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A^y allele promotes azoxymethane-induced colorectal carcinogenesis by macrophage migration in hyperlipidemic/diabetic KK mice

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The incidence of colorectal cancer has been increasing and is associated with obesity and diabetes. We have found that type 2 diabetes model KK-A^y/TaJcl (KK-A^y) mice develop tumors within a short period after treatment with azoxymethane (AOM). However, factors that contribute to the promotion of carcinogenesis have not been clarified. Therefore, we looked at the genetic background of KK-A^y, including two genetic characteristics of KK/TaJcl (KK) mice and C57BL/6J-Ham-A^y/+ (A^y) mice, compared with other non-obese and non-diabetic mouse strains C57BL/6J and ICR, and induced colorectal premalignant lesions, aberrant crypt foci (ACF), and tumors using AOM (150 μg/mouse/week for 4 weeks and 200 μg/mouse/week for 6 weeks, respectively). The mice with a diabetes feature, KK-A^y and KK, developed significantly more ACF, 67 and 61 per mouse, respectively, whereas ICR, A^y, and C57BL/6J mice developed 42, 24, and 18 ACF/mouse, respectively, at 17 weeks of age. Serum insulin and triglyceride levels in KK-A^y and KK mice were quite high compared with other non-diabetic mouse strains. Interestingly, KK-A^y mice developed more colorectal tumors (2.7 ± 2.3 tumor/mouse) than KK mice (1.2 ± 1.1 tumor/mouse) at 25 weeks of age, in spite of similar diabetic conditions. The colon cancers that developed in both KK-A^y and KK mice showed similar activation of β-catenin signaling. However, mRNA levels of inflammatory factors related to the activation of macrophages were significantly higher in colorectal cancer of KK-A^y mice than in KK. These data indicate that factors such as insulin resistance and dyslipidemia observed in obese and diabetic patients could be involved in susceptibility to colorectal carcinogenesis. In addition, increase of tumor-associated macrophages may play important roles in the stages of promotion of colorectal cancer. (*Cancer Sci* 2013; 104: 835–843)

Excessive accumulation of visceral adipose tissue can induce many disorders, such as type 2 diabetes mellitus (elevated fasting glucose, insulin, and insulin-like growth factor levels), and dyslipidemia (elevated triglyceride or low high-density lipoprotein cholesterol levels). Obesity is common in Western countries, and is currently increasing almost ubiquitously across the globe. Recently, obesity has attracted much interest as a risk factor for colorectal cancer. The World Cancer Research Fund and American Institute for Cancer Research have evaluated causal relationships between accumulation of visceral adipose tissue and cancer, and concluded "confident evidence" for colorectal cancers.[1] In males in Japan, an overweight condition and obesity (body mass index ≥ 25) are reported to be associated with colorectal cancer.[2,3]

We have reported that obese KK-A^y mice are highly susceptible to induction of colorectal premalignant lesions, ACF, and to development of colorectal cancers by AOM treatment.[4] The KK-A^y mice feature severe hyperinsulinemia, severe hypertriglyceridemia, excessive abdominal obesity, and resultant elevation of serum adipocytokines, such as IL-6, leptin, and Pai-1 compared with values for lean C57BL/6J mice. Such a consequent abnormality is suggested to be involved in the promotion of colorectal carcinogenesis, and is partly considered as a factor for high cancer susceptibility.

The KK-A^y mice were established by cross-mating KK mice, a type 2 diabetes mellitus model, with A^y mice,[5,6] which carry the *Agouti yellow* (A^y) gene and feature severe hyperphagia, hyperinsulinemia, and dyslipidemia. Non-diabetic or non-obese mice could be set as control, e.g., young A^y mice or C57BL/6J-Ham-+/+ (+/+) mice. In addition to this, the body weight of ICR mice is almost the same as KK-A^y mice at a young age, and they are also susceptible to induction of colorectal carcinogenesis by AOM treatment. The strains and features that could be the most susceptible to induction of colorectal carcinogenesis by AOM treatment have not been clarified. Thus, we aimed to investigate and find the features and molecules involved in obesity-associated cancer by comparing mouse strains KK-A^y, KK, A^y, +/+, C57BL, and ICR. In the present study, we showed colorectal ACF development was strongly affected by diabetic conditions, and additional features of inflammation derived from agouti gene overexpression may lead to further promotion of cancer development.

Materials and Methods

Animals. Female 5-week-old KK-A^y, KK, C57BL, and ICR mice were purchased from CLEA Japan (Tokyo, Japan), and A^y and +/+ mice were purchased from SLC Japan (Shizuoka, Japan). All animals were acclimated to laboratory conditions for 1 week. Three to four mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 22 ± 1°C and 55% humidity on a 12:12 h light:dark cycle, and fed AIN-76A powdered basal diet (CLEA Japan). Food and water were available *ad libitum*. The animals were observed daily for clinical signs, including anal bleeding and mortality. Body weights and food and water consumption were measured weekly. The experiments were carried out according to the Guidelines for Animal Experiments in the National Cancer Center (National Cancer Center, Tokyo, Japan) and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Azoxymethane-induced colorectal ACF development. For the induction of ACF by AOM (Nard Institute, Amagasaki, Japan), 6-week-old female KK-A^y (n = 13), KK (n = 13), A^y (n = 12), +/+ (n = 12), C57BL/6J (n = 13), and ICR (n = 13) mice

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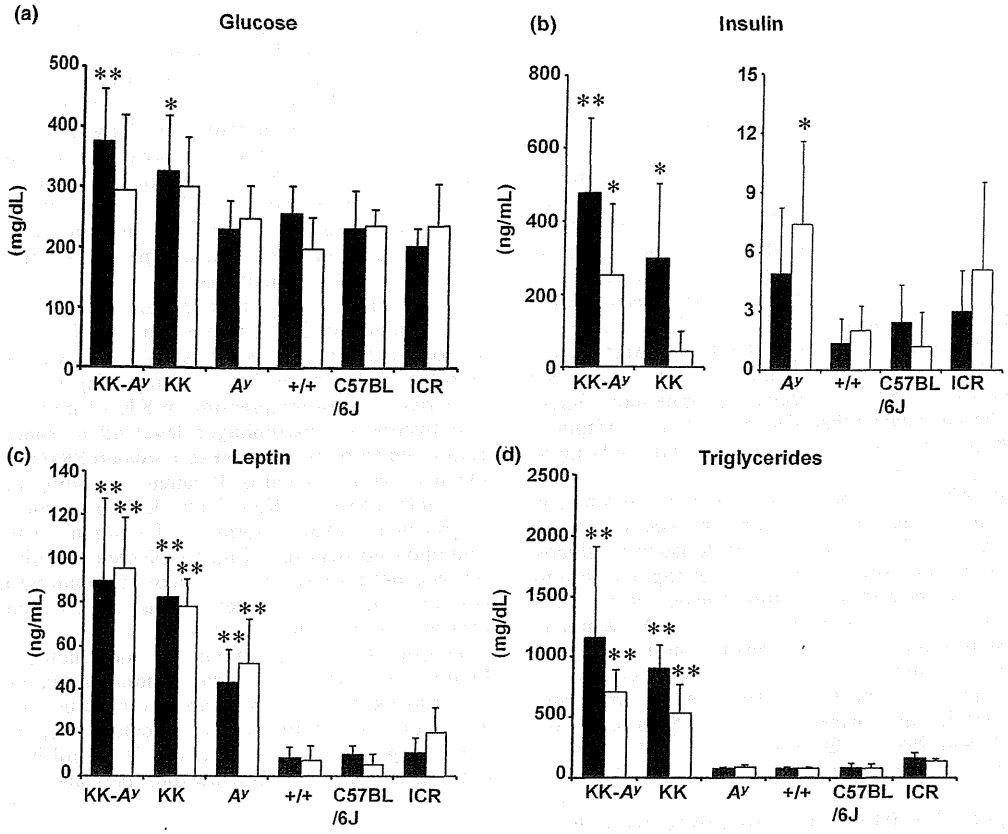

Fig. 2. Parameters for diabetes in KK-A^y/TaJcl (KK-A^y), KK/TaJcl (KK), and ICR mice treated with or without azoxymethane (AOM). The data of the group treated with AOM six times are shown in an open box, and without AOM are shown in a closed box. Parameters for diabetes, such as blood glucose (a), serum insulin levels (b), serum leptin levels (c), and serum triglyceride levels (d) are shown in the indicated mice strain. Data are mean ± SD. *$P < 0.05$, **$P < 0.01$ *vs* ICR mice. +/+, C57BL/6J-Ham-+/+; A^y, C57BL/6J-Ham-A^y/+.

Table 1. Development of colorectal aberrant crypt foci (ACF) in six strains of mice treated with azoxymethane

Strain of mice	No. of mice with ACF	No. of ACF/colorectum				
		Proximal	Middle	Distal	Rectum	Total
KK-A^y	13/13	0.3 ± 0.5	16.7 ± 9.0**	36.0 ± 24.0**	13.7 ± 7.1*	66.7 ± 35.4**
KK	13/13	0.8 ± 1.0*	14.2 ± 6.2**	31.4 ± 8.2**	14.6 ± 7.3**	61.0 ± 17.4**
A^y	12/12	0.0 ± 0.0	1.9 ± 2.1	15.7 ± 6.7*	6.1 ± 2.3	23.7 ± 8.6
+/+	12/12	0.0 ± 0.0	3.4 ± 1.9	12.3 ± 4.6	6.6 ± 4.2	22.3 ± 6.9
C57BL/6J	13/13	0.1 ± 0.3	1.6 ± 2.3	9.3 ± 3.0	7.0 ± 2.2	18.0 ± 5.4
ICR	13/13	0.1 ± 0.3	7.7 ± 4.4*	27.2 ± 12.0**	6.9 ± 4.8	41.9 ± 18.7**

Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$ versus C57BL/6J-Ham-+/+ (+/+). A^y, C57BL/6J-Ham-A^y/+; KK, KK/TaJcl; KK-A^y, KK-A^y/TaJcl.

