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.

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.

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.

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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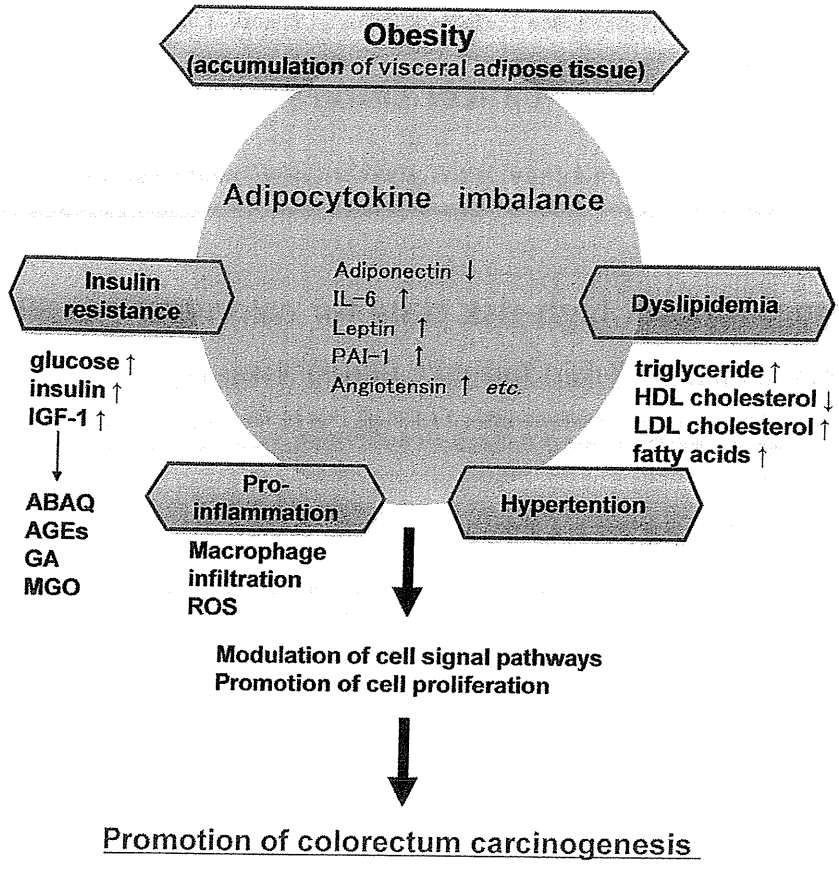

Fig. 1. Assumed relationship between metabolic syndrome and imbalance of adipocytokine production linked to colorectal cancer development. AGEs, advanced glycation end products; GA, glyceraldehydes; HDL, high-density lipoprotein; IGF-1, insulin like growth factor-1; IL-6, interleukine-6; LDL, low-dencity 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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Sesamol suppresses cyclooxygenase-2 transcriptional activity in colon cancer cells and modifies intestinal polyp development in *Apc*$^{Min/+}$ mice

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Excessive prostaglandin production by cyclooxygenase-2 in stromal and epithelial cells is a causative factor of colorectal carcinogenesis. Thus, compounds which inhibit cyclooxygenase-2 transcriptional activity in colon epithelial cells could be candidates for anti-carcinogenic agents. A cyclooxygenase-2 transcriptional activity in the human colon cancer cell line DLD-1 has been measured using a β-galactosidase reporter gene system. Using this system, we demonstrated that the decrease in basal cyclooxygenase-2 transcriptional activities at 100 μM sesamol, one of the lignans in sesame seeds, was 50%. Other compounds in sesame seeds such as sesamin, sesamolin, ferulic acid, and syringic acid did not exhibit significant suppression of cyclooxygenase-2 transcriptional activity at up to 100 μM. In a following experiment, 6-week-old male *Min* mice, *Apc*-deficient mice, were divided into a non-treated and 500 ppm sesamol groups. At the age of 15 weeks, it was found that treatment with sesamol decreased the number of polyps in the middle part of small intestine to 66.1% of the untreated value. Moreover, sesamol suppressed cyclooxygenase-2 and cytosolic prostaglandin E_2 synthase mRNA in the polyp parts. The present findings may demonstrate the novel anti-carcinogenetic property of sesamol, and imply that agents that can suppress cyclooxygenase-2 expression may be useful cancer chemopreventive agents.

