Chemopreventive effects of silymarin against 1,2-dimethylhydrazine plus dextran sodium sulfate-induced inflammation-associated carcinogenicity and genotoxicity in the colon of gpt delta rats

Naomi Toyoda-Hokaiwado, Yumiko Yasui, Mina Muramatsu, Kenichi Masumura, Makiko Takamune, Masami Yamada, Toshihiro Ohta, Takuji Tanaka, and Takehiko Nohmi

Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan. 1Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa 920-2093, Japan. 2School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan. 3Department of Physiology and Nutrition, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido 060-8501, Japan and 4The Tokai Cytopathology Institute: Cancer Research and Prevention, Gifu 500-8285, Japan.

*To whom correspondence should be addressed. Tel: +81 3 3700 9872; Fax: +81 3 3700 2348; Email: nohmi@nih.go.jp

Silymarin, a natural flavonoid from the seeds of milk thistle, is used for chemoprevention against various cancers in clinical settings and in experimental models. To examine the chemopreventive mechanisms of silymarin against colon cancer, we investigated suppressive effects of silymarin against carcinogenicity and genotoxicity induced by 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS) in the colon of F344 gpt delta transgenic rats. Male gpt delta rats were given a single subcutaneous injection of 40 mg/kg DMH and followed by 1.5% DSS in drinking water for a week. They were fed diets containing silymarin for 4 weeks, starting 1 week before DMH injection and samples were collected at 4, 20 and 32 weeks after the DMH treatment. Silymarin at doses of 100 and 500 p.p.m. suppressed the tumor formation in a dose-dependent manner and the reduction was statistically significant. In the mutation assays, DMH plus DSS enhanced the gpt mutant frequency (MF) and the silymarin treatments reduced the MFs by 20%. Silymarin also reduced the genotoxicity of DMH in a dose-dependent manner in bacterial mutation assay with Salmonella typhimurium YG7108, a sensitive strain to alkylating agents (21,22), to examine whether silymarin inhibits genotoxicity of DMH and its metabolite AOM in vivo. From the results, we conclude that silymarin suppresses the inflammation-associated colon carcinogenesis and suggest that the antigenotoxic property contributes to the chemopreventive effects at least partly.

Materials and methods

Introduction

Silymarin, an extract from the milk thistle fruit (Silibum marianum, Family Asteraceae), has been utilized for remedy of liver diseases such as cirrhosis or hepatitis for many years (1). Silymarin is actually the collective name of the extract and composed of at least seven flavonolignans and one flavonoid, and silybin is the major active constituent (2,3). Silymarin inhibits proliferation of various cancer cells and reduces carcinogenesis in various animal models (4,5). Therefore, it has been used in the experimental therapy of cancer and chemoprevention and even in human clinical trials. Because silymarin possesses a variety of biological properties, such as antioxidant and anti-inflammatory activities, induction of phase II enzymes and apoptosis (1), it may suppress cancer development via multiple mechanisms. However, few studies that evaluate antigenotoxic properties of silymarin are available and the contribution to the chemopreventive effects remains elusive.

In this study, we explored the antigenotoxic and chemopreventive effects of silymarin in the colon of rats. We chose colon because silymarin is highly distributed in colon mucosa when it is administered to humans orally (6) and the colon cancer is one of the most frequent human cancers worldwide (7). In fact, silymarin inhibits growth of colorectal carcinoma cells in vitro (8,9) and suppresses colon carcinogenesis induced by methylating agents in vivo (10,11). The anti-inflammatory and anticancer effects in chemically induced and spontaneous intestinal carcinogenesis in mice are also reported (12,13). To evaluate the antigenotoxic and anticarcinogenic properties, we employed F344 gpt delta transgenic rats treated with 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS). DMH and its metabolite azoxymethane (AOM) are potent genotoxic agents and the following treatment with a non-genotoxic agent, i.e. DSS, strongly induces inflammation in the colon, thereby enhancing colon carcinogenesis in mice (14,15) and rats (16,17). gpt delta transgenic rats carry approximately five copies of λE1G10 DNA at a single site in the chromosome 4 (18). The λDNA carries reporter genes for in vivo mutagenesis, and thus point mutations and deletions can be identified in any organs of rats at the sequence levels (19,20). Because the transgene is not expressed in vivo, the transgenic rats are expected to display very similar sensitivity to chemical carcinogens to non-transgenic F344 rats. We also conducted bacterial mutation assay with Salmonella typhimurium YG7108, a sensitive strain to alkylating agents (21,22), to examine whether silymarin inhibits genotoxicity of DMH and its metabolite AOM in vitro. From the results, we conclude that silymarin suppresses the inflammation-associated colon carcinogenesis and suggest that the antigenotoxic property contributes to the chemopreventive effects at least partly.

