Jpn. J. Cancer Res. 87, 1045–1051, October 1996

Inhibitory Effect of Chlorophyllin on Diethylnitrosamine and Phenobarbital-induced Hepatocarcinogenesis in Male F344 Rats

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Modifying effects of chlorophyllin (CHL) on the diethylnitrosamine (DEN)-phenobarbital (PB) hepatocarcinogenesis model were examined in rats. Five-week-old male F344 rats were divided into 8 groups. Groups 1 through 5 were given i.p. injections of DEN (100 mg/kg body weight) once a week for 3 weeks beginning one week after the start of the experiment, while groups 6 through 8 received vehicle treatment. Groups 1, 2, 3 and 7 received drinking water with 500 ppm PB from one week after the end of carcinogen or vehicle treatment. CHL-containing diet (2000 ppm) was given to group 2 during the initiation phase and to groups 3 and 5 during the promotion and the post-initiation phase, respectively. Group 6 was given the experimental diet alone throughout the experiment (24 weeks). Liver neoplasms were present in DEN-treated groups and PB treatment promoted liver tumorigenesis. The incidences of adenoma in groups 2 and 3 were significantly smaller than in group 1 ($P<0.05$ and $P<0.02$), although the reductions in the incidences of liver cell cancer were not significant. The average numbers of liver neoplasms/rat in group 2 were significantly smaller than in group 1 ($P<0.05$–$P<0.005$). Glutathione *S*-transferase placental form-positive foci were also significantly decreased by CHL treatment ($P<0.05$ and $P<0.001$). DEN and PB exposure increased liver ornithine decarboxylase activity and this increase was significantly inhibited by feeding of CHL during the initiation phase ($P<0.001$). These results suggest that CHL is a chemopreventive agent for liver neoplasia.

Key words: Chlorophyllin — Hepatocarcinogenesis — Chemoprevention — Ornithine decarboxylase — Rat

An inverse relationship between cancer risk and consumption of green and yellow vegetables has been observed.[1,2] Based on such epidemiological data, anti-mutagenic or carcinogenic effects of naturally occurring chemicals present in edible plants have been disclosed.[3,4] For example, natural chlorophylls, constituents of the human diet, are protective, and the anti-mutagenic activities of certain vegetable extracts have been considered to correlate with the content of chlorophylls.[5,6]

Chlorophyllin (CHL), the man-made sodium-copper salt of chlorophyll, is used for treatment of several human ailments and as a food additive for coloration. It also has potent anti-mutagenic activity.[2,7–13] In *in vitro* assay, anti-mutagenic activity of CHL was demonstrated using chemicals such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, aflatoxin B_1 (AFB_1), 2-aminoanthracene, benzo[*a*]pyrene, dimethylnitrosamine (DEN) and tobacco-related nitrosamines, and complex mixtures such as cigarette smoke, tobacco snuff, coal dust, diesel emission particles, airborne particles, fried meat, black pepper and red wine.[7–13] Furthermore, anti-mutagenic activity of CHL was demonstrated *in vivo* in *Drosophila*.[14–16] CHL inhibited DNA binding of AFB_1 in rainbow trout liver[8] and of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in rat liver.[17,18]

Notwithstanding many reports about the anti-mutagenic effects of CHL, there have been few studies of the modifying effect of CHL on experimental carcinogenesis. So far, anticarcinogenic effects of CHL have been reported in AFB_1-induced hepatocarcinogenesis in rainbow trout, IQ-induced tumorigenesis in male F344 rats[19,20] and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced mammary carcinogenesis in rats.[21] Promoting activity of this compound has been reported in dimethylhydrazine-induced colon carcinogenesis in rats.[22]

We examined the modifying effects of CHL on DEN and phenobarbital (PB)-induced hepatocarcinogenesis. In this study, analysis of glutathione *S*-transferase placental form (GST-P)-positive foci, as a preneoplastic lesion of the liver, was also done. Ornithine decarboxylase (ODC) activity, which is considered to correlate to cell proliferation[23] was also measured in the liver tissues.