Key Words: **cyclooxygenase-2, reporter gene assay, sesame, sesamol, *Min* mice**

The sesame plant (*Sesamun indicum*, Linn.) is well known for its edible seeds and oil.[1] Sesame seeds are characterized by the presence of fatty acids (linoleic acid, linolenic acid, oleic acid, palmitic acid and stearic acid), oil-soluble lignans (episesamin, sesamin, sesaminol, sesamol and sesamolin) and other phenol compounds (γ-tocophenol, ferulic acid and syringic acid). The nonfat portion of sesame seed is only 1–2% by wet weight. Recently, multiple biological functions of sesame seeds, such as inhibition of inflammation and carcinogenesis, have been elucidated. In experimental studies, sesamol was shown to inhibit development of spontaneous development of preneoplastic hepatocytic foci in rats.[2] Sesamin reduced the incidence of chemically induced rat mammary gland cancers.[3] Moreover, sesame oil has been reported to inhibit growth of human colon cancer cells *in vitro*.[4] These effects of sesame seed and its constituents were partly associated with its hydroxyl radical scavenging activity, inhibitory activity of lipid peroxidation and anti-mutageneic activity.[5–7] However, the desirable biological functions have not entirely been elucidated yet.

Recent accumulating evidence has indicated that prostaglandins (PGs) are implicated in colon carcinogenesis.[8] Expression levels of cyclooxygenase-2 (COX-2) are increased in colon carcinoma tissues compared to that of normal colonic mucosa. Therefore, inhibitors against COX-2 have been studied extensively for their ability to suppress colon carcinogenesis. It has been also reported that COX-2 gene knockout causes significant reduction in number and size of intestinal polyps in a mouse model for human familial adenomatous polyposis, *Apc*-deficient *Min* mice.[9] Thus, it is likely that agents that can suppress COX-2 expression at the transcriptional level may be equally advantageous.

As reported in previous papers,[10–12] we have constructed a β-galactosidase reporter gene system to test the effects of compounds on COX-2 transcriptional activity in a human colon cancer cell line, DLD-1 cells. In the present study, effects of five sesame seeds constituents on the transcriptional activity of COX-2 were investigated and one constituent, sesamol, was found to suppress basal COX-2 transcriptional activity. In a further experiment, we investigated the suppressive effect of sesamol on intestinal polyp development and on COX-2 expression levels in *Min* mice.

Materials and Methods

Chemicals. Sesamin were obtained from Cayman Chemical, (Ann Arbor, MI), ferulic acid, sesamol and syringic acid were from Sigma-Aldrich Co. (St. Louis, MO). Sesamolin was from Nagara, Ltd. (Gifu, Japan).

Cell culture. DLD-1 cells, a human colon adenocarcinoma cell line, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Construction of DLD-1/COX-2-B2-βGal-BSD cells has been reported in our previous papers.[13] The cells were maintained in DMEM medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37°C in 5% CO_2.

Measurements of cell viability. Cell viability in each culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells at a density of 2.0×10^4 cells per well were seeded in 96-well tissue culture plates

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and treated with sesame constituents for 48 h. After treatment, the cells were further incubated in a medium containing 0.5 mg/mL of MTT for 1 h. The MTT formazan produced by living cells was dissolved in dimethyl sulfoxide and absorbance at 595 nm was measured on a microplate Reader (Bio-Rad Laboratories, CA).

Reporter gene assay for COX-2 promoter-dependent transcriptional activity. DLD-1/COX-2-B2-βGal-BSD cells were seeded at a density of 2.0×10^4 cells per 96-well tissue culture plate and precultured for 24 h. After treatment with the test reagents, the total β-galactosidase activities of the cells in each well were determined by colorimetric assay using *o*-nitrophenyl-β-d-galactopyranoside (ONPG) as described previously.[10,11] The background β-galactosidase activity of DLD-1 cells was determined in non-treated DLD-1/B2-βGal-BSD cells, and the value was set as 0. Basal β-galactosidase activity of non-treated DLD-1/COX-2-B2-βGal-BSD cells was set as 100%. The percent β-galactosidase activity of each treatment was calculated from triplicate wells. The viable cell number was assessed by the MTT assay. All assays, including MTT assay, were carried out in triplicate and each experiment repeated at least three times.

