RESEARCH COMMUNICATION

White, but not Red, Ginseng Inhibits Progression of Intestinal Carcinogenesis in Rats

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Abstract

Ginseng is a well known traditional medicine in Asian countries which has attracted attention as a potential chemopreventive agent. In the present study, inhibitory effects of white and red ginseng on tumor development were examined using medium-term liver and multi-organ carcinogenicity bioassay systems. No modifying potential of the ginseng preparations were evident in terms of the numbers or areas of glutathione S-transferase placental form (GST-P)-positive foci in rat livers. However, white ginseng, although not its red counterpart, was found to decrease the incidences of adenocarcinoma of the small intestine and colon in the medium-term multi-organ carcinogenesis model, without any affect on the numbers of aberrant crypt foci (ACF). These results indicate that white ginseng may have inhibitory effects on the progression stage of rat intestinal carcinogenesis, but the influence is not strong. Ginseng did not appear to have promoting or inhibitory effects in other organs under the present experimental conditions. Possible application on ginseng for chemoprevention of colon cancer in humans, can be concluded given the lack of obvious adverse effects.

Key Words: Ginseng - Carcinogenesis - Chemoprevention - Medium-term - Colon

Asian Pacific J Cancer Prev, **3**, 243-250

Introduction

Ginseng (the root of Panax ginseng) is well known as a traditional medicine in Asian countries. It is classified into three types depending on how it is processed: fresh ginseng; white ginseng (dried after peeling); and red ginseng (steamed and dried). Both white and red ginseng are commonly used, each type being ingested in various forms, for example as a juice, extract, powder, tea, tablet or capsule. Ginseng is taken not only as a medicine but in dietary therapy as a health-giving vegetable with several pharmacological activities reported (Bhattacharya and Mitra, 1991; Shibata et al., 1985; Tanaka et al., 1984).

Cancer preventive effects of ginseng in human populations have been documented (Yun and Choi, 1990; 1995), including the results of a case-control study in which ginseng intake was associated with a decreased risk for most cancers, like carcinomas of the esophagus, stomach, colon, pancreas, lung and liver. Clinical trials have also been designed to evaluate the chemopreventive effects of red ginseng on hepatocellular carcinoma development in patients with chronic hepatitis C infection.

Experimentally, several investigators have shown inhibitory effects of ginseng on carcinogenesis, tumor growth and metastasis (Yun et al., 1983; Tode et al., 1993; Bespalov et al., 1993; Mochizuki et al., 1995; Nakata et al., 1998). For example a medium term (9 weeks) model system revealed anti-carcinogenic activity of ginseng extract against pulmonary adenoma induction by benzo(a)pyrene in newborn mice (Yun, 1991). Nishino et al. further demonstrated extracts of red ginseng to inhibit the development of spontaneous liver tumors in C3H/He mice [unpublished data] and Xiu-gan et al.(Xiu-gan and Da-he, 1990) described inhibitory effects on the development of liver cancers in diethylnitrosamine-treated rats. The present investigation was carried out to evaluate the modifying effects of ginseng on chemical carcinogenesis in three different models in rats.

Experiment 1 was performed to investigate the influence of white and red ginseng on rat hepatocarcinogenesis in a

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medium-term liver bioassay system for the detection of carcinogens and tumor promoters or inhibitors in the second stage of hepatocarcinogenesis, developed by Ito et al. (Ito et al., 1988; Henry and Yvonne, 2001). This model allows rapid screening of promoting or inhibitory activity of chemicals using a putative preneoplastic lesion, the glutathione S-transferase placental form (GST-P)-positive focus, as the end point.

In experiment 2, the dose dependence of red ginseng effects on the initiation and post-initiation stages of hepatocarcinogenesis was further studied using the Ito test and an initiation bioassay system (Takada et al., 1997) . Since transforming growth factor-alpha (TGF-α) is a strong growth factor for hepatocytes (Mead and Fausto, 1989), with overexpression playing an important role in hepatocarcinogenesis (Dragan et al., 1995; Steinmetz and Klaunig, 1996; Kitano et al., 1998), TGF-α-positive foci in the liver were also assayed.

