58]
3-(4'-Geranyloxy-3'-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline	Mice (1% DSS)	Inhibition	PMID: 18791932 (2008)
NNK (10 μM)	Mice (1.5% DSS)	Enhancement	PMID: 18853746 (2008)
Zerumbone (100, 250, 500 ppm in diet)	Mice (1.5% DSS)	Inhibition	PMID: 19003968 (2009)
Melatonin (0.4, 2, 10 ppm in drinking water)	Rats (1% DSS)	Inhibition	[52]
β-Cyclodextrin inclusion compound of auraptene (100, 500 ppm in diet)	Mice (1.5% DSS)	Inhibition	[59]
β-Cyclodextrin inclusion compound of 4'-geranyloxyferulic acid (100, 500 ppm in diet)	Mice (1.5% DSS)	Inhibition	[59]
Freeze-dried yam sanyaku (20, 100, or 500 ppm in diet)	Mice (1.5% DSS)	Inhibition	PMID: 21367960 (2011)
Diosgenin (20, 100, or 500 ppm in diet)	Mice (1.5% DSS)	Inhibition	PMID: 21367960 (2011)
Astaxanthin (100, 200 ppm in diet)	Mice (1.5% DSS)	Inhibition	[61]
Silymarin (100, 500 ppm in diet)	*Gpt* delta rats (1.5% DSS)	Inhibition	[54]
Morin (50, 250, 1000 ppm in diet)	Rats (1.5% DSS)	Inhibition	[43]
Bezafibrate (50, 100, 500 ppm in diet)	Mice (1.5% DSS)	Inhibition	[43]
Valproic acid (50, 250, 1000 ppm in diet)	Mice (2% DSS)	Inhibition	[43]

NNK=4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

* All the animals except $Apc^{Min/+}$ mice were males. Rats (F344) and mice (ICR) were initiated with a single s.c. injection of AOM (20 mg/kg body weight) and a single i.p. injection of AOM (10 mg/kg body weight), respectively; *Gpt* delta rats were initiated with a single s.c. injection of DMH (40 mg/kg BW). $Apc^{Min/+}$ mice (both sexes) were not initiated with a colonic carcinogen.

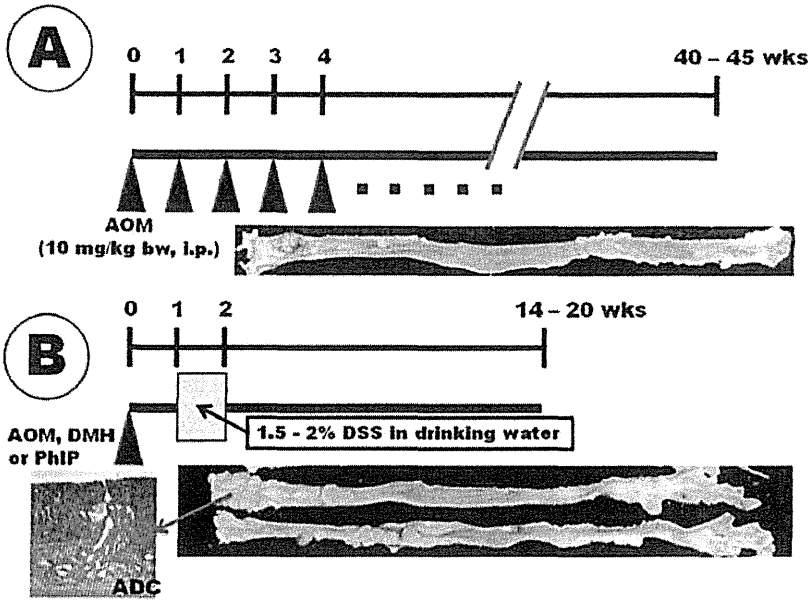

Fig. (2). (A) A standard protocol for experimental induction of colorectal tumors in mice and macroscopic view of the tumors. (B) An experimental protocol of inflammation-associated colorectal tumors and macroscopic view of the tumors. Histopathology of adenocarcinoma (ADC) that invades into the muscularis propria.

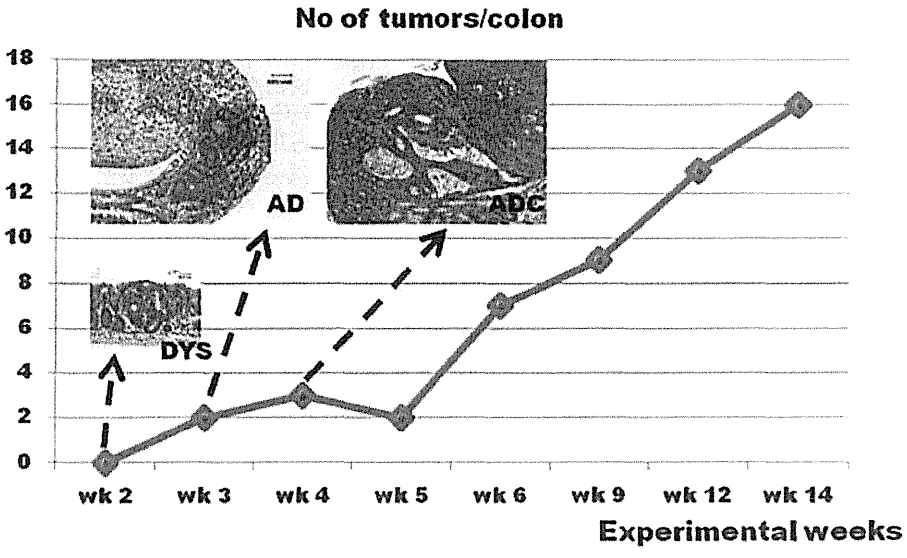

Fig. (3). Time course observation of colon tumor development in AOM/DSS-induced mouse colon carcinogenesis. About 65% of the tumors are histopathologically tubular adenocarcinoma (ADC) and the remains are tubular adenoma (AD). AD and ADC could be observed at weeks 3 and 4, respectively. Dysplastic crypts (DYS) are present at week 2.

only a few colonic AD [29]. Thus, AOM followed by DSS is important for induction of a number of colorectal tumors. We found strain differences of male mice (Balb/c, C3H/HeN, C57BL/6N and DBA/2N) to AOM/DSS-induced colorectal carcinogenesis among male mice: Balb/c > C57 BL/6N > C3H/HeN > DBA/2N [46]. The differences may have been directly influenced by the response to nitrosation stress due to the inflammation caused by DSS [46]. Regarding the molecular weight of DSS, 36,000 to 50,000 are suitable for inducing colitis that acts as a tumor-promoter [29,

46-48]. As to the dosage of DSS, 1.5%~2% in drinking water is effective for promotion of colorectal carcinogenesis in mice [48]. Moreover, PhIP [49, 50] and DMH [51] can be used as tumor-initiators in the colorectum of mice. High frequency of multiple colorectal tumors was also produced in mice within a short-term period in rats that are initiated with AOM [52, 53] or DMH [54]. These inflammation-related mouse or rat colorectal carcinogenesis model, where colorectal tumors and multiple DYS are induced within 20 weeks [40, 41, 43], can be used for detecting the modification effect (inhibiting and promoting effect) [55] of various compounds and the tumor-initiation agents (colonic carcinogens) [56] in the environment. The cancer chemopreventive effects of anti-inflammatory compounds, such as non-steroidal anti-inflammatory drugs (NSAIDs) [57], inhibitors of inducible nitric oxide synthase (iNOS) [58] and cyclooxygenase (COX)-2 selective inhibitors [57], are well-known. As shown in (Table 1), we consider that the screening of anti-inflammatory chemopreventive agents using this model is significant and meaningful for identifying candidate chemopreventive compounds against colorectal neoplasms in the inflamed colon [6, 24, 25, 43, 52, 59].

3. CHEMOPREVENTIVE STUDIES ON INFLAMMATION-RELATED COLORECTAL CARCINOGENESIS

We have been conducting chemopreventive research with natural products and synthetic compounds using our colorectal carcinogenesis model with inflamed colon as described above. Among these, the results of pitavastatin [60] and astaxanthin (AX) [61] are introduced. In addition, the experimental findings regarding colon carcinogenesis and the suppression thereof using the familial adenomatous polyposis (FAP) and *Apc*$^{Min/+}$ mouse model [58, 62] will be discussed. In our chemoprevention studies, we administered chemicals tested after the carcinogen exposure to rodents since adults are already initiated with carcinogen(s) and thus the effects of chemicals on promotion/progression stages are considered to be important. However, the effects of the chemicals on initiation of the carcinogenic process are of interest.

3.1. AOM-DSS Colon Carcinogenesis Suppression by Pitavastatin [60]

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), are used as a therapeutic agent for hypercholesterolemia. Recent research has clearly indicated that statins not only have a hypocholesterolemic activity, but also various bioactivities such as anti-inflammatory actions, with reports on its anti-carcinogenesis effects [63-65]. Accordingly, using pitavastatin (usually as a calcium salt) that developed in Japan [66], we investigated the suppressing effect of statins on inflammation-associated colon carcinogenesis using AOM/DSS mouse model [60]. Pitavastatin is fat-soluble, unlike many other statins.

When 1 ppm to 10 ppm pitavastatin in diet was administered to male ICR mice (5-week-old) for 17 weeks, starting one week after the AOM/DSS treatment, the frequencies and numbers of mucosal ulceration and ADC in the colorectum were significantly decreased when compared the AOM/DSS group [60]. Furthermore, growth inhibition and the apoptosis induction of the ADC cells were increased by feeding with pitavastatin. In the group treated with pitavastatin, the serum triglyceride and total cholesterol levels were lowered. Decrease in the number of nitrotyrosine-positive cells in the colonic mucosa was also observed. These finding suggest that pitavastatin has a potential as a candidate chemopreventive compound against colorectal carcinogenesis in inflamed colorectum [60].

3.2. AOM-DSS Colon Carcinogenesis Suppression by AX [61]

AX, which is widely distributed in aquatic organisms, is a type of carotenoid having a strong anti-oxidative effect such as the scavenging activity of a singlet oxygen, radical acquisition effect, etc [5]. Reports on the acceleration effect of adjuvanticity, anti-inflammatory action, and detoxification enzyme activity of AX have been published [5]. Moreover, we found cancer chemopreventive effects against carcinogenesis in the colon [67], tongue [68] of rats, in addition to mouse urinary bladder [69] carcinogenesis. However, the effects on inflammation-related carcinogenesis remain unknown. Therefore, we investigated the effects of AX on this inflammation-related mice colon carcinogenesis [61].