Quantitative real-time polymerase chain reaction (PCR) analysis. Total RNA was isolated using TRIzol Reagent (Invitrogen, NY), treated with DNase (Invitrogen, Grand Island, NY) and 1 μg aliquots in a final volume of 20 μL were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using Fast Start Universal SYBR Green Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for COX-1 (5'-TGA TGC TCT TCT CCA CGA and 5'-GCA GGA AAT AGC CAC TCA AG), COX-2 (5'-GTG CCA ATT GCT GTA CAA GC and 5'-TAC AGC TCA GTT GAA CGC CT), cPGES (5'-AGT CAT GGC CTA GGT TAA C and 5'-TGT GAA TCA TCA TCT GCT CC), EP1 (5'-ACC CTG CAT CCT GAG CAG CAC TGG CCC TCT and 5'-CGA TGG CCA ACA CCA CCA ACA CCA GCA GGG), EP2 (5'-AGG ACT TCG ATG GCA GAG GAG AC and 5'-CAG CCC CTT ACA CTT CTC CAA TG), EP3 (5'-TGA CCT TTG CCT GCA ACC TG and 5'-AGA CAA TGA GAT GGC CTG CC), EP4 (5'-TCC CGC TCG TGG TGC GAG TGT TC and 5'-GAG GTG GTG TCT GCT TGG GTC AG), mPGES-2 (5'-AAG ACA TGT CCC TTC TGC and 5'-CCA AGA TGG GCA CTT TCC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-TGT CAG CAA TGC ATC CTG CA and 5'-TTA CTC CTT GGA GGC CAT GT) were employed. For evaluation of human mRNA levels, indicated primers as shown below were used. Human EP1 (5'-TCT ACC TCC CTG CAG CGG CCA CTG and 5'-GAA GTG GCT GAG GCC GCT GTG CCG GGA), human EP2 (5'-ATG GGC AAT GCC TCC AAT GAC TCC CAG and 5'-CTC CAG GGA ACA ATT TCA AAA T), human EP4 (5'-CCT CCT GAG AAA GAC ACT GCT and 5'-AAG ACA CTC TCT GAG TCC T), and human GAPDH (5'-CCA CCC ATG GCA AAT TCC and 5'-TGG GAT TTC CAT TGA TGA CAA). To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Western blot analysis. EP1–4 protein levels were analyzed by western blot. DLD-1/COX-2-B2-βGal-BSD cells were seeded at a density of 2×10^5/well in 24-well plates, and incubated with 50 and 100 μM sesamol for 24 and 48 h. After treatment, cells were lysed in 100 μl lysis buffer [0.0625 M Tris–HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate]. Samples were separated in 10% polyacrylamide gel electrophoresis–sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Millipore, MA). Abs against the EP1, EP2, EP4 (Cayman Chemical Co. Ann Arbor, MI) and EP3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a 1:2,000 dilution. Peroxidase-conjugated secondary Abs for anti-rabbit IgG were obtained from GE Healthcare (Buckingham shire, UK). Blots were developed with ECL western blotting detection reagents (GE Healthcare).

Animals. Female C57BL/6-*Apc*$^{Min/+}$ mice (*Min* mice) were purchased from The Jackson Laboratory (ME). Mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24 ± 2°C and 55% humidity on a 12 h light/dark cycle. Sesamol was well mixed at a concentration of 500 ppm in AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan).

Protocol for Animal experiments. Ten female *Min* mice at 5 weeks of age were given 500 ppm sesamol, for 8 weeks. The animals in each cage were all in the same treatment group. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured weekly. The intestinal tract was removed and separated into the small intestine, cecum and colon. The small intestine was divided into the proximal segment (4 cm in length) and then the proximal (middle) and distal halves of the remainder. Polyps in the proximal segments were counted and all

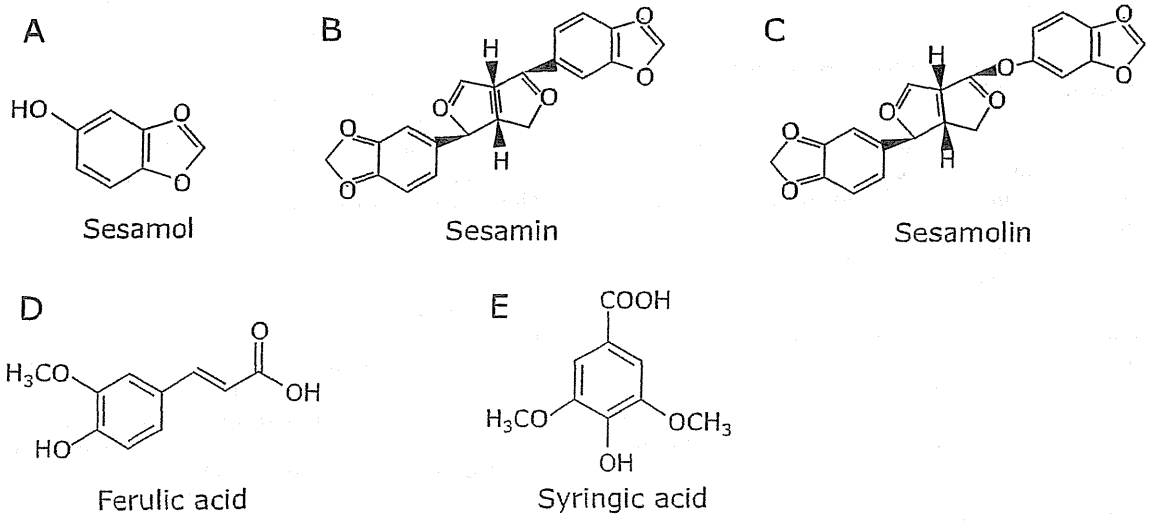

Fig. 1. Chemical structures of the five constituents in the sesame seed. (A), sesamol; (B), sesamin; (C), sesamolin; (D), ferulic acid; (E), syringic acid.