For chemoprevention, it is important to gain data on modifying effects in all major organs. In experiment 3, the effects of white and red ginseng preparations on tumor development were therefore investigated using a medium-term multi-organ carcinogenesis bioassay, the DMBDD model, already documented to be a useful tool for detection of carcinogens or chemopreventive agents in various organs within a relatively short period (Takahashi et al., 1992; Hagiwara et al., 1993; Hirose et al., 1993; Hasegawa et al., 1994; Yamamoto et al., 1995; Ito et al., 1996; Hirose et al., 1997).

Colon carcinogenesis models using azoxymethane or 1,2-dimethylhydrazine (DMH) with putative preneoplastic lesions, aberrant crypt foci (ACF) as surrogate marker lesions, have recently been used for assessing promoting or inhibitory activity of chemicals by many authors. A number of natural compounds that inhibit ACF after exposure to several colon carcinogens have proven to be protective against colon cancer in rodents (Pereira et al., 1994; Tanaka and Mori, 1996). Our previous experiment also demonstrated that 22-oxa-calcitriol, a synthetic analog of 1α ,25-dihydroxyvitamin D_3, significantly decreased the formation of DMH-induced ACF as well as small and large intestinal carcinomas in a multi-organ carcinogenesis model (Otoshi et al., 1995). Recently, we have shown that dietary administration of red ginseng powder concomitant with DMH inhibited the induction of DMH-induced ACF in the colon of rats (Li et al., 2000). In experiment 3, possible inhibitory effects of ginseng on colon carcinogenesis were therefore also assessed in terms of quantitative values for ACF.

Materials and Methods

Animals

A total of 331 male F344/DuCrj rats were obtained at 5 weeks of age from Charles River Japan (Hino, Shiga, Japan), housed five to a plastic cage with hard wood chips for bedding, and fed a powdered diet CE2 (Clea Japan Inc., Osaka, Japan) and water ad libitum. The animals were maintained in an environmentally controlled room at a temperature of 22±2°C, and a relative humidity of 55±10% with a 12-h light/dark cycle. They were used in this study after a one-week acclimation period.

Chemicals

Diets containing ginseng were kindly provided by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan). Diethylnitrosamine (DEN)(Wako Pure Chemical Industries Ltd., Osaka, Japan), N-methyl-N-nitrosourea (MNU)(Sigma Chemical Co.,St.Louis,MO), N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)(Tokyo Kasei Kogyo Co.Ltd.,Tokyo, Japan), DMH(Tokyo Kasei Kogyo Co.Ltd.,Tokyo, Japan) and dihydroxy-di-N-propylnitrosamine (DHPN)(Nacalai Tesqe Inc., Kyoto, Japan) were employed for initiation. 2-Acetylaminofluorene (2-AAF) was obtained from Tokyo Kasei Kogyo Co.Ltd.

Experiment 1

Experiment 1 was performed to investigate the modifying effects of white and red ginseng on the second stage of hepatocarcinogenesis using the Ito test. The experimental design is shown in Fig. 1. A total of 100 rats were divided randomly into 10 groups (15 rats each in groups 1~5, 5 rats each in groups 6~10) for treatment with test compounds as shown in Fig.1. Animals in groups 1 to 5 were given a single intraperitoneal (i.p.) injection of DEN (200 mg/kg body wt.) dissolved in saline to initiate hepatocarcinogenesis. Groups 6-10 received saline alone. After 2 weeks, the rats in groups 1 and 6 were maintained on basal diet without ginseng. Animals in groups 2-5 and 7-10 received diet containing 1% white ginseng powder, 0.3% white ginseng extract, 1% red ginseng powder or 0.3% red ginseng extract, respectively, for the remaining experimental duration of 6 weeks. The level of the original ginseng in the 0.3% extract diet was equal to that in the 1% powdered diet. All rats were subjected to two-thirds partial hepatectmy at week 3. Throughout the experiment, the animals had free access to food and water, and body weights were measured once per week, along with food and water consumption. Surviving rats in each group were killed for examination at week 8. At autopsy, livers were excised and 3-mm thick slices were cut with a razor blade and fixed in 10% buffered formalin for immunohistochemical demonstration of GST-P-positive foci.