After administration of AOM/DSS to male ICR mice (5 weeks old), they were fed the diets containing AX at 3 dosages of 50, 100, and 200 ppm. As a result, dietary administration with AX significantly suppressed development of colonic mucosal ulceration and DYS at all dosages. The occurrence of colon ADCs was significantly suppressed by 200 ppm AX administration. Furthermore, the immunohistochemical expression of tumor necrosis factor (TNF)-α, nuclear factor-kappaB (NF-κB), interleukin (IL)-1β, PCNA, and survivin was significantly decreased in ADCs. mRNA expression of pro-inflammatory cytokines, TNF-α, NF-κB, and IL-1β was also significantly decreased in non-lesional colonic mucosa by feeding with AX. The results suggest that AX has strong suppressing effects against inflammation-associated colorectal carcinogenesis with anti-inflammatory activities in the colorectum. However, there still remain unclear regarding the cancer preventing effects of carotenoids [5]. There are reports showing that β-carotene administration to smokers with a high risk of lung cancer increases lung cancer development as well as mortality [5]. Therefore, a safety evaluation of long-term use of AX is warranted.

3.3. *Apc*$^{Min/+}$ Mouse Colon Carcinogenesis Model by Inflammatory Stimuli and the Inhibition of its Carcinogenesis [58, 62]

Apc$^{Min/+}$ mice are widely used as a mouse model for human FAP [70]. FAP is an autosomal dominant disease that develops colonic ADC from adenomatous polyps (tubular AD) in almost all cases, with *APC* genetic mutation as the cause thereof. At present, means to avoid colon cancer development includes removal of the colorectum where malignant epithelial tumors develop [71]. In addition to genetic mutation, lifestyle such as exercise, food, and smoking are involved in the cancerization of FAP [72, 73].

The *Apc*$^{Min/+}$ mice are characterized by severe hypertriglyceridemia and hyperlipidemia that develop with age [74]. This alteration of serum biochemistry in *Apc*$^{Min/+}$ mice that is well-correlated with the occurrence of numerous intestinal polyps (ADs) is similar to that observed in patients

with CRCs [75]. However, the most common site of tumors in the intestinal tract of $Apc^{Min/+}$ mice is mainly the small intestine [40, 41, 70]. In the colorectum, a small number of precursor lesions, such as aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), of colonic ADCs are observed [76, 77], although a few ADC is observed in aged $Apc^{Min/+}$ mice [76, 77]. Since $Apc^{Min/+}$ mice are *Apc* gene hetero-deficient and ACF and BCAC are found in their colorectum, they are already initiated. Therefore, we considered that when given tumor-promotion stimuli, colonic cancer will develop.

To test this hypothesis, DSS at a dose of 2% in drinking water was administered to $Apc^{Min/+}$ mice of both sexes for a week [62] Fig. (**4A**). Surprisingly, when 2% DSS was administered in the drinking water of $Apc^{Min/+}$ mice for a week, colonic ADs developed 2 weeks after the commencement of the experiment (a week after the cession of DSS administration), and by 5 weeks after the start of the experiment (4 weeks after the termination of DSS exposure), multiple colonic tumors, mainly ADCs, occurred in the inflamed colonic mucosa in all cases Fig. (**4A**) [62]. Colonic ADC cells were immunohistochemically positive for β-catenin, p53, iNOS, and nitrotyrosine. The number of polyps (histologically AD) in the small intestine also increased by 2% DSS administration. Moreover, there were no colorectal tumors in $Apc^{+/+}$ (wild type) mice that received 2% DSS in drinking water. These findings suggest that inflammation in the colon induced by DSS exposure exerted strongly promoting effects on the growth of existing precancerous lesions, ACF and BCAC, with oxidative and nitrosative stress involved in the occurrence of colorectal tumors in $Apc^{Min/+}$ mice [62].

Subsequently, using this experimental system with male $Apc^{Min/+}$ mice and DSS, we determined whether a specific iNOS inhibitor, ONO-1714, suppresses DSS-induced colon carcinogenesis in $Apc^{Min/+}$ mice [58] in a short-term experiment of 5 weeks. As a result, dietary administration with ONO-1714 at doses of 50 and 100 ppm significantly suppressed the occurrence of ADCs Fig. (**4B**). In addition, mRNA expression of COX-2, IL-1β, and TNF-α in colonic mucosa, as well as serum triglycerides level significantly was significantly lowered by ONO-1714 administration Fig. (**4B**). The findings suggest that iNOS inhibitors may be effective in the suppression of FAP-related colon carcinogenesis. The improvement of hypertriglyceridemia is also beneficial for reducing risks of colorectal carcinogenesis in FAP patients.

4. CONCLUSION

From the basic, clinical, and epidemiologic studies, inflammations, particularly chronic inflammation, are deeply involved in different tissues, including CRC. Indeed, most reported candidate chemopreventive agents have an anti-inflammatory action. However, inflammation responses, particularly acute inflammation, are essential for host defense. With regard to cancer chemoprevention, chemopreventive agents require a long period of administration/intake; therefore, using compounds with strong anti-inflammatory action without careful consideration is risky. For example, the long-term administration of NSAIDs may cause side effects, such as ulcer and perforated ulcer of the gastrointestinal tract. A COX-2 selective inhibitor was also reported to have a valid effect against progressive colorectal ADs in a clinical study with a high risk group. However, side effects, such as cardiovascular diseases, may occur [78]. As a method of avoiding these side effects of NSAIDs and COX-2 selective inhibitors, we would like to pay attention to che-

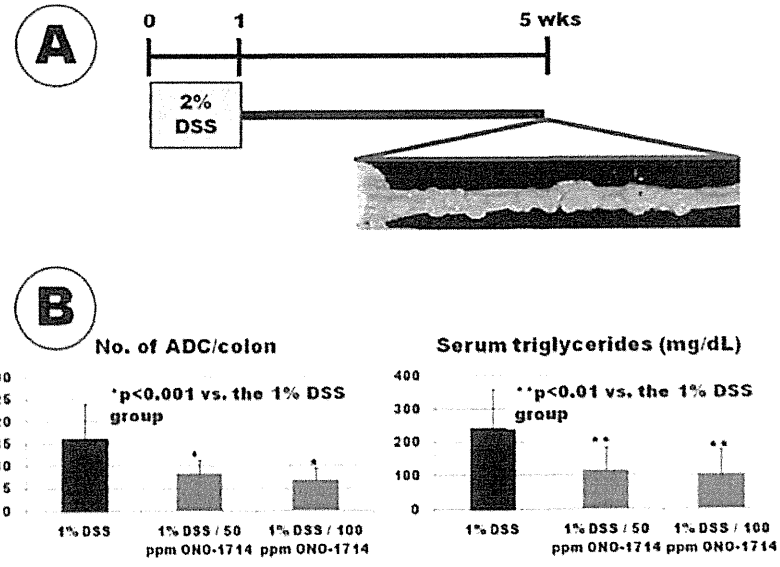

Fig. (4). (**A**) Experimental protocol for induction of colorectal tumo0rs in $Apc^{Min/+}$ mice that received 2% DSS in drinking water for 7 days. (**B**) No. of ADC/colon (*left*) and serum level of triglycerides (*right*) in the $Apc^{Min/+}$ mice that received 2% DSS with or without ONO-1714.

moprevention by complex administration in combination with other agents [8, 13, 79, 80]. To obtain greater chemopreventive effects at lower concentrations of different chemopreventive agents may be achieved by concomitantly using agents that are effective by themselves due to synergistic effects because of their different mechanisms of action, thus further leading to the suppression of any side effects. In Japan, due to the changes in lifestyles with dietary habits following the Second World War and due to the acceleration of aged society, we should overcome "cancer". We might take "translational cancer chemoprevention" into account. Narrowing-down the high risk group for cancerogenesis and a detailed analysis of the oncogenic mechanism in individual cases is required for lower side effects of chemoprevention. As mentioned above, careful consideration is required for selecting candidate chemicals for chemoprevention; however, considering the improvement of quality of life and reduced burden of medical expenses, we believe that the role which cancer (chemo)prevention plays is quite large.

CONFLICT OF INTEREST

The author confirms that this article content has no conflicts of interest.

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ABBREVIATIONS

ACF	=	Aberrant Crypt Foci
AD	=	Adenoma
ADC	=	Adenocarcinoma
AOM	=	Azoxymethane
AX	=	Astaxanthin
BCAC	=	β-catenin Accumulated Crypts
CD	=	Crohn's Disease
COX	=	Cyclooxygenase
CRC	=	Colorectal Cancer
DMH	=	1,2-dimethylhydrazine
DSS	=	Dextran Sodium Sulfate
DYS	=	Dysplastic Crypts
FAP	=	Familial Adenomatous Polyposis
HMG-CoA	=	3-hydroxy-3-methylglutary coenzyme A
IBD	=	Inflammatory Bowel Disease
IdUrd	=	5-iodo-2'-deoxyuridine
IL	=	Interleukin
iNOS	=	Inducible Nitric Oxide Synthase
NF-κB	=	Nuclear Factor-kappaB
NSAIDs	=	Non-Steroidal Anti-Inflammatory Drugs
PhIP	=	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
TNBS	=	Trinitrobenzene Sulfonic Acid
TNF	=	Tumor Necrosis Factor
UC	=	Ulcerative Colitis

