Table 7. Biomarkers used for detection of chemopreventive compounds against colon carcinogenesis

	Biomarkers
Proliferation activity	BrdU-labeling index, PCNA-labeling index, AgNORs number, Apoptotic index, Tumor-angiogenesis, etc.
Biochemistry	ODC activity, Polyamine levels, GST activity, QR activity, MDA, 4-HNE
Histopathology	ACF, BCAC, MDF, Adenoma, Adenocarcinoma

BrdU, 5'-bromodeoxyuridine; PCNA, proliferative nuclear antigen; AgNORs, silver-stained nucleolar regions; ODC, ornithine decarboxylase; GST, glutathione *S*-transferase; QR, quinone reductase; MDA, malondialdehyde; 4-HNE, 4-hydroxy-2(E)-nonenal; ACF, aberrant crypt foci; BCAC, β-catenin accumulated crypts; and MDF, mucin-depleted foci.

Inhibition of colon carcinogenesis by dietary capsaicin and rotenone in rats

Capsaicin (Fig. 4a) is widely consumed as a food additive throughout the world, particularly in South-East Asia and Latin American countries. The Korean people are large consumers of capsicum fruit: average daily per capita consumption of capsaicin may reach 50 mg (Buch and Burks, 1983). The content of capsaicin in capsicum is about 0.02% in fresh fruit and 0.5-1.0% in dried ripe fruit (Yun, 1999). It is currently used as a versatile tool for the study of pain mechanisms and also for pharmacotherapy to treat several pain disorders because of its selective effects on the functions of a defined subpopulation of sensory neurons (Tominaga and Julius, 2000). The intake of excessive hot peppers containing capsaicin has been considered an irritant for the gastric mucosa and may be a risk factor for several gastrointestinal lesions including gastric ulcer and cancer. However, some studies suggested that capsaicin may have a beneficial effect on human peptic ulcer and certain type of cancer (Yun, 1999). In animal carcinogenesis, capsaicin was able to inhibit cancer development in a multiple organ such as stomach, lung, and liver (Yun, 1999).

Rotenone (Fig. 4b) is a naturally occurring pesticide derived from *Derris* and *Lonchorcarpus* species root and back, relatively harmless for mammals, especially after oral administration. Rotenone, deguelin and related compounds (rotenoids) are the active ingredients of botanical insecticides used for at least 150 years to control crop pests. They have been used even longer as fish poisons by native tribes to obtain food in South America and East Africa and more recently in fish management to achieve the desired balance of species. The acute toxicity of rotenone to insects, fish, and mammals is attributable to inhibition of mitochondrial NADH:ubiquinone oxidoreductase activity as the primary target. Rotenoids are known not only as toxicants but also as candidate chemopreventive agents against liver tumors in mice (Cunningham et al., 1995), mammary tumors in rats (Hansen et al., 1965), and skin tumors in mice (Udeani et al., 1997). Also, rotenoids could inhibit cell proliferation induced by peroxisome proliferators in mouse liver (Cunningham et al., 1995), and deguelin and three of its derivatives could inhibit phorbol ester-induced ODC activity as a biomarker of cancer chemopreventive potency (Gerhauser et al., 1997; Gerhauser et al., 1995).

In order to determine, we investigated the modifying effects of dietary feeding with administration of capsaicin and rotenone on AOM-induced colon tumorigenesis were investigated in male F344 rats. Gavage with capsaicin and rotenone significantly elevated phase II enzymes, GST and QR, in the liver and colon. In an ACF bioassay, feeding with capsaicin and rotenone at a dose of 500 ppm for 4 weeks significantly inhibited ACF formation induced by AOM (20 mg/kg body weight, once a week for 2 weeks). In a subsequent long-term study designed to confirm the protective effects of both compounds on ACF development, one group was treated with AOM alone and four other groups received the carcinogen treatment plus diets containing 500 ppm test compounds for 4 weeks (initiation phase) and for 34 weeks (post-initiation phase). Two groups were treated with capsaicin or rotenone alone (500 ppm in diet) and one group was maintained on the basal diet. At the termination of the study, dietary exposure of capsaicin during the initiation phase was found to significantly reduce the incidence of colonic adenocarcinoma (60% vs. 24%, 60% reduction, $P<0.05$). Feeding with rotenone during the post-initiation phase also reduced the frequency of colonic adenocarcinoma (60% vs. 19%, 68% reduction, $P<0.05$).

In this study, both test compounds capsaicin and rotenone elevated phase II enzymes GST and QR in the liver and colon. Dietary feeding with capsaicin and rotenone for 4 weeks during AOM exposure significantly decreased ACF development induced by AOM at weeks 4 and 12. Also, at week 12 the treatments with capsaicin and rotenone significantly suppressed the number of large ACF containing 4 or more crypts, which strongly correlates with tumor formation (Pretlow et al., 1992). These results suggested that the two chemicals could inhibit the growth of colonic ACF and suppress the progression of preneoplasia to malignancy. Subsequent long-term experiments confirmed the results of the pilot study. The data on the incidence of colonic adenocarcinoma indicated that capsaicin could inhibit AOM-induced colon carcinogenesis when fed during the initiation phase, while rotenone exerted its chemopreventive action when fed during the post-initiation phase. This is the first report describing the preventive effects of capsaicin and rotenone in an animal model of colon carcinogenesis. Our data suggest that capsaicin and rotenone are possible new dietary preventive agents against colon cancer development.

In the study, suppressing effects of rotenone on AOM-induced colon tumorigenesis rather than its blocking effects. The mechanism(s) by which rotenone exert its inhibitory action when fed during the post-initiation are not known, but feeding with rotenone reduced PCNA-labeling index in colonic adenocarcinoma and polyamine level in colonic epithelium. These results may indicate that modulation of cell proliferation by feeding with rotenone accounts in part for its chemopreventive action. Our results suggest possible cancer chemopreventive ability of rotenone. However, Betarbet et al recently reported that chronic exposure of rotenone reproduces several features of human Parkinson's disease in rats (Betarbet et al., 2000), Therefore, chronic toxicity studies of rotenone should be conducted prior to its use as a chemopreventive drug.

Inhibition of rat colon carcinogenesis by dietary feeding with citrus limonoids, obacunone and limonin

Limonoids are a group of triterpene derivatives present in the *Rutaceae* and *Maliaceae* families. Limonoids, including obacunone (Fig. 4c) and limonin (Fig. 4d), are also found in citrus seeds, commercial citrus juice and *Philodendron amurense* (Kihada). For example, commercial orange juice contains an average of 320 ppm limonoid glucosides (Fong et al., 1989). These glucosides are responsible for delayed bitterness in citrus juices and processed products (Miller et al., 2004). Obacunone and limonin have been reported to enhance GST activity in various organs of mice (Miller et al., 2004). Limonin and nomilin are reported to inhibit forestomach, buccal pouch, lung, and skin carcinogenesis in rodents (Miller et al., 2004). However, the modifying effects of the citrus limonoids obacunone and limonin on large bowel carcinogenesis have not been reported. Therefore, we investigated the modifying effects of dietary administration of the citrus limonoids obacunone and limonin on AOM-induced colon tumorigenesis were investigated in two experiments in male F344 rats. In a pilot study, we examined the modifying effects of obacunone and limonin on AOM-induced (20 mg/kg body wt, once a week for 2 weeks) formation of ACF. Dietary feeding with both compounds at dose levels of 200 and 500 ppm during AOM exposure for 4 weeks ('initiation' feeding) or after AOM treatment for 4 weeks ('post-initiation' feeding) significantly inhibited ACF formation (55-65% reduction by 'initiation' feeding, $P<0.001$; 28-42% reduction by 'post-initiation' feeding, $P<0.05$). In a long-term study designed to confirm the protective effects of obacunone and limonin on ACF development, one group was treated with AOM alone and another four groups received the carcinogen treatment plus diets containing 500 ppm of test compounds for 3 weeks (initiation phase) or 29 weeks (post-initiation phase). Two groups were treated with obacunone or limonin alone (500 ppm in diet) and one group was maintained on the basal diet. At the termination of the study, dietary exposure to obacunone or limonin during the initiation phase was found to have significantly reduced the incidence of colonic adenocarcinoma (72% vs. 25% or 6%, $P<0.005$). Feeding with obacunone or limonin during the post-initiation phase also reduced the frequency of colonic adenocarcinoma (72 versus 13%, $P<0.001$).