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polyps in the proximal segment were picked up under a stereoscopic microscope and the remaining intestinal mucosa (non-polyp part) was removed by scraping, and then both stored at −80°C for further analysis. Other segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. The numbers and sizes of polyps and their distributions in the intestine were assessed with a stereoscopic microscope. The experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Statistical analysis. All the results are expressed as mean ± SD. values, with statistical analysis using the Student's *t* test, except for the COX-2 promoter activity investigation and mRNA examination in the human cell line. The Bonferroni *z* test was used for statistical analyses of the COX-2 promoter activity and of human mRNA levels. Differences were considered to be statistically significant at $p<0.05$.

Results

Suppression of COX-2 promoter activity in human colon cancer cells by sesamol. Five compounds, shown in Fig. 1, were tested at various concentrations up to 100 μM with regard to their effects on COX-2 promoter activity. Remarkable suppression of cell proliferation rates by five compounds was not observed at the concentrations up to 100 μM in MTT assay. Among five constituents in the sesame seed, only sesamol significantly suppressed COX-2 prompter activity in a dose-dependent manner. Decrease in COX-2 promoter activities by sesamol at 100 μM was 50% (Fig. 2A). The other four compounds, ferulic acid, sesamin, sesamolin, syringic acid exhibited weak or no suppression of COX-2 promoter activity (Fig. 2B–E).

Suppression of intestinal polyp formation in *Min* mice by sesamol. Administration of 500 ppm sesamol to *Min* mice for 8 weeks did not affect body weights, food intake or clinical signs throughout the experimental period. Average daily food intake did not significantly differ among the groups, being 3.9 and 3.5 g per mouse per day for the 0 and 500 ppm group of *Min* mice, respectively. In addition, there were no changes observed in any organ weights that might have been attributable to toxicity.

Table 1 summarizes data for the number and distribution of intestinal polyps in the basal diet and sesamol-treated groups. Almost all polyps developed in the small intestine, with only a few in the colon. The total number of polyps tended to be decreased by administration of 500 ppm sesamol to 75% of the untreated control value. Reduction of polyps was observed in the middle part, and was by 66% ($p<0.05$ vs 0 ppm). In the other parts of small intestine and colon, treatment with sesamol lowered the number of polyps without significant difference.

Fig. 3 shows the size distribution of intestinal polyps in the basal diet and sesamol-treated groups. The maximal number of polyps was observed in the size range between 0.5 and 2.0 mm in diameter. Administration of sesamol significantly reduced the numbers of polyps sized <0.5 mm in diameter.

Decrease of inflammation-related factors mRNA levels in intestinal polyp parts by sesamol. Inflammation-related factors mRNA expressions in intestinal polyp parts and non-polyp parts were investigated (Fig. 4). Real-time PCR revealed that treatment with 500 ppm sesamol for 8 weeks significantly suppressed COX-2, and cPGES, mRNA levels in the intestinal polyp parts to 48% ($p<0.01$) and 54% ($p<0.05$) of sample value, respec-