MATERIALS AND METHODS

Animals, diet, water and carcinogen Weanling male F344 rats were purchased from Shizuoka SLC Co., Shizuoka. CE-2 (CLEA Japan Inc., Tokyo) was used as

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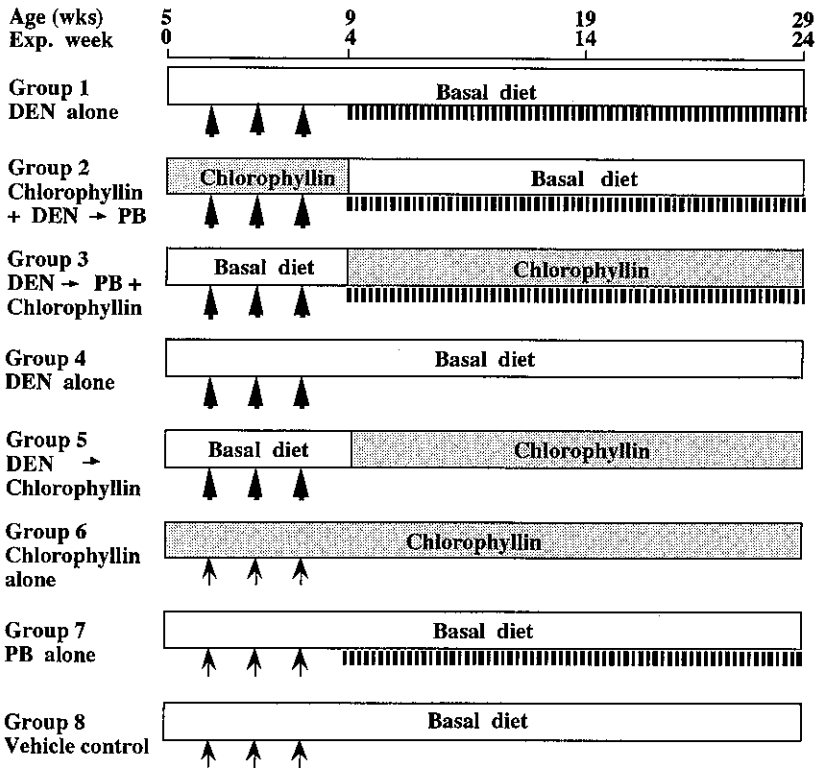

Fig. 1. Experimental design. ↓ DEN 100 mg/kg body weight, once a week for 3 weeks, ↑ Saline, ▮▮▮▮ PB 500 ppm in drinking water, ▒▒ Chlorophyllin 2000 ppm in diet.

a basal diet. DEN and CHL were purchased from Nacalai Tesque Inc., Kyoto. PB was obtained from Maruishi Pharm. Co., Osaka.

The experimental design is shown in Fig. 1. A total of 134 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 2000 ppm CHL in the initiation phase and 500 ppm PB; group 3, 20 rats for DEN and 500 ppm PB and 2000 ppm CHL in the promotion phase; group 4, 24 rats for DEN alone; group 5, 20 rats for DEN and CHL in the promotion phase; group 6, 8 rats for CHL alone; group 7, 8 rats for PB alone; and group 8, 14 rats for vehicle control. All animals were housed in wire cages (3 rats/cage). They had free access to water and diet under controlled environmental conditions of humidity (50±10%), lighting (12h light/dark cycle) and temperature (23±2°C). The experimental diets mixed with CHL were prepared weekly and stored in a cold room.

Experimental procedure Rats in groups 2 and 6 were given 2000 ppm CHL-containing diet from the start of the experiment and animals in the other groups were fed the basal diet. Animals of groups 1 through 5 were given i.p. injections of DEN (100 mg/kg body weight) once a week for 3 weeks after the start of the experiment, and groups 6 through 8 received a single i.p. injection of saline as vehicle treatment. Rats of groups 1, 4 and 8 were fed the basal diet alone throughout the experiment (24 weeks). The experimental diet in group 2 was changed to the basal diet, which was continued 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 2000 ppm CHL from one week after the end of DEN or vehicle treatment. At the termination of the experiment, all animals were killed by ether inhalation. At autopsy, the location, number and size of liver tumors were recorded. Liver tissues were sliced into three pieces from each lobe. One set of slices was fixed in cold acetone and another set was fixed in 10 % buffered formalin, embedded in paraffin blocks, and processed for routine histological observation with the use of hematoxylin and eosin stain. The liver sections from acetone-fixed tissues were stained for GST-P stain. An 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, Japan. The areas of GST-P-positive foci and number of foci/cm^2 were measured by means of an image analyzer with a microscope (IPAP, Sumitomo Chemical Co., Ltd., Osaka). GST-P-positive lesions composed of more than 11 cells were designated as altered liver cell foci.