Materials and methods

Materials

DMH and silymarin (silymarin group, a mixture of isomers, molecular weight = 482-442) were purchased from Sigma-Aldrich Co. (St Louis, MO), AOM, N-methyl-N-nitrosurea (MNU) and dimethyl sulfoxide were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Animals, diet and housing conditions

Male 6-week-old F344 gpt delta transgenic rats (20) were obtained from Japan SLC and housed three or four animals per polycarbonate cage under specific pathogen-free standard laboratory conditions: room temperature, 23 ± 2°C; relative humidity, 60 ± 5%; with a 12:12 h light–dark cycle and free access to Charles River formula-1 basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water.

Treatments of animals

The protocol for this study was approved by the Animal Care and Utilization Committee of Kanazawa Medical University. One-hundred rats were randomly divided into seven groups (Figure 1). Groups 1–4 received single subcutaneous injection of DMH (40 mg/kg body wt). Groups 5–7 received no injections. One week after the carcinogen treatment, Groups 1–3 and 5 were treated with 1.5% DSS in drinking water for a week. Groups 4, 6 and 7 had just drinking water instead of 1.5% DSS solution. Groups 2, 3 and 6 were fed diets containing 100 or 500 p.p.m. silymarin for 4 weeks, starting 1 week before DMH injection. Group 7 served as an untreated control. All rats were carefully observed for clinical welfare and weighed weekly and experimental diet consumptions were recorded. Animals were killed at 4 weeks (short), 20 weeks (medium) and the experiment was terminated at 32 weeks (long).

Histological analysis

At autopsy, liver, kidneys, spleen and intestine were macroscopically examined for the presence of pathologic lesions and then isolated. The intestine was...
excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. Colon were fixed in 10% buffered formalin and processed to hematoxylin- and eosin-stained sections. Neoplastic lesions of colorectal mucosa were histopathologically classified into dysplastic foci, adenomas and adenocarcinomas. At 4 weeks periods, 5 cm long colorectal tissues from distal segment were excised and frozen in liquid nitrogen for mutation assay. Then, colon were fixed in 10% buffered formalin and then processed for aberrant crypt foci analysis by conventional methods (23). One centimeter-long slice from stump was processed into serial paraffin sections by en face preparation and stained with hematoxylin and eosin and immunohistochemistry. Remaining tissues were routinely embedded in paraffin and hematoxylin and eosin stained and histopathologically examined by light microscopy. The histological analysis of β-catenin-accumulated crypts and ulcer was performed based on the criteria described previously (24–26). Tumor incidence (%) means number of rat with colon tumors per total number in the experimental group and tumor multiplicity indicates number of colon tumors per rat in the experimental group.

Immunohistochemical procedures
Paraffin sections of colon were immunostained with a polyclonal anti-β-catenin antibody. Antigen retrieval was carried out by autoclaving for 15 min in 10 mmol/L citrate buffer (pH 6.0). Immunohistochemical staining was done by the avidin–biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1:200. Sections were lightly counterstained with hematoxylin for microscopic examination.

DNA isolation, in vitro packaging and gpt mutation assay
High-molecular weight genomic DNA was extracted from the colon using the RecoverEase DNA Isolation Kit (Stratagene by Agilent Technologies, Santa Clara, CA). Legion phages were rescued using Transpack Packaging Extract (Stratagene). The gpt assay was conducted according to previously published methods (27,28). The mutant frequencies (MFs) of the gpt gene (gpt MFs) in the colon were calculated by dividing the number of confirmed 6-thioguanine (6-TG)-resistant colonies by the number of rescued plasmids. DNA sequencing of the gpt gene was performed with the BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems by Life Technologies, Carlsbad, CA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All of the confirmed gpt mutants recovered from the carcinogen-untreated colon and selected confirmed gpt mutants recovered from the carcinogen-treated colon (basically, 10 mutants per animal were analyzed) were sequenced; identical mutations from the same rat were counted as one mutant.

Bacterial reverse mutation test (Ames test)
The mutagenic activities of DMH and silymarin were assayed in a bacterial reverse mutation assay using S. typhimurium tester strains YG7108, as TA1535 but is <delta><gamma><sigma>ta (21,22). The test was conducted by the preincubation method with modification (29). Briefly, silymarin was dissolved in dimethyl sulfoxide and mixed with DMH or AOM, dissolved in distilled water. In the case of MNU, it was dissolved in dimethyl sulfoxide. The chemicals were mixed with overnight culture of YG7108 in the presence or the absence of S9 mix and incubated for 20 min at 37°C. The reaction mixture containing bacteria, an alkylating agent and silymarin was poured onto agar plates with soft agar and incubated for 2 days at 37°C. Assays were performed on triplicates.

