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ORIGINAL ARTICLE

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Suppressive effect of *Astragalus membranaceus* Bunge on chemical hepatocarcinogenesis in rats

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Abstract *Astragalus membranaceus* (AM) has been widely used for treating liver diseases in traditional Chinese medicine. Experimental evidence indicates that it has antitumor potential. In this study, the effect of AM on hepatocarcinogenesis induced by diethylnitrosamine (DEN), two-thirds partial hepatectomy, and 2-acetylaminofluorene (2-AAF) (DEN-PH-AAF) was evaluated using glutathione S-transferase placenta form (GST-P) as marker. First, rats were injected intraperitoneally (i.p.) with DEN (200 mg/kg in saline), a two-thirds partial hepatectomy was carried out 2 weeks later, and the rats were then placed on a basal diet containing 0.02% AAF from week 3 to week 8 to induce hepatocarcinogenesis. The rats were given AM (90 mg/kg or 180 mg/kg body weight) by gavage from week 3 to week 8 (treatment groups). The formation of GST-P-positive foci and the expression of GST-P protein and mRNA caused by DEN-PH-AAF were reduced in the treatment groups, which clearly suggests that AM is effective in delaying DEN-PH-AAF-induced hepatocarcinogenesis.

Keywords *Astragalus membranaceus* Bunge · Chemical-induced hepatocarcinogenesis

R.C. and J.H. contributed equally to this work.

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Introduction

Chemical-induced hepatocarcinogenesis is a multistep process which can be divided into at least three stages: (1) initiation, (2) promotion, and (3) progression [1]. Genotoxic carcinogens induce irreversible DNA damage, generating initiated cells that can proliferate clonally in the presence of promoter substances until they acquire the capacity for autonomic growth. All of these steps involve interactions with host biochemical, endocrinological, immunological, and microenvironmental regulatory systems. It is likely that inhibiting or delaying hepatocarcinogenesis takes place during the initiation stage.

The formation of hepatocellular foci or nodules, which show a specific biochemical/enzymatic phenotype, is considered a preneoplastic or precancerous step [2, 3, 4, 5]. The most widely used methods for their identification are immunohistochemical demonstration of the placental form of glutathione-S-transferase (GST-P) [6, 7, 8, 9, 10, 11]. GST-P is considered to be the most accurate marker enzyme for detection of initiated cells [6, 8, 12, 13]. Although not all the liver foci detected may necessarily develop into tumors, a good correlation (93% concordance) has been obtained between different hepatocarcinogens and the incidence of hepatocellular carcinomas in parallel long-term studies [14, 15, 16].

Astragalus membranaceus (AM) is used as a natural herbal medicine in east Asia for preventing severe side effects of chemotherapy in patients with cancer [17]. It is also a common component in many mixed herbal medicines for preventing liver fibrosis [18]. In the present study, we induced preneoplastic foci in livers from female Sprague-Dawley rats by administration of

diethylnitrosamine (DEN), followed by a two-thirds partial hepatectomy (PH), and finally the dietary administration 2-acetylaminofluorene (2-AAF) [19, 20], and then investigated the effects of AM Bunge on formation of preneoplastic foci.

Material and methods

Chemicals

The reagents were obtained from the following sources: 2-AAF and DEN from Sigma Chemicals (St. Louis, Mo.), and rabbit anti-rat GST-P from Medical and Biological Laboratories (Nagoya, Japan). Dr. M. Muramatsu and Dr. M. Sakai (Department of Biochemistry, Hokkaido University School of Medicine) kindly provided the probe for GST-P [21, 22].

Animal and animal care

Female Sprague-Dawley rats at 30 days of age obtained from the Chinese Medical Science Academy were maintained under a 12-h light/dark cycle at 23 °C and a humidity of 60 ± 10%. The animals were allowed 4 weeks to acclimatize. The animal experiments were performed in accordance with local institutional and governmental regulations on the use of experimental animals. The experimental design was based on the resistant hepatocyte model of Solt and Farber [19, 20] and is outlined in Fig. 1. Body weights were measured once a week.

A total of 48 rats were divided into four groups, including model (M group), high-dose treatment (H group), treatment (T group) and control (C group). Rats in the M, H and T groups were injected intraperitoneally (i.p.) with DEN (200 mg/kg in saline) on day 0 and given a basal diet for the first 2 weeks. Rats in the C group were given i.p. saline injections on day 0. All rats were subjected to a two-thirds PH during week 3. The rats in the M, H and T groups were placed on a basal diet containing 0.02% AAF from week 3 to week 8. At the same time, rats in the M and C groups received 3 ml distilled water intragastrically, rats in the H group were given AM intragastrically at a dose of 180 mg/kg body weight, and rats in the T group were given AM intragastrically at a dose of 90 mg/kg body weight, All rats were treated intragastrically once a day. The doses in the T and H groups were based on the dose used clinically, and 7 and 14 times the clinical dose respectively.