Experiment 2

Experiment 2 was performed to investigate the dose dependence of any effects of red ginseng on the post-initiation stage using the Ito test, partly for the purpose of confirmation of the results of experiment 1. A total of 78 rats were divided into five groups, all given a single i.p. injection of DEN. After 2 weeks, the rats in group 1 received basal diet, while groups 2, 3,4 and 5 were given diet containing 0.03,0.1,0.3 and 1% powdered red ginseng, respectively, for the remaining experimental duration of 6

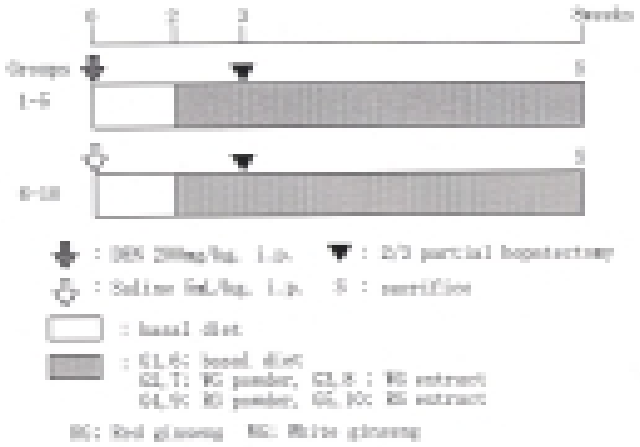

Figure 1. The Medium-term Liver Bioassay Protocol.

weeks. Surviving rats in each group were killed for examination at week 8, 1 hour after receiving an i.p. injection of 5-bromo-2'-deoxyuridine (BrdU) (100 mg/kg body wt.). The livers were examined immunohistochemically for GST-P-positive foci, TGF-α-positive foci and BrdU-labeled cells.

Experiment 2 was also planned to examine the modifying effects of red ginseng on the initiation stage using an initiation bioassay system (Takada et al., 1997). The experimental design is shown in Fig. 2. A total of 78 rats were divided into five groups, all given a single i.p. injection of DEN (20 mg/kg body wt.) dissolved in saline. They received diet containing powdered red ginseng at doses of 0, 0.03,0.1,0.3 or 1% from 6 days prior to DEN injection to one day after. The animals were fed 0.01% 2-AAF in powdered diet from weeks 2 to 4 and subjected to two-thirds partial hepatectomy at week 3. Throughout the experiment the animals had free access to food and water, and body weights were recorded once per week, along with food and water consumption. Surviving rats in each group were killed for examination at week 5. Immunohistochemical demonstration of GST-P-positive foci and TGF-α-positive foci was performed as described for experiment 1.

Experiment 3

Experiment 3 was conducted to assess the modifying

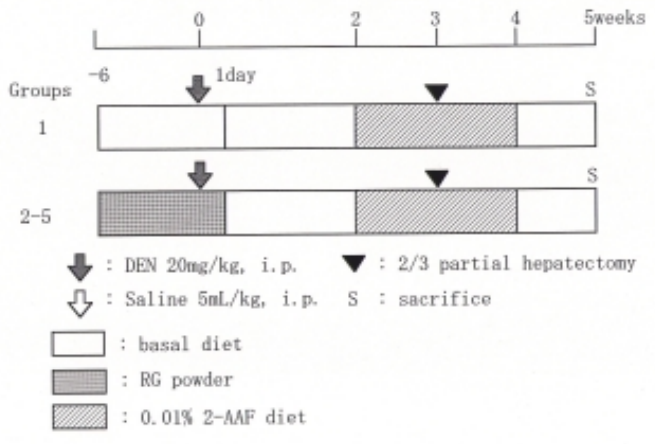

Figure 2. Initiation Bioassay Protocol.