Statistical analysis
The statistical significance of the difference in the value of MFs between treated groups and negative controls was analyzed by the Student’s t-test. A P value <0.05 denoted the presence of a statistically significant difference. Variances in values for body weight, organ weight and pathological data were examined by Tukey multiple comparison post-test using GraphPad InStat (GraphPad Software, La Jolla, CA) to compare the differences. The tumor incidence was examined by Yates x²-test.

Results
General conditions of animals
No marked clinical symptoms were observed during experimental periods. Body weight gain and food consumption were similar between each group. Final body weights at killing were not significantly different among groups (supplementary Tables I–III are available at Carcinogenesis Online).

Silymarin suppressed preneoplastic lesions
At 4 weeks necropsy, no obvious macroscopic changes were detected. Pathological findings are shown in Table I. Aberrant crypt foci developed in rats treated with DMH and DSS. The frequency of aberrant crypt foci/colon in Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500), which received DMH + DSS and silymarin at 100 and 500 p.p.m., respectively (Figure 1), was significantly lower than that of Group 1 (DMH/DSS), which received DMH + DSS alone (P < 0.001). The frequency of β-catenin-accumulated crypt was also reduced by dietary silymarin treatments (Group 2, P < 0.05; Group 3, P < 0.01).
P < 0.01). In addition, number of colon mucosal ulcer was significantly reduced in these groups (Group 2, Group 3, P < 0.001). On microscopic observation, major changes were observed in the distal colon.

**Gene mutation assay in vivo**

DMH treatments enhanced gpt MF in the colon 100 times over the control levels (Table II). Silymarin itself was non-genotoxic [Group 6 (S500) in Figure 1]. DSS treatments did not show marked effects on the MFs. The dietary administration of silymarin at 100 and 500 p.p.m. [Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500) in Figure 1] reduced the DMH-induced MF by 20%. Because of the large standard deviation, however, the difference was not statistically significant. Dominant base substitution induced by DMH treatment was G:C to A:T transitions and silymarin treatments did not change the mutation spectra substantially (supplementary Table IV is available at *Carcinogenesis* Online).

**The colon neoplasms were reduced by silymarin treatment**

The incidence and multiplicity of tumors at 20 and 32 weeks are shown in Tables III and IV, respectively. Most of adenomas and adenocarcinomas were observed in the distal colon. Although 20 week observation did not show any statistical significance between groups, the number of tumors per rat was significantly reduced by dietary silymarin administration in a dose-dependent manner at 32 weeks. In the silymarin-treated groups [Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500) in Figure 1], incidence and multiplicity of proliferative lesions were markedly reduced compared with Group 1 (DMH/DSS).

**Silymarin inhibited genotoxicity of DMH in S. typhimurium YG7108**

To further characterize the suppressive effects of silymarin against DMH-induced genotoxicity, bacterial mutation assay was performed. Silymarin itself was non-genotoxic either with or without S9 activation (Figure 2 and supplementary Figure S1 is available at *Carcinogenesis* Online).

**Table I. Pathological findings in colon (4 weeks)**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>No. of BCAC/rat</th>
<th>No. of ACF/rat</th>
<th>No. of foci containing</th>
<th>No. of foci containing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;4 crypts</td>
<td>≥4 crypts</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>4.50 ± 1.38a</td>
<td>3.33 ± 1.21</td>
<td>34.83 ± 1.40</td>
<td>27.17 ± 6.46</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>1.33 ± 1.21***</td>
<td>1.67 ± 1.03*</td>
<td>17.50 ± 2.51***</td>
<td>16.17 ± 2.64**</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.83 ± 0.75***</td>
<td>0.67 ± 0.82**</td>
<td>13.83 ± 3.82***</td>
<td>13.50 ± 4.04***</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

BCAC, β-catenin-accumulated crypt; ACF, aberrant crypt foci.

*Mean ± SD.

**, ***: Significantly different from group 1 at P < 0.05, P < 0.01 and P < 0.001, respectively, by Tukey multiple comparison post-test.