In the pilot study, dietary feeding with obacunone and limonin for 4 weeks both during or after AOM exposure significantly decreased development of ACF, suggesting that the two chemicals tested could inhibit the growth of colonic ACF and suppresses the progression of preneoplasia to malignancy. Subsequent long-term experiments confirmed the results of the pilot study and indicated that the suppressing effects of both compounds fed to rats during either the initiation or post-initiation phase were significant. It should be noted, however, that since commercial orange juice contains 320 ppm limonoid glucosides (Fong et al., 1989), the concentrations necessary to achieve the effects observed in our study would be ~12- to 30-fold higher than those obtained from normal dietary ingestion of these limonoids. Our data suggest that obacunone and limonin are possible new dietary preventive agents against colon cancer development.

One possible mechanism for the suppression of colonic tumor development might be through the control of cell proliferation in ACF and/or 'normal appearing' crypts of rats exposed to AOM. Increased cell proliferation is suggested to play an important role in multistage carcinogenesis (Dictor et al., 1999), including colon tumorigenesis (Lipkin, 1988). There is a greater correlation between ACF and reduction in PCNA labeling index in ACF than between ACF and reduction in size of the proliferative component of ACF in rats (Zheng et al., 1997). Over-expression of cyclin D1 has been reported in ACF and adenocarcinoma in the mouse colon (Wang et al., 1998). Over-expression of cyclin D1 plays an important role and is an early event in colon tumorigenesis. Therefore, we suspect that dietary administration of obacunone and limonin post-AOM injections might lower cell proliferation activity in ACF and/or colonic tumors.

The results of our study clearly demonstrate the inhibitory effects of dietary obacunone and limonin on AOM-induced colon tumorigenesis. Further experiments, including pre-clinical efficacy and mechanistic studies, are warranted to fully evaluate these natural compounds for their cancer preventive properties and to understand their mode of action. Additional toxicity studies, such as genotoxicity, reproduction toxicity, acute oral toxicity, and 2-year carcinogenicity trials should also be conducted prior to their use as chemopreventive drugs. One advantage of these compounds as chemopreventive agents in human trials is that, unlike synthetic chemopreventive agents, they are naturally occurring compounds that are produced endogenously in edible plants and are present in human foods.

Inhibition of rat colon carcinogenesis by dietary feeding with a citrus polymethoxy flavonoid, nobiletin

Citrus fruit is a rich source of cancer inhibiting agents (Tanaka et al., 2001a). Nobiletin (5,6,7,8,3',4'-hexamethoxyfiavone) is a polymethoxy flavonoid extracted from citrus fruits (Fig. 4e) (Montanari et al., 1998). The compound is reported to inhibit proliferation of human cancer cells (Kandaswami et al., 1991) and exert anti-mutagenic activity (Wall et al., 1988). These findings suggest a possible inhibitory effect of nobiletin on colon carcinogenesis. In the current study, the possible modifying effect of nobiletin on AOM-induced rat colon tumorigenesis was investigated. Also, several biomarkers for cancer chemoprevention studies were assayed for mechanistic investigation.

Firstly, we conducted the ACF bioassay to determine the modifying effects of dietary feeding with a polymethoxyflavonoid nobiletin isolated from *Citrus unshiu* on the development of AOM-induced colonic ACF in male F344 rats. We also assessed the effects of nobiletin on cell proliferation activity of ACF using a monoclonal antibody MIB-5. Rats were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for 3 weeks to induce ACF, They also received the experimental diet containing 100 ppm or 500 ppm nobiletin for 5 weeks, starting one week before the first dosing of AOM. AOM exposure produced 139 ± 35 ACF/rat at the end of the study (week 5). Dietary administration of nobiletin caused significant reduction in the frequency of ACF: 70 ± 15 (50% reduction, $P<0.001$) at a dose of 100 ppm and 63 ± 10 (55% reduction, $P<0.001$) at a dose of 500 ppm. Feeding with nobiletin significantly lowered MIB-5-index in ACF. Also, dietary administration of nobiletin significantly reduced prostaglandin (PG) E_2 content in the colonic mucosa. These findings might suggest possible chemopreventive ability of nobiletin, through suppression of cell proliferating activity of ACF, in the development of ACF.

Subsequently the experiment was conducted to investigate the inhibitory effects of dietary feeding with citrus nobiletin on AOM-induced rat colon carcinogenesis using a long-term bioassay. Five-week old male F344 rats were initiated with two weekly subcutaneous injections of AOM (20 mg/kg bw) to induce colonic tumors. They were also given the diets containing 100 ppm or 500 ppm nobiletin for 34 weeks, starting one week after the last dosing of AOM. At the end of the study, the incidence of colonic adenocarcinoma were 67% in the AOM alone group, 55% in the AOM→100 ppm nobiletin group, 35% ($P<0.05$) in the AOM→500 ppm nobiletin group. Also, feeding with nobiletin reduced the cell-proliferation activity, increased the apoptotic index, and decreased the PGE_2 content in colonic adenocarcinoma and/or colonic mucosa. These findings might suggest that citrus nobiletin has chemopreventive ability against AOM-induced rat colon carcinogenesis.

The results indicate that dietary feeding with nobiletin effectively suppresses AOM-induced large bowel carcinogenesis in rats. Nobiletin was reported to inhibit increased cell-proliferation activity (Kandaswami et al., 1991). In this study, feeding with nobiletin caused reduction in expression of cell proliferation biomarkers such as PCNA-labeling index in colonic tumors and polyamine level in non-lesional colonic mucosa. In addition, dietary nobiletin increased apoptotic index in the colonic adenocarcinoma, as found in an in vitro study (Zheng et al., 2002). Thus, it likely that the inhibition of AOM-induced colonic adenocarcinoma formation for animals consuming nobiletin is due in part to the alteration of cell proliferating activity in the colonic mucosa and neoplasms.

In this study, administration of nobiletin reduced biosynthesis of PGE_2 in colonic adenocarcinoma and in their surrounding mucosa. Eicosanoids including PGE_2, the metabolites of arachidonic acid (AA) through the lipoxygenase (LOX) and cyclooxygenase (COX) pathways, have a variety of biological activities. AA products synthesized via these pathways could modulate colon carcinogenesis (Bennett et al., 1987) and some inhibitors of the AA cascade possess chemopreventive activity in colon carcinogenesis (Cuendet and Pezzuto, 2000; Steele et al., 1999). Although, we did not investigate expression of COX and LOX in colonic mucosa in the current study, nobiletin, reported to suppress the COX-2 expression in RAW 264.7 cells treated with lipopolysaccharide (Murakami et al., 2000) and interferon (IFN)-γ, suggesting that nobiletin may affect both pathways of AA. The results of this study suggest that dietary nobiletin has a beneficial effect on chemically induced rat colon carcinogenesis. Our findings and recent studies on possible anti-metastatic ability of nobiletin (Minagawa et al., 2001; Sato et al., 2002) may suggest need for further investigations of biological functions and its mechanisms of nobiletin for fighting cancer development. In this context, our recent study indicating that nobiletin has anti-genotoxic effects against tobacco-specific nitrosamine-induced mouse lung tumorigenesis is of interest (Ikeda M, et al., submitted).

Suppression of colon carcinogenesis by feeding with a polyphenolic anti-oxidant flavonoid, silymarin in rats

Silymarin, the collective name for an extract from milk thistle [*Silybum marianum* (L.) Gaertneri] is a naturally occurring polyphenolic flavonoid anti-oxidant (Valenzuela et al., 1986). It is composed mainly (~80%, w/w) of silybin (also called silybinin, silibin or silibinin), with smaller amounts of other stereoisomers, such as isosilybin, dihydrosilybin, silydianin and silychristin. Silymarin protects experimental animals against the hepatotoxin a-amanitin and has a strong anti-oxidant property. Other biologic properties of silymarin and its components have been reported, including inhibition of LOX (Fiebrich and Koch, 1979a) and PG syntheses (Fiebrich and Koch, 1979b). For over 20 years, silymarin has been used clinically in Europe for the treatment of alcoholic liver disease and as an anti-hepatotoxic agent. As a therapeutic agent, it is well tolerated and largely free of adverse effects (Comoglio et al., 1990). It might be a potent anti-carcinogen against in vitro and in vivo carcinogenesis. However, animal chemopreventive studies have been mainly limited to skin (Katiyar et al., 1997; Lahiri-Chatterjee et al., 1999) and only few studies have involved the digestive organs, including colon. The silymarin group of flavonoids (silybin, silychristin and silydianin) inhibits xanthine oxidase (Sheu et al., 1998). Silymarin induces G1 arrest in human prostate carcinoma DU 145 cell and causes growth inhibition by inactivation of erbB1-SHC signaling pathway leading to up-regulation of Kip1/p27 followed by its increased binding with CDK causing a decrease in CDK- and cyclin-associated kinase activity (Zi et al., 1998b). These findings led us to evaluate the possible suppressing effects of dietary silymarin on the development of ACF, and early biomarker of colorectal carcinogenesis and colorectal tumors in rats.

