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Inhibitory Effects of S-Methylcysteine and Cysteine on the Promoting Potential of Sodium Phenobarbital on Rat Liver Carcinogenesis

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The effects of S-methylcysteine (SMC) and cysteine on the promotion stages of rodent hepatocarcinogenesis in a medium-term bioassay previously developed by Ito were examined. Initiation was induced by a single dose of diethylnitrosamine (DEN), followed by dietary administration of the promoter sodium phenobarbital (NaPB) 2 weeks later, for 6 weeks. Partial hepatectomy was conducted on all the animals at week 3. Inhibitory potential was evaluated by analyzing two markers of carcinogenesis, namely numbers of glutathione S-transferase placental form (GST-P)-positive foci, and proliferating cell nuclear antigen (PCNA). In addition, the level of ornithine decarboxylase (ODC), one of the rate-limiting enzymes of polyamine metabolism induced by promoters, was analyzed. SMC and cysteine induced significant reduction in the areas of GST-P-positive foci. A significant reduction in the PCNA index was observed in the entire liver as well as in GST-P-positive areas. SMC also induced down-regulation of the ODC enzyme activity. Thus, SMC and cysteine were found to inhibit the promotion stage of DEN-induced hepatocarcinogenesis. No cocarcinogenic effects were evident on administration of either of these chemicals with NaPB.

Key words: S-Methylcysteine — Cysteine — Rat hepatocarcinogenesis — GST-P — PCNA

Diet plays a major role in the prevention of many diseases, including cancer. A well-known vegetable having a wide range of medicinal qualities is garlic, which can reduce serum cholesterol and triglyceride levels. People who consume garlic regularly have a lower gastric cancer death rate.[1, 2] Further, it has numerous chemical constituents which inhibit the progression of different types of cancer in experimental chemical carcinogeneses.[1–11] Many reports have emphasized the preventive potential of oil-soluble orangosulfur compounds (OSCs) present in the allium family, on chemical carcinogenesis.[1–5, 8–10] Water-soluble OSCs, on the other hand, have not been extensively studied.

S-Methylcysteine (SMC), a water-soluble OSC, is present in garlic, beans, and cabbage.[12–14] S-Allylcysteine (SAC), an analog of SMC, has been found to inhibit experimental colon carcinogenesis in mice.[15] Cysteine esters have protective potential against toxicity induced by sulfur mustard in cultured rodent lung slices.[16] Earlier studies in our lab have revealed the capacity of SMC and its analog cysteine to inhibit initiation, post-initiation and promotion by 2-acetylaminofluorene (2-AAF) of diethylnitrosamine (DEN)-induced hepatocarcinogenesis.[17, 18] SMC and cysteine also reduce the number of putative preneoplastic lesions of the colon in multi-organ carcinogenesis bioassay.[17]

The present study was designed to evaluate the modulating effects exerted by SMC and cysteine on hepatocarcinogenesis initiated with DEN and promoted with sodium phenobarbital (NaPB), during replicative DNA synthetic phase induced by partial hepatectomy.[19] Another aim of this experiment was to investigate if these two compounds exhibited similar patterns of inhibition to those obtained in an earlier report.[18] The medium-term liver bioassay system was employed since it allows early and reliable observation of the post-initiation modifying effects of chemicals within a short duration of 8 weeks.[19, 20] Immunohistochemical analysis of glutathione S-transferase placental form (GST-P)-positive foci, one of the widely accepted end-point markers of hepatocellular carcinoma,[19] was conducted. In addition, the effects of the compounds on proliferating cell nuclear antigen (PCNA), a marker for cell proliferation,[21] and on ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine metabolism which is induced by promoters,[22, 23] were examined.

MATERIALS AND METHODS

Chemicals SMC (H_3C-S-$CH_2CH(NH_2)COOH$) and cysteine (H-S-$CH_2CH(NH_2)COOH$) were kindly provided by Wakunaga Pharmaceutical Co., Ltd., Osaka. DEN and

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NaPB were obtained from Tokyo Chemical Industry Co., Ltd., Tokyo. [1-^{14}C]Ornithine was obtained from Moravek Biochemicals, Inc., Brea, CA.

Animals Eighty male F344 rats (5 weeks old) were purchased from Charles River Japan, Inc., Shiga. They were housed 5/steel cage and fed MS basal powdered diet (Oriental East Co., Ltd., Tokyo) and water *ad libitum*. They were maintained under controlled conditions of temperature (23±1°C), and relative humidity (36±6%), with a 12 h light and 12 h dark cycle. Animals were acclimatized for a week before the start of the experiment.