effects of white and red ginseng in a multi-organ carcinogenesis model. A total of 75 rats were divided randomly into 6 groups (20 rats each in groups 1-3, 5 rats each in groups 4-6) for treatment with test compounds as shown in Fig.3. Those in groups 1 to 3 received combined treatments with a single i.p. injection of 100 mg/kg body weight of DEN, four i.p. injections of 20 mg/kg body weight of MNU, four s.c. injections of 40 mg/kg body weight of DMH, together with 0.05% BBN for 2 weeks, and then 0.1% DHPN for 2 weeks (both given in the drinking water), during the initial 4-week period for multiple organ initiation (DMBDD treatment). After this treatment with five carcinogens, all animals were given basal diet for 2 weeks and then those in groups 1 to 3 were administered basal diet, diet containing 1% powdered white ginseng or 1% powdered red ginseng, for 30 weeks. Groups 4 to 6 received vehicles without carcinogens in the first step followed by the respective test chemicals. Animals were observed daily to assess general health, and body weight and food consumption were measured weekly for the first 14 weeks and once every 4 weeks thereafter. At the beginning of week 37, all surviving animals were killed under ether anesthesia

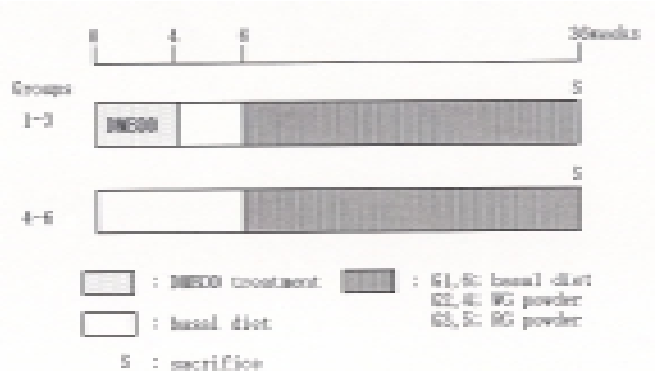

Figure 3. The Medium-term Multi-organ Bioassay Protocol.

and subjected to complete necropsy.

All major organs were excised, fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination. In the DMBDD treated groups, five rats each were used for examination of ACF. The colons were removed and after 10% buffered formalin had been injected into the lumens, were cut along the longitudinal median axis and stained with methylene blue (0.2% in D.W.) for 3-5 min. After staining, ACF (>4 crypts) were recorded according to standard procedures (McLellan et al., 1991). Livers fixed in 10% buffered formalin were examined after immunohistochemical demonstration of GST-P-positive foci.

Immunohistochemical Staining

The avidin-biotin-peroxidase complex (ABC) method was used to demonstrate GST-P-positive foci and TGF-α-positive foci. For the former, after deparaffinization, liver

sections were treated sequentially with normal goat serum, anti-rabbit GST-P antibody (MBL Co., Ltd., Nagoya, Japan; 1:2000, overnight), biotin-labeled goat anti-rabbit IgG (1:400) for 1 hour and ABC. For the latter, sections underwent microwave processing in 10mM citrate buffer (10min) before incubation with anti-mouse antibody against TGF-α (Oncogene science, Uniondate, NY; 1:100, overnight) at 4°C overnight, biotin-labeled horse anti-mouse IgG (1:400) for 1 hour and ABC. In both cases the sites of peroxidase binding were visualized by the diaminobenzidine method and the nuclei were counter-stained with hematoxylin. The numbers and the areas of GST-P positive foci and TGF-α-positive foci >0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (IPAP, Sumika Technos Corp., Osaka, Japan). Immunohistochemical staining for BrdU was also performed using the ABC method. Briefly, sections were treated with 2N HCl for 5 min, actinase E

Table 1. Numbers and Areas of Liver GST-P-positive Foci in the Livers of Rats in Experiment 1

