RESEARCH COMMUNICATION

Preventive Effects of a Flavonoid Myricitrin on the Formation of Azoxymethane-induced Premalignant Lesions in Colons of Rats

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Abstract

   The preventive effect of dietary exposure to a flavonoid myricitrin of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and beta-catenin-accumulated crypts (BCAC) formation was investigated in male F344 rats. Thirty-four rats were divided randomly into five experimental groups. Rats in groups 1-3 were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for 3 weeks. Starting 1 week before the first injection of AOM, rats in groups 2 and 3 were fed a diet containing 500 or 1000 ppm myricitrin, respectively, for 11 weeks. Rats in group 4 were fed a diet containing 1000 ppm myricitrin. Rats in groups 1 and 5 were given the basal diet alone during the study. The experiment was terminated 11 weeks after the start. The frequency of ACF per colon in group 3 treated with AOM and 1000 ppm myricitrin was significantly lower than that in group 1 treated with AOM alone ($p<0.01$). Furthermore, dietary myricitrin at both doses (groups 2 and 3) significantly inhibited the formation of BCAC when compared to group 1 ($p<0.05$). These results indicate that myricitrin had possible chemopreventive effects in the present short-term colon carcinogenesis bioassays and suggest that longer exposure may cause suppression of tumor development.

Key words: Myricitrin - beta-catenin accumulated crypts - aberrant crypt foci – prevention - colon cancer.

Introduction

   Colorectal cancer is the third most malignant neoplasm in the world (Landis et al., 1999). Although the cause of it is not completely understood, dietary factors appear to be important for the development of colorectal cancer (Willett, 1989). One such example is that the progressive introduction of Western dietary habits in Japan, especially an increasing fat intake and decreasing carbohydrate intake, has increased the incidence of colorectal cancer (Weisburger, 1991). Some epidemiological data also indicate that the increased consumption of vegetables and fruits is consistently associated with a low risk of colorectal cancer (Hebert et al., 1993; Block et al., 1992; Boyle et al., 1985). Generally speaking, most foods we daily consume consist of macro- and micronutrients, some of which have anti-tumorigenic effects as well as mutagenic and/or carcinogenic (Ames, 1983). Currently, many studies have been directed to identifying such preventive agents against cancer development, especially those that are found in natural foods (Kelloff et al., 1994). As making use of them, a primary preventive approach against cancer

development, including chemoprevention, is now regarded as an effective and promising strategy (Henderson et al., 1991).

   Myricitrin (myricetin-3-O-a-L-rhamnopyranoside), a kind of flavonoid, is a rhamnose glycoside of myricetin consisting in various plants. From the biological point of view, myricitrin is reported to display high antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl assay (Luo, 2002; Zhong et al., 1997).). In addition, the mutagenicity induced by tert-butyl hydroperoxide or cumene hydroperoxide in Salmonella typhimurium TA102 was effectively reduced by myricitrin, suggesting its anti-mutagenic effects (Edenharder and Grunhage, 2003). These interesting findings in vitro suggest the possible application of the compound to chemopreventive approaches against cancer. Actually, flavonoids deserve special attention among the candidates of the chemopreventive compounds because they are present practically in all dietary plants, fruits and roots, consumed daily in considerable amounts and are heat stable and nontoxic (Kleijnen and Knipschild,

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1992; McGregor, 1998). Besides anti-carcinogenic and anti-mutagenic, they also show a variety of favorable biological properties, such as anti-allergic, anti-inflammatory, scavenging of free radicals, or modulation of enzymatic activities, to varying degrees (Robak and Gryglewski, 1988; Stavric, 1994).

Many, but not all, of the animal studies provided experimental evidence on the assumption that aberrant crypt foci (ACF) might be a putative preneoplastic lesion in colon (Bird, 1995; Corpet and Tache, 2002; Pretlow et al., 1991). Recently, focal lesions that have accumulation of beta-catenin protein and frequent beta-catenin gene mutations, termed beta-catenin-accumulated crypts (BCAC), have been detected in azoxymethane (AOM)-exposed colonic mucosa of rats (Yamada et al., 2000; Yamada et al., 2001). They are considered to be an independent precursor lesion of AOM-induced colon carcinogenesis. Recently, many short-term bioassays using animal models have utilized ACF and/or BCAC as valuable intermediate biomarkers of colon carcinogenesis (Corpet and Tache, 2002; Hirose et al., 2003). In particular, these are useful to examine possible chemopreventive effects of a wide variety of candidate agents on colon carcinogenesis.

In the present study, possible chemopreventive effects of myricitrin on colorectal carcinogenesis initiated with AOM were investigated in male F344 rats by determining the incidences of two different intermediate biomarkers, ACF and BCAC.