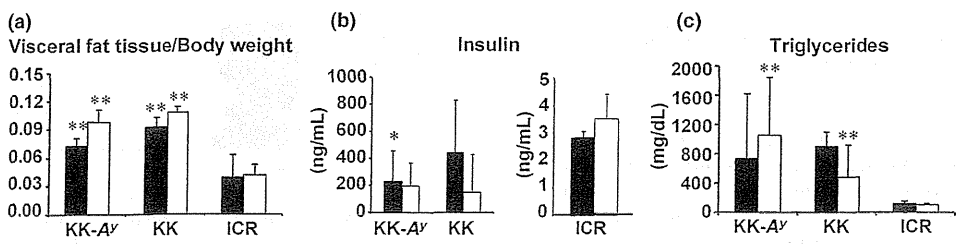

Fig. 3. Parameters for diabetes in KK-A^y/TaJcl (KK-A^y), KK/TaJcl (KK), and ICR mice treated with or without azoxymethane (AOM). The data of the group treated with AOM six times are shown in an open box, and without AOM are shown in a closed box. Parameters for diabetes, such as visceral fat weight/body weight (a), serum insulin levels (b), and serum triglyceride levels (c) are shown in the indicated mice strain. Data are mean ± SD. *$P < 0.05$, ** $P < 0.01$ *vs* ICR mice.

and C57BL/6J mice at 6 weeks of age (18–16 g), but markedly higher than those mice at the end of the experiment. In the ACF experiment with 4-times AOM treatment (ACF experiment), the final mean body weights at 17 weeks of age of KK-A^y, KK, ICR, A^y, +/+, and C57BL/6J mice with AOM treatment were 48.3 ± 4.7 g (mean ± SD), 44.8 ± 2.4, 40.6 ± 5.9, 30.8 ± 4.5, 20.4 ± 1.4, and 22.1 ± 1.3 g, respectively, and mean daily food intakes were 4.2 ± 1.1, 3.8 ± 0.3, 3.8 ± 0.3, 3.3 ± 0.6, 2.9 ± 0.1 and 2.9 ± 0.1 g/mouse/day, respectively. Thus, KK-A^y and A^y mice ate more than the respective control mouse strains. In the colorectal cancer experiment with 6-times AOM treatment (colorectal cancer experiment), the final mean body weights at 25 weeks of age of KK-A^y and KK mice with AOM treatment were significantly higher than those of ICR mice, being 54.2 ± 4.8, 46.9 ± 4.8, and 41.1 ± 5.9 g, respectively; furthermore, KK-A^y mice were significantly heavier than KK mice. Of note, weights of visceral fat in KK-A^y and KK mice were also significantly higher than those of ICR mice (Fig. 1b).

Diabetes status observed in KK-A^y and KK mice. Serum concentrations of glucose, insulin, leptin, and triglycerides at age 17 weeks were measured to evaluate characteristics of glucose metabolism between the strains (Fig. 2). Among the strains, KK-A^y and KK mice showed high serum glucose, insulin, and triglyceride levels, a feature of diabetes, in non-AOM-treated mice. Serum insulin and triglyceride levels in non-diabetic mice (A^y, +/+, C57BL/6J mice, and ICR) were all less than 15 ng/dL and 250 mg/dL, respectively. However, serum insulin levels in KK-A^y and KK mice were 478.3 ± 201.5 (mean ± SD) ($P < 0.01$ *vs* A^y) and 299.4 ± 201.6 ng/dL ($P < 0.01$ *vs* A^y), respectively. Serum triglyceride levels in KK-A^y and KK mice were 1158 ± 748 (mean ± SD) ($P < 0.01$ *vs* A^y) and 910 ± 191.0 mg/dL ($P < 0.01$ *vs* A^y), respectively. Serum leptin levels in A^y mice were also elevated compared to those of +/+ mice, but lower than those of KK-A^y and KK mice. Similar results were obtained in the mice treated with AOM.

Increased number of colorectal aberrant crypt foci in KK-A^y and KK mice. All KK-A^y, KK, A^y, +/+, C57BL/6J, and ICR mice developed ACF in the colon and rectum at 17 weeks with AOM treatment (Table 1). In spite of the treatment of all mice with AOM at the same dosage (150 μg/mouse [≈10 mg/kg], weekly for 4 weeks), induction of colorectal ACF was much greater in KK-A^y and KK mice compared to other non-diabetic mice, that is, A^y, +/+, C57BL/6J, and ICR mice. The numbers of total ACF per mouse in KK-A^y and KK mice were 66.7 ± 35.4 and 61.0 ± 17.4 (mean ± SD), which were almost three times higher than those in +/+ mice. Significantly higher numbers of colorectal ACF in KK-A^y and KK mice were observed in all portions of the colorectum compared with +/+ mice, but they were most abundant in the distal portion. The ICR mice were also susceptible to induction of ACF (41.9 ± 18.7/mouse), but the value was less than KK-A^y and KK mice. Saline-treated mice did not develop colorectal ACF.

Increased incidence, number, and size of colorectal tumors in KK-A^y mice. Serum concentrations of glucose, insulin, leptin, and triglycerides at the age of 25 weeks were similar to those at the age of 17 weeks. Serum insulin and triglyceride levels in KK-A^y, KK, and ICR mice at 25 weeks of age are shown in Figure 3.

As shown in Table 2, the incidence of colorectal tumors developed in the KK-A^y and KK mice by AOM treatment was 87% and 73%, respectively, the value in KK-A^y mice tending to be higher. No tumors were observed in ICR mice by 25 weeks of age. Incidences of colorectal tumors in A^y and +/+ mice were both 0 out of 3 at 30 weeks of age (0%), and 0 out of 15 (0%) and 3 out of 14 (20%) at 55 weeks of age, respectively, showing that A^y mice with a C57BL/6J background were not susceptible to colon carcinogenesis. Most colorectal tumors developed in KK-A^y and KK mice were distributed in the middle to distal portion (Fig. 4a–d). Most large tumors developed in KK-A^y mice were red-colored (Fig. 4a,b), and red blood cells were observed to be abundant in those tumors (Fig. 4f). Histopathological examination revealed most AOM-induced colorectal tumors to be adenocarcinomas (Fig. 4e–h). Table 2 also summarizes data on multiplicity (number of tumors/mouse). The average number of tumors in KK-A^y mice was more than twice that in KK mice ($P < 0.05$). The predominant histological types of carcinomas in KK-A^y mice were well-differentiated.

The number of large tumors (diameter ≥ 5 mm) was significantly higher in KK-A^y mice than in KK mice, and average tumor volume per mouse in KK-A^y mice was more than threefold that in KK mice (Fig. 5), suggesting tumor promotion due to *agouti* gene overexpression in KK-A^y mice.

Activation of macrophages involved in cancer promotion is suggested to be the difference between KK-A^y and KK mice. To evaluate the activation of β-catenin signaling in AOM-induced colorectal cancer in KK-A^y and KK mice, nuclear localization of β-catenin (active form of β-catenin) was examined by immunohistochemistry (Fig. 6). Nuclear localization of β-catenin was not observed in the tumor surrounding normal parts of colorectal mucosa. β-catenin was clearly activated only in cancerous parts of the colorectum.

Translocation of β-catenin to the nucleus, as shown in Figure 6(a,b), suggested transcriptional activation of β-catenin-responsive genes. Thus, we carried out semiquantitative RT-PCR to confirm the activation of β-catenin signaling pathways. Cyclooxygenase-2, cyclin D1, and Pai-1 mRNA, the β-catenin target genes, in the non-cancerous part and cancerous part of

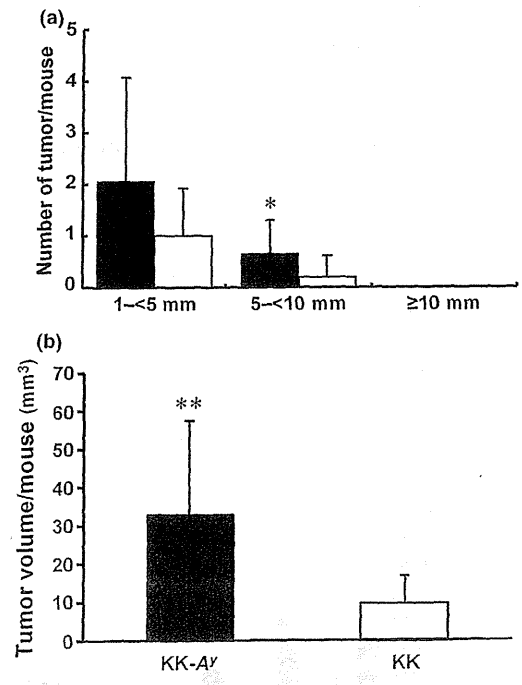

Fig. 5. Tumor size difference observed in azoxymethane-treated KK-A^y/TaJcl (KK-A^y) and KK/TaJcl (KK) mice at the age of 25 weeks. (a) Azoxymethane-developed tumors were divided into three groups by longitudinal diameter, 1–<5 mm, 5–<10 mm, and ≥ 10 mm. (b) *Tumor volume* was calculated using the formula, V = length × diameter (short) × diameter (long). ■, KK-A^y mice; □, KK mice. Data are mean ± SD. *$P < 0.05$, **$P < 0.01$ *vs* tumor volume of KK mice.