Group	Treatment	Effective No. of rats	GST-P-positive Foci No./cm²	Area(mm²/cm²)
1	DEN→basal diet	14	2.48 ± 0.92[a]	0.19 ± 0.08
2	DEN→WG powder, 1%	12	2.52 ± 1.22	0.21 ± 0.11
3	DEN→ WG extract, 0.3%	14	2.62 ± 1.28	0.24 ± 0.18
4	DEN→ RG powder, 1%	14	3.48 ± 1.80	0.31 ± 0.21
5	DEN→ RG extract, 0.3%	12	2.48 ± 1.02	0.29 ± 0.20

[a]Mean ± S.D. ; WG, white ginseng ; RG, red ginseng

Table 2. Numbers and Areas of Liver GST-P-positive Foci and TGF-α-positive Foci in the Livers of Rats in Experiment 2 (Ito test)

Group	Treatment	Effective No. of rats	GST-P-positive Foci No./cm²	GST-P-positive Foci Area(mm²/cm²)	TGF-α-positive Foci No./cm²	TGF-α-positive Foci Area(mm²/cm²)
1	DEN→ basal diet	17	3.39 ± 1.65[a]	0.33 ± 0.22	0.21 ± 0.29	0.029 ± 0.068
2	DEN→ RG, 0.03%	15	2.89 ± 1.32	0.31 ± 0.19	0.09 ± 0.21	0.020 ± 0.055
3	DEN→ RG, 0.1%	14	3.55 ± 1.51	0.30 ± 0.15	0.13 ± 0.18	0.014 ± 0.027
4	DEN→ RG, 0.3%	14	3.08 ± 1.16	0.30 ± 0.11	0.20 ± 0.33	0.034 ± 0.062
5	DEN→ RG, 1%	15	3.82 ± 2.08	0.37 ± 0.22	0.27 ± 0.32	0.031 ± 0.035

[a]Mean ± S.D. ; RG, red ginseng

Table 3. BrdU Labeling Indices in the Livers of Rats in Experiment 2 (Ito test)

Group	Treatment	Effective No. of rats	BrdU Labeling Index %
1	DEN→ basal diet	12	0.83 + 0.31[a]
2	DEN→ RG, 0.03%	9	0.71 + 0.28
3	DEN→ RG, 0.1%	9	0.75 + 0.35
4	DEN→ RG, 0.3%	9	1.00 + 0.76
5	DEN→ RG, 1%	10	0.81 + 0.53

[a]Mean + S.D. ; RG, red ginseng

Table 4. Numbers and Areas of Liver GST-P-positive Foci and TGF-α-positive Foci in the Livers of Rats in Experiment 2 (Initiation bioassay)

Group	Treatment	Effective No. of rats	GST-P-positive Foci No./cm²	GST-P-positive Foci Area(mm²/cm²)	TGF-α-positive Foci No./cm²	TGF-α-positive Foci Area(mm²/cm²)
1	DEN	13	17.11 + 8.73[a]	1.84 + 1.42	0.16 + 0.32	0.019 + 0.052
2	DEN+RG, 0.03%	14	15.32 + 11.26	1.49 + 1.50	0.10 + 0.28	0.026 + 0.072
3	DEN+RG, 0.1%	9	13.40 + 6.25	1.42 + 1.18	0.14 + 0.30	0.022 + 0.051
4	DEN+RG, 0.3%	10	15.49 + 9.73	1.55 + 1.08	0.26 + 0.35	0.049 + 0.084
5	DEN+RG, 1%	14	13.51 + 6.73	1.35 + 1.10	0.08 + 0.16	0.017 + 0.050

[a]Mean ± S.D. ; RG, red ginseng

(200 unit/ml) for 5 min, anti-mouse BrdU antibody (DAKO Japan Co., Ltd., Tokyo, Japan; 1:1000) at 4°C overnight, horse anti-mouse IgG (1:400) for 1 hour and ABC. The sites of peroxidase binding were demonstrated by the diaminobenzidine method. BrdU-labelled nuclei were counted under a microscope and indices were calculated as percentages of labeled cells among ~5000 hepatocytes.

