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

Nami Asano¹, Toshiya Kuno², Yoshinobu Hirose¹*, Yasuhiro Yamada¹, Koujirou Yoshida¹, Hiroyuki Tomita¹, Yoshiyuki Nakamura³, Hideki Mori¹

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,
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 (4Â°C) 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 neoplasms. 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.

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% H2O2 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/cm2 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Kidney weight (g)</th>
<th>Relative liver weight (g)</th>
<th>Relative kidney weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>224.0±21.2a</td>
<td>6.5±0.71</td>
<td>2.2±0.42</td>
<td>2.91±0.28</td>
<td>0.99±0.20</td>
</tr>
<tr>
<td>2</td>
<td>227.8±21.6</td>
<td>6.88±0.83</td>
<td>2.25±0.46</td>
<td>3.02±0.31</td>
<td>0.99±0.21</td>
</tr>
<tr>
<td>3</td>
<td>241.5±23.8</td>
<td>7.38±0.74</td>
<td>2.13±0.35</td>
<td>3.07±0.31</td>
<td>0.89±0.19</td>
</tr>
<tr>
<td>4</td>
<td>240.5±19.7</td>
<td>6.25±0.50</td>
<td>2.25±0.50</td>
<td>2.62±0.41</td>
<td>0.93±0.16</td>
</tr>
<tr>
<td>5</td>
<td>233.0±12.3</td>
<td>7.00±0.82</td>
<td>2.13±0.25</td>
<td>3.02±0.44</td>
<td>0.91±0.06</td>
</tr>
</tbody>
</table>

*a Mean±SD.

Table 2. Incidence and Multiplicity of ACF

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of AC /colon</th>
<th>No. of crypts /ACF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>10</td>
<td>240.1±74.2</td>
<td>3.2±1.6</td>
</tr>
<tr>
<td>2</td>
<td>AOM+500ppm myricitrin</td>
<td>8</td>
<td>194.1±52.6</td>
<td>3.0±1.3</td>
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<tr>
<td>3</td>
<td>AOM+1000ppm myricitrin</td>
<td>8</td>
<td>143.8±44.1</td>
<td>3.2±1.4</td>
</tr>
<tr>
<td>4</td>
<td>1000ppm myricitrin</td>
<td>4</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>No treatment</td>
<td>4</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

*a Mean±SD.

Table 3. Incidence and Multiplicity of BCAC

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of AC /colon</th>
<th>No. of crypts /ACF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>10</td>
<td>1.19±0.41</td>
<td>3.2±0.8</td>
</tr>
<tr>
<td>2</td>
<td>AOM+500ppm myricitrin</td>
<td>8</td>
<td>0.78±0.32</td>
<td>3.9±0.9</td>
</tr>
<tr>
<td>3</td>
<td>AOM+1000ppm myricitrin</td>
<td>8</td>
<td>0.73±0.43</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>4</td>
<td>1000ppm myricitrin</td>
<td>4</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>No treatment</td>
<td>4</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

*a Mean±SD.*Significantly different from group 1 by Student’s t-test, P<0.01.

Discussion

The results in the current study clearly indicate that dietary feeding of flavonoid myricitrin effectively suppressed the occurrence of colon 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., 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 glyceraldehyde I activity from yeast and bovine liver. (Iio et al., 1983) Glyceraldehyde 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. Importantly, it is reported that dietary myricitrin is metabolized and which function(s) it modulates in colonic mucosa, resulting in the tumor-preventive effects to be applied to a chemopreventive trial against colon cancer of human.

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

Acknowledgements

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References

Nami Asano et al