The modifying effect of dietary administration of the polyphenolic anti-oxidant flavonoid silymarin, isolated from milk thistle [*Silybum marianum* (L) Gaertnerf], on AOM-induced colon carcinogenesis was investigated in male F344 rats. In the short-term study, the effects of silymarin on the development of AOM-induced colonic ACF, being putative precursor lesions for colonic adenocarcinoma, were assayed to predict the modifying effects of dietary silymarin on colon tumorigenesis. Also, the activity of detoxifying enzymes, GST and QR, in liver and colonic mucosa was determined in rats that were gavaged with silymarin. Subsequently, the possible inhibitory effects of dietary feeding with silymarin on AOM-induced colon carcinogenesis were evaluated using a long-term animal experiment. In the short-term study, dietary administration of silymarin (100, 500 and 1,000 ppm in diet), either during or after carcinogen exposure, for 4 weeks caused significant reduction in the frequency of colonic ACF in a dose-dependent manner. Silymarin had given by gavage elevated the activity of detoxifying enzymes in both organs. In the long-term experiment, dietary feeding with silymarin (100 and 500 ppm) during the initiation or post-initiation phase of AOM-induced colon carcinogenesis reduced the incidence and multiplicity of colonic adenocarcinoma. The inhibition by feeding with 500 ppm silymarin was significant ($P<0.05$ by 'initiation' feeding and $P<0.01$ by 'post-initiation' feeding). Also, silymarin administration in the diet lowered the PCNA labeling index and increased the number of apoptotic cells in adenocarcinoma. β–Glucuronidase activity, PGE_2 level and polyamines content were decreased in colonic mucosa. These results clearly indicate a chemopreventive ability of dietary silymarin against chemically induced colon tumorigenesis and will provide a scientific basis for progression to clinical trials of the chemoprevention of human colon cancer.

These results clearly indicate that dietary feeding with silymarin effectively suppresses the occurrence of colonic ACF and adenocarcinoma induced by AOM when administered during or after the carcinogen treatment. The results described here are basically in agreement with those of Gershbein (Gershbein, 1994), who found that dietary feeding with silymarin (1,000 ppm) during the entire period of 1,2-dimethylhydrazine (DMH)-induced rat intestinal carcinogenesis significantly inhibited the development of large and small intestinal adenocarcinomas. We did not observe the inhibitory effect of silymarin on the incidence of small intestinal neoplasms. This may due to their low incidence and the use of a different carcinogen. Silymarin inhibited the growth of human breast (Zi et al., 1998a) and prostate (Zi et al., 1998b) cancer cell lines. A chemopreventive effect of silymarin on mouse bladder carcinogenesis (Vinh et al., 2002), rat tongue carcinogenesis, and rat prostate tumorigenesis (Kohno et al., 2005) has been found. Thus, silymarin may possess cancer chemopreventive ability in multiple organs.

Several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis could be considered. AOM is an intermediate of the colonic carcinogen DMH and is metabolized by cytochrome P-450 2E1 and, possibly, P450 1A, as well as by the phase II carcinogen-detoxifying enzyme GST (Sohn et al., 1991). However, silymarin has no influence in liver P4502E1 (Miguez et al., 1994). We (Tanaka et al., 2001a) and others (Reddy et al., 1993) have reported that certain chemopreventive agents inhibit the development of ACF and carcinoma induced by AOM through induction of GST and QR. Also, epidemiologic observations suggest that consumption of certain cruciferous vegetables reduces the risk of colon cancer in individuals with GSTM1 null type (Lin et al., 1998). Our results on GST and QR activities in liver and colon could explain the decrease in ACF formation and the colon cancer development in rats given silymarin during the initiation phase. In conclusion, dietary administration of silymarin significantly suppressed the development of AOM-induced rat colonic carcinoma, in conjunction with modulation of cell growth in the colonic adenocarcinoma and induction of the phase II enzymes QR and GST in the liver and large intestine, and reduced the levels of β–glucuronidase and PGE_2 in the colorectal mucosa. Further studies to assess the chemopreventive ability of silymarin are needed in different carcinogenesis models. The results described and those reported by others suggest that silymarin has cancer chemopreventive effects in several organs through several mechanisms.

DISCUSSION

As described above, our recent data on the chemopreventive effects of naturally occurring compounds, capsaicin, rotenone, obacunone, limonin, nobiletin, and silymarin, present in edible plants against AOM-induced colon tumorigenesis are described. All these compounds are anti-oxidants. In general, plants are complicated mixtures of numerous chemicals, and interactions with their components may affect the effectiveness of the anti-oxidant. The effectiveness of tested compounds as *in vivo* anti-oxidants has been reported, but the metabolic pathway and action of naturally occurring anti-oxidative compounds is not clear. Flavonoids compounds, which are widely distributed in the plant kingdom and occur in considerable quantities, show a wide range of pharmacological activities other than their anti-oxidative properties. These compounds have been used to treat various pathological conditions including allergies, inflammation, and diabetes. Experimental data including this report showing their anti-tumor activities is accumulating; their chemopreventive potential, however, has not been fully proven clinically. Their behavior and fate should be investigated *in vivo*.

As reported, commonly consumed foods contain non-nutritive compounds capable to inhibit colon cancer in an animal model. The diet provides a rich abundance of these compounds that have the ability to intervene in all phases of carcinogenesis. Mechanisms of action include effects of Phase I and Phase II enzymes activities, interception of DNA mutating agents, and influences on cell proliferation and oncogene activation. Each of these mechanisms has been study in isolation. For explanation of reduced risk for cancer in populations with a greater reliance on fruits and vegetables in the daily diet, future research should focus on potential combinations of foods and the protective components within them.

The association of certain malignancies with chronic inflammation has been recognized for many years (Gordon and Weitzman, 1993). The link between inflammation and subsequent malignancy in visceral sites is known. Examples include large bowel cancer after ulcerative colitis or Crohn's disease (Collins et al., 1987; Gordon and Weitzman, 1993). Central to the concept of inflammation and cancer is the finding that chronic irritation of squamous or glandular epithelium will result in migration of inflammatory cells to the injured site by a mechanism dependent on neutrophils adhesion molecules. These cells, stimulated to produce reactive oxygen species (including superoxide radicals, NO and/or hydroxy radicals) via the respiratory burst and NADPH activation, can function as facilitators in the process of carcinogenesis. There is convincing evidence from animal model systems that prolonged exposure of cells to these products of activated oxygen can result in cell injury and play a role in several stages of carcinogenesis (Tanaka et al., 2005a; Tanaka et al., 2003; Tanaka et al., 2005b). Recently, up-regulation of COX-2, but not COX-1, gene expression was reported in human colorectal neoplasms (Eberhart et al., 1994). New drugs, specific for inhibition of COX-2, may provide effective tumor prevention with reduced side effects (Oshima et al., 1996; Reddy et al., 1996; Sheng et al., 1997). The elevation of COX-2 expression can protect intestinal epithelial cells from apoptosis (Tsuji and DuBois, 1995). Certain COX-2 inhibitors can induce apoptosis (Hara et al., 1997) and inhibit tumor angiogenesis (Tsuji et al., 1998). Elegant review on chemopreventive ability of NSAIDs including COX-2 inhibitors against colon tumorigenesis has been published in this journal (Wakabayashi, 2000). More recently, synthetic anti-oxidants have reported to reduce COX-2 expression PG production, and cell proliferation of colorectal cancer cells (Chinery et al., 1998). This may suggest that COX-2 may provide a new chemopreventive target in colorectal malignancies (Rustgi, 1998), if there are the natural products being a specific inhibitor of COX-2 expression in edible plants.

From the evidence mentioned above, our search for chemopreventives against colon cancer focuses on several flavonoids and some other compounds possessing certain biological activities including anti-inflammatory and/or anti-oxidative properties present in foods. Approximately 2,000 individual members of the flavonoid class have been described and the flavonoids are consumed in rather large amounts through dietary vegetables and fruits.