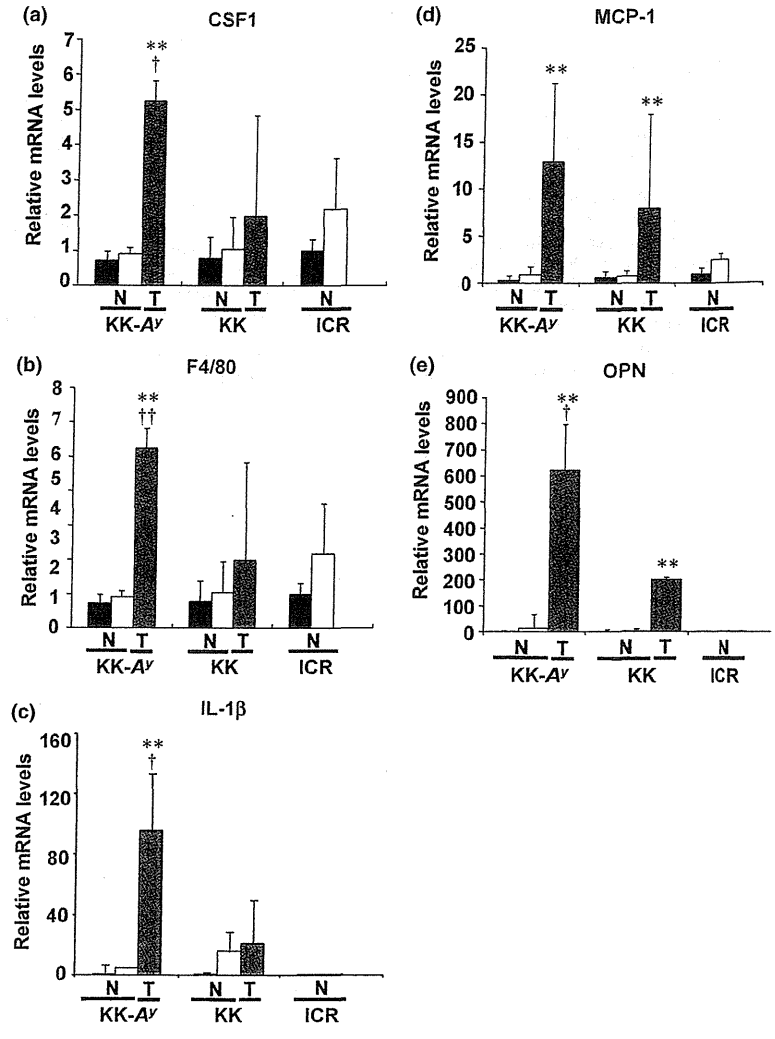

Fig. 7. Relative expression levels of mRNA regarding macrophage activation. Messenger RNA levels of colony-stimulating factor 1 (CSF1) (a), F4/80 (b), interleukin-1β (IL-1β) (c), monocyte chemotactic protein 1 (MCP1) (d), and osteopontin (OPN) (e) in non-cancerous (N) and cancerous (T) parts of colorectum in KK-A^y/TaJcl (KK-A^y), KK/TaJcl (KK), and ICR mice aged 25 weeks were examined by RT-PCR analysis. Data from the mucosa from the saline-treated group were set as 1. β-Actin mRNA level was used to normalize the data. ■, Mucosa from saline-treated group (n = 3); □, mucosa from azoxymethane-treated group (n = 2–4); ▨, cancerous part (n = 5–6). Data are mean ± SD. $^{**}P < 0.01$ *vs* tumor tissue from KK/TaJcl (KK) mice. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ *vs* mucosa from saline-treated group.

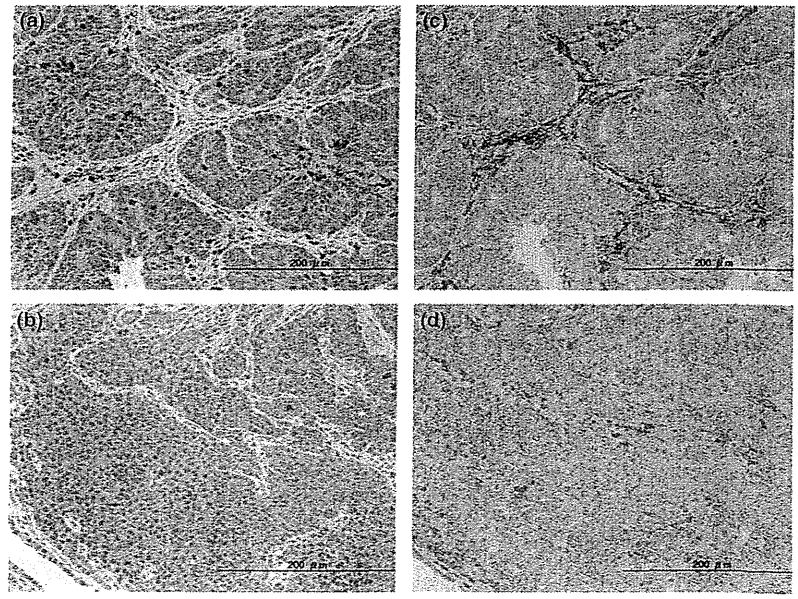

Fig. 8. Accumulation of macrophages in the colon tumor tissue of KK-A^y/TaJcl (KK-A^y) mice. Representative examples of colorectal cancer tissue immunostained with anti-colony-stimulating factor 1 antibodies (a,b) and anti-F4/80 antibodies (c,d) of KK-A^y (a,c) and KK/TaJcl (KK) (b,d) mice are shown. Bar = 200 μm.

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Review Articles

Prevention and Intervention Trials for Colorectal Cancer

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Prevention and Intervention Trials for Colorectal Cancer

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There have been a number of candidates for chemopreventive agents from synthetic drugs and natural compounds suggested to prevent colorectal cancer. However, they have shown modest efficacy in humans. The reason for this could be partly explained by the use of inappropriate models *in vitro* and *in vivo,* and the limitation of chemoprevention trials. In Japan, there are no cancer chemopreventive medicines, and few cancer chemoprevention trials to date. In contrast, an increase in the prevalence of colorectal cancer in Japan has forced us to develop more efficient chemopreventive strategies. It is now a good time to review in detail the current status and future prospects for chemoprevention of colorectal cancer with respect to the future development of chemopreventive medicines, particularly using synthetic drugs and natural compounds in Asian populations. The role and mode of action of available synthetic drugs, mainly aspirin and metformin, are reviewed. In addition, the possible impact of natural compounds with anti-inflammatory/immunosuppressive properties, such as ω3 polyunsaturated fatty acid and lactoferrin, are also reviewed.

Key words: chemoprevention – aspirin – metformin – ω6-PUFAs – lactoferrin

INTRODUCTION

The prevalence of colorectal cancer (CRC) is increasing in Asia, including Japan, believed to be caused by changing dietary habits and lifestyle, interacting with genetic characteristics. Many Asian countries have experienced a 2- to 4-fold increase in CRC incidence over the past few decades (1). Fortunately, a natural history of sporadic CRC, evolution from normal mucosa to developing overt cancer, spans on average 10–20 years, thereby allowing us an opportunity for effective prevention and intervention. CRC can be prevented by lifestyle modification, i.e. taking regular physical activity, abstaining from smoking and taking healthy nutrition. Moreover, there are population screening methods for the early detection of CRC and adenomatous polyps, precursor lesions for CRC, such as using fecal occult blood testing and endoscopy. However, the efficacy of such screening and surveillance strategies for patient uptake is often suboptimal, limiting real effectiveness. Thus, there is a clear imperative to consider alternative preventative strategies, such as using cancer chemopreventive agents.

In contrast to 'chemotherapy', the term 'chemoprevention' was first introduced by Sporn (2). Chemoprevention is now defined as the use of specific agents, including natural and chemical compounds, to suppress, delay or reverse carcinogenesis, and thereby to prevent the development of cancers (3). As the user of cancer chemopreventive agents is not a cancer patient, the ideal cancer chemopreventive agent needs to meet several criteria: (i) it should have a convenient dosing schedule; (ii) it should be easily administered; (iii) it should be low cost; and most importantly; (iv) it should have very low side effects.

Subjects adopted for cancer chemopreventive trials are general populations or those who are in cancer high-risk

groups. Generally speaking, it is considered that patients in cancer high-risk groups, that is well-defined and representative for common CRC, are suitable subjects. Two conditions fall under this consideration, i.e. familial adenomatous polyposis (FAP) and Lynch syndrome. FAP is a rare autosomal dominant inherited disorder due to *APC* gene mutation, characterized by the occurrence of many polyps in the colorectum and other parts of the intestine. It has been reported that half of the patient population develops adenocarcinoma from intestinal polyps by the age of 40 years (4). Lynch syndrome is also known as a hereditary non-polyposis colon cancer, carrying a breakdown in DNA mismatch repair gene. Of note, there are other polyposis syndromes, but they are very rare and lack clear relevance to the general population, and hence not suitable as trial subjects. For instance, these are cases of juvenile polyposis, in which the responsible gene is *SMAD4*, Peutz–Jeghers syndrome, in which it is *STK11*, and Cowden syndrome, in which it is *PTEN*.

Based on reports of chemopreventive activity in the literature and/or efficacy data from *in vitro* models, animal models and human trials, the most promising drugs are aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). The effectiveness of these may be attributed to their potent inhibition of cyclooxygenase (COX) enzymes. In this review, important aspects of the current status and future prospects for chemoprevention of CRC, particularly using synthetic drugs (aspirin and metformin) and natural compounds (ω3 polyunsaturated fatty acid and lactoferrin), involving data of Asian populations, are summarized. Meanwhile, recent clinical trials have been requested to be registered in public trial registries (www.clinicaltrials.gov, www.actr.org.au, www.ISRCTN.org, www.umin.ac.jp/ctr/index/htm or www.trialregister.nl) so that anyone can obtain updated trial records in the database after the publication of this review.

CHEMICAL COMPOUNDS

Aspirin

A large number of epidemiological and experimental studies have indicated that NSAIDs reduce the risk of CRC. As COX-2 expression and prostaglandin (PG) E_2 synthesis is elevated in CRC, PGE_2 is more likely to enhance colorectal carcinogenesis than other prostanoids. The COXs/PGH synthases have two enzymes, COX-1 and COX-2, which catalyze both oxidative and reductive reactions in the PG synthesis pathway. The constitutive enzyme COX-1 is detectable, but has low expression in normal human colorectal tissue, whereas for the inducible enzyme COX-2, its expression is elevated under conditions of inflammation and cancer.

Aspirin is a conventional NSAID, and irreversibly inhibits COX-1 and COX-2, through selective acetylation of a specific serine residue of Ser^{529} and Ser^{516}, respectively (5). Low-dose aspirin (70–100 mg/day) is widely used for cardiovascular disease prevention. Aspirin has a short half-life (~20 min) and it preferentially inhibits platelet COX-1 in the presystemic circulation when administered at low doses once a day. Moreover, aspirin is a medicine that has been in use for a long time and its adverse events are well-defined; it meets all the criteria of chemopreventive agents, and thus has become the most widely studied pharmacological agent for the prevention of CRC.

Observational Studies

Kune et al. (6) first reported in humans that there is an inverse association between use of aspirin and the risk of CRC, in a study conducted in Australia. They investigated the relationship between risk of CRC and several chronic illnesses in 715 CRC cases and 727 age/sex-matched controls, and it was found that those who had used aspirin-containing medications in the past were less likely to develop CRC (relative risk, RR = 0.53, 95% confidence interval, CI 0.40–0.71). Moreover, a prospective cohort study (7) was conducted in the USA with 47 900 middle-aged male health professionals, who responded to a mailed questionnaire in 1986. The questionnaires aimed to assess the use of aspirin and other variables, including the occurrence of cancer in 1986, 1988 and 1990; 251 new patients were diagnosed with CRC during the study period. Regular users of aspirin (≥2/week) in 1986 had a lower risk of total CRC (RR = 0.68, CI 0.52–0.92). A meta-analysis of the case–control studies, on available data on the association between aspirin use and CRC by 2007 that included 20 815 cases of CRC, revealed that there was significantly lower use of aspirin or NSAIDs in cases than in control studies (8). These results support the contention that regular use of aspirin decreases the risk of CRC.