REFERENCES

[1] Tanaka, T. Effect of diet on human carcinogenesis. Crit Rev Oncol Hematol 1997; 25: 73-95.
[2] Tanaka T, Tanaka M, Ishigamori R. Biomarkers for colorectal cancer. Int J Mol Sci 2010; 11: 3209-25.
[3] Tanaka T. Chemoprevention of human cancer: biology and therapy. Crit Rev Oncol Hematol 1997; 25: 139-74.
[4] Tanaka T. Cancer chemoprevention. Cancer J 1992; 5: 11-6.
[5] Tanaka T, Shimizu M, Moriwaki H. Cancer chemoprevention by carotenoids. Molecules 2012; 17: 3202-42.
[6] Tanaka T, Tanaka T, Tanaka M, Kuno T. Cancer chemoprevention by citrus pulp and juices containing high amounts of beta-cryptoxanthin and hesperidin. J Biomed Biotechnol 2012; 2012: 516981.
[7] Weisburger JH. Worldwide prevention of cancer and other chronic diseases based on knowledge of mechanisms. Mutat Res 1998; 402: 331-7.
[8] Mehta RG, Murillo G, Naithani R, Peng X. Cancer chemoprevention by natural products: how far have we come? Pharm Res 2010; 27: 950-61.
[9] Tanaka T, Oyama T, Yasui Y. Dietary supplements and colorectal cancer. Curr Topics Neutraceut Res 2008; 6: 165-88.
[10] Sporn MB, Dunlop NM, Newton DL, Smith JM. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed Proc 1976; 35: 1332-8.
[11] Albini A, Pennesi G, Donatelli F, Cammarota R, De Flora S, Noonan DM. Cardiotoxicity of anticancer drugs: the need for cardio-oncology and cardio-oncological prevention. J Natl Cancer Inst 2010; 102: 14-25.
[12] Ferrari N, Tosetti F, De Flora S, *et al.* Diet-derived phytochemicals: from cancer chemoprevention to cardio-oncological prevention. Curr Drug Targets 2011; 12: 1909-24.
[13] Saldanha SN, Tollefsbol TO. The role of nutraceuticals in chemoprevention and chemotherapy and their clinical outcomes. J Oncol 2012; 2012: 192464.
[14] Corpet DE, Pierre F. Point: From animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. Cancer Epidemiol Biomarkers Prev 2003; 12: 391-400.
[15] Corpet DE, Tache S. Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr Cancer 2002; 43: 1-21.
[16] Tanaka T, Ishigamori R. Understanding carcinogenesis for fighting oral cancer. J Oncol 2011; 2011: 603740.
[17] Tanaka T, Miyazawa K, Tsukamoto T, Kuno T, Suzuki K. Pathobiology and chemoprevention of bladder cancer. J Oncol 2011; 2011: 528353.
[18] Tanaka T, Tanaka M, Tanaka T. Oral carcinogenesis and oral cancer chemoprevention: a review. Pathol Res Int 2011; 2011: 431246.
[19] Oyama T, Yasui Y, Tanaka T. Breast cancer chemoprevention: current perspectives. Curr Enzyme Inhibition 2009; 5: 198-208.
[20] Bruce WR. Counterpoint: From animal models to prevention of colon cancer. Criteria for proceeding from preclinical studies and choice of models for prevention studies. Cancer Epidemiol Biomarkers Prev 2003; 12: 401-4.
[21] Carroll C, Booth A, Cooper K. A worked example of "best fit" framework synthesis: a systematic review of views concerning the taking of some potential chemopreventive agents. BMC Med Res Methodol 2011; 11: 29.

[22] Cooper K, Squires H, Carroll C, *et al.* Chemoprevention of colorectal cancer: systematic review and economic evaluation. Health Technol Assess 2010; 14: 1-206.
[23] Shimizu M, Kubota M, Tanaka T, Moriwaki H. Nutraceutical approach for preventing obesity-related colorectal and liver carcinogenesis. Int J Mol Sci 2012; 13: 579-95.
[24] Tanaka T. Development of an Inflammation-Associated Colorectal Cancer Model and Its Application for Research on Carcinogenesis and Chemoprevention. Int J Inflamm 2012; 2012: 658786.
[25] Tanaka T, Hosokawa M, Yasui Y, Ishigamori R, Miyashita K. Cancer chemopreventive ability of conjugated linolenic acids. Int J Mol Sci 2011; 12: 7405-09.
[26] Tanaka T, Tanaka T, Tanaka M. Potential cancer chemopreventive activity of protocatechuic acid. J Exp Clin Med 2011; 3: 27-33.
[27] Tanaka T, Yasui Y, Ishigamori-Suzuki, R Oyama T. Citrus compounds inhibit inflammation- and obesity-related colon carcinogenesis in mice. Nutr Cancer 2008; 60 Suppl 1: 70-80.
[28] Sung JJ, Lau JY, Goh KL, Leung WK. Increasing incidence of colorectal cancer in Asia: implications for screening. Lancet Oncol 2005; 6: 871-6.
[29] Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci 2003; 94: 965-73.
[30] Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001; 357: 539-45.
[31] Tanaka T, Suzuki R. Inflammation and cancer. In: Tanaka, T., editor. Cancer: Disease progression and Chemoprevention 2007. Kerala (India): Research Signpost: 2007. pp. 27-44.
[32] Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984; 1: 1311-5.
[33] Gonzalez CA, Agudo A. Carcinogenesis, prevention and early detection of gastric cancer: where we are and where we should go. Int J Cancer 2012; 130: 745-53.
[34] Tatematsu M, Tsukamoto T, Toyoda T. Effects of eradication of Helicobacter pylori on gastric carcinogenesis in experimental models. J Gastroenterol 2007; 42 Suppl 17: 7-9.
[35] Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001; 48: 526-35.
[36] Lakatos PL, Lakatos L. Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies. World J Gastroenterol 2008; 14: 3937-47.
[37] Munkholm P. Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. Aliment Pharmacol Ther 2003; 18 Suppl 2: 1-5.
[38] Tanaka T, Kohno H, Murakami M, Shimada R, Kagami S. Colitis-related rat colon carcinogenesis induced by 1-hydroxyanthraquinone and methylazoxymethanol acetate (Review). Oncol Rep 2000; 7: 501-8.
[39] Waldner MJ, Neurath MF. Chemically induced mouse models of colitis. Curr Protoc Pharmacol 2009; Chapter 5: Unit 5 55.
[40] Rosenberg DW, Giardina C, Tanaka T. Mouse models for the study of colon carcinogenesis. Carcinogenesis 2009; 30: 183-96.
[41] Tanaka T. Colorectal carcinogenesis: Review of human and experimental animal studies. J Carcinog 2009; 8: 5.
[42] Tanaka T, Sugie S. Inhibition of colon carcinogenesis by dietary non-nutritive compounds. J Toxicol Pathol 2007; 20: 215-35.
[43] Tanaka T. Preclinical cancer chemoprevention studies using animal model of inflammation-associated colorectal carcinogenesis. Cancers 2012; 4: 673-700.
[44] Suzuki R, Miyamoto S, Yasui Y, Sugie S, Tanaka T. Global gene expression analysis of the mouse colonic mucosa treated with azoxymethane and dextran sodium sulfate. BMC Cancer 2007; 7: 84.
[45] Yasui Y, Tanaka T. Protein expression analysis of inflammation-related colon carcinogenesis. J Carcinog 2009; 8: 10.
[46] Suzuki R, Kohno H, Sugie S, Nakagama H, Tanaka T. Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. Carcinogenesis 2006; 27: 162-9.
[47] Suzuki R, Kohno H, Sugie S, Tanaka T. Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate. Cancer Sci 2004; 95: 721-7.
[48] Suzuki R, Kohno H, Sugie S, Tanaka T. Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane. Histol Histopathol 2005; 20: 483-92.
[49] Nakanishi M, Tazawa H, Tsuchiya N, Sugimura T, Tanaka T, Nakagama, H. Mouse strain differences in inflammatory responses of colonic mucosa induced by dextran sulfate sodium cause differential susceptibility to PhIP-induced large bowel carcinogenesis. Cancer Sci 2007; 98: 1157-63.
[50] Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, Wakabayashi K. Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and dextran sodium sulfate in male ICR mice possess beta-catenin gene mutations and increases immunoreactivity for beta-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. Carcinogenesis 2005; 26: 229-38.
[51] Kohno H, Suzuki R, Sugie S, Tanaka T. Beta-Catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. Cancer Sci 2005; 96: 69-76.
[52] Tanaka T, Yasui Y, Tanaka M, Oyama T, Rahman KM. Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats. Chem Biol Interact 2009; 177: 128-36.
[53] Yoshimi K, Tanaka T, Takizawa A, *et al.* Enhanced colitis-associated colon carcinogenesis in a novel Apc mutant rat. Cancer Sci 2009; 100: 2022-7.
[54] Toyoda-Hokaiwado N, Yasui Y, Muramatsu M, *et al.* Chemopreventive effects of silymarin against 1,2-dimethylhydrazine plus dextran sodium sulfate-induced inflammation-associated carcinogenicity and genotoxicity in the colon of gpt delta rats. Carcinogenesis 2011; 32: 1512-7.
[55] Hata K, Tanaka T, Kohno H, *et al.* Lack of enhancing effects of degraded lambda-carrageenan on the development of beta-catenin-accumulated crypts in male DBA/2J mice initiated with azoxymethane. Cancer Lett 2006; 238: 69-75.
[56] Kohno H, Totsuka Y, Yasui Y, *et al.* Tumor-initiating potency of a novel heterocyclic amine, aminophenylnorharman in mouse colonic carcinogenesis model. Int J Cancer 2007; 121: 1659-64.
[57] Kohno H, Suzuki R, Sugie S, Tanaka T. Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands. BMC Cancer 2005; 5: 46.
[58] Kohno H, Takahashi M, Yasui Y, *et al.* A specific inducible nitric oxide synthase inhibitor, ONO-1714 attenuates inflammation-related large bowel carcinogenesis in male Apc(Min/+) mice. Int J Cancer 2007; 121: 506-13.
[59] Tanaka T, de Azevedo MB, Duran N, *et al.* Colorectal cancer chemoprevention by 2 beta-cyclodextrin inclusion compounds of auraptene and 4'-geranyloxyferulic acid. Int J Cancer 2010; 126: 830-40.
[60] Yasui Y, Suzuki R, Miyamoto S, *et al.* A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis. Int J Cancer 2007; 121: 2331-9.
[61] Yasui Y, Hosokawa M, Mikami N, Miyashita K, Tanaka T. Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice *via* modulation of the inflammatory cytokines. Chemico Biol Interact 2011; 193: 79-87.
[62] Tanaka T, Kohno H, Suzuki R, *et al.* Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int J Cancer 2006; 118: 25-34.
[63] Jacobs RJ, Kodach LL, Hardwick JC. The potential of statins for individualized colorectal cancer chemoprevention. Curr Drug Targets 2011; 12: 1903-8.
[64] Yasuda Y, Shimizu M, Shirakami Y, *et al.* Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db obese mice. Cancer Sci 2010; 101: 1701-7.
[65] Shimizu M, Yasuda Y, Sakai H, *et al.* Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-db/db obese mice. BMC Cancer 2011; 11: 281.
[66] Mukhtar RY, Reid J, Reckless JP. Pitavastatin. Int J Clin Pract 2005; 59: 239-52.
[67] Tanaka T, Kawamori T, Ohnishi M, *et al.* Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis 1995; 16: 2957-63.

[68] Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K, Hara A. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res 1995; 55: 4059-64.

[69] Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A, Mori H. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 1994; 15: 15-9.

[70] McCart AE, Vickaryous NK, Silver A. Apc mice: models, modifiers and mutants. Pathol Res Pract 2008; 204: 479-90.

[71] Smith KD, Rodriguez-Bigas MA. Role of surgery in familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer (Lynch syndrome). Surg Oncol Clin N Am 2009; 18: 705-15.