OTHER NON-NUTRITIVES THAT MAY EXERT SUPPRESSIVE EFFECTS ON RAT COLON TUMORIGENESIS

Our recent studies demonstrated that juices rich in hesperidin and β-cryptoxanthin could inhibit AOM-induced rat colon tumorigenesis (Kohno et al., 1999; Tanaka et al., 2000a). Juices rich in hesperidin and β-cryptoxanthin also inhibit lung tumorigenesis in mice (Kohno et al., 2001a). Thus, citrus fruit is a rich source of cancer inhibiting agents (Tanaka et al., 2001a). The rhizomes of *Zingiber zerumbet* Smith are used for anti-inflammatory folk medicine in Indonesia (Elliott and Brimacombe, 1987). A sesquiterpene zerumbone isolated from the rhizome is a potent inhibitor of 12-*O*-tetradecanoyl-13-acetate-induced Epstein-Barr virus activation (Murakami et al., 1999), and expression of inducible nitric oxide synthase (iNOS) and COX-2 expression in RAW 264.7 macrophages treated with lipopolysaccharide and interferon-γ and NO/O_2^- generation in leukocytes (Murakami et al., 2002). We demonstrated that dietary feeding with zerumbone is able to suppress AOM-induced ACF formation in rats (Tanaka et al., 2001d). A polyisoprenylated benzophenone, garcinol (also named camboginol) is present in *Guttiferae*. Dried rind of *G. indica* ('Kokum') containing garcinol (2-3%, w/w) is used as a garnish for curry and in traditional medicine in India. We have recently found the inhibitory effects of garcinol on AOM-induced ACF (Tanaka et al., 2000b). On-going long-term experiments will provide the data showing that these compounds could modify (possibly inhibit) colonic carcinoma development. Ferulic acid (FA), widely found in bran from rice, wheat and barley, vegetables, and other edible plants, is able to inhibit chemically-induced colon carcinogenesis (Kawabata et al., 2000). Recently, Tsuda's group synthesized a new chemical, 3-(4'-geranyloxy-3-methoxyphenyl)-2-propenoate (EGMP), from the parent compound FA by adding a geranyl chain. They tested the chemopreventive efficacy of EGMP and FA in AOM-induced ACF, since the compound is more potent anti-oxidant than FA. They concluded that both compounds are effective in reducing ACF formation and the effect of EGMP is more potent than FA (Han et al., 2001).

FUTURE PROSPECTIVE

An important component of the chemopreventive agent development research is the identification and characterization of intermediate biomarkers (Armstrong and Taylor, 2005; Tanaka, 1997a) that may serve as surrogate end points for cancer incidence reduction in chemoprevention clinical trials. Such effort is critical to the progress of chemoprevention and potential for cost-effective development of chemopreventive research.

ACF were first reported in rodents injected with AOM by Bird in 1987 (Bird, 1987) and similar lesions were characterized in humans in 1991 (Pretlow et al., 1991) and 1994 (Pretlow et al., 1994b) by Pretlow; since then, the AOM-induced ACF model has been the most widely used animal model system for evaluating naturally occurring compounds (flavonoids, carotenoids, green tea, etc.) as well as synthetic chemicals COX-2 inhibitors, iNOS inhibitors and peroxisome proliferators-activator receptor (PPAR)-γ agonists for their colon cancer chemopreventive efficacy (Corpet and Pierre, 2003; Corpet and Tache, 2002; Tanaka et al., 2001a). The growth dynamics, morphological and molecular features of ACF support the contention that ACF are putative preneoplastic lesions. For instance, ACF have a hyper-proliferative epithelium and their size increases with time (Dashwood et al., 2001; McLellan et al., 1991a; McLellan et al., 1991b; Roncucci et al., 1993). The nuclear atypia observed in some ACF are similar to those seen in the crypts of adenocarcinomas in colons (McLellan et al., 1991a). Furthermore, identification of dysplasia and monoclonality strongly lnks this lesion to neoplastic progression

(Siu et al., 1999). Recently, two new types of lesion have been described in the AOM-induced ACF model. Yamada *et al.*, identified new possible precursor lesions β-catenin accumulated crypts (BCAC, Fig. 1e) for colon carcinoma in the whole-mount preparations of the colon in rats exposed to AOM by using immunohistochemical method (Yamada et al., 2000; Yamada et al., 2001). The lesions are different in their morphology and location from ACF. In the lesions, accumulation are different is more prominent than did not present a ACF-like appearance (Yamada et al., 2000). Cell proliferation activity estimated by counting the number of AgNORs/nucleus in the lesions is also greater than in ACF (Yamada et al., 2001). In addition, Cademi et al. (Caderni et al., 2003; Femia et al., 2005; Femia et al., 2004) have identified mucin depleted foci (MDF) in unsectioned colon stained with high-iron diamine alcian blue (HID-AB). These newly described lesions are not yet well characterized and we do not know if BCAC and MDF are related lesions. It is interesting to note that BCAC, like MDF, have a low production of mucins and are thought to be premalignant lesions rather than preneoplastic lesions. A recent review article described significance of these three lesions (ACF, BCAC, and MDF) in colon carcinogenesis (Mori et al., 2005). Since, ACF are widely accepted as a reliable end point in experimental colon carcinogenesis, this study reports the effects of herbal supplements on the 'classical' ACF. We should thus estimate chemopreventive efficacy of non-nutritives in edible plants reported using both ACF and these new lesions as biological markers for colon carcinogenesis in future studies. Since ligands for PPARs can inhibit AOM-induced ACF, which weakly express PPARγ (Fig. 1d) (Kohno et al., 2001b; Tanaka et al., 2001b), we are now searching natural compounds acting as a ligand for PPARs (Kohno et al., 2002b; Kohno et al., 2004a; Kohno et al., 2004b). In the near future, we would like to provide promising non-nutritive compounds (including citrus compounds, auraptene and nobiletin) with less toxicity in Asian edible plants (Tanaka, 1976; Yun, 1999) for use in clinical colon cancer chemoprevention trials. Also, new compounds with more effective chemopreventive effects can be synthesized from the non-nutritive compounds, including collinin in edible plants (Kohno et al., 2006), when a small amount of the parent compound can be isolated (Curini et al., 2004). Known non-nutritive chemopreventive agents with low dose in combination can be considered to obtain a pronounced chemopreventive effect against colon cancer development in future.

Epidemiological studies showed that obesity or diabetes might be one of the risk factors for colon cancer development (McTiernan, 2005). An animal study using *db/db* mice, which have obese and diabetic phenotypes because of disruption of the leptin receptor, demonstrated that they have high susceptibility of colon carcinogenesis (Hirose et al., 2004). *Citrus unshiu* segment membrane (CUSM) contains fiber, flavonoids, and pectin, but its biological activity is unknown. Therefore, we conducted a short-term experiment to determine whether dietary CUSM affects the development of AOM-induced ACF and BCAC in the colon in C57BL/KsJ-*db/db* mice (Suzuki et al. 2006). Male db/db mice were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for 5 weeks. From one week after the last dosing AOM, they received the diet containing 200, 1,000, or 5,000 ppm CUSM for 7 weeks. At week 12, dietary administration of CUSM caused a reduction in the frequency of ACF (53-59% reduction). In addition, the number of BCAC was lowered by the treatment with CUSM (29-62% reduction). Also, pathological alterations (fibrosis) in liver, which were resembled a metabolic disorder, non-alcoholic steatohepatitis (NASH) (Pessayre and Fromenty, 2005) were reduced by feeding with CUSM. NASH may in some cause fibrosis, cirrhosis, and premature death resulting from liver failure. Its prevalence is increasing, and it is probably underestimated as a cause for cirrhosis and/or liver cell cancer. The need for an effective treatment is clear and urgent using an animal model of NASH (Sahai et al., 2004). Our data may indicate that CUSM is useful for reducing the risk for colon carcinogenesis in obesity or diabetes and that for NASH.

CONCLUSION

In conclusion, certain flavonoids and other substances with biological activity including anti-oxidative and/or anti-inflammatory properties, which are present in edible plants including vegetables and fruits, could exert chemopreventive action in rat colon carcinogenesis as shown here. However, more work needs to be done to better understand the underlying mechanism(s) of action and to confirm their safety for use in humans. Since plants are complex mixtures of chemicals, the potential for finding new chemopreventive agents in plants is high. Studies are underway to identify new compounds in edible plants with chemopreventive potential. For screening chemopreventive agents based on different mechanisms, new *in vitro* co-culture model might be useful (Mace et al., 1998) and microarray analysis (Hokaiwado et al., 2004). The effects of these agents on colon carcinogenesis should be carefully studies to assist the discovery and development of new chemopreventive agents, and to understand carcinogenesis mechanisms. Our goal is to develop chemopreventive agents that could be effective in decreasing the risk of colon cancers in general and/or high-risk populations. The strategy was only partially successful; it could give a significant impact on reduction of colon cancer mortality.