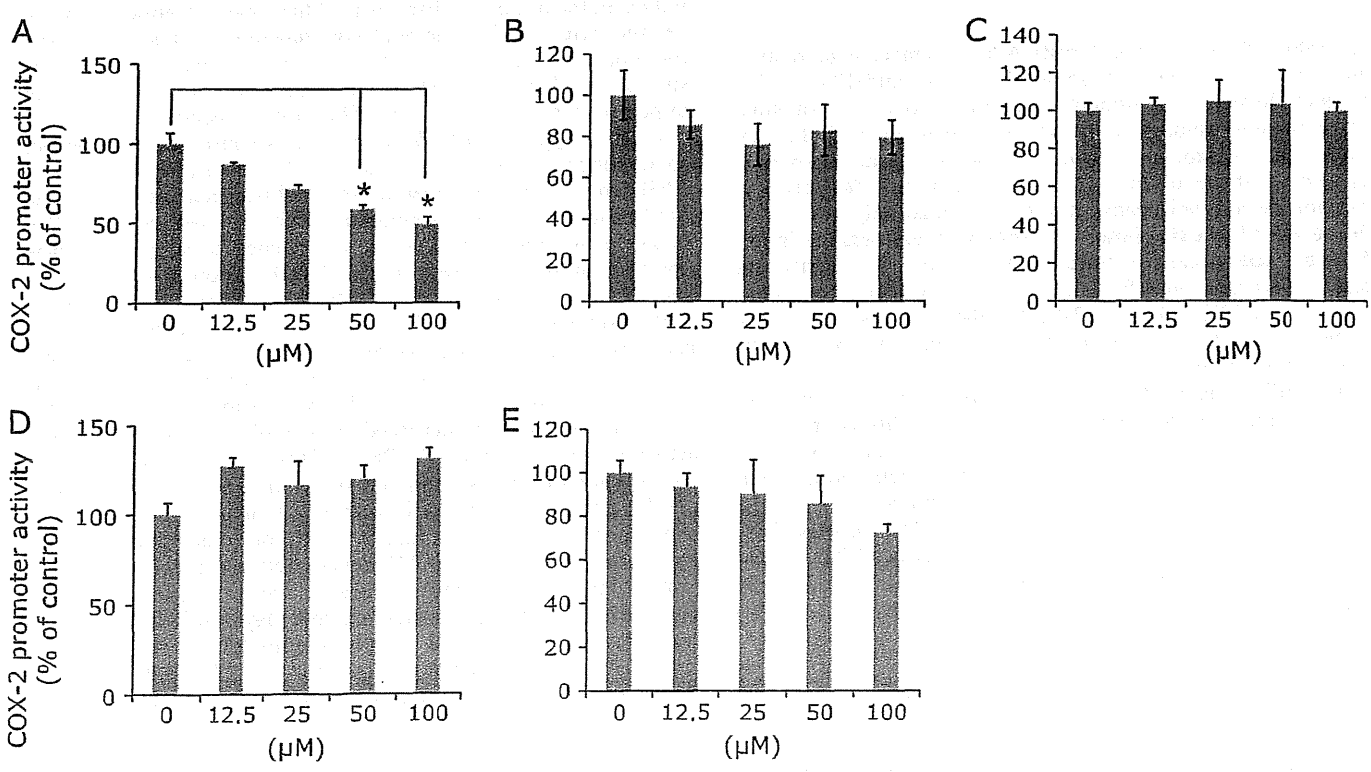

Fig. 2. Effects of treatment with sesame constituents on reporter gene activity in DLD-1/COX-2-B2-β-gal-BSD cells. DLD-1/COX-2-B2-β-gal-BSD cells were seeded in 96-well multiwell plates at a density of 2×10^5 cell/well and cultured in medium containing sesame constituents at concentrations up to 100 μM. After 48 h, the COX-2 promoter activity was evaluated by β-galactosidase activity and was normalized for viable cell numbers assessed by MTT assay. The columns indicate the values of the mean percentages of triplicate wells of promoter activity of DLD-1/COX-2-B2-β-gal-BSD cells. The data are representative of more than three independent experiments. Bars indicate the SD. *$p<0.05$. (A), sesamol; (B), sesamin; (C), sesamolin; (D), ferulic acid; (E), syringic acid. COX; cyclooxygenase, cPGES; cytosolic PGES, mPGES; microsomal PGES, PGES; prostaglandin E synthase.

Table 1. Number of intestinal polyps/mouse in *Min* mice

Sesamol (ppm)	No. of mice	Small intestine			Colon	Total
		Proximal	Middle	Distal		
0	9	4.9 ± 4.6	17.1 ± 5.6	22.1 ± 11.7	0.7 ± 1.3	44.8 ± 15
500	8	2.4 ± 1.5	11.3 ± 5.6*	19.9 ± 4.4	0.1 ± 0.4	33.6 ± 9.2#

Data are mean ± SD. *Significantly different from the control untreated group at $p<0.01$. #$p = 0.087$.

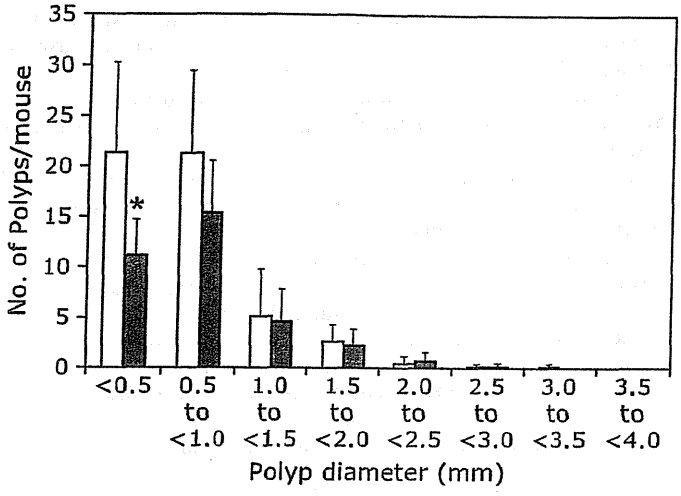

Fig. 3. Effects of sesamol on the size distribution of intestinal polyps in *Min* mice. *Min* mice were fed a basal diet (open box) or a containing 500 ppm (black filled box) sesamol for 8 weeks. The number of polyps per mouse in each size class is given as a mean ± SD. *$p<0.05$.