**Table II. gpt MF in colon (4 weeks)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>MF (×10^-6) (mean ± SD)</th>
<th>P value* (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td>557.7 ± 213.4 (100%)b</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td>423.2 ± 246.3 (75.9%)</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Silymarin 100 p.p.m.</td>
<td>6</td>
<td>457.7 ± 185.6 (82.1%)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Silymarin 500 p.p.m.</td>
<td>6</td>
<td>646.8 ± 231.1 (116%)</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>Silymarin 500 p.p.m.</td>
<td>4</td>
<td>9.8 ± 11.0 (1.8%)</td>
</tr>
<tr>
<td>6</td>
<td>Silymarin 500 p.p.m.</td>
<td>4</td>
<td>5.3 ± 5.2 (1.0%)</td>
<td>0.1346</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>–</td>
<td>4</td>
<td>5.6 ± 6.4 (1.0%)</td>
</tr>
</tbody>
</table>

*P values were calculated by the Student’s t-test.

bPercentage of the MF of each Group against that in Group 1.

**Discussion**

In this study, silymarin inhibited colon cancer development significantly, suggesting that it might be a quite efficient chemopreventive agent. Previously, the efficacy of silymarin against colon carcinogenesis was reported in several animal models (10–13). Kohno et al. (10) report that oral administration of silymarin enhances glutathione S-transferase activity in liver. In addition, cell proliferation in colonic mucosa is reduced and apoptosis is significantly increased by silymarin administration. Here, we reported that tumor number and incidences were greatly reduced and the MF induced by DMH was reduced by the silymarin treatments (Tables II–IV). It suggests that the antigenotoxic efficiency might contribute to the tumor reduction at least partly in vivo.

In *in vivo* situation, DMH is first oxidized to azomethane, which appears in the exhaled air of DMH-treated animals (30). Azomethane is oxidized to AOM, which is hydroxylated to methylazoxymethanol (MAM). AOM and MAM are also detected in the urine. MAM is unstable and decomposes to methylidyazonium, which is a highly reactive methylating intermediate (30). DMH and its metabolites, i.e. AOM and MAM, are potent carcinogens that induced colorectal carcinomas in rodent. Here, we revealed that silymarin was clearly antigenotoxic against potent alkylating carcinogens DMH, AOM and MNU *in vitro* (Figure 2). Silymarin showed similar inhibitory effects against DMH-induced genotoxicity with or without S9 mix
supplementary Figure S1 is available at Carcinogenesis Online. It suggests that the antigenotoxic efficacy is not reduced by the metabolism in vivo. Nevertheless, the efficacy of antigenotoxic activity of silymarin was less pronounced in vivo than in vitro. For the bacterial mutation assays, each chemical and silymarin were directly mixed in the medium. Therefore, we speculate that the route of exposure, i.e. oral administration in rats, and the effective concentration of silymarin in the colon might account for the different efficacy between in vivo and in vitro.

When DMH is administrated by single subcutaneous injection, N7-methylguanine and O6-methylguanine are detected in colon, kidney and liver in mice (31). O6-Methylguanine DNA adduct is a potent...
detrimental lesion for colorectal cancer and induces G:C to A:T transitions. The levels of O\textsuperscript{6}-methylguanine are highly distributed in the distal colon by DMH treatment (32) and histologically altered crypts often have β-catenin gene mutations (33). In the present study, the dominant base substitution in the gpt gene induced by DMH treatment was G:C to A:T transitions (supplementary Table IV is available at Carcinogenesis Online) and most of the tumors developed in distal area. To induce DNA mutations, DNA replication is required. Silymarin is known to inhibit cancer cell proliferation and induce apoptosis (8). Hence, the inhibitory effects on cell proliferation might play roles in the reduction of genotoxicity and carcinogenicity in the colon of silymarin-treated rats (Tables II–IV).

Silymarin is also reported to possess the anti-inflammatory activity (1). DMH initiation followed by DSS modification model is an established medium-term colorectal bioassay for mice (14,15) and rats (16,17). DSS induced massive inflammation on colonic mucosa by drinking administration (14,34). Under the inflammatory environment, infiltrating mast cells produced genotoxic superoxide anions. In this study, genotoxicity was not induced by DSS treatments (Table II). However, in the silymarin-treated groups, the number of colorectal mucosal tumors was reduced in a dose-dependent manner of silymarin (Tables III and IV). Oral administrated silymarin might prevent mucosal tumors was reduced in a dose-dependent manner of silymarin (14,34).

In summary, the current study revealed the antigenotoxic potency of silymarin against alkylating agents, and suggests that the antigenotoxic efficiency along with its inhibitory effects on cell proliferation and inflammation might contribute to the effective tumor reduction in vivo. Our results also indicate that F344 gpt delta rats are useful for screening cancer chemopreventive compounds as well as environmental genotoxic carcinogens (20).

**Supplementary material**

Supplementary Tables I–IV and Figure S1 can be found at http://carcin.oxfordjournals.org/

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**References**


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