[72] Hopper JL. Application of genetics to the prevention of colorectal cancer. Recent Results Cancer Res 2005; 166: 17-33.

[73] Booij KA, Mathus-Vliegen EM, Taminiau JA, *et al.* Evaluation of 28 years of surgical treatment of children and young adults with familial adenomatous polyposis. J Pediatr Surg 2010; 45: 525-32.

[74] Niho N, Takahashi M, Kitamura T, *et al.* Concomitant suppression of hyperlipidemia and intestinal polyp formation in Apc-deficient mice by peroxisome proliferator-activated receptor ligands. Cancer Res 2003; 63: 6090-5.

[75] Mutoh M, Niho N, Wakabayashi K. Concomitant suppression of hyperlipidemia and intestinal polyp formation by increasing lipoprotein lipase activity in Apc-deficient mice. Biol Chem 2006; 387: 381-5.

[76] Yamada Y, Hata K, Hirose Y, *et al.* Microadenomatous lesions involving loss of Apc heterozygosity in the colon of adult Apc(Min/+) mice. Cancer Res 2002; 62: 6367-70.

[77] Yamada Y, Mori H. Multistep carcinogenesis of the colon in Apc(Min/+) mouse. Cancer Sci 2007; 98: 6-10.

[78] Rostom A, Dube C, Lewin G, *et al.* Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force. Ann Intern Med 2007; 146: 376-89.

[79] Reddy BS, Wang CX, Kong AN, *et al.* Prevention of azoxymethane-induced colon cancer by combination of low doses of atorvastatin, aspirin, and celecoxib in F 344 rats. Cancer Res 2006; 66: 4542-6.

[80] Russo GL. Ins and outs of dietary phytochemicals in cancer chemoprevention. Biochem Pharmacol 2007; 74: 533-44.

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ORIGINAL ARTICLE

Early-stage formation of an epigenetic field defect in a mouse colitis model, and non-essential roles of T- and B-cells in DNA methylation induction

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Epigenetic fields for cancerization are involved in development of human cancers, especially those associated with inflammation and multiple occurrences. However, it is still unclear when such field defects are formed and what component of inflammation is involved in induction of aberrant DNA methylation. Here, in a mouse colitis model induced by dextran sulfate sodium (DSS), we identified three CpG islands specifically methylated in colonic epithelial cells exposed to colitis. Their methylation levels started to increase as early as 8 weeks after DSS treatment and continued to increase until colon cancers developed at 15 weeks. In contrast to the temporal profile of DNA methylation levels, infiltration of inflammatory cells spiked immediately after the DSS treatment and then gradually decreased. Exposure of cultured colonic epithelial cells to DSS did not induce DNA methylation and it was indicated that inflammation triggered by the DSS treatment was responsible for methylation induction. To clarify components of inflammation involved, severe combined immunodeficiency (SCID) mice that lack functional T- and B-cells were similarly treated. Even in SCID mice, DNA methylation, along with colon tumors, were induced at the same levels as in their background strain of mice (C.B17). Comparative analysis of inflammation-related genes showed that *Ifng*, *Il1b* and *Nos2* had expression concordant with methylation induction whereas *Il2*, *Il6*, *Il10*, *Tnf* did not. These results showed that an epigenetic field defect is formed at early stages of colitis-associated carcinogenesis and that functional T and B cells are non-essential for the formation.

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Introduction

Epigenetic alterations are critically involved in human carcinogenesis (Jones and Baylin, 2007; Esteller, 2008). Especially, DNA methylation is stably inherited upon somatic cell replication (Riggs and Xiong, 2004) and methylation of promoter CpG islands (CGIs) consistently inactivates their downstream genes, including tumor-suppressor genes, such as *CDK2A*, *HIC1* and *SOCS1* (Ushijima, 2005). In spite of its importance, inducers of aberrant DNA methylation are still not fully understood. Among the few established inducers, importance of chronic inflammation, such as ulcerative colitis, hepatitis and gastritis due to *Helicobacter pylori* infection, has been shown by multiple studies (Hsieh *et al.*, 1998; Kondo *et al.*, 2000; Issa *et al.*, 2001; Niwa *et al.*, 2010; Hur *et al.*, 2011). In addition to chronic inflammation, aging and exogenous DNA, such as viruses, have been considered as inducers (Ushijima and Okochi-Takada, 2005).

Chronic inflammation-associated cancers are characterized by frequent occurrence of multiple cancers, suggesting that non-cancerous tissues exposed to chronic inflammation have already accumulated genetic and epigenetic alterations, forming a field for cancerization (Braakhuis *et al.*, 2003; Ushijima, 2007). Accumulation of genetic alterations is difficult to assess because their frequency in non-cancerous tissues is very low (Nagao *et al.*, 2001). In contrast, aberrant DNA methylation of various genes is accumulated at high levels in non-cancerous tissues exposed to chronic inflammation (Hsieh *et al.*, 1998; Kondo *et al.*, 2000; Issa *et al.*, 2001), and such accumulation levels are correlated with cancer risk (Schulmann *et al.*, 2005; Maekita *et al.*, 2006; Nakajima *et al.*, 2006; Garrity-Park *et al.*, 2010), forming an epigenetic field for cancerization (epigenetic field defect) (Ushijima, 2007). Methylation levels of marker genes, which show high methylation levels, are correlated with those of tumor-suppressor genes, which tend to show very low methylation levels (Maekita *et al.*, 2006; Ushijima, 2007).

However, it is still unclear when such an epigenetic field defect is formed during chronic inflammation-associated carcinogenesis, and how aberrant DNA methylation is induced by chronic inflammation. As inflammation involves multiple types of cells at different

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time points, it is important to investigate mechanisms of how such a field defect is formed *in vivo*. Such *in vivo* analysis has been hampered by the lack of appropriate animal models in which chronic inflammation and aberrant DNA methylation are involved, except for a *Helicobacter pylori* infection-induced gastritis model in Mongolian gerbils (Niwa *et al.*, 2010; Hur *et al.*, 2011). In mouse models, aberrant DNA methylation itself has been reported in lung (Vuillemenot *et al.*, 2004), skin (Fraga *et al.*, 2004), small intestine (Hahn *et al.*, 2008), and prostate (Yamashita *et al.*, 2008) cancers and in hematological malignancies (Yu *et al.*, 2005).

In this study, we focused on a mouse colitis model induced by dextran sulfate sodium (DSS), which share some aspects with human ulcerative colitis (Rosenberg *et al.*, 2009), as a possible model in which an epigenetic field defect is involved. After identification of CGIs aberrantly methylated in azoxymethane (AOM) and DSS-induced mouse colon cancers, we clarified when aberrant DNA methylation is induced in colonic epithelial cells during colitis and what cells are critically involved in the induction.

Results

Identification of CGIs methylated in colitis-associated mouse colon tumors

We started by identifying CGIs specifically methylated in mouse colon tumors associated with colitis. AOM/DSS-induced colon tumors and untreated normal colon epithelial samples were obtained from the animal experiment described in Figure 1a. Methylated DNA immunoprecipitation (MeDIP)-CGI microarray analysis was performed using a pool of the two tumors and a pool of two normal epithelial samples, and we isolated 23 candidate CGIs methylated in the tumors. Their methylation statuses were analyzed by methylation-specific PCR (MSP) of the samples used for the MeDIP-microarray analysis, three additional colon tumors and normal epithelial samples obtained from untreated mice. Fifteen of the 23 CGIs were specifically methylated in four or more of the five tumors (Figure 1b; Table 1). The presence of densely methylated DNA molecules was further confirmed by bisulfite sequencing for three CGIs (*Fosb*, *Hoxa5* and *Krt7*) in the five tumors (Figure 1c).

DNA methylation induction in colonic epithelial cells and its temporal profiles

DNA methylation of the 15 CGIs was then analyzed in epithelial samples exposed to AOM and/or DSS by quantitative MSP (qMSP) (Figure 1d and Supplementary Figure S1). Fourteen of them had significantly increased methylation levels in epithelial samples of AOM/DSS-treated and DSS-treated mice but not in those of AOM-treated mice. This showed that DNA methylation of these CGIs was associated with DSS treatment.

Epithelial samples might be contaminated with infiltrating blood cells although they were prepared by the crypt isolation technique. We therefore analyzed methylation levels of the 14 CGIs in peripheral blood, and three (*Fosb*, *Msx1* and *Sox11*) had low methylation levels (0–5.1 percentage of the methylated reference (PMR)) whereas the other 11 CGIs had high methylation levels (11–78 PMR; shown as numbers in parentheses in Figure 1d and Supplementary Figure S1). We further purified epithelial cells and blood leukocytes by fluorescence-activated cell sorting using Epcam (a pan-epithelial cell marker) and Cd45 (a pan-leukocyte marker), respectively, from colonic epithelial samples. At 15 weeks, methylation levels of the three CGIs with low methylation in peripheral blood (*Fosb*, *Msx1*, and *Sox11*) were significantly higher in the epithelial (Epcam-positive) cells of the DSS-treated mice than in those of untreated mice (7.3- to 19.3-fold; Supplementary Figure S3). These three CGIs were located inside the genes or in the 5′ far upstream region of the gene.

Using these three CGIs, temporal profiles of methylation induction were analyzed in the course of DSS-induced colitis (Figure 2a). All the three CGIs showed gradual increases of DNA methylation levels, starting at 8 weeks after DSS treatment and reaching 7.4- to 9.2-fold high levels, compared with untreated controls, at 15 weeks (Figure 2b). When the remaining 11 CGIs with high DNA methylation levels in peripheral blood were analyzed (Supplementary Figure S2), three (*Fut4*, *Hoxa5* and *Mex3a*) showed similar profiles to the above three CGIs and eight (*5730596B20Rik*, *Bcl6b*, *Epcam*, *Fmnl1*, *Irf2bp*, *Nav1*, *Rara* and *Sh2d3c*) showed a spiked increase immediately after the DSS treatment and a gradual decrease to control levels by 15 weeks.