Measurement of ODC activity At necropsy, macroscopic non-tumor tissues were trimmed from the liver of DEN-treated animals, and liver tissues from vehicle controls were sampled randomly. Liver samples were immediately frozen in liquid nitrogen for subsequent measurement of ODC activity and stored at −70°C for ODC activity. The specimens were pooled and homogenized in 0.25 ml of homogenizing buffer containing 0.25 *M* sucrose, 50 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 0.4 m*M* pyridoxal 5′-phosphate, and 1 m*M* dithiothreitol in an Ultra-Turrax tissue homogenizer. They were then centrifuged at 15000*g* for 30 min at 4°C. The supernatant was assayed for ODC activity by a modification of the micromethod of Lans *et al.*[24] in an Eppendorf microfuge in a final volume of 40 μl. The reaction mixture (final concentration) contained 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 1 m*M* EDTA, 0.25 m*M* pyridoxal 5′-phosphate, 1 m*M* dithiothreitol, and 130 μM [1-^{14}C]ornithine (40.6 mCi/m mol; Amersham International plc, Amersham, UK). The reaction (at 37°C for 60 min) was initiated with 20 μl of the supernatant, and the liberated $^{14}CO_2$ was collected at the top of the microtubes on paper filter with 10 μl of 10% potassium hydroxide. It was terminated by adding 10 μl of 6 *N* hydrochloric acid. After the addition of hydrochloric acid, the sample was incubated for 15 min again to collect $^{14}CO_2$ completely. At the end of this period, the paper-filter including $^{14}CO_2$ was removed, immersed in a scintillation vial, and counted for radioactivity in a 10-ml scintillation cocktail. One enzyme unit was defined as 1 pmol of $^{14}CO_2$ released/mg protein/h. Protein content was measured with a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA), utilizing bovine serum albumin as the standard.

Statistical analysis Differences of incidence or density of pathological lesions in the liver between groups were analyzed by use of the χ^2 test, Fisher's exact probability test or Student's *t* test.

RESULTS

General observations There was no clear evidence of CHL toxicity in animals (Table I). Four rats in group 3 and four rats in group 5 died of pneumonia before termination of the experiment, and no neoplasms were found in them. CHL-containing diet reduced the body weights and liver weights compared to the other groups. Relative liver weights were significantly increased by PB, and decreased by CHL compared to the appropriate controls ($P<0.001$ and $P<0.02$, respectively).

Tumor incidence Liver tumors were recognized only in DEN-treated groups. The neoplasms were those of hepatocellular origin (Table II). The incidences of adenoma in groups 2, 3 and 4 were significantly lower than that of group 1 ($P<0.02$, $P<0.0001$ and $P<0.0001$, respectively). The incidences of total liver neoplasms of groups 3 and 4 were lower than that of group 1 ($P<0.005$ and $P<0.0001$, respectively), and the incidence of hepatocellular carcinoma of group 4 was also lower than that of group 1 ($P<0.01$). No significant difference in incidences was found between groups 4 and 5. The incidence of total hepatocellular neoplasms in group 2

Table I. Body and Liver Weights of Rats in Each Group

Group	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (%)
1	DEN→PB	20	322.7±29.5[a]	15.9±1.9	4.96±0.42
2	Chlorophyllin+DEN→PB	20	319.3±43.8	15.4±2.6	4.81±0.35
3	DEN→Chlorophyllin+PB	16	319.3±15.0	15.4±1.6	4.83±0.45
4	DEN alone	24	325.8±18.8	11.7±1.2[b]	3.59±0.47[b]
5	DEN→Chlorophyllin	16	317.1±18.6	10.9±0.7[c]	3.45±0.21
6	Chlorophyllin alone	8	352.3±16.2	11.0±0.8	3.12±0.19
7	PB alone	8	357.0±23.9	16.1±2.4	4.51±0.59
8	Vehicle control	14	351.6±13.8	11.9±0.9[d, e]	3.39±0.24[e, f]

a) Mean±SD.
b) Significantly different from group 1 by Student's *t* test ($P<0.001$).
c) Significantly different from group 4 by Student's *t* test ($P<0.05$).
d, f) Significantly different from group 6 by Student's *t* test ([d]$P<0.05$, [f]$P<0.02$).
e) Significantly different from group 7 by Student's *t* test ($P<0.001$).