Material and Methods

Animals, diets and carcinogen

Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) aged 4 weeks were used. All animals were housed in wire cages (three or four rats per cage) with free access to drinking water and diets, under controlled conditions of humidity (50+/-10%), lighting (12 h light/dark cycle) and temperature (23+/-3˚C). They were quarantined for 14 days and randomized into experimental and control groups. Powdered CE-2 (Clea Japan, Tokyo, Japan) diet was used as basal diet throughout the study. Myricitrin was obtained from Funakoshi (Tokyo, Japan) and its purity (over 99%) was measured by high-performance liquid chromatography. The experimental diet was made by mixing CE-2 with myricitrin at the concentration of 500 or 1000 ppm. The experimental diet was stored in a cold room (4Åé) until used. AOM was obtained from Sigma Chemical Co. (St Louis, MO, USA) and given by subcutaneous injection (15mg/kg body weight) between 10:00 and 11:00 a.m. to produce colorectal preneoplastic lesions.

Experimental procedure

A total of 34 male F344 rats were divided into five groups as shown in Figure 1. Groups 1-3 were initiated with AOM by three weekly subcutaneous injections. Rats in groups 2 and 3 were fed the diet containing 500 and 1000 ppm of myricitrin, respectively, through the experiment, starting 1 week before the first dosing of AOM. Group 4 was fed the diet of 1000 ppm myricitrin during the study. Groups 1 and 5 were given basal diet alone

throughout the study. When the experiment was terminated, all animals were sacrificed to assess the incidences of ACF and BCAC in the large bowel.

Identification of ACF

After fixation of the resected colons in 10% buffered formalin for at least 24 h at room temperature, they were stained in 0.5% methylene blue (in saline) for 1-3 min. After the staining, ACF were counted and recorded according to the procedure described previously (Bird, 1987). In this study, we divided the colon into three portions (proximal, middle and distal) and used middle and distal colon for the analysis.

Identification of BCAC

After ACF counting, colonic tissues were embedded in paraffin and processed for evaluation of BCAC. Briefly, serial sections (4mm thick) were prepared to include the mucosal part between the surface and the bottom of the crypt. Some of these sections were used for immunohistochemistry of beta-catenin and routine hematoxylin & eosin staining. Immunohistochemistry was performed with treatment of sections in 3% H_2O_2 for 20 min to block the endogenous peroxidase activity and incubated them with a primary antibody of the beta-catenin protein (1:100 dilution, Transduction Laboratories, Lexington, KY, USA) at room temperature for 60 min. Then, the sections were routinely stained using a LSAB kit (DAKO, Kyoto, Japan).

Statistical analysis

Statistical analysis of the data on weights (total body, liver and kidney) and incidence and multiplicity of the lesions was carried out by Student's t-test. The results were considered statistically significant if the P value was less than 0.05.

Results

General observations

The rats were tolerated well the AOM injection and myricitrin feeding. During the study, no clinical signs of toxicity were present in any group. There is no significant difference in the weights of total body, liver and kidney between groups (Table 1). Histologically there were no pathological alterations indicative of toxicity of myricitrin in major organs, such as liver or kidney.

Inhibitory effects of myricitrin on ACF and BCAC

All rats in groups 1-3 developed ACF and BCAC in the colonic mucosa. There were no ACF and BCAC seen in any of the rats of groups 4 and 5. The number of ACF in AOM–treated rats fed 1000 ppm myricitrin diet was significantly lower than that in rats treated AOM alone (p<0.01; Table 2). In groups 2 and 3, the treatment of rats with both dose levels of myricitrin caused a significant decrease in mean numbers of BCAC/cm^2 colon, when compared with the control rats treated with AOM alone (group 1) (p<0.05; Table 3). These results indicate that dietary myricitrin significantly inhibited the ACF and BCAC formation induced by AOM in F344 rats.

Table 1. Body, Liver and Kidney Weights in Each Group

Group	Body weight (g)	Liver weight (g)	Kidney weight (g)	Relative liver weight (g)	Relative kidney weight (g)
1	224.0±21.2a	6.50±0.71	2.20±0.42	2.91±0.28	0.99±0.20
2	227.8±21.6	6.88±0.83	2.25±0.46	3.02±0.31	0.99±0.21
3	241.5±23.8	7.38±0.74	2.13±0.35	3.07±0.31	0.89±0.19
4	240.5±19.7	6.25±0.50	2.25±0.50	2.62±0.41	0.93±0.16
5	233.0±12.3	7.00±0.82	2.13±0.25	3.02±.44	0.91±0.06

a Mean±SD.