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Dextran sodium sulfate strongly promotes colorectal carcinogenesis in $Apc^{Min/+}$ mice: Inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms

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The mouse model for familial adenomatous polyposis, $Apc^{Min/+}$ mouse, contains a truncating mutation in the *Apc* gene and spontaneously develops numerous adenomas in the small intestine but few in the large bowel. Our study investigated whether dextran sodium sulfate (DSS) treatment promotes the development of colonic neoplasms in $Apc^{Min/+}$ mice. $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes were exposed to 2% dextran sodium sulfate in drinking water for 7 days, followed by no further treatment for 4 weeks. Immunohistochemistry for cyclooxygenase-2, inducible nitric oxide synthase, β-catenin, p53, and nitrotyrosine, and mutations of *β-catenin* and *K-ras* and loss of wild-type allele of the *Apc* gene in the colonic lesions were examined. Sequential observation of female $Apc^{Min/+}$ mice that received DSS was also performed up to week 5. At week 5, numerous colonic neoplasms developed in male and female $Apc^{Min/+}$ mice but did not develop in $Apc^{+/+}$ mice. Adenocarcinomas developed in $Apc^{Min/+}$ mice that received DSS showed loss of heterozygosity of *Apc* and no mutations in the *β-catenin* and *K-ras* genes. The treatment also significantly increased the number of small intestinal polyps. Sequential observation revealed increase in the incidences of colonic neoplasms and dysplastic crypts in female $Apc^{Min/+}$ mice given DSS. DSS treatment increased inflammation scores, associated with high intensity staining of β-catenin, cyclooxygenase-2, inducible nitric oxide synthase and nitrotyrosine. Interestingly, strong nuclear staining of p53 was specifically observed in colonic lesions of $Apc^{Min/+}$ mice treated with DSS. Our results suggest a strong promotion effect of DSS in the intestinal carcinogenesis of $Apc^{Min/+}$ mice. The findings also suggest that strong oxidative/nitrosative stress caused by DSS-induced inflammation may contribute to the colonic neoplasms development.

Key words: $Apc^{Min/+}$; mice; dextran sodium sulfate; colon carcinogenesis; p53; nitrotyrosine

Carcinogenesis and inflammation are pathological consequences of injury and repair at the cellular and molecular levels[1,2] and are influenced by several life style factors, including dietary factors.[3] Recent studies suggest inflammation in enhancing the risk of various types of cancer[2] including colon cancer.[4] In fact, individuals suffering with inflammatory bowel disease (IBD) are at high risk of developing colon cancer.[5,6] We recently proposed a novel mouse colon carcinogenesis model and demonstrated the powerful tumor-promoting effects of dextran sodium sulfate (DSS), which can induce colonic mucosal inflammation, resembling the histopathology of one of the IBD ulcerative colitis (UC),[7] on colon carcinogenesis initiated with azoxymethane (AOM),[8–10] 1,2-dimetylhydrazine (DMH)[11] or heterocyclic amines (HCAs)[12] in mice. Thus, inflammation/inflammatory stimuli induced by a short-term (for a week) treatment with 2% DSS in drinking water after initiation with a low-dose of carcinogens is effective for rapid induction of colon neoplasms possessing β-catenin gene mutations in mice.[11,12] Similarly, Cooper *et al.*[13] found that inflammation plays an important role in the dysplasia-cancer sequence in the colon. They also reported the development of colon cancer in 60-day-old $Apc^{Min/+}$ mice that received 4% DSS alone.[14] In addition, Barbour *et al.*[15] suggested that a relationship between chronic inflammation and small intestinal tumorigenesis in $Apc^{Min/+}$ mice.

Cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) play an important role in colon tumor growth and progression. COX catalyzes the committed step in the conversion of arachidonic acid to protumorigenic eicosanoids, such as prostaglandin E_2, which are involved in the maintenance of tumor integrity.[16] COX-2 is frequently undetectable in normal tissues but is induced by cytokines, growth factors, reactive oxygen species and tumor promoters.[17] Gene expression of COX-2 is upregulated in 80–85% of human colonic adenocarcinomas,[18] in colonic tumors induced by AOM in rodents[19] and in 80–85% of $Apc^{Min/+}$ mouse adenomas.[20] Nitric oxide (NO) is endogenously produced by a family of enzymes. NO is reported to cause mutagenesis[21] and DNA deamination,[22] and is implicated in the inflammatory responses and in the production of vascular endothelial growth factor.[23] Several studies also report that iNOS is up-regulated in human cancers, including colon cancer[24,25] and in AOM-induced colon tumors in rodents.[26] In addition, one study reported that iNOS inhibitors suppress the development of AOM-induced aberrant crypt foci in rats.[27] Although the role of iNOS plus NO and related radical species in intestinal polyposis is still controversial,[28,29] NO/iNOS may be involved in intestinal tumorigenesis.[30–33] The interaction between iNOS and p53 as a crucial pathway in inflammatory-mediated carcinogenesis is also suggested.[34] An increased cancer risk occurs in the tissues undergoing chronic inflammation.[35] Thus, NO is a candidate free radical, and the *p53* tumor suppressor gene is a candidate molecular target.[36]

Familial adenomatous polyposis (FAP) is an inherited form of human colon cancer characterized by the development of 100–1,000 adenomas in the large intestine.[37] If not removed, these benign epithelial neoplasms inevitably progress to carcinomas.[37] FAP can be caused by germline mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene.[38] Min mice were a germline mutation in the *Apc* gene and develop multiple polyps in the intestine.[39] *Apc*-deficient mice including Min mice are considered to be good models of FAP and have been used for investigating the influence of environmental factors, such as dietary factors, carcinogens, chemopreventive agents and other xenobiotics.[40] However, unfortunately, unlike human FAP, most of the neo-

Abbreviations: AOM, azoxymethane; APC, adenomatous polyposis coli; COX, cyclooxygenase; DMH, 1,2-dimethylhydrazine; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; H&E, hematoxylin and eosin; HCAs, heterocyclic amines; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; LOH, loss of heterozygosity; NO, nitric oxide; UC, ulcerative colitis.

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plasms occur predominantly in the small intestine of these genetically altered mice. Yamada *et al.*[41] recently reported that a number of adenomatous lesions together with a few tumors are present in the colon of old *Apc*$^{Min/+}$ mice. The finding suggests the presence of precursor cryptal lesions for colonic epithelial malignancies and the possibility of progression of the lesions to epithelial neoplasms under appropriate experimental conditions. Mutations of several genes, including *Apc*, *β-catenin*, *K-ras*, *DCC*, *p53* and alterations proteins' expression, such as COX-2, β-catenin, iNOS and Wnt/Apc/β-catenin signaling, play important roles in both chemically induced colon carcinogenesis and human cancer development.[33] Thus, colon carcinogenesis is characterized by a succession of molecular changes involving basic cellular process such as cell proliferation, cell signaling and DNA integrity, but it is poorly understood what sifts the balance between them, causing a cryptal cell to lose its normal phenotype. Such knowledge could be crucial for the first step in fighting colon cancer development.

In our study, we investigated whether acute inflammation induced by DSS enhances small and large intestinal carcinogenesis in *Apc*$^{Min/+}$ mice. Mutational analysis of *β-catenin* and *K-ras* genes and immunohistochemical analysis of Apc, β-catenin, COX-2, iNOS and p53 expression were also performed in the colonic neoplasms. The immunohistochemistry of nitrotyrosine, a good marker for oxidative stress caused by inflammation,[42] was performed on the colonic mucosa of mice given DSS. In addition, sequential pathological alteration of the large intestines of female *Apc*$^{Min/+}$ mice exposed to DSS was investigated to test our hypothesis that inflammation induced by DSS promotes the growth of the early colonic cryptal lesions, dysplastic aberrant crypt foci[43] or adenomatous lesions[41] and the treatment resulted in the high frequency of colonic neoplasms in the short-term (5 weeks).

Material and methods

Animals, chemicals and diets

Male and female C57BL/6J *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice aged 3 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were genotyped to identify carriers of the *Min* allele of *Apc* with a PCR assay as described.[44] They were housed in plastic cages (4 or 5 mice/cage) under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle) and temperature (23 ± 2°C). Drinking water and a pelleted basal diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan) were available *ad libitum*. They were quarantined for 7 days after arrival and then randomized by body weights into experimental and control groups. DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH).