Table II. Incidences of Liver Tumors in Rats of Each Group

Group	Treatment	Incidence (%)			Multiplicity		
		Ad.[a]	Ca.[b]	Total	Ad.	Ca.	Total
1	DEN→PB	95	70	100	3.2±2.4[c]	2.0±1.9	5.2±3.9
2	Chlorophyllin+DEN→PB	70[d]	60	80	1.3±1.4[e]	0.9±0.9[f]	2.2±1.9[g]
3	DEN→Chlorophyllin+PB	63[h]	63	88	1.8±1.6	1.3±1.5	3.1±2.6
4	DEN alone	13[i]	29	42[j]	0.1±0.3[k]	0.4±0.6[k]	0.5±0.7[k]
5	DEN→Chlorophyllin	13	31	44	0.1±0.3	0.4±0.6	0.5±0.6
6	Chlorophyllin 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,h,i,j) Significantly different from group 1 by Fisher's exact probability test or the χ^2 test ([d]$P<0.05$, [h]$P<0.02$, [i]$P<0.001$, [j]$P<0.01$).
e–g,k) Significantly different from group 1 by Student's *t* test ([e]$P<0.01$, [f]$P<0.05$, [g]$P<0.005$, [k]$P<0.0001$).

Table III. Results of the Quantitative Analysis of GST-P-Positive Foci in Rats of Each Group

Treatment	Density (No./cm^2)	Average area ($\times 10^4$ μm^2)	Unit area ($\times 10^{-3}$)
DEN→PB	46.6±7.6[a]	13.9±3.5	65.7±18.1
Chlorophyllin+DEN→PB	39.8±9.9[b]	13.8±3.7	56.5±20.2
DEN→Chlorophyllin+PB	39.0±8.1[c]	11.7±2.1[d]	46.7±12.6[e]
DEN alone	21.6±6.3[d]	9.4±3.1[d]	21.0±10.0[d]
DEN→Chlorophyllin	15.6±5.7[f]	9.9±3.0	15.7± 7.3

a) Mean±SD.
b–e) Significantly different from the rats treated with DEN→PB by Student's *t* test ([b]$P<0.05$, [c]$P<0.01$, [d]$P<0.001$, [e]$P<0.005$).
f) Significantly different from the rats treated with DEN alone by Student's *t* test ($P<0.01$).

was rather lower than that of group 1, but the difference was not significant. The multiplicities of adenoma, carcinoma and total tumors of groups 2 and 4 were significantly smaller than those of group 1 ($P<0.001$, $P<0.05$ and $P<0.005$, and $P<0.0001$, $P<0.0005$, and $P<0.0001$, respectively). The multiplicity of adenoma and total tumor of group 3 were significantly smaller than that of group 1 ($P<0.0001$ and $P<0.005$). The multiplicity of hepatocellular carcinoma of group 3 was rather reduced compared to that of group 1, but the difference was not significant. No significant differences in the incidence and multiplicity of tumors were seen between groups 4 and 5.

Expression of hepatocellular foci A number of GST-P-positive foci appeared in the groups exposed to DEN. A few liver cell foci were also found in some animals of the vehicle controls, in which no tumors were detected. 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 were highest in group 1. The values of groups 2, 3 and 4 were slightly smaller than those of group 1 (Table III).

Results of ODC assay Table IV gives the ODC activities in the liver tissues without macroscopic tumors. CHL exposure during DEN injections, followed by PB treatment (group 2) significantly reduced the liver ODC level when compared with the DEN→PB group ($P<0.0001$). In this experiment, DEN or PB treatment significantly increased the ODC activity of the liver ($P<0.0001$ or $P<0.01$, respectively). PB exposure after DEN treatment (group 1) slightly increased ODC activity compared to the DEN alone group (group 4). CHL administration in the promotion or post-initiation phase after DEN exposure (group 1 or 5) tended to reduce liver ODC activity (Table IV).

