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Chemopreventive Effects of Scordinin on Diethylnitrosamine and Phenobarbital-induced Hepatocarcinogenesis in Male F344 Rats

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Modifying effects of scordinin, a biological active component in garlic, on diethylnitrosamine (DEN)- and phenobarbital (PB)-induced hepatocarcinogenesis were examined in rats. Male F344 rats, 5 weeks old, were divided into 8 groups. After a week, groups 1–5 were given DEN (100 mg/kg body weight, i.p.) once a week for 3 weeks, whereas groups 6–8 received vehicle treatment. Group 2 was given 600 ppm scordinin-containing diet in the initiation phase. From 4 weeks after the start of experiment, groups 3 and 5 were fed scordinin, and groups 1–3 and 7 received drinking water containing 500 ppm PB. Group 6 was given scordinin diet alone throughout the experiment (24 weeks). The incidences of hepatocellular adenoma and carcinoma were significantly smaller in group 3 than those in group 1 ($P<0.005$ and $P<0.05$, respectively). The average numbers of liver neoplasms in groups 2 and 3 were significantly smaller than in group 1 ($P<0.01$ and $P<0.0001$, respectively). Glutathione *S*-transferase placental form-positive foci were also significantly decreased by scordinin treatment in the initiation or promotion phase. Scordinin significantly decreased the mean number of nucleolar organizer regions' protein (AgNORs)/nucleus in hepatocellular adenoma and carcinoma. AgNORs/nucleus in the non-lesional area was also significantly decreased by scordinin treatment during the promotion phase. These results suggest that scordinin is a promising chemopreventive agent for liver neoplasia.

Key words: Scordinin — Chemoprevention — Hepatocarcinogenesis — GST-P — AgNORs

Epidemiological studies suggest that consumption of green and yellow vegetables is inversely related to cancer risk.[1–4] Organosulfur compounds that are present abundantly in a group of cruciferous vegetables or allium species have been shown to possess chemopreventive properties.[5] We have reported protective effects of organosulfur compounds such as benzyl isothiocyanate, benzyl thiocyanate, *S*-methylmethane thiosulfonate and taurine on hepatocarcinogenesis in animal models.[6–8]

Garlic (*Allium sativum*) and related allium foods have been widely used as a popular remedy for various ailments and physiological disorders.[9–11] Many organosulfur compounds have been purified from garlic and they have been reported to have antioxidant, antibiotic and detoxifying properties.[9–11] In epidemiological studies, protective activity of garlic has been proved in some organs.[12–16] Animal studies have also provided evidence that garlic and associated organosulfur compounds inhibit the development of neoplasms in skin, lung, stomach, esophagus, colon, mammary gland, liver and oral cavity.[17, 18] Garlic contains a complex mixture of allyl sulfur compounds that have been shown to afford protection against carcinogenesis. Diallyl sulfide (DAS) and diallyl disulfide (DADS), which are representative lipid-soluble allyl sulfur compounds present in garlic, have been extensively studied from the viewpoint of chemopreventive effects. On the other hand, enhancing effects of some organosulfur compounds in diethylnitrosamine (DEN)-induced hepatocarcinogenesis were also reported.[19–21]

Scordinin (Fig. 1) is a thioglycoside, isolated from boiled garlic (0.03% in garlic).[22, 23] Two basic structures, scordinin A and scordinin B are known for scordinin. However, the biological activities of these chemicals have not been well defined.

We examined the possible chemopreventive effects of scordinin in a DEN/phenobarbital (PB)-induced hepatocarcinogenesis model. Numbers of silver-stained nucleolar organizer regions' protein (AgNORs), recognized as one of the parameters of cell proliferation in preneoplastic and neoplastic lesions, were also measured in this study.[24–26]

MATERIALS AND METHODS

Animals, diet, water and carcinogen Weanling male F344 rats (Shizuoka SLC, Co., Shizuoka) were used. CE-2 (CLEA Japan Inc., Tokyo) was used as a basal diet. DEN and scordinin were purchased from Nacalai Tesque Inc., Kyoto and Riken-Kagaku-Kogyo Ltd., Kyoto, respectively. PB was obtained from Maruishi Pharm. Co., Osaka.