Statistical Analysis

Comparisons of numerical data for statistically significant differences were performed using the two tailed Student's t-test. For the incidences of lesions, the significance of differences observed between the control and treated groups was evaluated with the Fisher's exact probability test. The analysis was carried out with the aid of the Stat View statistical package (Abacus Concepts, Inc., CA, U.S.A.). The levels of significance were set at $P<0.05$ and 0.01.

Results

Experiment 1

Final body weights were significantly increased in rats fed white ginseng without DEN initiation compared to the appropriate controls (data not shown). Numbers and areas of GST-P-positive foci in the livers of groups 1 to 5 in experiment 1 are summarized in Table 1. Values per unit area of liver section after DEN initiation were increased in rats treated with red ginseng powder, but without statistical significance. GST-P-positive foci were not seen in the non-initiated groups.

Table 5. Histopathological findings in a variety of organs in Experiment 3

Organ and Finding	Group 1:Control (n=20)	2:WG (n=20)	3:RG (n=20)
Thyroids			
Follicular hyperplasia	19[a]	18	18
Follicular adenoma	12	9	10
Follicular carcinoma	4	9	9
Lungs			
Alveolar hyperplasia	20	20	20
Adenoma	11	13	15
Adenocarcinoma	8	12	9
Esophagus			
Squamous cell hyperplasia	19	20	20
Squamous cell papilloma	3	4	2
Squamous cell carcinoma	1	0	3
Stomach			
Squamous cell hyperplasia	13	12	14
Squamous cell papilloma	6	2	5
Squamous cell carcinoma	2	1	1
Small intestine			
Adenoma	3	8	2
Adenocarcinoma	6	1	4
Large intestine			
Adenoma	1	4	3
Adenocarcinoma	5	1	3
Liver			
Hepatocellular adenoma	2	0	2
Hepatocellular carcinoma	1	1	1
Kidneys			
Altered tubules	20	19	20
Adenoma	11	13	16
Transitional cell carcinoma	2	1	2
Nephroblastoma	19	15	20
Urinary bladder			
PN hyperplasia c	6	2	3
Transitional cell papilloma	1	2	1
Transitional cell carcinoma	2	1	1

[a] Number of animals bearing lesions [b] Significantly different from group 1 at $p<0.05$
[c] PN, papillary or nodular WG, white ginseng ; RG, red ginseng

Table 6. Effects of ginseng on ACF formation in the Colons of Rats in Experiment 3

Group	Treatment	Effective No. of rats	No. of ACF[b]	4 ≤ACF No. of AC[c]	AC/ACF
1	DMBDD→ basal diet	5	50 + 12[a]	284 + 80	5.68 + 0.54
2	DMBDD→ WG, 1%	5	31 + 17	180 + 107	5.72 + 0.83
3	DMBDD→ RG, 1%	5	35 + 18	190 + 97	5.50 + 0.48

[a]Mean + S.D.
[b]Aberrant crypt foci
[c]Aberrant crypt
WG, white ginseng
RG, red ginseng

Experiment 2

Dietary administration of red ginseng did not affect the growth curves, final body weights and relative liver weights in the Ito test or the initiation bioassay system (data not shown). During the period of the experiment, no intergroup differences in daily food and water consumption were apparent in either case.

The numbers and areas of GST-P-positive foci and TGF-α-positive foci in the rats given red ginseng at any dose after DEN were not significantly altered from the values for animals treated with DEN alone (Table 2). Data for BrdU labeling indices in the livers are summarized in Table 3, no effects being evident for the red ginseng treatment.

Data for the numbers and areas of GST-P-positive foci and TGF-α-positive foci in the liver in the initiation bioassay are summarized in Table 4. The numbers and areas of GST-P-positive foci and TGF-α-positive foci in the rats given red ginseng after DEN were not significantly affected as compared to the values for animals treated with DEN alone.

Experiment 3

Dietary administration of white and red ginseng did not affect the growth curves of rats, mortalities and relative organ weights(liver, kidney, spleen).