Experimental procedure

Forty-seven *Apc*$^{Min/+}$ mice (16 males and 31 females) and 50 *Apc*$^{+/+}$ mice (29 males and 21 females) were used. Animals of the experimental groups were given 2% (w/v) DSS in drinking water for 1 week, starting 4 weeks of age. The control group (9 male and 10 female *Apc*$^{Min/+}$ mice, and 17 male and 11 female *Apc*$^{+/+}$ mice) were given the tap water without DSS throughout the experiment. Among them, 14 female *Apc*$^{Min/+}$ mice exposed to 2% DSS were sequentially sacrificed at weeks 2 (4 mice), 3 (5 mice) and 4 (5 mice) to monitor the pathological alterations in the large intestine. All the remaining animals were sacrificed at week 5. At sacrifice, all organs were removed, and the small and large intestines were cut open along their longitudinal axis, and fixed flat in 10% buffered formalin for 24 hr at room temperature after macroscopic inspection. Longitudinal sections of the large intestine were made, and then processes for histopathological examination were performed by routine procedures. Small intestine was divided into 3 equal segments (proximal, middle and distal parts), the number and distribution were determined under a dissecting microscope Nikon SMZ1000 (Nikon Co., Tokyo, Japan). After counting, cross sections of the small intestine were made at 2 mm intervals and processed for histopathological evaluation of the polyps by routine procedures. Histological examination was performed on hematoxylin and eosin (H&E)-stained sections. On H&E-stained sections, histological alterations, such as mucosal dysplasia and colonic tumors, were examined. Colonic mucosal dysplasia was diagnosed according to the criteria described by Paulsen *et al.*[43] Colonic tumors were diagnosed according to the description by Ward.[45]

Scoring of inflammation in the intestinal mucosa

Mucosal inflammation with or without ulceration in the entire intestine was analyzed on H&E-stained sections. Small and large intestinal inflammation with or without mucosal ulceration was graded according to the following morphological criteria described by Cooper *et al.*:[46] grade 0, normal appearance; grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium and grade 4, presence of mucosal ulcer with severe inflammation (neutrophil, lymphocyte and plasma cell infiltration) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

Immunohistochemistry

Immunohistochemical analyses for β-catenin, COX-2, iNOS, p53 and nitrotyrosine were carried out with 4 μm-thick paraffin-embedded sections as previously described[8,9,47] or a report by Mollersen *et al.*[48] As the primary antibodies, anti-β-catenin mouse monoclonal antibody (diluted 1:1,000, Transduction Laboratories, Lexington, KY), anti-COX-2 mouse monoclonal antibody (diluted 1:200, Transduction Laboratories), anti-iNOS mouse monoclonal antibody (diluted 1:250, Transduction Laboratories), anti-p53 rabbit polyclonal antibodies (CM5, diluted 1:100, Novocastra Laboratories, Ltd., Newcastle, UK) and rabbit polyclonal anti-nitrotyrosine (diluted 1:500, Upstate Biotechnology, Lake Placid, NY) were used. To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Inc., Burlingame, CA) was applied. For p53 and nitrotyrosine immunohistochemistry, normal rabbit serum was used to block background staining. Nonspecific binding was blocked by incubating the slides with a blocking solution (0.1 M PBS containing 0.1% triton X-100 and 2% normal goat serum) for nitrotyrosine. Staining was performed using a LSAB KIT or DAKO EnVision kit (DAKO, Glostrup, Denmark) or Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). At the last step, the sections were counterstained with hematoxylin. As a negative control, omission of the primary antibody was used. To quantitate the degree of nitrotyrosine stainability, the grading system (Grade 0–4) was used according to the following criteria described by Zingarelli *et al.*[49]: Grade 0, no immunoreactivity; Grades 1–3, increasing degrees of intermediate immunoreactivity and Grade 4, extensive immunoreactivity.

Apc allelic loss analysis

Seventeen tissues (14 colonic adenocarcinomas and 3 colonic mucosa) from male *Apc*$^{Min/+}$ mice that received 2% DSS, and 5 tissues (2 colonic adenocarcinomas and 3 colonic mucosa) from male *Apc*$^{Min/+}$ mice that received tap water without DSS were selected at random for *Apc* allelic loss analysis. They were digested overnight at 50°C in 20 μl of lysis buffer containing 500 μg/ml proteinase K, 10 mmol/liter Tris-HCl (pH 8.0), 50 mmol/liter KCl, 0.45% NP40 and 0.45% Tween 20. The proteinase K was heat inactivated (10 min at 95°C). The tubes were centrifuged for 5 min, and the supernatant was transferred to new tubes. Loss of heterozygosity (LOH) of the *Apc* gene was checked using PCR with mismatched primers, as described previously.[50]

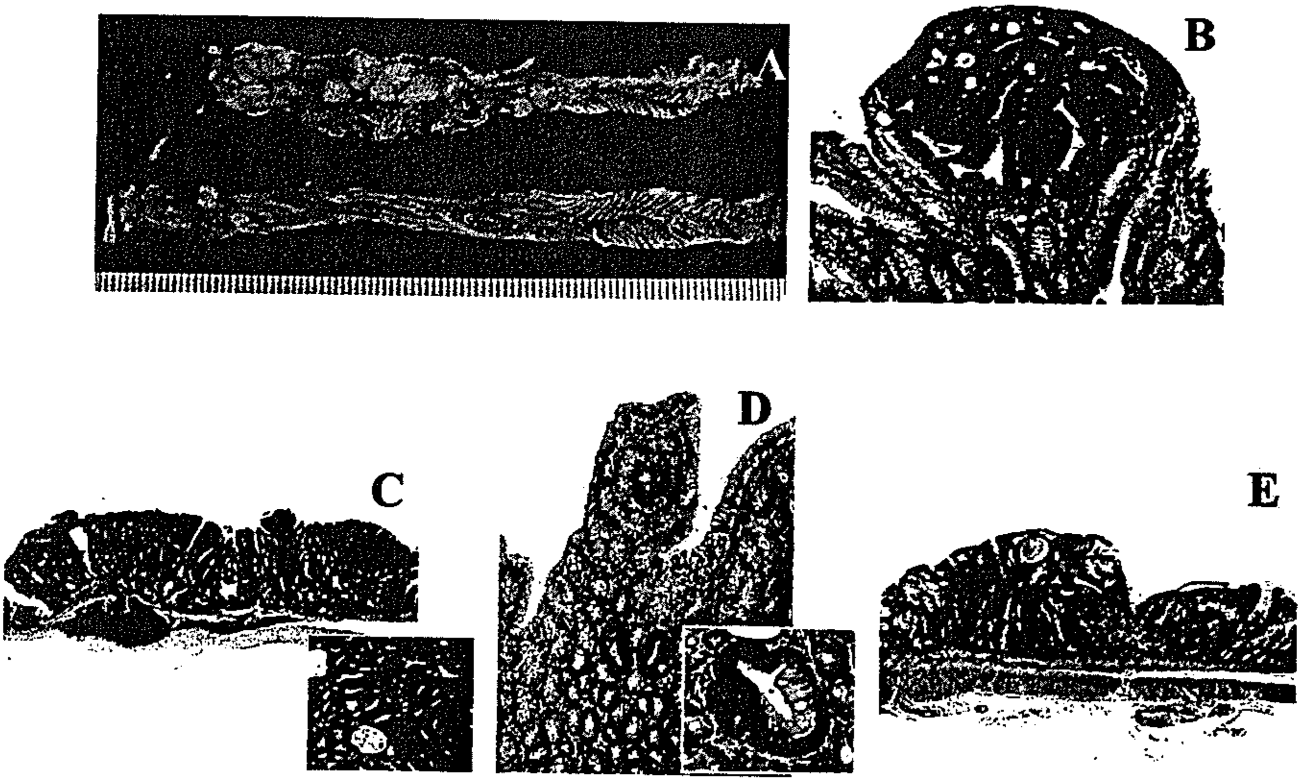

FIGURE 1 – Macroscopic view of the large bowel (*a*) and histopathology of the colonic lesions (*b–e*) of male $Apc^{Min/+}$ mice treated with 2% DSS. (*a*) Male $Apc^{Min/+}$ mice given 2% DSS had multiple colonic tumors (upper), while male $Apc^{Min/+}$ mice given tap had a few colonic tumors (lower); (*b*) A polypoid tumor is diagnosed as tubular adenoma compressing surrounding crypts; (*c*) A nodular tumor is diagnosed as well-differentiated tubular adenocarcinoma (insert: cancer cells with tubular pattern); (*d*) Three dysplastic crypts with hyperchromatic nuclei (insert: a dysplastic crypt with bud formation) are noted in the colonic mucosa; and (*e*) Colonic mucosal ulcer with regenerative hyperplasia is seen in the colonic mucosa. H&E stain, original magnification: (*b*), (*d*), ×100; (*c*), ×10; (*e*) ×20; (*c*, insert), ×100; and (*d*, insert), ×200.