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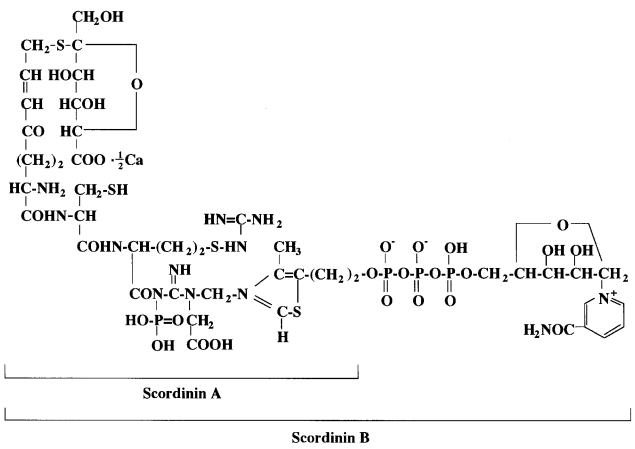

Fig. 1. Molecular structure of scordinin.

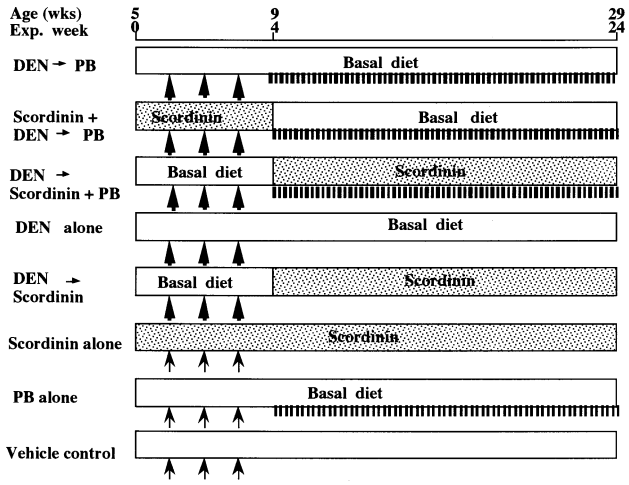

Fig. 2. Experimental design. ▲, DEN 100 mg/kg body weight, once a week for 3 weeks; ▲, saline; ▮▮▮, PB 500 ppm in drinking water; ▩, scordinin.

Experimental procedure The experimental design is shown in Fig. 2. A total of 126 rats, 5 weeks of age, were divided into 8 groups: group 1, 20 rats for DEN and 500 ppm PB; group 2, 20 rats for DEN and 600 ppm scordinin in the initiation phase and 500 ppm PB; group 3, 20 rats for DEN and 500 ppm PB and 600 ppm scordinin in the promotion phase; group 4, 20 rats for DEN alone; group 5, 20 rats for DEN and scordinin in the post-initiation phase; group 6, 8 rats for scordinin alone; group 7, 8 rats for PB alone; and group 8, 10 rats for vehicle control. All animals were housed three to four to a wire cage and had free access to water and diet under controlled environmental conditions of humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C). The experimental diet mixed with scordinin was prepared weekly and stored in a cold room.

Animals in groups 1 through 5 were given i.p. injections of DEN (100 mg/kg body weight) once a week for three weeks from one week after the start of the experiment, and groups 6 through 8 received i.p. injections of saline (vehicle). Rats in groups 2 and 6 were given 600 ppm scordinin-containing diet from the start of the experiment and the animals in the other groups were given the basal diet. Animals in groups 1, 4, 7 and 8 were fed the basal diet alone throughout the experiment (24 weeks). Animals in group 2 were transferred from the experimental diet to the basal diet and continued on this regimen to the end of the experiment. Groups 1, 2, 3 and 7 received drinking water containing 500 ppm PB from one week after the end of carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet with 600 ppm scordinin from one week after the end of DEN treatment. At termination of the experiment, complete autopsies were performed after sacrifice by ether inhalation. At autopsy, the location, number, and size of liver tumors were recorded. Two sets of liver sections were made from each lobe. One set of slices was fixed in cold acetone and the other set was fixed in 10% buffered formalin, embedded in paraffin blocks, and processed for routine histological observation.