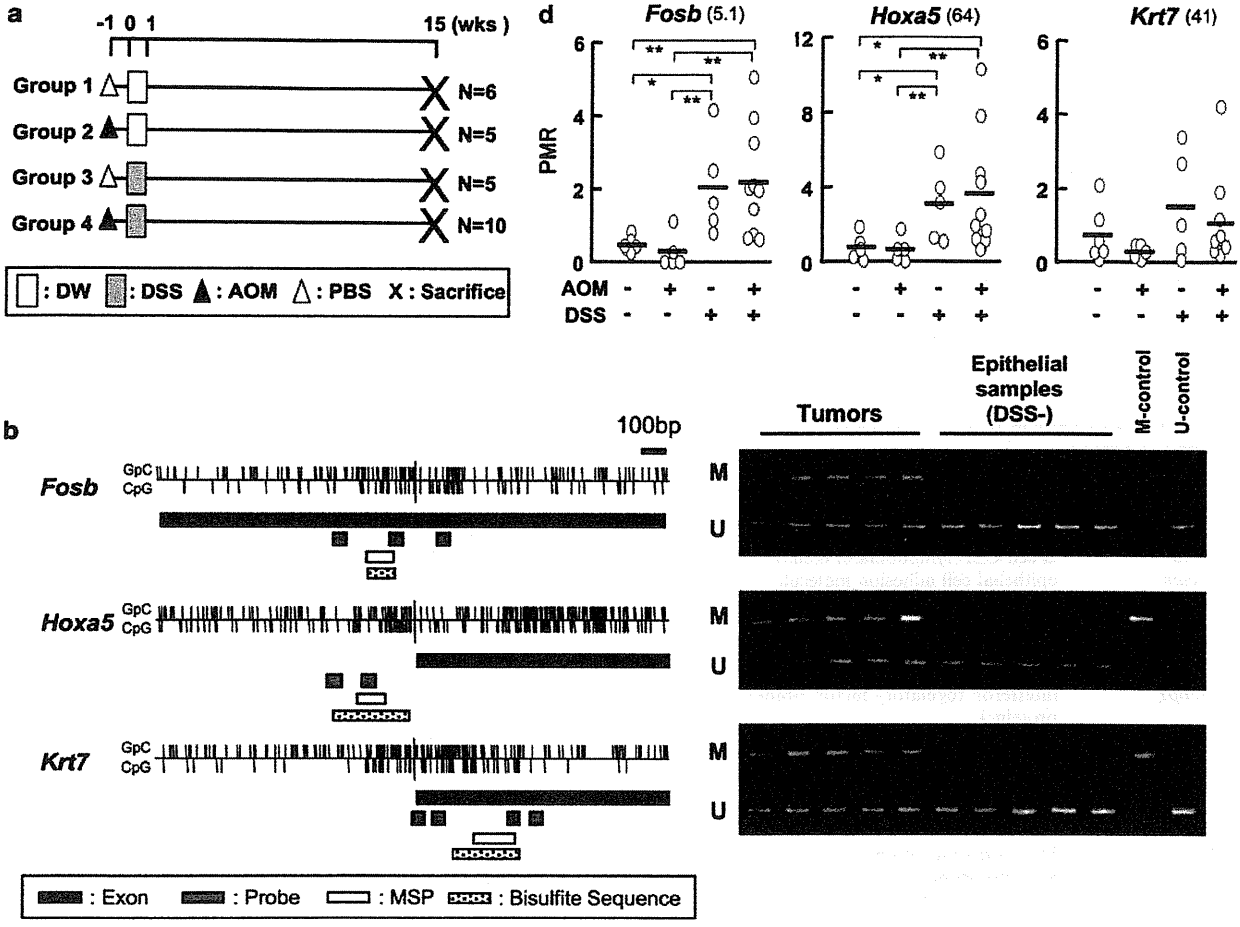
No induction of aberrant DNA methylation by treatment of colonic epithelial cells with DSS in vitro

DSS is known to have direct effects on epithelial cells, such as induction of cell growth arrest and production of cytokines (Ni *et al.*, 1996; Araki *et al.*, 2006), and this raised a possibility that DSS might directly induce aberrant DNA methylation in colonic epithelial cells. To address this possibility, immortalized normal mouse colonic epithelial cells (LIF-16) were treated with four concentrations of DSS that inhibited cell growth in a dose-dependent manner (Figure 3a). As the maximum dose (2%) did not affect cellular morphology (Figure 3b), the cells were cultured for 2, 5 and 8 weeks after 1 week of treatment with 2% DSS or in the presence of 2% DSS (Figure 3c). The methylation levels of the three CGIs (*Fosb*, *Msx1*, and *Sox11*) in the DSS-treated cells remained in the same range with those in untreated cells (Figure 3d). This showed that DSS itself was unlikely to induce DNA methylation in colonic epithelial cells.

Temporal profiles of inflammatory cell infiltration after the DSS treatment

In the previous study, we found that aberrant DNA methylation is induced in gastric epithelial samples exposed to specific kinds of inflammation (Hur *et al.*, 2011). Because a direct effect of DSS on methylation induction was unlikely, inflammation triggered by the

DSS treatment was suggested to be involved in the methylation induction. To identify specific inflammatory cells associated with DNA methylation induction, their infiltration was assessed by counting the number of infiltrating lymphocytes, macrophages, and neutrophils in colonic mucosae and submucosae (Figure 2c). Lymphocyte and neutrophil infiltration was most severe immediately after the DSS treatment, then gradually decreased, but was still present even at weeks 15. Macrophage infiltration stayed at high levels through-



out the experimental period. These results showed that inflammation was present in the DSS-treated colon throughout the experimental period despite DSS being transiently administered, and that dominantly infiltrating cells shifted from neutrophils/lymphocytes to macrophages. The number of inflammatory cells did not parallel the temporal profiles of gradually increasing DNA methylation levels of the six CGIs (*Fosb*, *Fut4*, *Hoxa5*, *Mex3a*, *Msx1* and *Sox11*).

Carcinogenicity and DNA methylation induction in severe combined immunodeficiency mice

As the infiltration of inflammatory cells in wild-type (BALB/c) mice after DSS treatment was extensive, identification of specific inflammatory cells and inflammation-related genes responsible for DNA methylation induction was difficult. Therefore, we adopted a strategy of analyzing carcinogenicity and DNA methylation induction in severe combined immunodeficiency (SCID) mice (Figure 4a), which lack functional T- and B-cells but have normal innate immunity (Bosma *et al.*, 1983). So far, there has been no report of aberrant methylation induction by DSS colitis in SCID mice. Tumor incidence and multiplicity showed no significant difference between SCID and its wild-type control, C.B17 (Figure 4b; Table 2), demonstrating that functional T- and B-cells are not essential for tumor induction in SCID mice. Methylation of *Fosb*, *Msx1* and *Sox11* was significantly induced in colonic epithelial samples of AOM/DSS-treated SCID mice to almost the same level as those in C.B17 mice (Figure 4c), demonstrating that functional T- and B-cells are dispensable for methylation induction.

Expression of inflammation-related genes in DSS-treated SCID mice

Infiltration of inflammatory cells and expression levels of inflammation-related genes were analyzed in the

Table 1 CGIs aberrantly methylated in mouse colon tumors

	Gene symbol	*Gene name*	*Accession numbers*	*Affected CGI region*[a]	*Chromosome*	*Position*[b]
1	*5730596B20Rik*	RIKEN cDNA 5730596B20 gene	NM_175261	52106690-52106900	6	5′ Proximal upstream
2	*Bcl6b*	B-cell CLL/lymphoma 6, member B	NM_007528	70042575-70042939	11	Inside
3	*Epcam*	epithelial cell adhesion molecule	NM_008532	87548510-87548927	17	Inside
4	*Fmnl1*	formin-like 1	NM_019679	103015371-103015514	11	3′ Downstream
5	*Fosb*	FBJ osteosarcoma oncogene B	NM_008036	18463460-18463668	7	Inside
6	*Fut4*	fucosyltransferase 4	NM_010242	14501158-14501694	9	Inside
7	*Hoxa5*	homeobox A5	NM_010453	52134331-52134506	6	5′ Proximal upstream
8	*Irf2bp1*	interferon regulatory factor 2-binding protein 1	NM_178757	18163624-18163938	7	Inside
9	*Krt7*[c]	keratin 7	NM_033073	101240421-101240949	15	Inside
10	*Mex3a*	mex3 homolog A	NM_001029890	88622212-88622469	3	Inside
11	*Msx1*	homeobox, msh-like 1	NM_010835	38109507-38109773	5	Inside
12	*Nav1*	neuron navigator 1	NM_175447	137401177-137401323	1	Inside
13	*Rara*	retinoic acid receptor, alpha	NM_009024	98752420-98752549	11	5′ Far upstream
14	*Sh2d3c*	SH2 domain-containing 3C	NM_013781	32563730-32563900	2	Inside
15	*Sox11*	SRY-box-containing gene 11	NM_009234	27929531-27929660	12	5′ Far upstream

Abbreviation: CGI, CpG island.
[a]Region in the corresponding chromosome annotated by UCSC mm8 (NCBI Build 36, February 2006).
[b]Relative position to the gene (5′ far upstream, a region more than 300 bp upstream of the transcription start site; 5′ proximal upstream, a region within 300 bp upstream of the transcription start site; inside, a region in the exon or intron; and 3′ downstream, a region within 10 kbp downstream of the last exon).
[c]A gene whose methylation in non-cancerous mucosae was not significantly elevated.

Figure 1 Identification of CGIs methylated in mouse colon tumors and colonic epithelial samples exposed to DSS. (**a**) Experimental protocol of tumor induction by AOM and DSS in BALB/c mice. Tumor incidence of this experiment is summarized in Supplementary Table S1. (**b**) CpG map of a CGI, and its methylation in tumor samples (data shown for representative three CGIs). Vertical lines, individual CpG or GpC sites; open boxes, positions of MSP products; closed boxes, positions of exons; gray boxes, locations of probes in CGI microarrays; and dotted boxes, positions of bisulfite sequencing. MSP was performed using two pairs of colon tumors and normal epithelial samples without DSS treatment used for MeDIP-CGI microarray analysis and three additional pairs. M, MSP using primers specific to methylated DNA; U, MSP using primers specific to unmethylated DNA; M-control, fully methylated genomic DNA; and U-control, fully unmethylated DNA. (**c**) The presence of dense methylation in AOM/DSS-induced mouse colon tumors. Bisulfite sequencing of *Fosb*, *Hoxa5* and *Krt7* was performed in epithelial samples of an untreated mouse aged 22 weeks (group 1, weeks 15) and two AOM/DSS-induced colon tumors (group 4, weeks 15). Ten to 12 DNA molecules were analyzed per sample for a gene. Three other tumors not presented here showed similar methylation patterns. Bars, CpG sites interrogated by MSP primers; open circles, unmethylated CpG sites; and closed circles, methylated CpG sites. (**d**) DNA methylation levels in colonic epithelial samples from AOM/DSS, AOM, DSS and untreated groups. DNA methylation levels were quantified by qMSP. Bold horizontal bars indicate average. Numbers in parentheses show methylation levels in peripheral blood of mice aged 22 weeks. Methylation levels were shown to be increased by DSS treatment. $*P<0.05$; $**P<0.01$.