tively. mPGES2, EP1 and EP2 mRNA levels tended to be reduced in intestinal polyp parts by sesamol. COX-1 and EP4 mRNA expression levels in non-polyp parts and polyp parts of small intestine were not suppressed by sesamol treatment. Only EP3 did not increase its expression levels in the intestinal polyp parts compared to those of mucosa parts, and rather decreased its expression by sesamol treatment in the intestinal polyp parts.

Decrease of prostaglandin E_2 receptor expression levels in human colon cancer cells by sesamol. To clarify the effects of sesamol on EP1 and EP2 mRNA levels in human cells, we treated DLD-1/COX-2-B2-β-gal-BSD cells with 50 and 100 μM sesamol for 48 h. Sesamol significantly suppressed EP1 and EP2 mRNA levels in a dose-dependent manner (Fig. 5). However, mRNA levels for EP4 were increased and EP3 was not clearly detected (data not shown). We further confirmed the EP1–4 protein expression levels in the cells with or without sesamol treatment. As shown in Fig. 5D, sesamol down-regulated EP1 and EP2 protein levels in dose- and time-dependent manner. As in the case of EP4, sesamol treatment for 24 h slightly increased its protein levels, whereas 48 h treatment decreased EP4 protein levels. EP3 expression levels seem to be very low in these cells, as shown in Fig. 5D.

Discussion

In the present study, sesamol was found to suppress basal transcriptional activity of the COX-2 gene in human colon cancer DLD-1 cells. Previously, we have reported that mono-benzonic compounds such as resorcinol and resacetophenone suppress COX-2 transcriptional activity,[10,11] but their inhibitory activities are almost 5-times less than that of sesamol. Thus, sesamol may have a notable potential to suppress COX-2 expression for a natural compound.

The underlying mechanism of suppression of COX-2 transcriptional activity by sesamol is not clear. Protein-tyrosine kinases (PTKs), including the epidermal growth factor receptor, are well known to be involved in the induction of COX-2 expression.[14,15] Signals from activated PTKs are transduced to the downstream transcription factor NF-κB, mainly by the Ras and mitogen-activated protein kinase pathways. It is also known that activation of NF-κB has been reported to play an important role in the regulation of COX-2 expression. However, our preliminary experiment that aimed to evaluate the effects of sesamol on NF-κB transcriptional activity failed to show its reduction at concentrations up to 100 μM in human colon cancer cells (data not shown). Further studies are needed to elucidate the molecular mechanisms responsible for the inhibition of COX-2 transcriptional activity by sesamol.

We next aimed to show the suppressive potential of sesamol on intestinal polyp development in *Min* mice. Administration of 500 ppm sesamol tended to reduce the total number of intestinal polyps development compared to that of the untreated group. Further analysis revealed that treatment with sesamol decreased the number of polyps in the middle part of the small intestine. It has been reported that indomethacin, a COX inhibitor, and nimesulide, a COX-2 selective inhibitor, mainly reduce the number of polyps in the middle to distal part of the small intestine.[16,17] Thus, sesamol with a COX-2 suppressive function has a similar inhibitory potential for polyp development. For instance, LPL inducers such as NO-1886 or PPAR ligands effectively reduce the number of polyps in the proximal part of the small intestine.[18,19]