Randomized Controlled Trials

Observational studies can powerfully identify causal associations, but much has been learned from intervention trials. Reports of the randomized controlled trials of aspirin, such as APACC (9), AFPPS (10), CALGB (11) and ukCAP (12), revealed that aspirin (75–325 mg/day for 3 years) reduces the risk of any recurrent colorectal adenoma by 17% and advanced adenoma by 28% (13) (Table 1). Moreover, meta-analysis of aspirin on the long-term risk of death due to CRC in randomized trials of aspirin vs control revealed that the use of aspirin for around 5 years reduces the incidence of and mortality due to CRC by 30–40% after 20 years of follow-up (14). In the case of trials in cancer high-risk patients, such as FAP (CAPP1 or J-FAPP study) and Lynch syndrome (CAPP2 study) patients, they also present evidence of the effectiveness of aspirin as shown below (Table 1).

CAPP1

The CAPP1 was a double-blind, randomized trial in FAP patients with four arms: aspirin for 600 mg/day plus matched

Table 1. Selected aspirin RCT performed for CRC prevention in the past

Study name (Drug)	Length of treatment	Subject (no. of enrollment; Mean ± SD age)	Primary objects/Results	Trial site (Ref.)
APACC (Aspirin: 160 or 300 mg/day)	4 Years	Recent history of sporadic colorectal adenomas ($n = 272$; 160 mg/day aspirin group, 59 ± 9)	Reduction in adenoma recurrence/ RR = 0.95 (95% CI: 0.75–1.21)	France (9)
AFPPS (Aspirin: 81 or 325 mg/day)	3 Years	Recent history of sporadic colorectal adenomas ($n = 1121$; 81 mg/day aspirin group, 58 ± 9)	Reduction in adenoma recurrence/ RR = 0.88 (95% CI: 0.77–1.02)	USA and Canada (10)
ukCAP (Aspirin: 300 mg/ day ± folic acid: 0.5 mg/day)	3 Years	Recent history of sporadic colorectal adenomas ($n = 939$; aspirin group, 58 ± 10)	Reduction in adenoma recurrence/ RR = 0.79 (95% CI: 0.63–0.99)	UK and Denmark (12)
CAPP1 (Aspirin: 600 mg/ day ± resistant starch: 30 g/day)	1–12 Years	FAP ($n = 227$; 300 mg/day aspirin group, 18 ± 8)	Prevention of disease progression/the size of the largest polyp was reduced	UK (15)
CAPP2 (Aspirin: 600 mg/day)	1–4 Years	Lynch syndrome gene carriers ($n = 861$)	Intention-to-treat analysis of time to first CRC/HR = 0.63 (95% CI: 0.35–1.13)	UK (16)
J-FAPP2 (Aspirin: 100 mg/day)	6–10 Months	FAP ($n = 17$; aspirin group, 40 ± 13)	Number of subjects with reduced polyps/ Response ratio = 2.33 (95% CI: 0.72–7.55)	Japan (17)

Response ratio = aspirin response rate (no. of subjects with reduced polyps/total)/placebo response rate (no. of subjects with reduced polyps/total).
AFPPS, Aspirin/Folate Polyp Prevention Study; APACC, Association pour la Prevention par l'Aspirine du Cancer Colorectal; CALGB, Cancer and Leukemia Group B; ukCAP, United Kingdom Colorectal Adenoma Prevention; CRC, colorectal cancer; FAP, familial adenomatous polyposis; HR, hazard ratio; RCT, randomized control trials; Ref., reference; RR, relative risk.

placebo ($n = 31$, 10–21 years old), resistant starch (RS) for 30 g/day plus matched placebo ($n = 30$), aspirin plus RS ($n = 31$) and placebo plus placebo ($n = 41$) (15). No significant trend of a reduced number of polyps in the colorectum was observed in the aspirin group compared with the non-aspirin group at the end of intervention. However, the size of the largest polyp was reduced in the overall aspirin group compared with the non-aspirin group. Furthermore, after more than 1 year of intervention, the diameter of the largest polyp recorded in the aspirin group (3 mm) was only half of that in the placebo group (6 mm; $P = 0.02$).

CAPP2

The CAPP2 was a 2X2 factorial randomized trial in 861 Lynch syndrome gene carriers. The subjects were divided into intervention groups of an aspirin enteric-coated tablet (600 mg/day for a minimum 2 years) and a matched placebo (427 participants) for between 1 and 4 years, with a pre-planned design for a 10-year follow-up (16). This trial is the first double-blind randomized trial of aspirin chemoprevention with cancer as a primary endpoint. After a mean observation period of 29 months of intervention, there was no evidence showing that aspirin influenced development of colorectal neoplasia. After a mean of 55.7 months, the hazard ratio for new CRC development for aspirin was 0.63 (CI 0.35–1.13 $P = 0.02$). Adverse events in the aspirin and placebo group were almost the same. This finding suggests that a follow-up for several years after a randomized trial is necessary for evaluating the effects of aspirin, and this may be true with other CRC chemopreventive agents.

J-FAPP2

In Japan, a double-blind randomized trial was performed, using a low-dose aspirin enteric-coated tablet (100 mg/day for 6–10 months) in 34 subjects with FAP (17 each in the aspirin and placebo groups) (17). This trial is the first double-blind randomized trial of aspirin in Japanese subjects. The J-FAPP2 trial resulted in a tendency of reduction in the size of colorectal polyps in FAP with aspirin administration, when compared with placebo administration. Furthermore, subgroup analysis indicated that the number of subjects with a small polyp with a mean baseline polyp diameter ≤ 2 mm was significantly reduced in the aspirin group. Adverse effects of aspirin, such as astomotic ulcer, aphtha in the colorectum and progression of anemia, occurred in three subjects. All of these subjects were non-smoking women, with an age lower than 40 years with high β-catenin staining of their polyps. Moreover, none of the subjects developed CRC.

Insights into the Mechanism of Aspirin Chemoprevention

In the CAPP2 study, aspirin reduced development of CRC long after cessation of exposure to aspirin. It is assumed that the primary action of aspirin on COX in colonic tumors is not likely to be the important mechanism, but that other mechanisms could exist. Several pieces of evidence have shown that aspirin can inhibit proliferation and induce apoptosis of colon cancer cells independently from its inhibitory effects on prostanoid biosynthesis (18). Reported COX-independent molecular mechanisms are: (i) the interruption of nuclear factor kappa B (NF-κB) (19, 20); (ii) the

interruption of extracellular signal-regulated kinases (21); (iii) the induction of caspase 8 and 9 (22,23); (iv) the inhibition of β-catenin signaling (24) and (v) the activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) (25) (Table 2).

Ongoing Trials Using Aspirin

CAPP3

To determine ideal doses of aspirin for all Lynch syndrome gene carriers, a CAPP3 study is recruiting 3000 gene carriers to test the relative benefits of 100, 300 or 600 mg/day.

J-CAPP Study

The aim of the study was to present the evidence that aspirin is useful as a chemopreventive agent in general Asian populations. The J-CAPP study aimed to investigate the effects of low-dose aspirin for 2 years in Japanese in a double-blind, randomized, placebo-controlled clinical study in patients whose colorectal tumors (one or more) were all excised by colon endoscopy. The research protocol of the J-CAPP study is described elsewhere (26).

Trials Using Other NSAIDs and Selective COX-2 Inhibitors

Related to PG biosynthesis, many human studies using various NSAIDs have been conducted. There are small, randomized clinical trials using sulindac as a chemopreventive agent. Forty-five FAP patients were enrolled in these studies, and sulindac showed a statistically significant decrease in the number of colorectal tumors (27,28). On the other hand, 77 FAP patients were enrolled in a double-blind placebo-controlled study using a selective COX-2 inhibitor, celecoxib, at a dose of 100 mg twice a day, and 400 mg twice a day for 6 months (29). The dose of 100 mg resulted in a 12% decrease in the number of colorectal tumors. Celecoxib at 400 mg reduced the number of colorectal tumors by 28% from the baseline, evaluated by endoscopy at the beginning of the trial.

Table 2. Summary of cyclooxygenase-independent targets of aspirin

Targets	Target-reactive molecules	Target-related bioactivity
NF-κB	COX-2, iNOS, IL-6, TNFα, etc.	Inflammation, cell survival, etc.
ERK	Elk1, AP-1	Cell growth, cell differentiation
Caspase-8 and-9	Caspase-3, -6 and -7	Apoptosis
β-catenin	c-Myc, Cyclin D1, etc.	Cell growth, cell survival, cell differentiation, etc.
AMPK	GLUT4, PGC-1α, PPAR, etc.	Cellular energy homeostasis, modulation of insulin secretion, etc.

AMPK, 5′-adenosine monophosphate-activated protein kinase; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinases; GLUT4, glucose transporter 4; iNOS, induced nitric oxide synthase; IL-6, interleukin-6; NF-κB, nuclear factor kappa B; PGC-1α, PPAR-γ-coactivator 1α; PPAR, peroxisome proliferater-activated receptor; TNF-α, tumor necrosis factor-α.

However, the promising use of coxibs in chemoprevention was halted abruptly due to the enhancement of cardiovascular risks. This could be explained partly by the inhibition of COX-2-dependent PGI_2 production, which plays an important role in vasoprotective and anti-thrombotic pathways. In addition, other major problems of some NSAIDs and COX-2 inhibitors are that the suppressive effects on tumorigenesis are transient and disappear soon after drug withdrawal.

To use such NSAIDs and selective COX-2 inhibitors for a long time, we need to give careful consideration by comparing the benefits of use and the risks of adverse effects, such as gastrointestinal bleeding and cardiovascular events. To prevent such adverse effects, several approaches can be considered: (i) reduction of doses, (ii) co-prescription of a proton-pump inhibitor and (iii) treatment to eradicate *Helicobacter pylori* infection possibly to overcome bleeding complications. Moreover, mPGES-1 inhibitors cause a selective inhibition of PGE_2 by affecting a PGE_2 synthase downstream of COX-2 and, thus, they may not affect the production of PGI_2 (30). Other ongoing studies are additionally listed in Table 3.

Metformin

As denoted above, NSAIDs and selective COX-2 inhibitors are the first candidates for CRC chemopreventive agents. In addition to those powerful and well-noted drugs, the anti-diabetic drug metformin has been thrown into the limelight recently (31).