Animal treatments Six-week-old animals were divided into eight groups with fifteen rats each in groups 1–4 and five rats each in groups 5–8. Animals in group 1 were given NaPB in the diet after being exposed to a single intraperitoneal (i.p.) administration of DEN. Groups 2–4 received a single i.p. administration of DEN while those in groups 5–8 received saline. This was followed by daily intake of NaPB through the diet and SMC (group 2)/cysteine (group 3) by intragastric gavage (i.g.) five times a week for 6 weeks, after a lag period of 2 weeks (Fig. 1). Groups 1–8 were subjected to partial hepatectomy at week 3. Group 4 was maintained as the DEN control while group 5 served as the NaPB control. Groups 6 and 7 were given NaPB in the diet, with i.g. administration of SMC in the former and cysteine in the latter. Group 8 was maintained as control animals and fed normal diet. Administration of the test chemicals by gavage was always carried out in the morning between 9 am and 10 am. DEN was administered at a dose of 200 mg/kg body weight in 0.9% saline. NaPB was mixed in the diet to obtain a final concentration of 500 ppm/rat and either SMC or cysteine, dissolved in saline, was administered at a dose of 100 mg/kg body weight. The weights of the animals were recorded each day to adjust the dose in accordance with the increase in body weight. The food trays were checked every day, cleared of debris, weighed and filled. The surviving animals were killed under ether anesthesia at the end of 8 weeks.

Tissue processing Macroscopic examination was conducted to see if any external pathology was evident in the liver. Livers were then excised and weighed. Two to three millimeter thick sections were cut with a sharp razor blade and representative samples from the right posterior, anterior and caudate lobes were fixed in ice-cold acetone for immunohistochemical examination of GST-P. Similar sections of the liver were fixed in 10% buffered formalin and used for the immunohistochemical analysis of PCNA. Remaining samples were frozen in liquid nitrogen to measure ODC.

Immunohistochemical staining of GST-P and PCNA The avidin-biotin-peroxidase complex (ABC) method of Hsu *et al.*[24] was employed to demonstrate GST-P-positive liver foci and PCNA-positive cells. Liver sections were

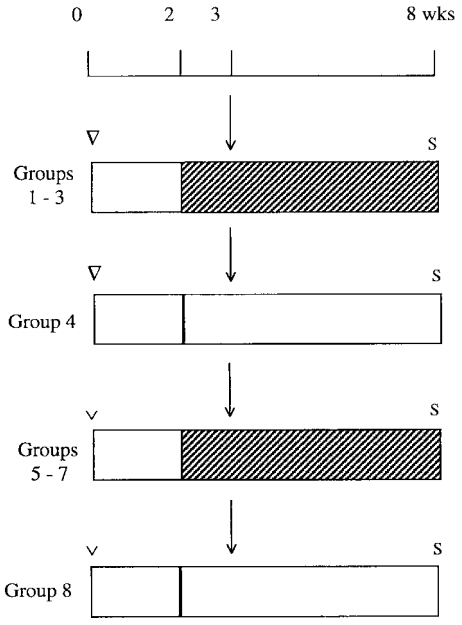

Fig. 1. Liver medium-term bioassay according to Ito.[19] ▽, DEN 200 mg/kg body weight (i.p.); ↓, partial hepatectomy; S, sacrifice; ▨, 500 ppm NaPB in the diet±SMC/cysteine (i.g.), 5 times a week; ⋁, 0.9% saline (i.p.), 5 ml/kg body weight; ▭, normal diet.

deparaffinized and treated sequentially with normal goat serum, anti-rabbit GST-P antibody (MBL Co., Ltd., Nagoya; 1:2000), or anti-PCNA antibody (DAKO Japan Co., Ltd., Kyoto; 1:400), biotin-labeled goat anti-rabbit IgG (1:400) and finally with ABC. The diaminobenzidine method[25] was used to demonstrate the sites of peroxidase binding. Sections were then counterstained with hematoxylin for microscopic examination. The numbers and the areas of GST-P-positive foci greater than 0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (Olympus-Ikegami VIP-21CH, Tokyo). The numbers of PCNA-positive cells were measured at random in 1000 cells and the proliferating indices were expressed as percentage values. The PCNA-positive cells within the GST-P-positive foci of the first four groups of rats were also scored.

Measurement of ODC activity ODC was measured by employing the method of Otani *et al.*[26] Rat liver samples, which had been frozen in liquid nitrogen, were suspended in 0.5 ml of Tris buffer (50 m*M*, pH 7.5) containing sucrose (0.25 *M*) and homogenized for 1 min. The homogenized supernatant was centrifuged at 100 000*g* for 30 min. The amount of radioactive CO_2 produced from [1-^{14}C]ornithine was assayed to estimate the ODC activity.

Statistical analysis Statistical analysis of data was performed by employing Student's *t* test. The Stat-View statistical package was used to aid in the analysis.