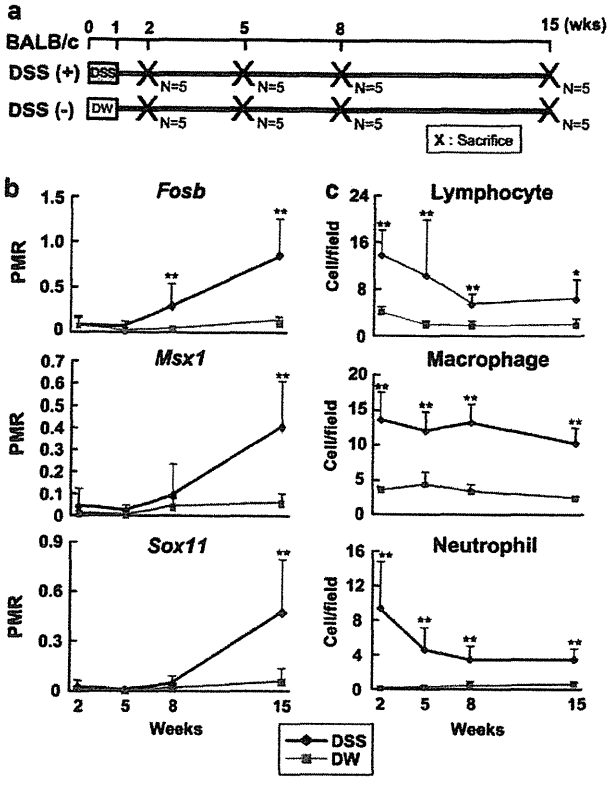


Figure 2 Temporal profiles of DNA methylation and inflammation after DSS treatment. (**a**) Experimental protocol for the time course analysis. (**b**) Temporal profiles of DNA methylation levels. (**c**) Temporal profiles of infiltration of inflammatory cells. Methylation levels and infiltration of inflammatory cells are shown as mean + s.d. $*P<0.05$; $**P<0.01$ when compared with untreated age-matched groups.

colon of DSS-treated SCID mice at 8 weeks (Figure 4a, d and e). This time point was chosen because DNA methylation was increasing at this point in the DSS-treated BALB/c mice and was considered to be actively being induced. Quantification of inflammatory cells confirmed that there was little infiltration of lymphocytes in SCID mice. In contrast, infiltration of macrophages and neutrophils were induced both in SCID and C.B17 mice by DSS treatment. Treatment with only DSS was conducted because DSS only was sufficient for methylation induction (Figure 1d and Supplementary Figure S1) and we wanted to avoid any additional expression changes caused by AOM.

Among the eight inflammation-related genes analyzed, upregulation of *Ifng*, *Il1b* and *Nos2* by DSS treatment was commonly observed in SCID and C.B17 mice (Figure 4e). On the other hand, upregulation of four genes (*Il2*, *Il6*, *Il10* and *Tnf*) was observed only in C.B17 mice, not in SCID mice. *Cox2* expression was not induced by DSS treatment either in SCID or in C.B17 mice. As inflammation-related genes upregulated commonly in SCID and C.B17 mice were likely to be involved in DNA methylation induction, *Ifng*, *Il1b* and *Nos2* were considered as candidates involved in DNA methylation induction.

Discussion

We here demonstrated that aberrant DNA methylation was induced in colonic epithelial cells as early as 8 weeks after DSS treatment, when no macroscopic tumors appeared, and the methylation level gradually increased until macroscopic tumors developed. The presence of aberrant DNA methylation in early stages of carcinogenesis was consistent with findings in *Gpx1/2* double knockout mice, a model for human inflammatory bowel disease (Hahn *et al.*, 2008) and in gastric epithelia of Mongolian gerbils exposed to *Helicobacter pylori* infection (Niwa *et al.*, 2010; Hur *et al.*, 2011). The present study is unique in that DNA methylation levels gradually increased even if inflammation gradually diminished.

To investigate components of inflammation involved in methylation induction, we utilized SCID mice, and found that DNA methylation and colon tumors were induced in them to almost the same level as those in C.B17 mice. This clearly showed that functional T- and B-cells are non-essential for DNA methylation and tumor induction. Induction of colitis by DSS in SCID mice has long been known (Dieleman *et al.*, 1994), but tumor incidence has not been analyzed. This is the first study that showed DNA methylation and tumors are induced in SCID mice by AOM and DSS to almost the same level as those in wild-type mice. In SCID mice, infiltration of macrophages and neutrophils was almost at the same levels as in C.B17 mice. Considering the importance of chronic inflammation, it was suggested that macrophages could be the proximate effector for DNA methylation induction. Among the eight inflammation-related genes examined, *Ifng*, *Il1b* and *Nos2* were upregulated by DSS treatment both in SCID and C.B17 mice.

The eight inflammation-related genes (*Cox2*, *Ifng*, *Il1b*, *Il2*, *Il6*, *Il10*, *Nos2*, and *Tnf*) examined here are known to show increased expression in inflamed human bowels (Cappello *et al.*, 1992; McLaughlan *et al.*, 1997; Autschbach *et al.*, 2002; Li *et al.*, 2009; Wang and Dubois, 2010). Especially, IL1β is produced at significantly high levels also in human ulcerative colitis (Ligumsky *et al.*, 1990). *In vitro*, administration of IL1β is reported to induce DNA methylation through induction of *Nos2* (Hmadcha *et al.*, 1999) and in *CDH1* promoter (Qian *et al.*, 2008). INFγ is reported to be involved in initiation of DSS-induced colitis (Ito *et al.*, 2006) and in development of colon tumors in *Socs1*-deficient mice (Hanada *et al.*, 2006), but its role in induction of aberrant DNA methylation is still unknown. Il6 is known to induce DNA methyltransferase expression (Hodge *et al.*, 2001), and its deficiency in mice leads to decreased tumor number and size after AOM and DSS treatment (Grivennikov *et al.*, 2009). *Il2* and *Il10* deficiency in mice leads to development of spontaneous colitis (Kuhn *et al.*, 1993; Sadlack *et al.*, 1993). Blocking of *Tnf* signal reduced tumor number after AOM and DSS treatment (Popivanova *et al.*, 2008).

Technically, the MeDIP-CGI microarray analysis isolated 23 candidate CGIs methylated in primary

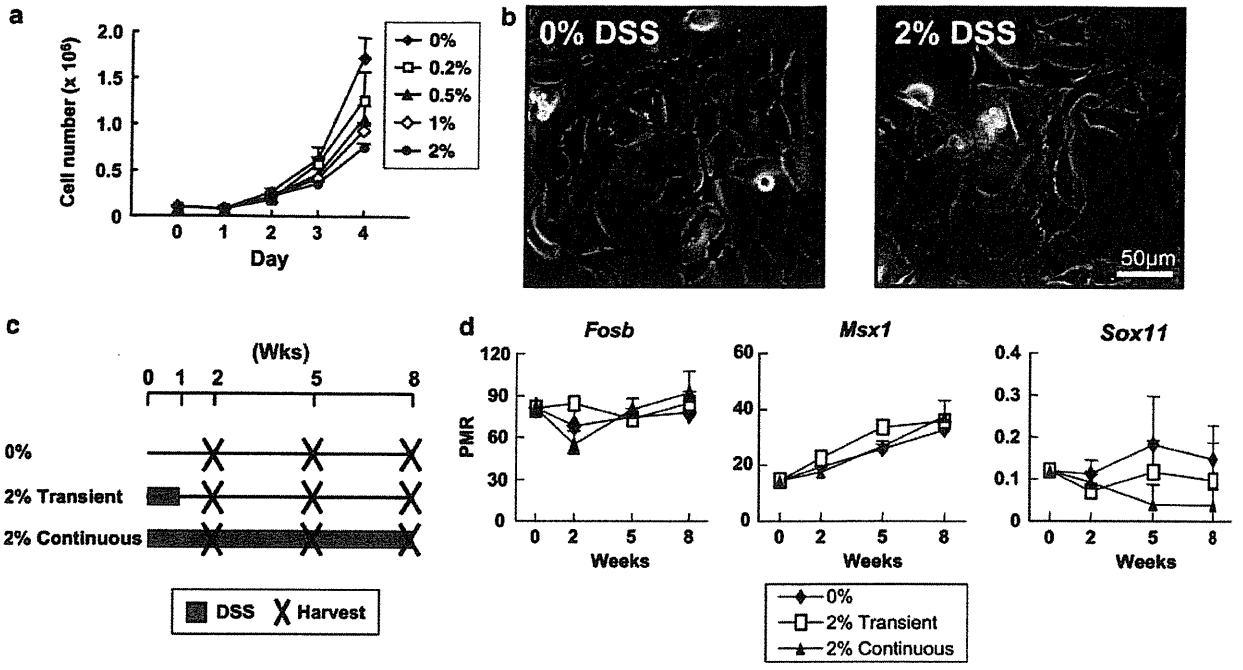


Figure 3 Direct effects of DSS on methylation induction. (**a**) Effect of four doses of DSS on cellular growth. Numbers are shown as mean + s.d. of three independent cultures. (**b**) Morphology of the cells (day 2). No morphological changes were induced with the highest dose. (**c**) Experimental protocol for time course methylation analysis. (**d**) Methylation levels in the DSS-treated epithelial cells. Cells on day 0 denote original cells before the plating. Methylation levels are shown as mean + s.d. of three independent cultures. DSS did not induce methylation directly, even after 8-week culture, although it affected the cellular growth.

mouse colon tumors. The number was smaller than expected from the known finding that 170–621 CGIs are methylated in human colon cancers (Keshet *et al.*, 2006; Kim *et al.*, 2011). However, using the same cutoff values used in this study, we were able to isolate 2339 methylated CGIs in a mouse colon cancer cell line, Colon26 (data not shown). Therefore, it was unlikely that there was a technical problem, and it was suggested that the mouse primary colon tumors had much smaller numbers of methylated CGIs than the Colon26 mouse colon cancer cell line and human colon cancers.

In summary, by DSS-induced inflammation, aberrant DNA methylation was shown to start to accumulate in epithelial cells at early stages of carcinogenesis. T- and B-cells were non-essential for DNA methylation induction.