Table IV. ODC Activity of Rat Livers in Each Group

Group	Treatment	No. of rats	ODC activity (pmol $^{14}CO_2$/mg protein/h)
1	DEN→PB	20	39.03±10.81[a]
2	Chlorophyllin+DEN→PB	20	15.83±16.69[b]
3	DEN→Chlorophyllin+PB	16	30.52±18.07
4	DEN alone	24	30.93±17.91
5	DEN→Chlorophyllin	16	21.14±13.52
6	Chlorophyllin alone	8	3.67± 5.48
7	PB alone	8	10.16± 6.37
8	Vehicle control	14	4.74± 1.27[c]

a) Mean±SD.
b) Significantly different from the rats treated with DEN→PB by Student's *t* test ($P<0.001$).
c) Significantly different from the rats treated with PB alone by Student's *t* test ($P<0.01$).

DISCUSSION

In the present study, CHL clearly inhibited DEN-PB-induced hepatocarcinogenesis in rats when administered during the initiation phase. Although the differences were not significant, incidence and multiplicity of liver tumors of the animals treated with CHL in the promotion phase tended to be decreased when compared with those of the appropriate control.

The results of quantitative analysis of altered liver cell foci using a phenotypic marker, GST-P, were also in agreement with the data on the incidence of liver neoplasms. CHL administration in the initiation phase reduced the density of GST-P-positive foci. CHL feeding in the promotion phase also decreased both the density and the average area of foci. An increased number of foci appears to reflect initiation activity and an increased average area of foci is considered to be a consequence of promotion activity. Many investigators have studied hepatocarcinogenesis by analyzing enzyme-altered foci, using markers such as GST-P, γ-glutamyltranspeptidase, adenosine triphosphatase, glucose-6-phosphatase and others. Among a number of markers of enzyme-altered foci, GST-P is considered to be more reliable and is used for short- and medium-term tests to detect carcinogens and carcinogenesis modifiers.[25,26]

In this study, dietary CHL during the initiation or promotion phase of the DEN-PB-induced hepatocarcinogenesis model decreased hepatic ODC activity. ODC induction is known to precede cell proliferation in many cells exposed to xenobiotics, including genotoxic carcinogens.[27] ODC activity is considered to be a biomarker for cell proliferation,[28,29] which is an important event during carcinogenesis.[30,31] In this study, ODC activities were enhanced by PB treatment. The data suggest an association between ODC activity and promoting action of PB in the liver. Cell proliferation is also believed to enhance the frequency of tumor initiation.[32-37] Hepatocyte proliferation is involved in several stages of liver cell tumorigenesis.[33,38,39] In this study, ODC activity was enhanced by DEN treatment. Similar findings have been reported by other investigators using different hepatocarcinogenesis models.[34-37]

The mechanism of the anticarcinogenic effect of CHL is not understood. However, antioxidative activity of sodium copper CHL has been reported,[40] and this could be a possible mechanism for the inhibitory effects in DEN-PB-induced hepatocarcinogenesis.

Other possible mechanisms for the effect of CHL include alteration of carcinogen metabolism.[8] Yun *et al.* reported that CHL inhibited all P450 activities in human and rat liver microsomes.[41] CHL is also considered to inhibit the bioactivation of carcinogens through P450s. There is evidence that chemopreventive agents function by modulating metabolism through phase I and phase II enzymes.

Hayatsu *et al.* has proposed that CHL traps carcinogens by forming a complex, thereby blocking the absorption of carcinogens from the digestive tract and inhibiting carcinogenesis.[42] Guo and Dashwood reported that simultaneous administration of CHL was much more effective than administration 1–24 h before IQ treatment.[43] However, there is evidence that post-initiation exposure to CHL promoted the colon cancer induced by DMH, and the effect of CHL may depend on the method of administration.[22] In this study, DEN was given by i.p. injection, and PB and CHL were administrated orally. The inhibitory effect of CHL was prominent in the group given CHL in the initiation phase, and a weak suppressive effect was recognized when CHL was given in the promotion phase. The results suggest CHL is basically effective in any phase of hepatocarcinogenesis.

In conclusion, the results of the present investigation suggest that CHL is a promising chemopreventive agent for liver neoplasia.

ACKNOWLEDGMENTS

We thank Dr. Kimihiko Satoh, Second Department of Biochemistry, Hirosaki University School of Medicine, for the gift of anti-GST-P antibody. We also thank Ms. K. Takahashi, Mrs. T. Hirose and Mr. K. Sato for technical support. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

(Received May 9, 1996/Accepted August 1, 1996)

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