In the polyp parts of *Min* mice, it was confirmed that sesamol could suppress expression levels of COX-2 mRNA. In addition, cPGES mRNA was reduced by sesamol treatment, and this is the first report that suggests suppressive effects of sesamol on cPGES as far as we know. Moreover, a tendency to suppression was observed in the expression levels of PGE_2 receptor subtypes EP1 and EP2 in the polyp parts of *Min* mice. Using PGE_2 receptor subtype-knockout mice, the roles of these receptors in colon carcinogenesis have been investigated.[20–23] These observations suggest that EP1, EP2 and EP4 are promotive receptors in colorectal carcinogenesis, and EP3 plays suppressive roles. EP1 signals transmitted by increased intracellular Ca^{2+} concentrations activate protein kinase C (PKC). However, the actual signal transduction mechanisms are not known in detail.[24,25] Stimulation of EP2 and EP4 receptors in both cases involves coupling with stimulatory G protein, leading to activation of adenylate cyclase. As a result, increased cAMP levels activate cAMP-dependent protein kinase (PKA) and increase a transcriptional factor that binds to cAMP-responsive element, that plays a role in cell growth and cell survival. Thus, it may be worthwhile to develop functional inhibitors or specific suppressors for EP1, EP2 and EP4. However, it is regrettable that there are a few inhibitors for PGE_2 receptor subtypes. To add to the novel potential of sesamol, we confirmed the effect of sesamol on human EP1 and EP2 mRNA levels. We found suppression of EP1 and EP2 mRNA levels by sesamol treatment. Down-regulation of EP1 and EP2 protein was also confirmed. These data imply a double suppressive potential exists in sesamol regarding cell growth function of PGE_2. Suppression of COX-2 may reduce production of growth lipid mediator PGE_2, and down regulation of PGE_2 receptors such as

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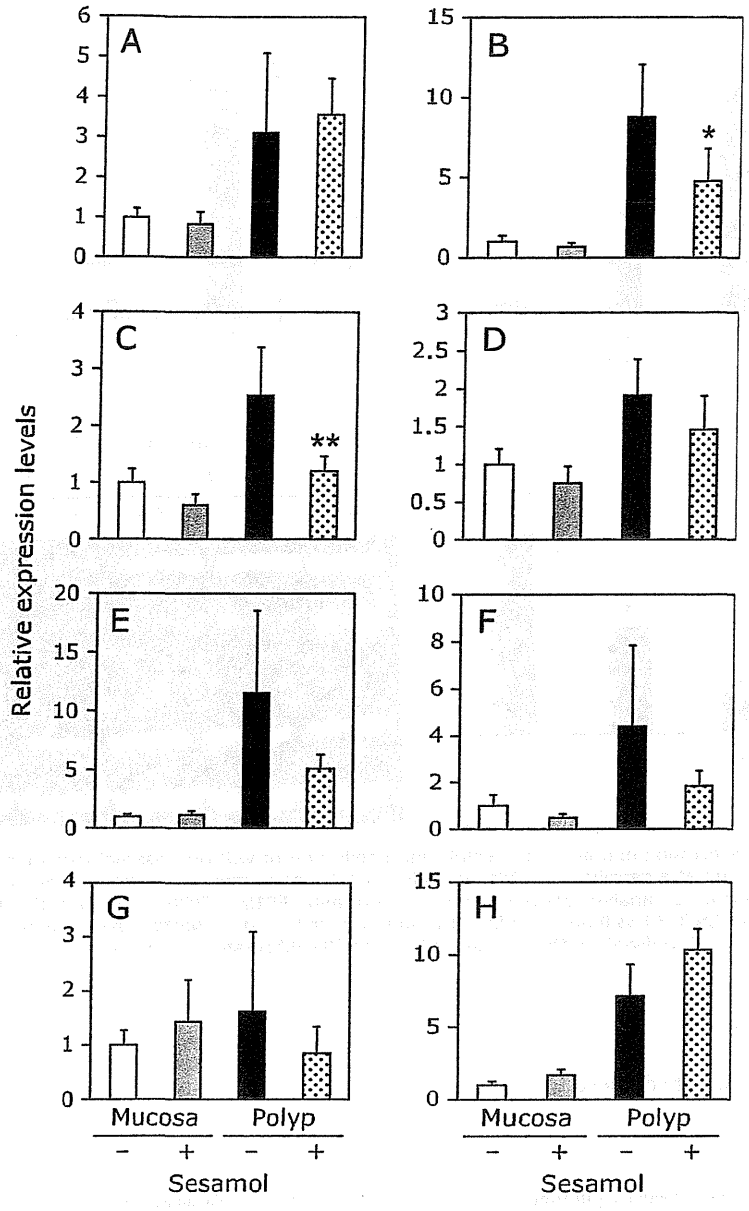

Fig. 4. Changes of inflammation-related factors in intestinal non-polyp mucosa parts and/or polyp parts of Min mice. Quantitative real-time PCR analysis were performed to determine COX-1 (A), COX-2 (B), cPGES (C), mPGES2 (D), EP1 (E), EP2 (F), EP3 (G), EP4 (H) mRNA expression levels in the polyps or non-polyp mucosa parts of *Min* mice, given diets containing sesamol at doses of 500 ppm for 8 weeks. Data are normalized with GAPDH expression level. Data are mean ± SD, $n = 6$. $^{**}p<0.01$, $^{*}p<0.05$ vs 0 ppm.

EP1 and EP2 may additionally suppress tumor growth through a transmembrane G protein-coupled receptor.