Metformin (dimethylbiguanide) was first discovered as a derivative of mono-substituted guanidine, which showed less lipophilic interaction and considerably safer disposition than the original European anti-diabetic agent Galegine, in 1922. Now, almost a century has passed from then, and metformin has become the most widely prescribed anti-hyperglycemic agent (32,33). Metformin has a powerful metabolic effect, especially for lowering blood triglyceride levels in diabetic patients, targeting phosphorylation/activation of AMPK. AMPK is one of the possible candidates for a carcinogenesis-associated molecule, as written in the aspirin section. A tumor-suppressor gene product, LKB1 kinase, has been proved to be the upstream regulator of AMPK, and therefore restraining AMPK signal pathway activation would affect carcinogenesis. Based on this logic, metformin was presumed to have anti-tumor activity (34).

Observational Studies

To test this hypothesis, many observational case–control studies have been performed. The first large cohort study was Diabetes Audit and Research in Tayside Scotland/

Table 3. Selected ongoing RCT whose primary purpose is prevention of CRC

Drug	Length of treatment	Subject (estimated enrollment; ages)	Phase	Primary objects	Protocol ID (trial site)
Erlotinib (75 mg/day) + sulindac (150 mg/day)	6 months	FAP ($n = 100$; 18–69)	II	Regression of adenoma	NCT01187901 (USA)
Celecoxib (16 mg/kg/day)	5 years	FAP ($n = 200$; 10–17)	III	Time reduction from randomization to treatment failure	NCT00585312 (USA, UK, Belgium and others)
Aspirin + DFMO	Treatment repeated every 28 days for 1 year	High risk of CRC ($n = 104$; 40–120)	II	Reduction of adenoma recurrence rate	NCT00983580 (USA)
DFMO (500 mg/day) + sulindac (150 mg/day)	2 years	FAP ($n = 150$; >19)	III	Delay time to the first occurrence of any FAP-related event.	NCT01483144 (USA)
EPA (465 mg/day) + DHA (375 mg/day)	6 months	History of >1 polyps + known genotype for rs174535 in *FADS1* ($n = 150$; 49–79)	II	Decrease in rectal epithelial cell proliferation indexes and markers of rectal crypt apoptosis	NCT01661764 (USA)

Information obtained December 2012 from the websites (www.clinicaltrials.gov). DFMO, difluoromethylornithine/eflornithine hydrochloride; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Medicines Monitoring Unit conducted in Scotland (35). This study demonstrated that diabetic patients taking metformin showed reduced all-cancer risk, compared with those taking other diabetes therapies (adjusted odds ratio, OR = 0.86, 95% CI 0.73–1.02). This study triggered scientific interest in this field, and more than dozens of studies have confirmed its effectiveness in cancer chemoprevention.

In the field of gastroenterological cancer, multiple studies indicated the anti-neoplastic capability of metformin. For example, Lee et al. (36) performed the first prospective cohort study in Asia, recruiting 800 000 Taiwanese diabetic patients treated with or without metformin. This study revealed that metformin was able to decrease incidence rates of CRC and hepatocellular carcinoma (HCC) close to the levels of non-diabetic individuals. Of note, there was a significant gender difference with metformin interaction, i.e. in CRC it favored women (HR = 0.36, 95% CI 0.13–0.98), and in HCC it favored men (HR = 0.06, 95% CI 0.02–0.16).

Some meta-analyses also certified that metformin could reduce cancer risk. Zhang et al. (37) reported that metformin treatment was associated with a significantly lower risk of colorectal neoplasm, analyzing five studies, with 108 161 patients (RR = 0.63, 95% CI 0.50–0.79, $P < 0.001$). In another meta-analysis, it was shown that metformin treatment was able to reduce risks of cancer mortality and incidence (38). In the observational cohort studies, the pooled RR for all-cancer mortality among metformin users was 0.62 (95% CI 0.46–0.82). Especially, the incidence of cancer risks was also significantly decreased for CRC, HCC and lung cancer. In contrast, prostate, breast, pancreatic and gastric cancer were not significant. Of note, some bias could be involved in the data of the meta-analysis. Thus, it is still worthwhile to describe that metoformin use was associated with a reduced risk of pancreatic cancer in a hospital-based case–control study (973 case vs 863 controls) (39). Almost all of these observational studies indicated that metformin treatment is associated with a reduced cancer risk and/or improved prognosis; however, these data are mostly from retrospective and non-randomized studies.

Randomized Controlled Trials

A pilot study was performed in Japan to evaluate the chemopreventive effect of metformin on rectal aberrant crypt foci (ACF), an endoscopic surrogate marker of CRC. Non-diabetic patients with ACF ($n = 26$) were prospectively randomized into a metformin group (250 mg/day, $n = 12$) or a non-treatment group (control, $n = 14$) for 1 month in a blinded manner. The metformin group had a significant decrease in the mean number of ACF, whereas the mean ACF number did not change significantly in the non-treatment group (40). Furthermore, Japanese researchers recently reported the trial protocol of an ongoing double-blind, randomized controlled trial of metformin against colorectal polyp formation (41).

If metformin were clearly proved to be effective for the prevention of CRC, and any other cancers, the impact would be extremely large, in the context of drug repositioning. Needless to say, more information is needed for making the design of clinical trials, i.e. evaluation of appropriate doses for metformin against CRC. It would be very effective to use metformin at conventional doses as an anti-diabetic agent, because attenuation of high levels of insulin may contribute to anti-neoplastic activity. In participants under 18 years old, no dosing or adverse event data are currently

available with regard to the use of metformin, which results in the exclusion of children in trials, but it will be eligible for future pediatric trials. Other desired information is the effects of metformin on the developing human fetus at recommended therapeutic doses. Therefore, a serum pregnancy test must be performed and be negative in all women of childbearing potential prior to starting the trials. The verification of these points may also explore more aggressive dosing of metformin.

NATURAL PRODUCTS

ω3 Polyunsaturated Fatty Acids

High-fat diets are generally associated with a high risk of colon cancer (42). However, there are several types of fat and the effects on carcinogenesis are different. Animal fat, rich in saturated fatty acids and cholesterol, and corn and safflower oils, rich in ω6 polyunsaturated fatty acids (PUFAs) such as linoleic acid, have been shown to promote colon carcinogenesis in animal studies. On the other hand, olive oil (rich in ω9 monounsaturated fatty acids) and fish oil (rich in ω3 PUFA) have been demonstrated to have no promotive effects on carcinogenesis; rather, fish oil suppresses colon carcinogenesis in animal models (43,44).

Docosahexaenoic acid (DHA, C22:6, ω3) and eicosapentaenoic acid (EPA, C20:4, ω3) are major components of fish oil. DHA and EPA have lowering effects on serum lipids (45). Thus, EPA has been approved as a therapeutic agent for the treatment of dyslipidemia, and suppressive effects have been demonstrated in the Japan EPA Lipid Intervention Study on the incidence of coronary events in hypercholesterolemia with impaired glucose metabolism (46). Besides, DHA has important physiological activities in the brain and retina (45), and thus capsules are sold as health supplements at drug stores.

Observational Studies

Epidemiologically, there is only limited, but suggestive evidence for the beneficial effects of fish intake or ω3 PUFA consumption on the risk of CRC. In a systematic review published in 2006 summarizing prospective cohort studies of estimated consumption of ω3 PUFA and risk of several cancers, nine studies of the risk of CRC from seven different cohorts were identified (47), but only one study, the New York University Women's Health Study, demonstrated a statistically significant reduction in the risk of CRC in the highest ω3 PUFA intake category compared with the lowest (RR = 0.49, 95% CI 0.27–0.89) (48). In addition, the Physicians' Health Study demonstrated that intake of fish and ω3 PUFAs was inversely associated with risk of CRC in men; multivariate RR for highest vs lowest category for fish intake was 0.60 (95% CI 0.40–0.91) and that for ω3 PUFAs was 0.74 (95% CI 0.57–0.95) (49). In a population-based case–control study in Caucasians and African Americans, increased consumption of long-chain ω3 PUFAs was associated with a reduced risk of distal large bowel cancer in Caucasians, but not in African Americans; multivariable odds ratio for the highest vs lowest category in Whites was 0.49 (95% CI 0.34–0.71) (50). Recently, a Japan Public Health Center-based prospective study has demonstrated that intake of ω3 PUFAs was inversely associated with cancer risk in the colon in women (RR for the highest vs lowest category = 0.60, 95% CI 0.31–1.14), and in the proximal colon in men (RR for highest vs lowest category = 0.35, 95% CI 0.14–0.88) (51).

The available observational evidence on the effect of ω3 PUFA exposure on risk of CRC has been summarized in detail in the Second Expert Report of the World Cancer Research Fund and American Institute for Cancer Research in 2007 (52), which has been updated as part of the Continuous Update Project of these organizations in 2011 (53). The results show heterogeneity of the effects of ω3 PUFA on the risk of CRC and remain inconsistent.

Animal Model Studies

There are many pre-clinical studies evaluating preventive effects of fish oil or ω3 PUFAs on colon carcinogenesis using rodent models, and they have recently been reviewed in detail by Cockbain et al. (54). EPA has been shown to decrease tumor incidence and multiplicity in a rat colon carcinogenesis model induced by azoxymethane (AOM) (55), and intestinal tumor number and size in $Apc^{Min/+}$ mice (56–58). DHA has also been demonstrated to decrease numbers of ACF, putative pre-neoplastic lesions and tumors in a rat colon carcinogenesis model induced by 1,2-dimethylhydrazine or AOM (59,60), and intestinal polyp number and size in female Apc^{D716} mice (61).

Clinical Studies

In seven of nine clinical studies of ω3 PUFA treatment on colorectal mucosa biomarkers, a reduction in the cell proliferation index was observed (54). There have been two reports of clinical studies of ω3 PUFA treatment with FAP patients using colorectal polyps as the primary endpoint for the risk of CRC (62,63). A small, open-label study in three patients with FAP, and two patients with multiple (more than 30) colorectal polyps demonstrated no significant change in the number of colorectal polyps by treatment with 2.2 g DHA + 0.6 g EPA daily for 1–2 years (62). A recent phase III randomized, double-blind, placebo-controlled trial of EPA-FFA 2g daily for 6 months in 55 FAP patients undergoing sigmoidoscopic surveillance of a rectal stump after total colectomy (EPA-FFA 28, placebo 27) demonstrated a 22.4% reduction in the number of polyps ($P = 0.012$), and a 29.8% decrease in the sum of polyp diameters ($P = 0.027$) in the EPA-FFA group, while the global polyp burden worsened over 6 months in the placebo group (63) (Table 4). The chemopreventive efficacy of EPA-FFA in FAP patients was similar to that previously observed with

Table 4. Selected RCT with natural products for CRC prevention

Natural products	Length of treatment	Subject (no. of enrollment; age)	Primary objects/Results	Trial site (Ref.)
EPA-FFA (2 g/day)	6 months	FAP ($n = 55$; 18–74)	Reduction in number and size of polyps/Polyp number and size were reduced 22.4% ($P = 0.012$) and 29.8% ($P = 0.027$), respectively.	UK (63)
bLF (1.5 or 3 g/day)	12 months	Patients with colorectal polyps (≤5 mm in diameter) ($n = 104$; 40–75)	Inhibition of the growth of colorectal polyps /3 g bLF inhibited growth of the polyps in patients less than 64 years old ($P = 0.006$).	Japan (84)

bLF, bovine lactoferrin; FFA, free fatty acid.

selective COX-2 inhibitors, and EPA-FFA was safe and well tolerated (63).