Briefly, the amplification of the Apc^{Min} allele resulted in a 155 bp PCR product with 1 *Hind*III site, whereas the 155 bp product from the Apc^{+} allele contained 2 *Hind*III sites. *Hind*III digestion of PCR-amplified DNA from $Apc^{Min/+}$ heterozygous tissue resulted in a 123 bp product from the Apc^{+} allele and a 144 bp product from the Apc^{Min} allele. Therefore, PCR products from tissue with LOH displayed only 1 band (144 bp) from the Apc^{Min} allele. Samples were assayed at least twice, independently.

DNA sequencing and mutation analysis of β-catenin and K-Ras genes

A total of 17 tissues (14 colonic adenocarcinomas and 3 colonic mucosa) from male $Apc^{Min/+}$ mice that received 2% DSS were subjected to analysis of β-*catenin* and K-*ras*. Also, a total of 5 tissues (2 colonic adenocarcinomas and 3 colonic mucosa) from male $Apc^{Min/+}$ mice that received tap water without DSS were subjected to analysis of these genes. PCR was performed in β-*catenin* and K-*ras* genes and the statuses were determined by direct sequencing. Exon 3 of the β-*catenin* gene (McatF, 5′-TCTCCTTGG CTGGCCTTTCTA-3′; McatR, 5′-GTCACACAGCCCTGTCAAGA-3′) and exon 1 of the k-*ras* gene (MrasF, 5′-GCCTGCTGAAAATGACTGAG-3′; MrasR, 5′-CTTTACAAGCGCACGCAGAC-3′) were amplified by PCR. Primers were included in the following PCR reaction mixture, which contained in a total volume of 20 μl: 20 μM of each primer, 200 μM of each deoxynucleotide triphosphate, 1 unit of *Taq* polymerase in 1 × PCR buffer (Promega, Madison, WI) and template DNA. The mixture was heated at 94°C for 5 min and subjected to 30 cycles of denaturation (94°C, 45 sec), annealing (57°C, 45 sec) and extension (72°C, 1 min) using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The products were sequenced directly after gel-purification in both directions using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's recommendations. Reactions were analyzed on an ABI Prism 3100 DNA Sequencer (Applied Biosystems).

Statistical analysis

Statistical significance of differences was evaluated by one-way ANOVA with Bonferroni correction or Fisher's exact probability test. Values were considered significantly different when $p < 0.05$.

Results

Pathological findings

$Apc^{Min/+}$ mice, but not $Apc^{+/+}$ mice, of both sexes exposed to 2 % DSS had bloody stools during DSS exposure. Other animals were healthy during the study. At week 5, macroscopically, a number of nodular, polypoid or caterpillar-like colonic tumors (Fig. 1*a*) were observed mainly in the middle and distal colon of male and female $Apc^{Min/+}$ mice treated with 2% DSS, but few in those treated with tap water. Microscopically, they were tubular adenoma (Fig. 1*b*) or well-/moderately-differentiated tubular adenocarcinoma (Fig. 1*c*). Similarly, dysplastic crypts (Fig. 1*d*) were frequently observed in all $Apc^{Min/+}$mice of both sexes. Also, mucosal ulcer was noted in mice given 2% DSS in drinking water (Fig. 1*e*).

TABLE I – INCIDENCE AND MULTIPLICITY OF LARGE INTESTINAL TUMORS AND DYSPLASTIC CRYPTS AT WEEK 5

Genotype	Sex	Colonic tumors: incidence (multiplicity)						Dysplastic crypts	
		Total: incidence (multiplicity[1])		AD[2]		ADC[2]			
		2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water	2% DSS	Tap water
$Apc^{min/+}$	Male	7/7, 100%[3] (9.43±3.31[4])	3/9, 33% (0.44±0.73)	7/7, 100%[4] (3.86±2.19[4])	2/9, 22% (0.22±0.44)	7/7, 100%[4] (5.57±2.37[4])	2/9, 22% (0.22±0.44)	7/7, 100% (18.86±2.18[4])	9/9, 100% (6.56±1.67)
	Female	7/7, 100%[5] (8.29±5.02)[5]	3/10, 30% (0.50±0.97)	5/7, 71% (3.29±3.04[6])	2/10, 20% (0.30±0.67)	7/7,100% (5.00±2.16[5])	2/10, 20% (0.20±0.42)	7/7, 100% (13.29±3.45[6])	10/10, 100% (7.70±4.14)
$Apc^{+/+}$	Male	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)	0/12, 0% (0)	0/17, 0% (0)
	Female	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)	0/10, 0% (0)	0/11, 0% (0)

[1]No. of tumors/mouse, Mean ± SD.–[2]AD, adenoma; and ADC, adenocarcinoma.–[3]Significantly different from $Apc^{Min/+}$ males received tap water by one-way ANOVA with Fisher's exact probability test ($P < 0.05$).–[4]Significantly different from $Apc^{Min/+}$ males received tap water by Fisher's exact probability test or one-way ANOVA with Bonferroni correction ($P < 0.01$).–[5]Significantly different from $Apc^{Min/+}$ females received tap water by Fisher's exact probability test or one-way ANOVA with Bonferroni correction ($P < 0.01$).–[6]Significantly different from $Apc^{Min/+}$ females received tap water by one-way ANOVA with Bonferroni correction ($P < 0.05$).

The incidences and multiplicities of colonic neoplasms (adenomas and adenocarcinomas) and total colonic tumors are summarized in Table I. The incidences of total tumors and adenocarcinomas in $Apc^{Min/+}$ mice of both sexes given 2% DSS were significantly greater than those given tap water alone (total tumors: males, 100% *vs.* 33%, $p < 0.05$ and females, 100% vs. 30%, $p < 0.01$; adenocarcinoma: males, 100% *vs.* 22%, $p < 0.01$ and females, 100% *vs.* 20%, $p < 0.01$). Treatment with 2% DSS significantly increased the incidence of colonic adenomas in male $Apc^{Min/+}$ mice when compared to that of male $Apc^{Min/+}$given tap water alone ($p < 0.01$). As for dysplastic foci (Table I), the frequencies in *ApcMin/+* mice of both sexes given 2% DSS were significantly greater than those given tap water alone ($p < 0.01$ for males and $p < 0.05$ for females).

Time-course observation of colonic tumors in female $Apc^{Min/+}$ mice revealed that the initial tumor (histologically tubular adenoma) developed at week 2 (Fig. 2*a*). The incidence of colonic adenomas reached 100% at week 3 and that of adenocarcinomas did at week 5, respectively (Fig. 2*a*), and their multiplicitis gradually increased up to week 5 (Fig. 2*b*). As for the frequency of dysplastic foci, there was no further increase in dysplastic crypts from week 4 to week 5 (Fig. 2*c*). The value at week 5 was significantly larger than that at week 2 ($p < 0.05$).

As summarized in Table II, a number of small intestinal polyps (histologically tubular adenoma) developed in all $Apc^{Min/+}$ mice with both sexes treated with or without 2% DSS, but not in $Apc^{+/+}$ mice with both sexes treated with or without 2% DSS. Their frequencies in $Apc^{Min/+}$ males and females given 2% DSS were significantly greater than in those given tap water alone ($p < 0.05$ for males and $p < 0.05$ for females). Considering the distribution of the polyps, significant increases in number were found at the distal region ($p < 0.01$ for males and $p < 0.01$ for females) in $Apc^{Min/+}$ mice of both sexes treated with 2% DSS when compared to those in $Apc^{Min/+}$ mice that received tap water. At the middle regions of small intestine the numbers of polyps were significantly decreased ($p < 0.05$ for males and $p < 0.01$ for females) in 2% DSS treated *ApcMin/+* mice of both sexes. Also, 2% DSS treatment increased the size (by 18%) of polyps in the small intestine. On the other hand, we could not find any polyps or tumors in the small intestine of wild type mice.