Table 2. Incidence and Multiplicity of ACF

Group	Treatment	No. of rats	No. of AC /colon	No. of crypts /ACF
1	AOM alone	10	240.1±74.2[a]	3.2±1.6
2	AOM+500ppm myricitrin	8	194.1±52.6	3.0±1.3
3	AOM+1000ppm myricitrin	8	143.8±44.1[b]	3.2±1.4
4	1000ppm myricitrin	4	0	-
5	No treatment	4	0	-

[a]Mean±SD. [b]Significantly different from group 1 by Student's t-test, P<0.01.

Table 3. Incidence and Multiplicity of BCAC

Group	Treatment	No. of rats	No. of AC /colon	No. of crypts /ACF
1	AOM alone	10	1.19±0.41[a]	3.2±0.8
2	AOM+500ppm myricitrin	8	0.78±0.32[b]	3.9±0.9
3	AOM+1000ppm myricitrin	8	0.73±0.43[b]	3.9±0.5
4	1000ppm myricitrin	4	0	-
5	No treatment	4	0	-

[a]Mean±SD. [b]Significantly different from group 1 by Student's t-test, P<0.05.

Discussion

The results in the current study clearly indicate that dietary feeding of flavonoid myricitrin effectively suppressed the occurrence of colonic lesions ACF and BCAC induced by colon carcinogen AOM when administered during and after the carcinogen treatment. ACF that Bird described originally in the unsectioned murine colon exposed to AOM have been widely used as an intermediate biomarker of colon carcinogenesis in the experimental animal models (Bird, 1987). BCAC, which we found histologically in the colons of rodents exposed to AOM, are dysplastic crypts with beta-catenin accumulation (Yamada et al., 2000). Sequential analysis indicates that BCAC have similar properties in histology and biology to colon tumors in animal models, suggesting BCAC as a precursor of colon tumors (Yamada et al., 2001). Moreover, BCAC are suggested to be a better biomarker than ACF, since BCAC appear to reflect the tumor outcomes of the corresponding long-term studies more sensitively and correctly than those of ACF (Hirose et al., 2005; Hirose et al., 2003). Importantly, BCAC and ACF are regarded as distinct and independent each other morphologically and biologically (Mori et al., 2004). That means that the preventive potentials against AOM-induced colon tumor development by dietary myricitrin were corroborated by the assays with two independent biomarkers. In addition, there was no toxicity or adverse effect caused by dietary myricitrin in the assays. Collectively, these results warrant this safe compound with potent tumor-preventive effects to be applied to a chemopreventive trial against colon cancer of human.

As regards the mechanisms, it is largely unknown how myricitrin is metabolized and which function(s) it modulates in colonic mucosa, resulting in the tumor-preventive action in vivo. It is reported that dietary myricitrin is absorbed in an intestine via the paracellular pathway in a time- and concentration-dependent manner, even after degradation by intestine environment (Yokomizo and Moriwaki, 2005a). Interestingly, myricitrin prepared under the same mildly alkaline conditions as those in the human intestines is shown to have a strong inhibitory effect on the low density lipoprotein oxidation induced by radical scavenging and metal-ion chelation (Yokomizo and Moriwaki, 2005b). Such antioxidative activity that some of the flavonoids have is sometimes involved in chemopreventive potential against tumorigenesis including colon (Ju et al., 2005; Suzuki et al., 2004; Nomoto et al., 2004). Furthermore, myricitrin is reported to suppress glyoxalase I activity from yeast and bovine liver. (Iio et al., 1983) Glyoxalase I converts methylglyoxal to D-lactic acid via an S-D-lactoylglutathione intermediate and its activity is higher in cancer cell lines from various tissues including colon. Interestingly, this enzyme overexpressed in the apoptosis-resistant tumor cells (Tsuruo et al., 2003), suggesting that it may promote carcinogenesis by inhibiting apoptosis. In addition, Kuntz et al. (1999) reported that myricetin, aglycon of myricitrin, could control the growth of HT-29 human colon cancer cell line. Accordingly, it is possible that dietary myricitrin might exert some of such effects as described above on the colonic mucosa, causing cancer prevention.

In conclusion, the results of our experiment clearly showed preventive effects of dietary myricitrin on the formation of AOM-induced colonic premalignant lesions in the short term bioassay using rats. Thus, myricitrin is suggested to be a putative chemopreventive agent against human colonic cancers and warrants further analysis.

Acknowledgements

References

Ames BN (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science*, **221**, 1256-64.

Bird RP (1995). Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett*, **93**, 55-71.

Bird RP (1987). Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett*, **37**, 147-51.

Block G, Patterson B, Subar A (1992). Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer*, **18**, 1-29.

Boyle P, Zaridze DG, Smans M (1985). Descriptive epidemiology of colorectal cancer. *Int J Cancer*, **36**, 9-18.

Corpet DE, Tache S (2002). Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer*, **43**, 1-21.