Insights into the Mechanism of Chemoprevention by ω3 Polyunsaturated Fatty Acids

There are several putative mechanisms underlying the anti-inflammatory and anti-neoplastic activity of ω3 PUFAs (54,64–68). (i) Inhibition of COX activity: COX-2 overexpressed in colon tumors stimulates cell proliferation and angiogenesis via PGE_2 production (69). Reduction of PGE_2 synthesis via inhibition of COX activity (54,64) is considered to be the main mechanism of the anti-neoplastic activity of ω3 PUFAs. (ii) Activation of PPARs and transrepression of NF-κB: PPARα and γ activation has the ability to inhibit expression of pro-inflammatory genes by inhibiting NF-κB activation. ω3 PUFAs have been implicated as PPAR-α/γ-agonists and inhibit NF-κB binding activity (54,65). (iii) Production of novel anti-inflammatory lipid mediators: ω3 PUFA-derived lipid mediators, resolvins and protectins, bind to G-protein-coupled receptors (GPCRs) and show anti-inflammatory and inflammation resolution activity. EPA and DHA can also act as direct ligands for GPCRs (54,64,65). (iv) Increase in membrane fluidity and alteration of lipid rafts and cell surface receptor function: lipid rafts are involved in modulating intracellular signaling cascades, including EGF receptor, insulin receptor, T cell receptor and B cell receptor. ω3 PUFAs are capable of suppressing CD4 + T cell proliferation and function via altering lipid rafts (66). (v) Increased oxidative stress: PUFAs are highly peroxidizable, and generated reactive oxygen species may induce apoptosis (54,67). (vi) Improvement of dyslipidemia: hyperlipidemia is a putative risk factor of colon cancer (70,71). ω3 PUFAs lower serum lipid levels via activation of PPARα (increase in FA oxidation) and suppression of SREBP-1c expression (decrease in triglyceride synthesis) (68). (vii) Activation of AMPK (72).

Lactoferrin

Lactoferrin is a component of whey/milk serum, which remains after milk has curdled and has been strained, i.e. a by-product of cheese or casein. The whey fraction also contains a large number of ingredients: α- and β-lactoalbumin, immunoglobulin, lactoferrin, etc. In humans, lactoferrin exists at relatively high concentrations in various secretions, i.e. tears, saliva and seminal fluid, with colostrum having particularly high levels (10 mg/ml) (73). We ingest bovine lactoferrin (bLF) as a component of cow's milk. Most ingested bLF is easily digested to lactoferricin (bLFcin) and its related peptides by acid pepsin hydrolysis (bLFH). bLFcin is detected in epithelial cells of the small intestine by immunohistochemical methods (74).

Animal Model Studies

Whey protein concentrate was found to exert a protective effect in a colon cancer models in rats (75), and the administration of whey protein to mice in the post-initiation stage resulted in a decrease in the colon tumor burden and prolongation of survival (76). These protective effects are thought to be due to a boost of the immune cells (77). Besides, α-lactoalbumin has been shown to be a calcium-elevating and apoptosis-inducing agent (78).

Bezault et al. (79) have shown protective effects of lactoferrin on the growth of solid tumors and the development of experimental metastases in mice. Moreover, we previously reported that bLF is a promising chemopreventor of colon carcinogenesis in rats (80,81). In rats administered AOM for initiation of colon carcinogenesis, the incidence of adenocarcinoma in the colorectum was markedly decreased (26%: $P < 0.01$ and 43%: $P < 0.05$ of the control in 2% and 0.2% bLF group, respectively) in rats fed bLF. The multiplicity (number of tumors per animal) was also significantly reduced in the bLF-fed groups. Cell proliferation in the carcinoma lesions, as assessed by 5-bromo-2′-deoxyuridine labeling indices, was significantly decreased in the 2 and 0.2% bLF-fed rats, compared with those in the control group. In addition to bLF, both bLFH and bLFcin also inhibited AOM-initiated colorectal carcinogenesis (82).

Randomized Controlled Trials

In 2002, a randomized, double-blind, placebo-controlled trial was conducted by the National Cancer Center Hospital, Tokyo to determine whether oral intake of bLF would inhibit the growth of adenomatous colorectal polyps in patients

(Table 4). Prior to the course of the 3-year trial, colorectal polyps were evaluated by colonoscopy. Target polyps were less than 5 mm in diameter with a pit pattern III (83). During the initial colonoscopic examination, the location of target polyps was marked, and the size of polyps was measured on the final day of one year of treatment.

Trial participants ingested 0, 1.5 or 3 g of bLF, and the results of the trial were published in 2009 (84). Participants aged 63 years or younger ingesting 3 g bLF had a significant reduction in target polyp size compared with the age-matched placebo subjects, and this group also had a significant increase in their levels of serum lactoferrin (hLF), but in participants 64 years or older, ingestion of bLF did not have a significant effect on the polyp size or serum hLF. Of note, serum bLF was undetectable in all the participants. The study also found that the participants ingesting 3 g bLF showed decreased induction of serum hLF with age.

Overall, participants with higher levels of NK cell activity had smaller polyps, but the effect of bLF ingestion on serum NK cell activity was inconclusive. A significant increase in NK cell activity was seen in the participants in the 1.5 g bLF group, but not in the 3 g bLF group. A larger study is needed to explore this point more conclusively.

No serious adverse effects associated with bLF ingestion occurred during the trial period, verifying the safety of bLF ingestion. Moreover, no malignant lesions were observed during the course of the trial.

bLF and bLFcin inhibit endothelial cell growth together with activation of immune cells that contribute to the anti-carcinogenesis and anti-metastatic activity. (i) bLF and bLFcin inhibit angiogenesis (85). In animal studies, bLF and bLFcin exhibited dose-dependent anti-angiogenesis effects on chick embryo chorioallantoic membrane. Human lactoferrin also exhibited strong anti-angiogenic effects. Moreover, bLF inhibited formation of tube-like structures by bovine pulmonary arterial endothelial cells in 1% FCS DMEM supplemented with VEGF in *in vitro* studies. (ii) During the examination aimed to inhibit tumor development and metastases by B16 melanoma and colon 26 tumor cells by bLF (86), marked increases in the number of cytotoxic T and NK cells in the mucosal layer of the small intestine and in the peripheral blood were found. This is possibly due to enhanced levels of interleukin-18 (87,88). Notably, in colon 26 tumor-cell-bearing SCID mice (origin BALB/c), which are deficient in T and B cells, bLF still showed significant inhibition of lung metastatic colony formation. On the other hand, anti-asialo GM1 antibody treatment results in markedly increased lung metastatic colonies in SCID mice with weakened NK cell activity. Those results suggest that inhibition of metastases by bLF is mediated through NK cells. (iii) In addition to the data in human trials, which show an increase in NK cell activity, we found that induction of serum hLF was associated with lower infiltration of polymorphonuclear leukocytes (PMNs) into target polyps. Moreover, lower infiltration of PMNs into polyp tissue was associated with growth suppression of polyps. Infiltration of PMNs into a polyp has been known to enhance tumor growth (89,90). All of these results suggest that bLF treatment can reduce the risk of colon carcinogenesis and have anti-tumor activity in humans. (iv) Lactoferrin is also reported to increase AMPK phosphorylation (91).

FUTURE ASPECTS

Other selected ongoing randomized control trials whose primary purpose is prevention of CRC are additionally listed in Table 1. Recent advanced technologies allow us to investigate further detailed mechanisms associated with the adenoma–carcinoma sequence and, thus, to obtain improved strategies to identify patients for CRC high-risk groups. Recently, genome-wide association studies identified four single nucleotide polymorphisms, such as *THADA*, *JAZF1*, *KCNJ11* and *TSPAN8*, as susceptibility loci for type II diabetes mellitus that affect the risk of CRC (92). Although the tools for an accurate estimation of cancer risk are increasing, problems still remain, such as lack of biomarkers for early detection and safe and effective chemopreventive agents. Taking a look at CRC management, the challenge of the next decade will be to explore paths for a double approach based on the development of innovative preventive strategies and anticancer therapies.

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Conflict of interest statement

None declared.

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Mini-review

Metabolic syndrome: A novel high-risk state for colorectal cancer

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ABSTRACT

Metabolic syndrome (MS) and related disorders, including cancer, are steadily increasing in most countries of the world. However, mechanisms underlying the link between MS and colon carcinogenesis have yet to be fully elucidated. In this review article we focus on the relationships between various individual associated conditions (obesity, dyslipidemia, diabetes mellitus type 2 and hypertension) and colon cancer development, and demonstrate probable related factors revealed by *in vivo* and *in vitro* studies. Furthermore, molecules suggested to be involved in cancer promotion are addressed, and the potential for cancer prevention by targeting these molecules is discussed.

1. Introduction

Many disorders can be induced by excessive accumulation of visceral adipose tissue, and the combination of related symptoms, so-called metabolic syndrome (MS), is attracting increasing attention as a major health problem since it can lead to conditions such as cardiovascular disease. Recently, MS has also attracted much interest as a risk factor for several cancers, including colon cancer. The World Cancer Research Fund and American Institute for Cancer Research have evaluated causal relationships between accumulation of visceral adipose tissue and cancer, and concluded 'confident evidence' for colorectum and pancreas cancers [1]. In Japan, overweight and obesity, defined as a body mass index (BMI) of 25 or more, are similarly reported to be associated with several cancers, such as colorectum cancer in males, breast cancer in postmenopausal females and liver cancer in those with a history of hepatitis C virus infection [2–4].