Histopathological findings for various organs in groups 1-3 are summarized in Table 5. The incidence of adenocarcinomas of the small intestine was significantly reduced in the white ginseng treatment group and similar tendency was noted for large intestinal adenocarcinomas. However, total incidences of tumors of the small and large intestine did not differ among groups 1-3. Incidences of tumors and preneoplastic lesions in other organs did not significantly differ among the groups receiving the DMBDD treatment. No tumors or preneoplastic lesions were observed in any of the rats given ginseng without DMBDD-treatment (data not shown).

The results of the effects of ginseng on ACF in the colons are summarized in Table 6. Administration of red ginseng or white ginseng did not affect the lesion numbers.

Discussion

The present study demonstrated that administration of white and/or red ginseng does not modify GST-P-positive foci development in the rat liver in either promotion or initiation stages of rat hepatocarcinogenesis. Moreover, treatment with red ginseng did not influence the volume of TGF-α-positive foci. These results indicate that ginseng lacks chemopreventive effects for hepatocarcinogenesis in the rat. However, dietary administration of white ginseng did appear to suppress cancer development in the small intestine and colon, exerting inhibitory effects on the progression stage.

Ogiso et al (1985) reported that the degree of induction of GST-P-positive foci in the presently applied bioassay system (Ito test) corresponds with the induction of hepatocellular carcinomas, as revealed by long-term in vivo studies. About 300 chemicals have already been analyzed and the efficacy of the system for detecting hepatocarcinogens, as well as chemopreventive agents has been well established (Ito et al., 1988). Experiment 1 demonstrated that dietary administration of any ginseng does not inhibit GST-P-positive foci development in the medium-term liver bioassay system. However, red ginseng powder slightly tended to increase the numbers and areas of GST-P-positive foci. Therefore, experiment 2 was performed but promotion was not confirmed.

TGF-α has been reported to play an important role in the regulation of cell proliferation (Mead and Fausto, 1989) and Dragan et al (1995) suggested that expression of TGF-α within altered hepatic foci might be a selective marker for quantitative analysis of the stage of progression in the rat multistage hepatocarcinogenesis. Only a few GST-P-positive foci expressed TGF-α (about 10%) in the present study, consistent with previous experiments (Kitano et al., 1998) and administration of red ginseng was without significant modifying potential. Furthermore, cell proliferation, which plays an important role in carcinogenesis (Cohen and Ellwein, 1990) was not influenced in terms of BrdU labeling, a specific measure of DNA synthesis and proliferation(Gratzner et al., 1975).

Nishino et al. (unpublished data) have demonstrated that extract of red ginseng in the diet may inhibit the development of spontaneous liver tumors in C3H/He mice. It can be speculated that the discrepancy with the present results is due to the different species. However, Xiu-gan et al. reported that red ginseng extract was found to inhibit induction of liver γ-GT-positive foci and cancer development in rats when given shortly after oral administration of DEN (Xiu-gan and Da-he, 1990). Whether variation in the administration route

of carcinogens, i.e., i.g. and i.p., is important in this regard remains unclear. It is possible that oral administration of red ginseng extract 1 hr after DEN suppressed absorbance of the carcinogen from the intestine and/or metabolism in their experiment.

While dietary administration of white ginseng suppressed cancer development in the small intestine and perhaps the colon, no decrease in numbers of ACF (≥4 crypts) was evident. Recently, we have also shown that dietary administration of red or white ginseng for 12 weeks after DMH-treatment was not effective at reducing development of ACF (≥4 crypts) in the colon (Li et al., 2000). Furthermore no inhibition was evident regarding the incidences of adenomas. Thus inhibitory effects of white ginseng on tumor development in the intestine might be due to an impact on the stage of progression.

The main component of ginseng is saponin, but there are many other components which depend on the processing. Further studies are needed to identify which might be active in the intestine. Ginseng did not have promoting or inhibitory effects in other organs under the present experimental conditions, so that our results suggest possible application for chemoprevention of colon cancer in humans, since no obvious adverse effects were noted.

Acknowledgements

The authors thank Hiromichi Sumiyoshi (Wakunaga Pharmaceutical, Hiroshima, Japan) for advice concerning ginseng. This investigation was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan.

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