Materials and methods

Cell line and DSS treatment

LIF-16, an embryonic colonic epithelial cell line established from *Trp53*$^{-/-}$ mice as described previously (Taniwaki *et al.*, 2007), was kindly provided by Dr Hiroshi Fukamachi at Tokyo Medical and Dental University and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For cell growth assay, 10^5 cells were plated. After attachment of the cells, the culture media were replaced by those containing DSS (molecular weight = 36 000–50 000; MP Biochemicals, Solon, OH, USA). The cell number was counted by a Countess automated cell counter (Invitrogen, Rockville, MD, USA). For methylation assay, 5×10^3 cells were plated on 6 cm dishes. Every third or fourth day, the cells were passaged using Dulbecco's modified Eagle's medium without DSS, and DSS was added after attachment of the cells during the duration described in Figure 3c.

Animals and cancer induction experiments

Male BALB/c mice were purchased from Charles River Laboratories (Yokohama, Japan). Male C.B17/Icr-scid/scid (SCID) and C.B17/Icr-$^{+/+}$ (C.B17) mice were purchased from CLEA Japan (Tokyo, Japan). AOM (10 mg/kg body weight; NARD Institute, Amagasaki, Japan) or phosphate-buffered saline (PBS) was administered by single intraperitoneal injection. DSS (molecular weight = 36 000–50 000) was administered to mice at 6 or 7 weeks of age in drinking water at a concentration of 1.5 or 2.0% w/v. Colon cancers and colonic epithelial samples for MeDIP-CGI microarray analysis were obtained from BALB/c mice treated with AOM and DSS (Figure 1a; Supplementary Table S1), which showed similar carcinogenicity to our previous reports (Tanaka *et al.*, 2003). All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

Sample preparation

The large bowel was cut open longitudinally and the number of macroscopic tumors whose major axes were more than 3 mm was counted. Large tumors were collected and half of each tumor was fixed with neutralized 10% formalin for histological analysis and the other half was kept frozen for

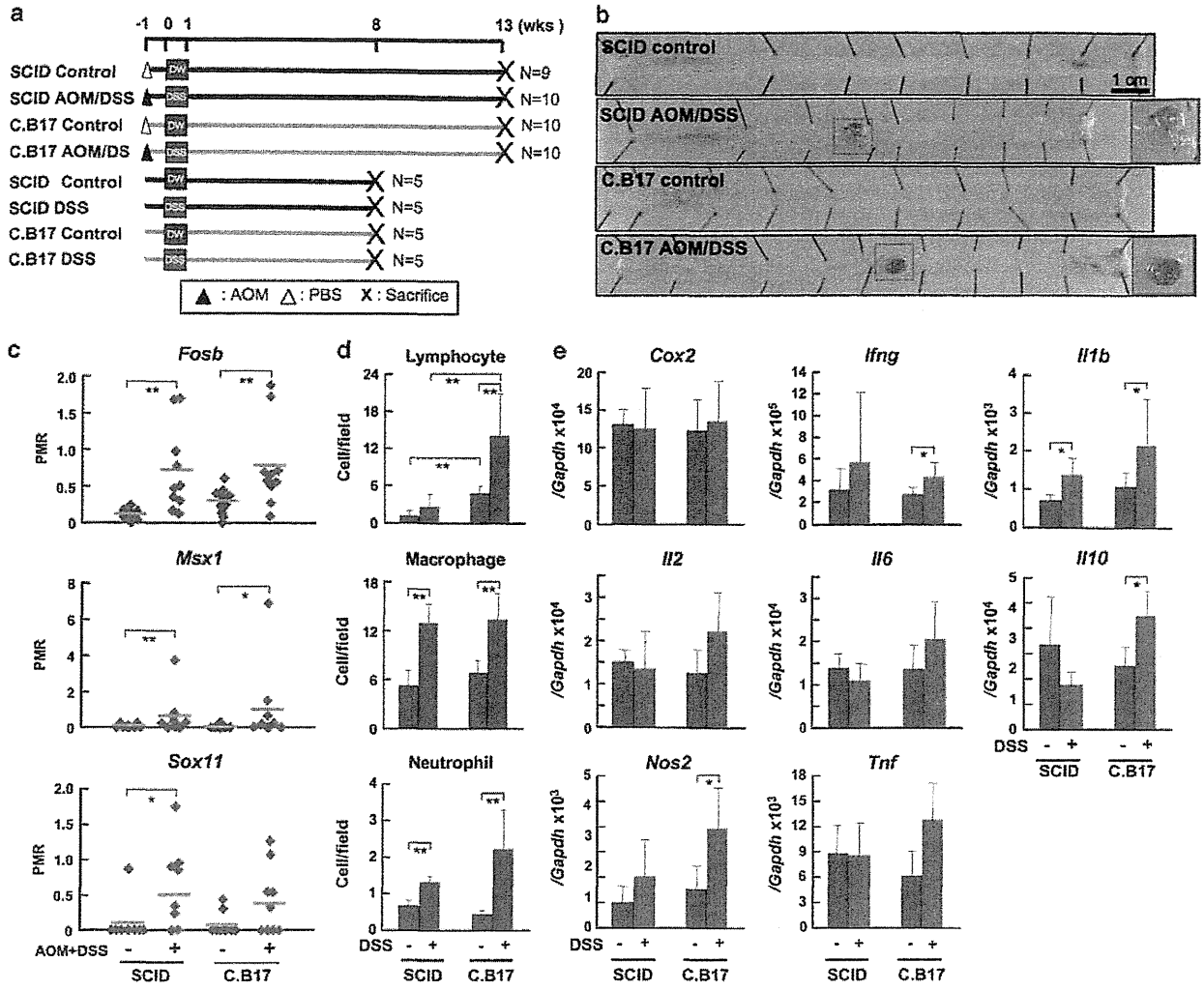


Figure 4 Tumor and DNA methylation induction in SCID mice. (**a**) Experimental protocol for AOM/DSS treatment in SCID and C.B17 mice. (**b**) Representative macroscopic appearance of the colon in the four groups. Colon tumors were induced at the same incidence in C.B17 and SCID mice. Right panels are the magnified view of tumors in blue rectangles. (**c**) DNA methylation levels at 13 weeks after DSS treatment. The levels were analyzed by qMSP of colonic epithelial samples from SCID and C.B17 mice treated with or without AOM/DSS. Bold horizontal bars indicate average. Similar levels of DNA methylation were induced in both SCID and C.B17 mice. (**d**) Infiltration of inflammatory cells and (**e**) expression levels of inflammation-related genes 8 weeks after DSS treatment in colonic tissues of SCID and C.B17 mice. Infiltration of inflammatory cells and gene expression levels are shown as mean + s.d. $^{*}P<0.05$; $^{**}P<0.01$.

Table 2 Tumor incidence and multiplicity in SCID and C.B17 mice

Strain	*Treatment*	*Incidence (%)*	*Number of tumors/mouse bearing tumors*[a]	*Size*[b]
SCID	AOM/DSS	9/10 (90)	2.8 ± 1.6	4.2 ± 0.69
	Control	0/9 (0)	—	—
C.B17	AOM/DSS	10/10 (100)	2.1 ± 1.2	4.6 ± 0.89
	Control	0/10 (0)	—	—

Abbreviations: AOM, azoxymethane; DSS, dextran sulfate sodium; SCID, severe combined immunodeficiency.
[a]Mean ± s.d.
[b]Mean ± s.d. in the major axis.

DNA and RNA isolation. Colonic epithelial samples were isolated from distal large bowels by the crypt isolation technique (Cheng *et al.*, 1984). Briefly, the distal half of the large bowel was incubated in a Hanks' balanced salt solution with 30 mM EDTA at 37 °C for 10 min. After the incubation, epithelium was collected by scraping off gently and washed with PBS. Peripheral blood was obtained from the inferior vena cava of DSS-treated and non-treated mice at 22 weeks of age.

Fluorescence-activated cell sorting
To dissociate single cells, epithelial samples were incubated in Hanks' balanced salt solution containing 10 mM HEPES (pH7.3), 1 mg/ml collagenase D (Roche Diagnostics, Penzberg, Germany) and 25 μg/mL DNase I (Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 80 min with gentle agitation. The cells were washed with PBS and then fixed with 80% acetone at 4 °C for 5 min. After washing with PBS, the fixed cells were incubated with a phycoerythrin-labeled anti-mouse Epcam antibody (eBiosciense, San Diego, CA, USA) and a

fluorescein isothiocyanate-labeled anti-Cd45 antibody (Miltenyi Biotech, Auburn, CA, USA) and sorted by FACSAria II cell sorter (BD, Franklin Lakes, NJ, USA).

Histological analysis
Macrophages and neutrophils were detected by immunohistochemistry of formalin-fixed tissues using a rabbit anti-F4/80 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rat anti-Ly6G antibody (Thermo Fisher Scientific, Fremont, CA, USA), respectively, as primary antibodies. Sections of 3 μm thickness were rehydrated and incubated in 10 mM citrate buffer (pH 6) at 120 °C for 5 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in PBS, sections were incubated with each primary antibody overnight. The immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and the sections were stained with hematoxylin. Mononuclear cells without F4/80 staining were considered as lymphocytes. The numbers of neutrophils, macrophages and lymphocytes in mucosae and submucosae were counted under 400 × magnification across seven random fields per colon. For immunofluorescent microscopy, fresh colonic tissues were embedded in O.T.C. compound (Sakura Finetek, Tokyo, Japan) and frozen by liquid nitrogen. Sections of 3 μm thickness were prepared and fixed with acetone at −20 °C for 5 min. The hydrated sections were incubated with the phycoerythrin-labeled anti-mouse Epcam antibody and the fluorescein isothiocyanate-labeled anti-Cd45 antibody.

Nucleic acid isolation
From colonic epithelial samples and cells purified by fluorescence-activated cell sorting, genomic DNA was extracted by the standard phenol/chloroform method, and RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). Genomic DNA of peripheral blood was extracted by a QuickGene DNA whole blood kit (Fujifilm, Tokyo, Japan).

MeDIP-CGI microarray
MeDIP-CGI microarray analysis was performed as previously described (Takeshima *et al.*, 2009; Yamashita *et al.*, 2009). Briefly, to immunoprecipitate methylated DNA, 5 μg of sonicated genomic DNA was incubated with an anti-5-methylcytidine antibody (Diagenode, Lié, Belgium) at 4 °C overnight. Immune complexes were collected with Dynabeads Protein A (Invitorogen Dynal AS, Oslo, Norway) and digested with proteinase K. Immunoprecipitated DNA was purified by phenol/chloroform extraction and isopropanol precipitation. Using an Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies, Santa Clara, CA, USA), the precipitated and input DNAs were labeled with Cy5 and Cy3, respectively, without any amplification. Labeled DNA was hybridized to a mouse CGI oligonucleotide microarray (Agilent Technologies) containing 97 652 probes covering 16 030 CGIs at 67 °C for 40 h with constant rotation and then scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). Scanned data were processed with Feature Extraction Ver.9.1 and Agilent G4477AA ChIP Analytics 1.3 software (Agilent Technologies). Probes with signal log ratio $\geqslant 0.5$ in a tumor sample and $\leqslant -0.2$ in a normal sample were considered to be methylated, and CGIs in which two or more continuous probes were methylated and at least one probe had a normalized log ratio more than 1.2 were considered as methylated.