In this review article, relationships between the symptoms of MS and colorectal carcinogenesis are focused on in animal models. Commonly used animals for MS models are rodents because of their size. The models are classified into three groups: diet-induced obesity models (C57BL/6J mice and F344 rats), monogenic models (*ob/ob* mice, *db/db* mice, ZDF rats and KK-A^y mice), and polygenic models (TSOD mice and OLETEF rats). A high-fat/-fructose diet, or mice with genetic alterations such as mutation of leptin, leptin receptor and agouti genes are commonly used. Suitable animal models of MS-associated carcinogenesis might be mice with intact leptin and leptin receptors because leptin signaling stimulates cell growth, and may affect carcinogenesis.

2. Metabolic syndrome

MS is common in Western countries, and is currently increasing almost ubiquitously across the globe. In addition to developed countries, MS is increasing in developing countries in adults and particularly in children [5]. Moreover, obesity and overweight are rapidly increasing in both urban and rural areas in the under developed countries of sub-Saharan Africa and South Asia [6].

Various diagnostic criteria for MS have been proposed by many national/international organizations [7–10]. Consensus statements for diagnosis of MS are almost the same, and these are the presence of any three abnormal findings out of five. i.e. (1) waist circumference (males: ⩾90 cm; females: ⩾80 cm), (2) blood triglyceride (TG) levels ⩾150 mg/dL (1.7 mmol/L), (3) blood high-density lipoprotein (HDL) cholesterol levels (males < 40 mg/dL (1 mmol/L); females <50 mg/dL (1.3 mmol/L), (4) blood pressure (systolic blood pressure ⩾130 mmHg and/or diastolic blood pressure ⩾85 mmHg or drug treatment for hypertension), and (5) blood sugar (fasting blood sugar ⩾100 mg/dL (5.6 mmol/L) or drug treatment for diabetes mellitus type 2 (T2DM)) [11]. However, further work is required for the components regarding waist circumference, which rely on population and country-specific definitions [12].

A major pathogenesis of this syndrome could be accumulation of visceral adipose tissue, characterized by increased numbers of macrophage infiltration along with low-grade inflammation [13]. In addition to low-grade inflammation, other factors that may contribute to colorectal cancer development would be dyslipidemia, insulin resistance, subsequent adipocytokine imbalance and activation of the renin-angiotensin system, which are further documented in detail in this paper Fig. 1.

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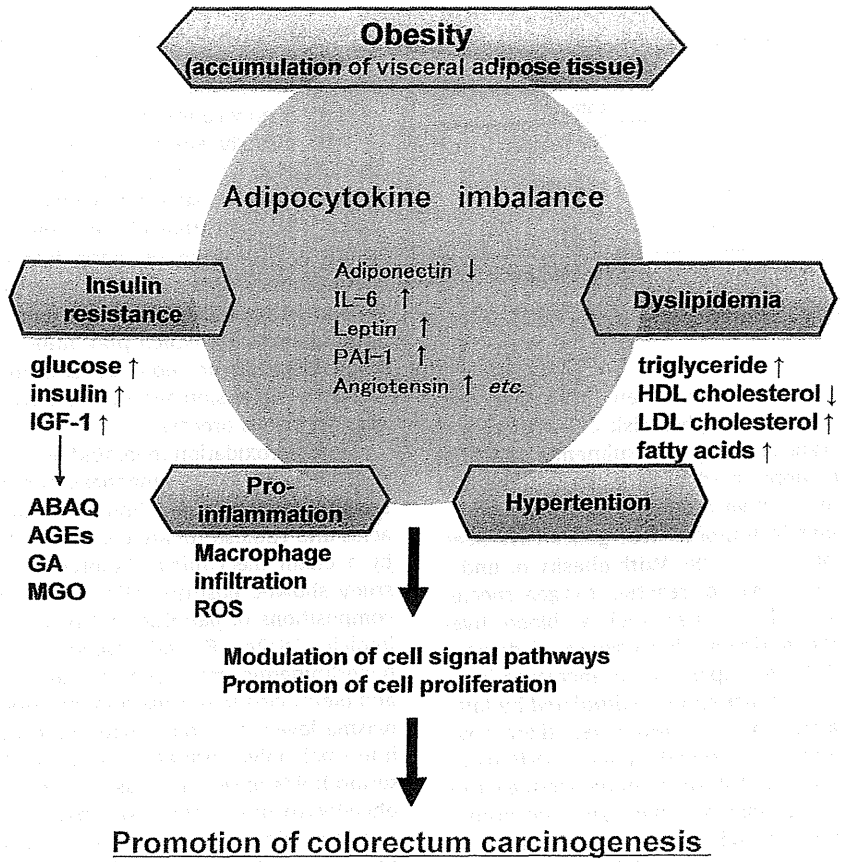

Fig. 1. Assumed relationship between metabolic syndrome and imbalance of adipocytokine production linked to colorectal cancer development. AGEs, advanced glycation end products; GA, glyceraldehydes; HDL, high-density lipoprotein; IGF-1, insulin like growth factor-1; IL-6, interleukine-6; LDL, low-dencity lipoprotein; MGO, methylglyoxal; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species.

3. Dyslipidemia

Hypertriglyceridemia is associated with an elevated risk (HR = 1.71) of colon cancer in Japanese men [14]. In the case of a precursor lesion of colorectal cancer, most epidemiological studies have consistently showed that serum TG levels are associated with their increase [15–18]. Thus, it is considered that serum TG, lipoprotein lipase (LPL), a key enzyme that catalyzes the hydrolysis of TG, could play important roles in carcinogenesis.

In animal models of human familial adenomatous polyposis (FAP), *Apc*^{1309} (C57BL/6J$^{Apc/ApcD1309}$) [19] and *Min* mice [20,21], elevated serum TG has been observed with suppression of mRNA levels for LPL in the liver and small intestine. Although no significant differences were observed between *Apc*^{1309} mice and wild-type mice at 6 weeks of age, the average serum TG value in *Apc*^{1309} mice at 12 weeks was found to be markedly increased almost 10-fold (~600 mg/dL) as compared to that at 6 weeks. A similar increase of TG levels (almost 400 mg/dL) was observed in *Min* mice at 15 weeks compared to the 8 weeks time point.

The anti-T2DM agent, pioglitazone, is a potent peroxisome proliferater-acitivated receptor γ (PPAR γ) ligand with a weak binding affinity for PPARα. PPAR responsive elements exist in the promoter region of the *LPL* gene, and pioglitazone has been confirmed to increase LPL mRNA levels in the liver and intestinal epithelial cells in *Apc*-deficient mice. Serum levels of TG at 12 weeks of the *Apc*^{1309} mice were reduced to 44% and 50% by 100 and 200 ppm of pioglitazone treatment, respectively, with a 33% decrease in the total numbers of polyps (Table 1) [19]. *Min* mice treated with 100–1600 ppm pioglitazone for 14 weeks also showed a decrease of intestinal polyps to 63–9% of the control number [20]. Administration of 100 and 200 ppm bezafibrate, a PPAR α ligand, which also elevates LPL mRNA, to *Apc*^{1309} mice reduced serum levels of TG dose dependently up to 55% ($P < 0.05$), with a reduction in the total numbers of polyps by 13% and 25% ($P < 0.05$), respectively [20]. We further treated *Min* mice with the LPL selective inducer NO-1886, demonstrated to possess no PPAR agonistic activity, unlike bezafibrate or pioglitazone [22,23], and showed 400 and 800 ppm doses to significantly decrease the total number of intestinal polyps to 48% and 42%, respectively, of the untreated control value, in mice (Table 1) [24]. Of note, NO-1886 caused a marked increase in *LPL* mRNA levels in the liver and the small intestine [24]. Based on these results, suppression of serum TG levels by increasing LPL activity is suggested to contribute to a reduction of intestinal polyp formation under *Apc*-deficient conditions, and both TG and LPL could be good molecular targets for colon cancer prevention.

Table 1
Summary of tumor suppressive effects of chemopreventive agents.

Agent	Dose (ppm)	Mouse model	Suppression to the untreated control group (%)	Refs.
Pioglitazone	200	*Apc*^{1309}	67	[48]
Pioglitazone	1600	Min	9	[49]
Bezafibrate	200	*Apc*^{1309}	75	[49]
NO-1886	800	Min	42	[53]
SK-216	100	Min	56	[42]

4. Diabetes

Insulin resistance is characteristic of metabolic syndrome, associated with high levels of fasting glucose, insulin and insulin-like

Table 2
Representative dicarbonyl compounds: occurrence and consequent mutations.

Compound[a]	Mutation spectrum	Target base	Increase in diabetic patients[b]
MGO	G:C → T:A [41] G:C → C:G	G, A [34]	3.5-fold [32]
GO	G:C → T:A [42] G:C → C:G	G, A, C [35]	2.2-fold [32]
GA	Unknown	G [36]	2-fold [38][c]

[a] Abbreviations: MGO, methylglyoxal; GO, glyoxal; GA, glyceraldehyde.
[b] Compared with healthy control.
[c] Detected as amino acid adducts.

growth factor (IGF-1) in the blood. It is considered that these conditions are linked to T2DM, and a higher risk of colon cancer [25,26]. For example, hyperglycemia, hyperinsulinemia and high level of IGF-1 have been demonstrated to increase cell viability and proliferation observed in an *in vitro* setting [27].

Multiple genetic alterations in tumor-related genes have been identified in various types of cancers [28]. With obesity or under T2DM conditions, an increased level of reactive oxygen species (ROS) has been reported in multiple sites, such as blood, liver and adipose tissue. In animal experiments, Furukawa et al. demonstrated that production of ROS in adipose tissue increased body weight-dependently, and ROS production was stimulated by fatty acids *via* NADPH oxidase activation [29]. Moreover, there have been several reports of significantly elevated oxidative DNA damage in blood of T2DM patients [30]. ROS attack of nucleotide bases in DNA yields a variety of alterations and damaged nucleosides that escape repair have the capacity to introduce mutations during DNA replication [31]. Based on these findings, T2DM may contribute to induction of mutations and colon carcinogenesis *via* increased oxidative stress.

In diabetes (both type 1 and type 2) patients, glucose concentrations in blood are at high levels compared with healthy subjects all day long. It has been reported that reduced sugars, including glucose, are non-enzymatically converted into dicarbonyl compounds, such as methylglyoxal (MGO), glyceraldehyde (GA), under physiological conditions [32]. Such dicarbonyl compounds react irreversibly with amino groups of physiological components, such as protein, DNA and lipid by the Maillard reaction, to form glycation adducts or so-called Advanced Glycation End products (AGEs) [33–36]. These have been detected as amino acid- [37,38], deoxyribonucleoside (nucleobase)- [39] and phospholipid-adducts [40], in both types of diabetic patients. Glycation products of DNA are known to induce mutations in mammalian [41,42] and bacterial cells [43], such as, for example, G:C to C:G and G:C to T:A transversions in the *supF* gene in simian kidney cells associated with N^2-(1-carboxyethyl)-2′-deoxyguanosine produced by the reaction of 2′-deoxyguanosine with MGO (Table 2) [41].