RESULTS

Table I shows the final body, absolute liver and relative liver weights of the rats in all eight groups. During the course of the experiment, a few rats died, though there was no mortality during PH. No significant differences in the body weights were observed between most of the treated groups except for groups 1 and 3, which showed significantly reduced body weights compared to the control group (group 8). Whole and relative liver weights, however, showed similar patterns of significant intergroup variations, with groups 1–3 and 5–7 exhibiting significantly greater weights than groups 4 and 8, as seen in Table I. However, there was no significant change in the rate of intake of food among the eight treatment groups (data not shown).

Quantitative data from the immunohistochemical analysis of the hepatic GST-P-positive foci are shown in Table II. While SMC and cysteine significantly reduced the areas of GST-P-positive foci (groups 2 and 3), neither had any potential to reduce the numbers of GST-P-positive foci.

Table III summarizes the data for PCNA-positive cell index and hepatic ODC activity. There was a significant reduction in the number of PCNA-positive cells scored

Table I. Final Body, Liver, and Relative Liver Weights of Treated Rats

Groups	Treatments	Effective no. of rats	Final body weight (g)	Liver weight	
				Absolute (g)	Relative (/100 g b.w.)
1	DEN→NaPB	9	262±9.6[a)]	9.5±0.9	3.6±0.4
2	DEN→NaPB+SMC	12	267±8.3	10.2±0.4[b)]	3.8±0.2
3	DEN→NaPB+Cysteine	13	263±11.0[a)]	9.9±0.7	3.8±0.2
4	DEN	11	263±15.1	7.7±0.6[c)]	2.9±0.2[e)]
5	Saline→NaPB	4	273±8.1	10.3±0.7	3.8±0.2
6	Saline→NaPB+SMC	4	268±17.2	10.3±0.6	3.8±0.2
7	Saline→NaPB+Cysteine	4	269±14.3	10.2±0.6	3.8±0.1
8	Saline	4	276±7.1	7.6±0.5[c, d)]	2.8±0.2[e, f)]

Values expressed are mean±SD (Student's *t* test).
a) $P<0.05$ (vs. group 8).
b) $P<0.05$ (vs. group 1).
c) $P<0.001$ (vs. groups 1–3; 5–7).
d) $P<0.01$ (vs. groups 1, 7).
e) $P<0.001$ (vs. groups 1–3; 5–7).
f) $P<0.01$ (vs. group 1).

Table II. Numbers and Areas of GST-P-positive Foci in the Liver

Groups	Treatments	Effective no. of rats	GST-P-positive foci	
			Number (no./cm^2)	Area (mm^2/cm^2)
1	DEN→NaPB	9	12.0±5.4[a)]	1.04±0.48[c)]
2	DEN→NaPB+SMC	12	10.1±4.1[a)]	0.57±0.23[d)]
3	DEN→NaPB+Cysteine	13	11.3±3.8[a)]	0.54±0.24[d)]
4	DEN	11	3.9±3.2[b)]	0.24±0.23[d, e)]
5	Saline→NaPB	4	0	0
6	Saline→NaPB+SMC	4	0	0
7	Saline→NaPB+Cysteine	4	0	0
8	Saline	4	0	0

Values expressed are mean±SD (Student's *t* test).
a) $P<0.001$ (vs. groups 4–8).
b) $P<0.01$ (vs. groups 6, 8).
c) $P<0.001$ (vs. all other groups).
d) $P<0.001$ (vs. groups 5–8).
e) $P<0.01$ (vs. groups 2, 3).

Table III. PCNA-positive Cells and ODC Activity in the Liver of Treated Rats

Groups	Treatments	Effective no. of rats	% PCNA-positive cells		ODC activity (pmol/h/mg pro)
			(/100 cells)	(/100 GST-P-positive cells)	
1	DEN→NaPB	9	4.0±1.0[a, b]	4.4±1.2	29.4±15.4
2	DEN→NaPB+SMC	12	1.4±0.9	1.9±1.4[c, d]	19.6±5.9[e]
3	DEN→NaPB+Cysteine	13	2.0±0.8	2.0±1.2[c]	22.5±10.1
4	DEN	11	3.4±2.3	4.1±2.5	21.8±4.8
5	Saline→NaPB	4	2.5±0.3		18.8±7.0
6	Saline→NaPB+SMC	4	2.9±0.4		16.2±7.5
7	Saline→NaPB+Cysteine	4	2.8±0.6		20.8±5.6
8	Saline control	4	2.5±0.1		20.2±6.7

Values expressed are mean±SD (Student's *t* test).
a) $P<0.001$ (vs. groups 2, 3, 8).
b) $P<0.01$ (vs. groups 5, 6, 7).
c) $P<0.001$ (vs. groups 1, 4).
d) $P<0.01$ (vs. group 4).
e) $P<0.001$ (vs. group 1).

both in the whole section and in the cells within the GST-P-positive foci (Table III) in groups 2 and 3 compared to groups 1 and 4. SMC exhibited a significant capacity to inhibit the ODC activity in group 2 while cysteine seemed to lower the enzyme activity, though not significantly.