Glutathione *S*-transferase placental form (GST-P) staining and counting The liver sections from acetone-fixed tissues were stained for GST-P. Immunohistochemical staining for GST-P was carried out using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame, CA). Anti-GST-P antibody was kindly provided by Dr. K. Satoh, Hirosaki University School of Medicine, Hirosaki. The areas of GST-P-positive foci and number of foci/cm^2 were measured by the image analyzer with a microscope (IPAP, Sumika Technos Ltd., Osaka). GST-P-positive foci were defined as lesions of the cells of more than 0.01 mm^2 in area. Unit areas of GST-P-positive foci were calculated as the area of foci (mm^2)/liver section (cm^2).

Determination of proliferative activity in the hepatocytes by AgNORs enumeration To assess the proliferative activity of the hepatocytes, the number of AgNORs per nucleus of five randomly selected animals from groups 1 through 5 was quantified according to the method described previously. The liver sections from 10% buffered formalin-fixed tissues were stained by a one-step silver colloid method for demonstration of AgNORs, and AgNORs counting was performed on 50 to 100 nuclei of interphase cells from all hepatocellular foci, adenomas, carcinomas, and non-lesional areas.

Statistical analysis Differences of incidence or density of pathological lesions in the liver between groups were ana-

lyzed by the χ^2 test, Fisher's exact probability test or Student's *t* test.

RESULTS

General observation In the DEN-treated groups, groups given scordinin had slightly decreased body and liver weights compared to the groups given basal diet (Table I). However, no significant difference of mean body weight was found among the groups given basal diet, scordinin or PB alone (groups 6–8). Mean liver weight and mean relative liver weight of the groups treated with PB were larger than those of the groups without PB treatment. No apparent toxic effects of scordinin were recognized in other organs.

Tumor incidence Liver tumors were only recognized in DEN-treated groups. The neoplasms were of hepatocellular origin (Table II). The incidences of adenoma and carcinoma of group 3 were significantly lower than those of group 1 ($P<0.005$ and $P<0.05$, respectively). The incidences of adenoma, carcinoma, and total liver neoplasms of group 5 were significantly lower than of group 4 ($P<0.05$, $P<0.05$, and $P<0.005$). The incidences of adenoma, carcinoma, and total neoplasms of group 2 were rather lower than those of group 1, although the differences were not statistically significant. The multiplicities of adenoma, carcinoma and total tumors of groups 2 and 3 were significantly smaller than the corresponding values of group 1 ($P<0.005$, $P<0.01$, $P<0.001$, $P<0.0001$, $P<0.0001$, and $P<0.0001$, respectively). The multiplicities

Table I. Body and Liver Weights of Rats

Group	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (%)
1	DEN→PB	20	332.1±14.2[a)]	16.4±1.1	4.94±0.38
2	Scordinin+DEN→PB	20	299.6±13.9[b)]	13.5±1.2[b)]	4.50±0.30[c)]
3	DEN→Scordinin+PB	20	317.5±14.4[d)]	14.1±1.2[b)]	4.45±0.29[b)]
4	DEN alone	20	339.8±20.0	11.4±0.9	3.35±0.25
5	DEN→Scordinin	20	327.7±12.7[d)]	10.2±0.6[e)]	3.12±0.14[f)]
6	Scordinin alone	8	351.3±12.2	11.0±0.5	3.13±0.15
7	PB alone	8	351.8±11.3	15.1±0.8[g)]	4.29±0.14[g)]
8	Vehicle control	10	346.0±14.5	10.8±0.7	3.11±0.11

a) Mean±SD.
b, *c*) Significantly different from group 1 by Student's *t* test (*b*) $P<0.0001$, *c*) $P<0.0005$, *d*) $P<0.005$).
d, *e*, *f*) Significantly different from group 4 by Student's *t* test (*d*) $P<0.05$, *e*) $P<0.0001$, *f*) $P<0.005$).
g) Significantly different from group 4 by Student's *t* test ($P<0.0001$).