Edenharder R, Grunhage D (2003). Free radical scavenging abilities of flavonoids as mechanism of protection against mutagenicity induced by tert-butyl hydroperoxide or cumene hydroperoxide in Salmonella typhimurium TA102. *Mutat Res*, **540**, 1-18.

Hebert JR, Landon J, Miller DR (1993). Consumption of meat and fruit in relation to oral and esophageal cancer: a cross-national study. *Nutr Cancer*, **19**, 169-79.

Henderson BE, Ross RK, Pike MC (1991). Toward the primary prevention of cancer. *Science*, **22**, 1131-8.

Hirose Y, Kuno T, Sheng HQ, et al (2005). Beta-catenin-accumulated crypts (BCAC) or aberrant crypt foci (ACF): which biomarker is more reliable? In "Carcinogenesis and modification of carcinogenesis" Eds Tanaka T and Tsuda H. Research Signpost, Kerala, pp 129-34.

Hirose Y, Kuno T, Yamada Y, et al (2003). Azoxymethane-induced beta-catenin-accumulated crypts in colonic mucosa of rodents as an intermediate biomarker for colon carcinogenesis. *Carcinogenesis*, **24**, 107-11.

Iio M, Himeno S, Miyauchi K, et al (1983). Effect of flavonoids on gloxalase I Activity. *Nippon Nogeikagaku Kaishi (Japanese)*, **57**, 765-9.

Ju J, Hong J, Zhou JN, et al (2005). Inhibition of intestinal tumorigenesis in Apcmin/+ mice by (-)-epigallocatechin-3-gallate, the major catechin in green tea. *Cancer Res*, **65**, 10623-31.

Kelloff GJ, Boone CW, Crowell JA, et al (1994). Chemopreventive drug development: perspectives and progress. *Cancer Epidemiol Biomarkers Prev*, **3**, 85-98.

Kleijnen J, Knipschild P (1992). *Ginkgo biloba. Lancet*, **34**, 1136-9.

Kuntz S, Wenzel U, Daniel H (1999). Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur J Nutr*, **38**, 133-42.

Landis SH, Murray T, Bolden S, et al (1999). Cancer Statistics. *CA Cancer J Clin*, **49**, 8-31.

Luo XD, Basile MJ, Kennelly EJ (2002). Polyphenolic antioxidants from the fruits of *Chrysophyllum cainito* L. (Star Apple). *J Agric Food Chem*, **50**, 1379-82.

McGregor D (1998). Diets, food components and human cancer. *Biotherapy*, **11**, 189-200.

Mori H, Yamada Y, Kuno T, et al (2004). Aberrant crypt foci and beta-catenin accumulated crypts; significance and roles for colorectal carcinogenesis. *Mutat Res*, **566**, 191-208.

Nomoto H, Iigo M, Hamada H, et al (2004). Chemoprevention of colorectal cancer by grape seed proanthocyanidin is accompanied by a decrease in proliferation and increase in apoptosis. *Nutr Cancer*, **49**, 81-8.

Pretlow TP, Barrow BJ, Ashton WS, et al. (1991). Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res*, **51**, 1564-7.

Robak J, Gryglewski RJ (1988). Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol*, **37**, 837-41.

Suzuki R, Kohno H, Murakami A, et al. (2004). *Citrus nobiletin* inhibits azoxymethane-induced large bowel carcinogenesis in rats. *Biofactors*, **22**, 111-4.

Stavric B (1994). Role of chemopreventers in human diet. *Clin Biochem*, **27**, 319-32.

Tsuruo T, Naito M, Tomida A, et al. (2003). Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Sci*, **94**, 15–21.

Weisburger JH (1991). Nutrition approach to cancer prevention with emphasis on vitamin, antioxidants and carotenoids. *Am J Clin Nutr*, **53**, 226s-37s.

Willett W (1989). The search for the causes of breast and colon cancer. *Nature*, **338**, 389-94.

Yamada Y, Yoshimi N, Hirose Y, et al (2001). Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res*, **61**, 1874-8.

Yamada Y, Yoshimi N, Hirose Y, et al. (2000). Frequent beta-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. *Cancer Res*, **60**, 3323-7.

Yokomizo A, Moriwaki M (2005a). Transepithelial permeability of myricitrin and its degradation by simulated digestion in human intestinal caco-2 cell monolayer. *Biosci Biotechnol Biochem*, **69**, 1774-6.

Yokomizo A, Moriwaki M (2005b). Myricitrin degraded by simulated digestion inhibits oxidation of human low-density lipoprotein. *Biosci Biotechnol Biochem*, **69**, 693-9.

Zhong X, Otsuka H, Ide T, et al. (1997). Three flavonol glycosides from leaves of Myrsine seguinii. *Phytochemistry*, **46**, 943-6.