MSP, qMSP and bisulfite sequencing
Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described (Yamashita *et al.*, 2008). The sample was resuspended in 40 μl of Tris–EDTA buffer, and an aliquot of 1 μl was used for MSP (qMSP) and bisulfite sequencing. Fully methylated and fully unmethylated DNA were prepared by amplifying mouse genomic DNA with GenomiPhi and by methylating it with *Sss*I methylase, respectively (Niwa *et al.*, 2005). Primers for MSP (Supplementary Table S2) were designed within ~100 bp from methylated probes. Primers for bisulfite sequencing were designed to cover the region amplified by MSP (Supplementary Table S2). qMSP was performed by real-time PCR using SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in duplicate. The number of molecules in a sample was determined by comparing its amplification with those of standard DNA prepared by purification of PCR products. The number was highly reproducible using the same samples (correlation coefficient >0.8). DNA methylation levels were expressed as a PMR, which reflected a fraction of the DNA molecules methylated at a specific locus (Kass *et al.*, 1997; Niwa *et al.*, 2010). PMR was calculated as ((no of molecules methylated at a target CGI in a sample)/(no of B2 SINE repeat in the sample))/((no of molecules methylated at the target CGI in a *SssI*-treated DNA)/(no of B2 SINE repeat in the *SssI*-treated DNA)) × 100.

Reverse transcriptase–PCR
Complementary DNA was synthesized from 2 μg of total RNA using a Superscript III kit (Invitrogen) with oligo dT primer. Real-time PCR was performed with gene-specific primers (Supplementary Table 3) as described in qMSP. The complementary DNA quantity of each gene was normalized to that of *Gapdh*.

Statistical analysis
Differences in DNA methylation and expression levels were analyzed by the Mann–Whitney *U* test using SPSS 13.0J. (SPSS Japan Inc., Tokyo, Japan).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

Araki Y, Sugihara H, Hattori T. (2006). *In vitro* effects of dextran sulfate sodium on a Caco-2 cell line and plausible mechanisms for dextran sulfate sodium-induced colitis. *Oncol Rep* **16**: 1357–1362.

Autschbach F, Giese T, Gassler N, Sido B, Heuschen G, Heuschen U *et al.* (2002). Cytokine/chemokine messenger-RNA expression profiles in ulcerative colitis and Crohn's disease. *Virchows Arch* **441**: 500–513.

Bosma GC, Custer RP, Bosma MJ. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature* **301**: 527–530.

Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. (2003). A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* **63**: 1727–1730.

Cappello M, Keshav S, Prince C, Jewell DP, Gordon S. (1992). Detection of mRNAs for macrophage products in inflammatory bowel disease by *in situ* hybridisation. *Gut* **33**: 1214–1219.

Cheng H, Bjerknes M, Amar J. (1984). Methods for the determination of epithelial cell kinetic parameters of human colonic epithelium isolated from surgical and biopsy specimens. *Gastroenterology* **86**: 78–85.

Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. (1994). Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* **107**: 1643–1652.

Esteller M. (2008). Epigenetics in cancer. *N Engl J Med* **358**: 1148–1159.

Fraga MF, Herranz M, Espada J, Ballestar E, Paz MF, Ropero S *et al.* (2004). A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. *Cancer Res* **64**: 5527–5534.

Garrity-Park MM, Loftus Jr EV., Sandborn WJ, Bryant SC, Smyrk TC. (2010). Methylation Status of Genes in Non-Neoplastic Mucosa From Patients With Ulcerative Colitis-Associated Colorectal Cancer. *Am J Gastroenterol.*

Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S *et al.* (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* **15**: 103–113.

Hahn MA, Hahn T, Lee DH, Esworthy RS, Kim BW, Riggs AD *et al.* (2008). Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. *Cancer Res* **68**: 10280–10289.

Hanada T, Kobayashi T, Chinen T, Saeki K, Takaki H, Koga K *et al.* (2006). IFNgamma-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice. *J Exp Med* **203**: 1391–1397.

Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. (1999). Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. *J Exp Med* **190**: 1595–1604.

Hodge DR, Xiao W, Clausen PA, Heidecker G, Szyf M, Farrar WL. (2001). Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells. *J Biol Chem* **276**: 39508–39511.

Hsieh CJ, Klump B, Holzmann K, Borchard F, Gregor M, Porschen R. (1998). Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. *Cancer Res* **58**: 3942–3945.

Hur K, Niwa T, Toyoda T, Tsukamoto T, Tatematsu M, Yang HK *et al.* (2011). Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation. *Carcinogenesis* **32**: 35–41.

Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. (2001). Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* **61**: 3573–3577.

Ito R, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J *et al.* (2006). Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol* **146**: 330–338.

Jones PA, Baylin SB. (2007). The epigenomics of cancer. *Cell* **128**: 683–692.

Kass DH, Kim J, Rao A, Deininger PL. (1997). Evolution of B2 repeats: the muroid explosion. *Genetica* **99**: 1–13.

Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E *et al.* (2006). Evidence for an instructive mechanism of *de novo* methylation in cancer cells. *Nat Genet* **38**: 149–153.

Kim YH, Lee HC, Kim SY, Yeom YI, Ryu KJ, Min BH *et al.* (2011). Epigenomic analysis of aberrantly methylated genes in colorectal cancer identifies genes commonly affected by epigenetic alterations. *Ann Surg Oncol* (doi:10.1245/s10434-011-1573-y).

Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. (2000). Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology* **32**: 970–979.

Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**: 263–274.

Li Y, de Haar C, Chen M, Deuring J, Gerrits MM, Smits R *et al.* (2009). Disease-related expression of the IL-6/STAT3/SOCS3 signaling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. *Gut* **59**: 227–235.

Ligumsky M, Simon PL, Karmeli F, Rachmilewitz D. (1990). Role of interleukin 1 in inflammatory bowel disease—enhanced production during active disease. *Gut* **31**: 686–689.

Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M *et al.* (2006). High levels of aberrant DNA methylation in Helicobacter pylori-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res* **12**: 989–995.

McLaughlan JM, Seth R, Vautier G, Robins RA, Scott BB, Hawkey CJ *et al.* (1997). Interleukin-8 and inducible nitric oxide synthase mRNA levels in inflammatory bowel disease at first presentation. *J Pathol* **181**: 87–92.

Nagao M, Ochiai M, Okochi E, Ushijima T, Sugimura T. (2001). LacI transgenic animal study: relationships among DNA-adduct levels, mutant frequencies and cancer incidences. *Mutat Res* **477**: 119–124.

Nakajima T, Maekita T, Oda I, Gotoda T, Yamamoto S, Umemura S *et al.* (2006). Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. *Cancer Epidemiol Biomarkers Prev* **15**: 2317–2321.

Ni J, Chen SF, Hollander D. (1996). Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. *Gut* **39**: 234–241.

Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T *et al.* (2010). Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res* **70**: 1430–1440.

Niwa T, Yamashita S, Tsukamoto T, Kuramoto T, Nomoto T, Wakazono K *et al.* (2005). Whole-genome analyses of loss of heterozygosity and methylation analysis of four tumor-suppressor genes in N-methyl-N'-nitro-N-nitrosoguanidine-induced rat stomach carcinomas. *Cancer Sci* **96**: 409–413.

Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S *et al.* (2008). Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* **118**: 560–570.

Qian X, Huang C, Cho CH, Hui WM, Rashid A, Chan AO. (2008). E-cadherin promoter hypermethylation induced by interleukin-1beta treatment or H. pylori infection in human gastric cancer cell lines. *Cancer Lett* **263**: 107–113.

Riggs AD, Xiong Z. (2004). Methylation and epigenetic fidelity. *Proc Natl Acad Sci USA* **101**: 4–5.

Rosenberg DW, Giardina C, Tanaka T. (2009). Mouse models for the study of colon carcinogenesis. *Carcinogenesis* **30**: 183–196.

Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. (1993). Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* **75**: 253–261.

Schulmann K, Sterian A, Berki A, Yin J, Sato F, Xu Y *et al.* (2005). Inactivation of p16, RUNX3, and HPP1 occurs early in Barrett's-associated neoplastic progression and predicts progression risk. *Oncogene* **24**: 4138–4148.

Takeshima H, Yamashita S, Shimazu T, Niwa T, Ushijima T. (2009). The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. *Genome Res* **19**: 1974–1982.

Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. (2003). A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* **94**: 965–973.

Taniwaki K, Fukamachi H, Komori K, Ohtake Y, Nonaka T, Sakamoto T *et al.* (2007). Stroma-derived matrix metalloproteinase (MMP)-2 promotes membrane type 1-MMP-dependent tumor growth in mice. *Cancer Res* **67**: 4311–4319.

Ushijima T. (2005). Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer* **5**: 223–231.

Ushijima T. (2007). Epigenetic field for cancerization. *J Biochem Mol Biol* **40**: 142–150.

Ushijima T, Okochi-Takada E. (2005). Aberrant methylations in cancer cells: where do they come from? *Cancer Sci* **96**: 206–211.

Vuillemenot BR, Pulling LC, Palmisano WA, Hutt JA, Belinsky SA. (2004). Carcinogen exposure differentially modulates RAR-beta promoter hypermethylation, an early and frequent event in mouse lung carcinogenesis. *Carcinogenesis* **25**: 623–629.

Wang D, Dubois RN. (2010). The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* **29**: 781–788.

Yamashita S, Hosoya K, Gyobu K, Takeshima H, Ushijima T. (2009). Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis. *DNA Res* **16**: 275–286.

Yamashita S, Takahashi S, McDonell N, Watanabe N, Niwa T, Hosoya K *et al.* (2008). Methylation silencing of transforming growth factor-beta receptor type II in rat prostate cancers. *Cancer Res* **68**: 2112–2121.

Yu L, Liu C, Vandeusen J, Becknell B, Dai Z, Wu YZ *et al.* (2005). Global assessment of promoter methylation in a mouse model of cancer identifies ID4 as a putative tumor-suppressor gene in human leukemia. *Nat Genet* **37**: 265–274.

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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.