Furthermore, we discovered a novel Maillard reaction product formed from L-tryptophan and glucose, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-de]quinolin-7-one, ABAQ, showing mutagenicity toward various Salmonella strains in the presence of S9 mix [44]. Because of a consistent increase in blood glucose levels under T2DM conditions its production might be enhanced in T2DM individuals. We are now investigating the presence of ABAQ *in vivo* using urine samples collected from DM rat models and DM patients.

5. ROS and inflammation

As mentioned in the previous section, DNA damage induced by ROS is likely to play an important role in carcinogenesis, and obesity increases ROS levels in adipose tissue and blood. In MS patients, abdominal fat tissue attracts macrophages by induction of several chemokines, such as monocyte chemoattractant protein-1 (MCP-1), and forms crown-like structures [13]. Activated macrophages are known to produce ROS and inflammatory cytokines, and thus obesity is now considered to be a pro-inflammatory condition.

ROS directly effects cell proliferation and apoptosis through modification of gene expression followed by activation of transcription factors, such as members of the AP-1 and NF-κB pathways [45]. Activation of AP-1 results in induction of cyclin D1, which in turn promotes entry into mitosis, while NF-κB induces inflammatory cytokines and growth factors, which enhance the inflammation status. A recent report demonstrated that ROS and prostaglandin E_2, which play important roles in inflammation in colon cancer tissue, modulate DNA methylation patterns [46], control gene expression and may thereby contribute to the multistage carcinogenesis process.

Lipid peroxidation mediated by ROS has also been recognized to play a key role in carcinogenesis, for example by activation of transcriptional factors [47]. Free and ester forms of unsaturated fatty acids and cholesterol are easily attacked by ROS, and are oxidized by a chain mechanism. Colorectal cancer risk in a case-control study showed positive relationships with erythrocyte membrane compositions of palmitic and oleic acids, but negative links with linoleic (18:2*n* − 6) and arachidonic acids [48]. *Min* mice with a hyperlipidemic state demonstrate elevated values for palmitic and oleic acids in plasma and erythrocyte membranes, and higher plasma levels of linoleic acid, indicating these to be important in intestinal polyp formation [49]. In addition, detailed analysis of serum lipids in *Min* mice using reverse-phase liquid chromatography/electrospray ionization mass spectrometry revealed that hydroperoxidizable TG precursors containing linoleic acid were deposited at the tips of villi with aging, and these hydroperoxidized TG were also increased in serum [50]. Such increases of oxidizable TG precursors in serum and small intestinal mucosa could be reduced by treatment with pitavastatin, a novel lipophilic statin [50], with concomitant reduction of intestinal polyp development [51]. These results indicated that quantitative and qualitative lipid changes affect the course of intestinal polyp formation in *Min* mice, and support the idea that oxidative stress might lead to the development of colon cancer.

6. Adipocytokine imbalance

Obese mice, such as the KK-A^y strain, are highly susceptible to induction of colon premalignant lesions, aberrant crypt foci (ACF), and development of colorectal carcinomas on exposure to azoxymethane (AOM) [52]. KK-A^y mice were established by crossmating KK, T2DM model mice, with C57BL/6J-A^y mice [53,54], which carry the *Agouti* gene (*Ay*), and feature severe hyperphagia, hyperinsulinemia and dyslipidemia. C57BL/6J mice are generally used as non-obese controls [55,56]. The numbers of AOM-induced ACF per mouse and tumor per mouse developing in KK-A^y mice (almost 70 and 8, respectively) also appeared higher than in other obese mice, *ob/ob* or *db/db* mice, not possessing intact leptin or leptin receptors [52]. In addition to severe hyperinsulinemia and hypertriglyceridemia, the KK-A^y mouse exhibits abdominal obesity, and resultant elevation of serum adipocytokines, such as interleukin-6 (IL-6), leptin and plasminogen activator inhibitor-1 (Pai-1) compared with values for lean C57BL/6J mice. In the visceral fat tissue, significant over-expression of pro-inflammatory adipocytokine mRNAs such as for IL-6, leptin, MCP-1, Pai-1 and tumor necrosis factor (TNF)-α were confirmed; in contrast, that for adiponectin was decreased. The consequent adipocytokine imbalance is suggested to be involved in the promotion of colon carcinogenesis.

Our recent findings for two adipocytokines, adiponectin and PAI-1, and their relevance to intestinal tumorigenesis provide

further support for this idea. Adiponectin is a 30 kDa protein, present at high levels in plasma (range, 3–30 μg/mL), inversely correlated with the BMI [57,58]. Moreover, low plasma adiponectin levels are associated with insulin resistance, high serum glucose levels, and coronary artery disease [59–61] as well as with increased risk of various cancers, including colorectal cancer [62,63].

Thus, we investigated how low levels of adiponectin might be involved in colon carcinogenesis using *Min* mice. Adiponectin-deficient *Min* mice of both sexes exhibited a 2- or 3-fold increase in the total number of intestinal polyps compared to those of adiponectin-wild *Min* mice at the ages of 9 and 12 weeks [64]. In addition, adiponectin-deficient C57BL/6 J mice treated with AOM showed increased incidences and multiplicities of colorectal adenomas and adenocarcinomas. AMPKα activation through the adiponectin receptor, AdipoR1, inhibits Akt activation followed by mammalian target of rapamycin (mTOR) inactivation [63,65], presumably through abolished signaling from AdipoR1, enhancing cell growth and tumor development.

In primary cell culture, fibroblasts from adiponectin-deficient C57BL/6J mice over-express Bcl-2 compared to those of adiponectin-wild C57BL/6J mice [64,66]. Adiponectin deficiency also affects production of other adipocytokines. Adiponectin-deficient *Min* mice exhibit an increase in serum Pai-1 levels with adiponectin gene dosage [64], in agreement with the tendency for elevation observed with adiponectin-deficiency at the age of 55 weeks in C57BL/6J mice [64]. Treatment with an AMPK activator, metformin, was also found to lower amounts of hepatic Pai-1 mRNA in *Min* mice, in line with earlier reports [67,68]. Thus, it is conceivable that Pai-1 levels are generally depressed by adiponectin.

PAI-1, a serine protease inhibitor (serpin) protein, which inhibits the function of tissue plasminogen activator and urokinase-type plasminogen activator by direct binding, demonstrates increased levels with obesity and the metabolic syndrome. PAI-1 can be induced by TG, very low-density lipoprotein, transforming growth factor β (TGFβ) and various growth factors [69–72]. There is also evidence that the serum PAI-1 concentration may be a reliable indicator of a poor prognosis in colorectal cancer [73–79].

In our experiments, serum Pai-1 levels in the 15-week-old male *Min* mice could be shown to be 8 times higher than in wild-type mice, while hepatic Pai-1 mRNA levels were 11-fold increased. Administration of a PAI-1 inhibitor, SK-216, at 25, 50 and 100 ppm doses in the diet for 9 weeks reduced serum Pai-1 levels and hepatic Pai-1 mRNA levels of *Min* mice compared to the wild-type levels. Moreover, *Min* mice receiving SK-216 at 50 and 100 ppm exhibited significantly reduced total numbers of intestinal polyps, to 64% and 56% of the untreated group value, respectively (Table 1). Serum TG levels were also decreased by 43% at the dose of 100 ppm [80]. These results indicate that Pai-1 induction associated with hypertriglyceridemia may contribute to intestinal polyp formation with *Apc* deficiency. Thus, adiponectin and PAI-1 are considered to be key molecules involved in obesity-associated cancers.

7. Angiotensin-renin system

Activation of the renin–angiotensin system (RAS) has been implicated in the etiology of hypertension, obesity and metabolic syndrome [81]. Angiotensin II (Ang II) elicits its biological activities through two well-defined receptors, type 1 (AT1R) and type 2 (AT2R), to elevate blood pressure, and agents that block AT1R, angiotensin-converting enzyme (ACE) activity and calcium influx block such elevation. It is not clear whether hypertension affects neoplasia, but accumulating evidence suggests that activation of RAS is involved in development of various cancers, such as in the breasts, colorectum, kidneys and lungs [82].

AT1R expressed in a wide variety of tissues activates downstream MAPK and STAT signal pathways [83]. Thus, Ang II- AT1R -mediated signals induce expression of protooncogenes such as *c-fos*, *c-myc* and *c-jun*, and thereby promote cell proliferation [84,85]. In animal models, the AT1R blockers (ARBs) captopril and telmisartan have been shown to suppress the development of ACF and more advanced preneoplastic lesions, β-catenin accumulated crypts, in male *db/db* obese mice [86]. Moreover, captopril or telmisartan decreased the mRNA levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 in the white adipose tissue of AOM-treated *db/db* mice.

ACE inhibitors block the formation of Ang II and have been demonstrated to attenuate tumor growth in experimental animals [87–90] and to reduce the risk of several human cancers [91]. AT2R expression is low in adult tissues, although detectable in heart, kidneys, pancreas, adrenal glands, uterus, ovaries and brain [92], and AT2R-mediated signals counteract AT1R-mediated actions [82,93]. It is interesting that down-regulation of cytochrome P450 2E1 expression in the liver of AT2R-null mice resulted in an increase in the number of AOM-induced colon tumors [94]. Calcium blockers are also primarily utilized to control peripheral blood pressure. Some of them, such as verapamil, are known to inhibit p-glycoprotein (encoded by *Mdr1a* gene), and the number of polyps in *Min* mice undergoing verapamil administration was significantly decreased [95].

The available findings with anti-hypertensive agents appear clinically significant because these drugs are widely used for patients with hypertension who frequently are obese. Inhibition of RAS might be an effective strategy for prevention of colon cancer.

8. Future aspects

Understanding the molecules involved in obesity-associated cancer may provide clues to cancer preventive strategies in obese individuals. There appears to be a convergence of effects of dyslipidemia, insulin resistance, inflammation and adipocytokines. Targeting related molecules and signaling pathways may therefore be a good preventive and/or therapeutic approach. Some studies suggest that weight loss after gastric bypass surgery is associated with a reduced incidence of cancer [96]. Its ability to reduce the risk of obesity-associated cancers needs to be confirmed in future investigations. In addition, factors reducing the risk of obesity-associated cancers with physical activity require clarification as a high priority.

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