Table II. Incidences of Liver Tumors in Rats Treated with DEN and/or Scordinin

Group	Treatment	Incidence (%)			Multiplicity		
		Ad.[a)]	Ca.[b)]	Total	Ad.	Ca.	Total
1	DEN→PB	100	95	100	6.2±3.7[c)]	4.1±2.4	10.3±5.7
2	Scordinin+DEN→PB	85	80	90	2.6±1.5[d)]	2.0±1.7[e)]	4.6±2.8[f)]
3	DEN→Scordinin+PB	60[g)]	70[h)]	85	1.1±1.1[i)]	1.0±0.7[i)]	2.1±1.5[i)]
4	DEN alone	70	50	80	1.0±1.1	0.7±0.8	1.7±1.7
5	DEN→Scordinin	30[j)]	15[j)]	35[k)]	0.3±0.5[l)]	0.2±0.4[l)]	0.5±0.8[m)]
6	Scordinin alone	0	0	0	—	—	—
7	PB alone	0	0	0	—	—	—
8	Vehicle control	0	0	0	—	—	—

a) Hepatocellular adenoma.
b) Hepatocellular carcinoma.
c) Mean±SD.
d–f, *i*) Significantly different from group 1 by Student's *t* test (*d*) $P<0.005$, *e*) $P<0.01$, *f*) $P<0.001$, *i*) $P<0.0001$).
g, *h*) Significantly different from group 1 by Fisher's exact probability test (*g*) $P<0.005$, *h*) $P<0.05$).
j, *k*) Significantly different from group 4 by Fisher's exact probability test (*j*) $P<0.05$, *k*) $P<0.005$).
l, *m*) Significantly different from group 4 by Student's *t* test (*l*) $P<0.05$, *m*) $P<0.01$).

of adenoma, carcinoma, and total neoplasms of group 5 were significantly smaller than those of group 4 ($P<0.05$, $P<0.05$, and $P<0.01$, respectively).

Frequency of hepatocellular foci Three types of preneoplastic hepatocellular foci (clear, eosinophilic and basophilic) which were positive for GST-P were found in all groups exposed to DEN. A few liver cell foci were also recognized in some animals given vehicle treatment. The results of quantitative analysis of the frequency of GST-P-positive foci are summarized in Table III. The density, average area, and unit area of GST-P-positive foci of groups 2, 3, and 4 were significantly smaller than those of group 1 ($P<0.0001$), except for the average area of foci of group 2. The parameters of group 5 were smaller than those of group 4 ($P<0.0001$, $P<0.05$, and $P<0.0001$, respectively).

Determination of proliferative activity in the hepatocytes by AgNORs enumeration Table IV summarizes data concerning AgNORs scores in each lesion. The mean number of AgNORs tended to show a stepwise increase from hepatocellular foci to carcinomas in each group. The number of AgNORs in cell nuclei of hepatocellular adenoma and carcinoma of groups 2 and 3 were significantly smaller than those of group 1 ($P<0.0001$). The numbers in cell nuclei of hepatocellular adenoma and carcinoma of group 5 were smaller than those of group 4 ($P<0.0001$). The number of AgNORs in cell nuclei of non-lesional areas of group 3 was also smaller than that from group 1 ($P<0.005$). The mean numbers of AgNORs of cell nuclei of neoplastic lesions in group 1 (DEN→PB) were similar to those of group 4 (DEN alone).

Table III. Quantitative Analysis of GST-P-positive Foci in Rats Treated with DEN and/or Scordinin

Treatment	Density (/cm^2)	Average area ($\times 10^{-2}$ mm^2)	Unit area (%)
DEN→PB	73.9±15.4[a)]	7.4±1.9	53.8±15.7
Scordinin+DEN→PB	36.3±11.4[b)]	8.6±2.6[b)]	29.9±9.4[b)]
DEN→Scordinin+PB	40.7±8.6[b)]	6.5±1.8[b)]	26.4±8.8[b)]
DEN alone	31.5±8.8[b)]	6.3±3.1[b)]	19.4±8.4[b)]
DEN→Scordinin	17.5±6.9[c)]	5.9±2.7[b)]	10.5±0.7[c)]

a) Mean±SD.
b) Significantly different from the rats treated with DEN→PB by Student's *t* test ($P<0.0001$).
c) Significantly different from the rats treated with DEN alone by Student's *t* test ($P<0.0001$).