Score for inflammation in the iintestine

Table III summarizes data on colonic inflammation scores at week 5. The values in $Apc^{Min/+}$and $Apc^{+/+}$ mice of both sexes treated with 2% DSS were significantly larger than those given tap water alone ($p < 0.01$). No significant differences on the degrees of colonic mucosal inflammation were noted between mice of 2 genotypes, $Apc^{Min/+}$ and $Apc^{+/+}$. Scoring of inflammation in the time-course study indicated that the value decreased after the cessation of 2% DSS (Fig. 2*d*). DSS exposure also produced small intestinal inflammation in both $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes: the inflammation scores in $Apc^{Min/+}$ mice were relatively greater than those in $Apc^{+/+}$ mice (data not shown). The scores of $Apc^{Min/+}$ mice that received 2% DSS were high in order of the distal (1.29±0.76 for males and 1.14±0.69 for females), middle (0.57±0.79 for males and 0.43±0.79 for females) and proximal (0.43±0.53 for males and 0.29±0.49 for females) parts.

Immunohistochemistry of β-catenin, COX-2, iNOS, p53 and nitrotyrosine

The immunoreactivities against β-catenin, COX-2, iNOS and nitrotyrosine were found in all colonic lesions including neoplasms and dysplastic crypts (Fig. 3) in the large intestine of $Apc^{Min/+}$ and $Apc^{+/+}$ mice of both sexes that received 2% DSS. Their intensity in the normal mucosa and the lesions induced in mice given tap water was relatively weaker than that in $Apc^{Min/+}$ mice treated with 2% DSS. p53 was positive in the nuclei of the colonic lesions developed in $Apc^{Min/+}$, while negative in those in $Apc^{+/+}$ mice. The immunoreactivity against 3 antibodies (β-catenin, COX-2 and iNOS) was also observed in the small intestinal polyps (tubular adenomas) in $Apc^{Min/+}$ mice of both sexes: the intensity in mice given tap water was lower than those treated with DSS.

β-Catenin staining in adenoma cells showed positive in their cell membrane and/or a few nuclei (Fig. 3*a*) in $Apc^{Min/+}$ mice treated with 2% DSS. Strong β-catenin expression was observed in the nucleus and cytoplasm of adenocarcinoma cells (Fig. 3*b*) in $Apc^{Min/+}$ mice given 2% DSS. The intensity of β-catenin staining in adenoma cells was relatively weak when compared to carcinoma cells. β-Catenin immunoreactivity was also observed in the cell membrane, cytoplasm, and a few nuclei of dysplastic cells (Fig. 3*c*). Nonlesional cryptal cells showed weak positivity of bgr;-catenin in their cell membrane. In addition, a positive reaction against β-catenin antibody was noted in the vascular endothelium, infiltrated inflammatory cells and ganglion cells in Auerbach's plexus.

Strong COX-2 immunoreactivity was present in adenoma (Fig. 3*d*) and adenocarcinoma cells (Fig. 3*e*) in their cytoplasm in $Apc^{Min/+}$ mice treated with 2% DSS. Dysplastic cells (Fig. 3*f*) showed relatively strong positivity for COX-2 when compared to neoplastic cells. Nonlesional cryptal cells at the lower part of crypts were weakly positive for COX-2, while strongly positive reaction of COX-2 was seen in the endothelium of small blood vessels and inflammatory cells infiltrated in the lamina propria. Smooth muscle cells and fibroblasts in inflamed large bowel showed weak reaction of COX-2.

iNOS-immunohistochemistry showed strong immunoreactivity in the cytoplasm of adenoma (Fig. 3*g*) and adenocarcinoma cells (Fig. 3*h*) in $Apc^{Min/+}$ mice given 2% DSS: the intensity was greater in carcinoma cells when compared to adenoma

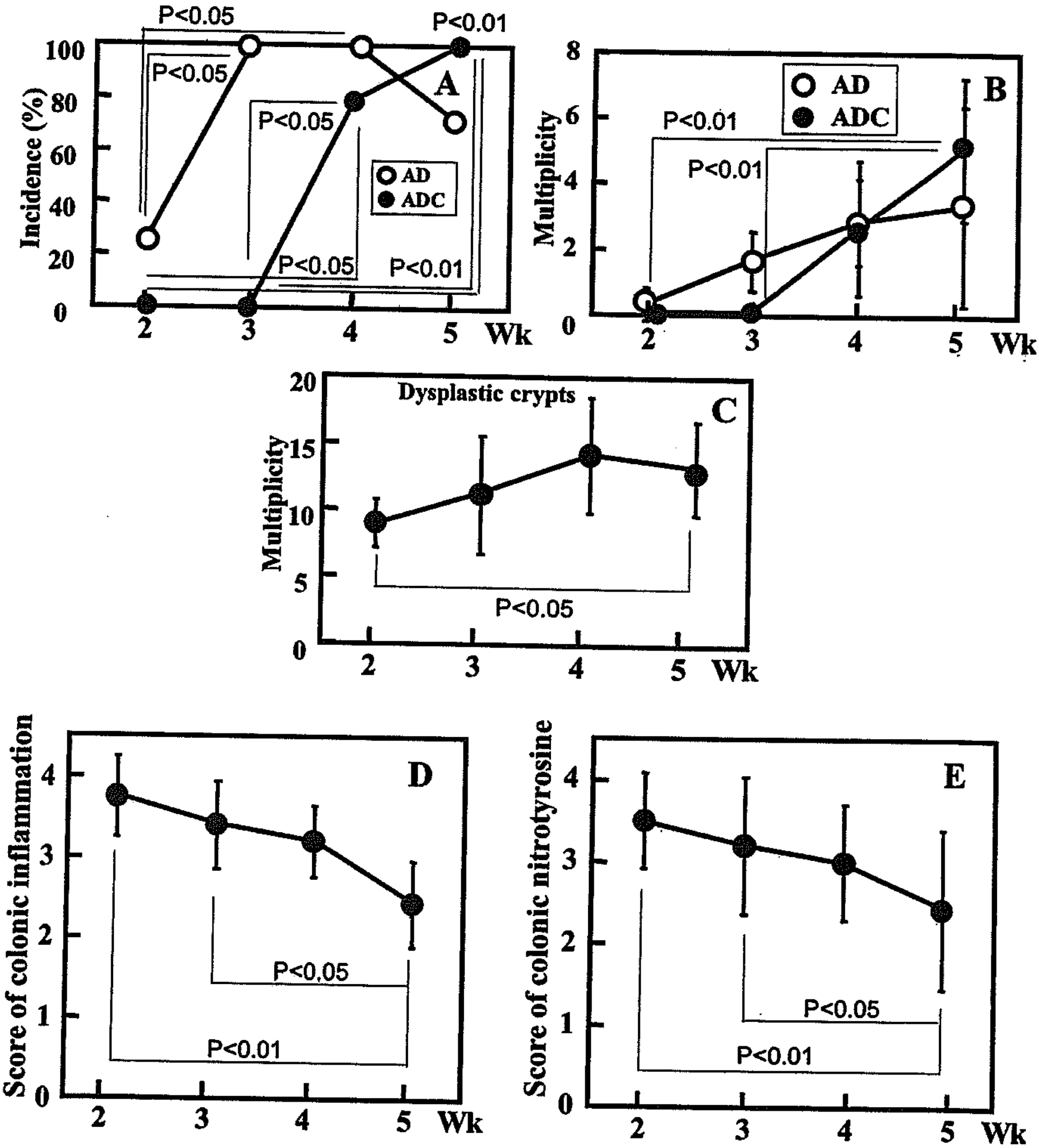

FIGURE 2 – Time-course observation of colonic lesions in female $Apc^{Min/+}$ mice treated with 2% DSS. The incidence (*a*) and multiplicity (*b*) of colonic neoplasms and the multiplicity (*c*) of colonic dysplastic crypt were gradually increased with time. Scores of colonic inflammation (*d*) and nitrotyrosine-positivity (*e*) gradually decreased after the cessation of DSS treatment with time. AD and ADC refer to adenoma and adenocarcinoma, respectively. Data represent mean±SD ($n = 4$ mice at week 2, 5 mice at week 3, 5 mice at week 4, and 8 mice at week 5). Statistical significance of differences was evaluated by Fisher's exact probability test (*a*) or one-way ANOVA with Bonferroni correction (*b–e*). Statistical significances of the squared correlation coefficients were found for the multiplicity of adenoma ($r = 0.9817$, $p < 0.05$), inflammation score ($r = -0.9618$, $p < 0.05$), and nitrotyrosine positive score ($r = -0.9764$, $p < 0.05$).

cells. Also, dysplastic cells (Fig. 3*i*) were positive for iNOS in their cytoplasm and the intensity was relatively greater than neoplastic cells. The faint positive reaction was found in the cytoplasm of nonlesional cryptal cells. Immunohistochemical iNOS expression was strong in the endothelial cells of small blood vessels and inflammatory cells in the lamina propria. COX-2- and iNOS-stained inflammatory cells were also frequently observed in the mucosa.