DISCUSSION

The present study revealed the inhibitory potential of SMC and cysteine on the promotion of DEN-initiated hepatocarcinogenesis by NaPB. It also supplements earlier findings[18] from our lab suggesting the inhibitory potential of these two compounds on hepatocarcinogenesis.

Although there are many reports regarding the inhibitory effects exerted by the oil-soluble OSCs on carcinogenesis in various organs,[1–5, 9, 27] few reports are available regarding similar effects of water-soluble OSCs.[17, 18, 28] Similarly, most of the reports deal with the inhibitory potential of OSCs when administered before or together with exposure to the carcinogen.[2–5, 9, 15, 27, 29] However, differences are evident in the potential to inhibit carcinogenesis after initiation between the different OSCs.[30–32] This has been attributed to the differences in the number of sulfur atoms.[30] Sulfhydryl compounds are known to be capable of scavenging free radicals.[9] The inhibitory influence of SMC and cysteine on the promotion stage of hepatocarcinogenesis may partly be due to this property, which enables them to deplete the free radicals generated during promotion. Though these two compounds inhibited the promotion stage, they did not exert any influence when either was coupled with only the promoter NaPB. Thus, no cocarcinogenic effect was evident on administration of either of these compounds with NaPB.

Increases in the weights of the liver were observed in the post-initiated groups treated with the promoter and SMC or cysteine, namely, SMC/cysteine+NaPB, and NaPB treatment. This is probably due to the mitogenic property of NaPB, as all the groups subjected to NaPB had greater liver weights compared to the DEN and saline group. Mitogenic compounds induce proliferation and not necrosis,[33] and hence in response to this insult, the liver increases in weight. In this study too, the DEN control group did not have increased liver weights, since DEN is necrogenic. On the other hand, the NaPB control group had significantly higher liver weights. Groups exposed to DEN and NaPB and cysteine coupled with NaPB in DEN-initiated rats showed reduced body weights. This observation is similar to the results of an earlier study on two other OSCs, which reduced the development of preneoplastic lesions, but had no influence on the increased liver and reduced body weights.[7]

Earlier experiments in our lab on the possible mechanisms of the influence of the OSCs on hepatocarcinogenesis employed the well-known Ito test[19] and assay of ODC, a key enzyme of polyamine synthesis.[34] Reduction was observed in the areas of GST-P-positive foci by SMC and cysteine treatment. These results are similar to those obtained earlier, i.e., decreased formation of GST-P-positive foci when administered in the initiation and promotion stages of rat hepatocarcinogenesis using DEN as the initiator and 0.01% AAF as the promoter.[17, 18] However, it is not clear why the chemicals failed to reduce the number of GST-P-positive foci in the present study. This discrepancy could be because of the difference in the chemicals, AAF and NaPB, employed for promotion. While AAF has the potential to initiate hepatocarcinogenesis and hence could

induce an additive effect with the initiator,[35] PB is a weak hepatocarcinogen,[20] and the detoxification metabolisms for these promoters could be different.

PCNA is an auxiliary protein of DNA polymerase δ,[36] and a biomarker of cell proliferation. SMC and cysteine remarkably inhibited proliferation in the present regimen. Earlier studies also found that a single administration of either of these compounds greatly inhibited cellular proliferation by reducing PCNA and the enzymes involved in polyamine synthesis.[18] The promoter NaPB, a potent mitogen, has the capacity to elevate cell turnover within enzyme-altered foci. To analyze the inhibitory potential of SMC/cysteine on the preferential growth of preneoplastic foci, PCNA-positive cells were scored in the GST-P-positive foci in the groups subjected to DEN administration. Both exhibited the capacity to regress proliferation of the enzyme-altered cells in the promotion stages.

We assayed ODC to see if the chemopreventive effects correlated with inhibition of polyamine synthesis were comparable to those obtained in an earlier study where the OSCs had significantly inhibited the formation of enzyme-altered foci with no corresponding effect on the rate-limiting enzymes of polyamines.[7] While SMC reduced the ODC enzyme activity significantly, cysteine did not. This may be because these compounds have different mechanisms of inhibition, either a) scavenging free radicals and superoxides,[8, 9] or b) inducing up-regulation of Phase II enzyme activity.[4] Further studies on the mechanisms of inhibition will be needed before these compounds can be used in pharmacological formulations aimed against environmental carcinogens.

Thus, this study has substantiated the inhibitory potential of SMC and cysteine on the promotion stages of hepatocarcinogenesis. Based on the classification of carcinogenic inhibitors,[37] they can be considered as "blocking agents" and "suppressing agents," as they induce inhibition in the initiation, post-initiation[18] and promotion stages of the disease.

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