DISCUSSION

The results of the present study indicate that scordinin, a component of garlic, has an anticarcinogenic potential. In this study, scordinin inhibited DEN and PB-induced hepatocarcinogenesis in rats when given during the post-initiation as well as initiation phase. The preventive effect of scordinin was more apparent in the post-initiation phase than in the initiation phase. Scordinin exposure in the initiation phase reduced the density of GST-P-positive foci, but not the average area of foci. Scordinin exposure in the promotion phase decreased both the density and the average area of the foci. It is suggested that increase in the number of foci reflects the initiation activity and increase of average area of foci indicates the promotion activity.[27–30] Thus, the results of quantitative analysis of altered liver cell foci using a phenotypic marker, GST-P, were in agreement with those of tumor induction.

In this study, exposure to scordinin decreased the number of AgNORs in the neoplastic hepatocytes. The decrease was in parallel with the reduction of tumor incidence. Since decrease in the number of AgNORs of hepatocytes in the non-lesional area was seen when scordinin was given during the post-initiation phase, it is suggested that scordinin is most effective in the promotion stage of hepatocarcinogenesis.

There is much evidence that cell proliferation plays an important role in carcinogenesis in different organs. In studies for cancer prevention, preventing agents are suggested to control carcinogen-induced hyperproliferation of the cells in target organs.[31–33] The enumeration of

Table IV. Average Number of AgNORs of Rats in Each Group

Group	Treatment	Hepatocellular foci	Hepatocellular adenomas	Hepatocellular carcinomas	Non-lesional area
1	DEN→PB	1.79±1.20[a)]	4.66±2.34	5.15±1.93	1.82±1.11
2	Scordinin+DEN→PB	1.75±1.21	2.16±1.33[b)]	2.82±1.77[b)]	1.56±1.50
3	DEN→Scordinin+PB	1.90±1.47	2.75±2.10[b)]	3.12±2.07[b)]	1.35±1.10[c)]
4	DEN alone	1.90±1.03	4.09±2.36	5.70±2.32	1.56±0.80
5	DEN→Scordinin	1.75±1.54	2.35±1.60[d)]	2.35±1.60[d)]	1.36±1.26

a) Mean±SD.
b, *c*) Significantly different from group 1 by Student's *t* test (*b*) $P<0.0001$, *c*) $P<0.005$).
d) Significantly different from group 4 by Student's *t* test ($P<0.0001$).

AgNORs is regarded as a useful method for evaluation of proliferative activity of preneoplastic and neoplastic cells.[24–26] Accordingly, our AgNORs scores suggest that the preventive action of scordinin is related to the suppression of cell proliferation in the liver. It is known that garlic extract or garlic oil has inhibitory effects on cell proliferation *in vitro*.[34–36]

In general, a number of mechanisms underlie the effects of chemopreventive agents. Modulation of phase I or II enzymes, and suppression of lipid peroxidation or DNA adduct formation are one of them.[37, 38] Reddy *et al*. reported that organosulfur compounds increase glutathione *S*-transferase (GST) and NAD(P)H-dependent quinone reductase (NAD(P)H:QR) in the liver.[39] It is also suggested that the protective effects of these organosulfur compounds may be accounted for, at least in part, by their ability to induce GST and other phase II enzymes that are involved in carcinogen detoxification. Garlic extract and some organosulfur compounds, diallyl sulfide, diallyl disulfide, allylmethyl trisulfide and *S*-allyl cysteine, of garlic were also reported to increase GST in some organs.[40, 41] In this context, further studies are needed to confirm the modulating effects of scordinin on phase I and phase II enzymes, in relation to its chemopreventive property.

The antioxidant activity against lipid peroxidation of scordinin was examined using the thiobarbituric acid (TBA) reaction in *in vitro* (unpublished data). Scordinin appears to have antioxidative activity, which may be important for its chemopreventive action.

Recently, it was suggested that ajoene, a major compound of crushed garlic, induces apoptosis in human leukemic cells via stimulation of peroxide production and activation of nuclear factor kB, which is a transcription factor known to respond to oxidative stress.[42] Furthermore, Singh *et al*. have shown that DADS, an organosulfide from garlic, suppresses the growth of H-*ras* oncogene-ransformed tumors in nude mice by inhibiting the membrane association of tumoral p21H-ras.[43] Whether or not scordinin also has these effects needs to be studied.

In summary, dietary administration of scordinin during the initiation and post-initiation phases effectively inhibited DEN and PB-induced hepatocarcinogenesis in rats. This thioglycoside compound contained in garlic seems to be a promising chemopreventive agent for human liver cancers.

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