In summary, sesamol suppressed the transcriptional activity of COX-2 gene in DLD-1 cells. Moreover, our *in vivo* data imply that agents that can suppress COX-2 expression at the gene level may be useful cancer chemopreventive agents. Further information of the mechanisms by which sesamol suppresses COX-2 expression may clarify the anti-inflammatory and anti-carcinogenetic properties of sesamol.

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Abbreviations

cPGES	cytosolic PGES
COX	cyclooxygenase
FBS	fetal bovine serum
mPGES	microsomal PGES
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor-κB
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PGs	prostaglandins
PGES	prostaglandin E synthase
PTKs	protein-tyrosine kinases

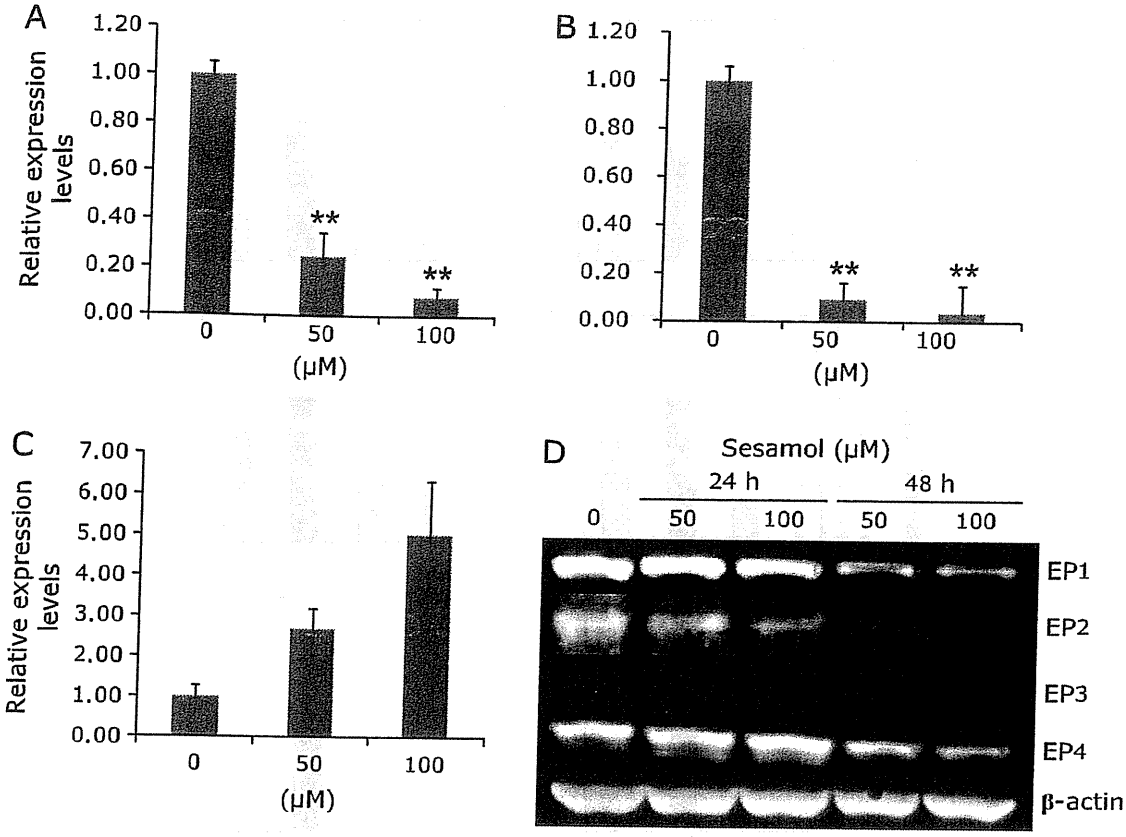

Fig. 5. Prostaglandin E_2 receptor expression in human colorectal cancer cells with or without sesamol treatment. DLD-1/COX-2-B2-β-gal-BSD cells were seeded in 6-well multiwell plates at a density of 2×10^6 cell/well and cultured in medium containing 50 and 100 μM sesamol for 48 h. After 48 h treatment, quantitative real-time PCR analysis was performed to determine EP1(A), EP2(B), EP4(C) mRNA levels. Data are normalized with GAPDH, Data are mean ± SD, $n = 3$.**$p<0.001$ vs 0 ppm. (D) EP1–4 protein was detected by western blot analysis, using DLD-1/COX-2-B2-β-gal-BSD cells (24-well plates at a density of 2×10^5 cell/well) with treatment of 50 and 100 μM sesamol for 24 and 48 h.

Conflict of Interest

No potential conflicts of interest were disclosed.

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