AM extract

Rhizomes of AM were cut into small pieces and 200 g of the material was boiled in 4 l of water until the liquid volume reduced to 2 l. The fluid was then filtered through a 1 mm pore size filter, the water-insoluble component was discarded, and the extract was lyophilized to a dry powder. The extract mainly contains saponin, polysaccharide, oflavonoids astragaloside IV, agroastragaloside II and glucopyranoside [23, 24, 25]. Jiangyin Herbal Company, China, kindly provided the lyophilized powder.

A 50-g sample of the dry powder was dissolved in 100 ml distilled water at 37 °C and stirred for 60 min, and the resulting solution was used for intragastric administration.

Histopathological analysis

Livers were removed and ten 5-mm thick slices were cut from the right lateral lobes of individual rats. The slices were fixed in paraffin for subsequent immunohistochemical evaluation of GST-P. Other slices were quickly frozen and kept in liquid nitrogen until RNA and GST-P extraction. Paraffin-embedded blocks were sectioned at a thickness of 3–4 μm and stained immunohistochemically for GST-P using the avidin-biotin peroxidase complex method [26, 27]. Polyclonal anti-GST-P antibody was used at a dilution of 1:1000. For quantitative assessment of lesions, the numbers and diameters of GST-P-positive foci >0.2 mm were measured using an image analyzer (SPICCA II, Nippon Avionics, Tokyo, Japan). For quantitative analysis, they were grouped into the following diameter classes: (1) 0.2–0.5 mm, (2) 0.5–1.0 mm, (3) 1.0–1.5 mm,

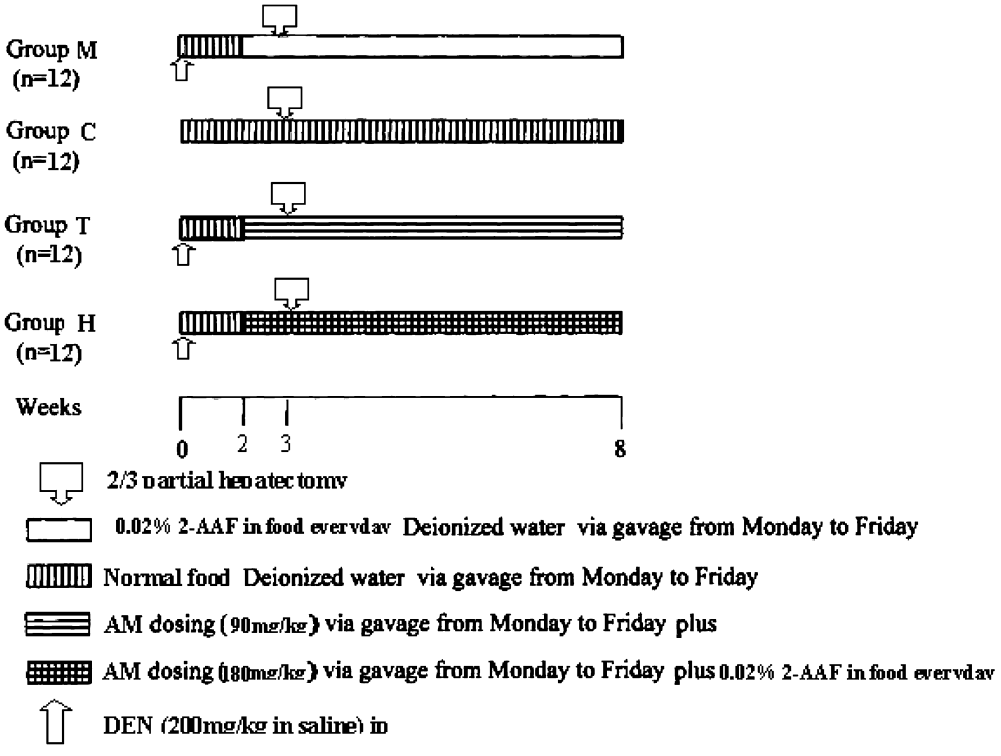

Fig. 1 Schematic representation of AM and DEN-PH-AAF treatment in Sprague-Dawley rats. *Group C* (control) rats were treated with two-thirds partial hepatectomy and deionized water via gavage; *group M* (model control) rats were treated with DEN-PH-AAF, *group T* (AM treatment) rats were treated with DEN and PH first, then gavaged with AM (90 mg/kg) for 6 weeks, and at the same time treated with 2-AAF (0.02% in food); and *group H* (AM high-dose treatment, AM treatment) rats were treated with DEN and PH first, then gavaged with AM (180 mg/kg) for 6 weeks, at the same time treated with 2-AAF (0.02% in food)

(4) > 1.5 mm [28, 29, 30]. This procedure made it inappropriate to count the number of discrete foci, so the percentage of section area occupied by the foci and the mean density of the foci staining were analyzed. We analyzed ten slices and obtained the mean as the end result for each rat. Liver lesions were diagnosed according to the criteria described by Squire and Levitt [31] and the descriptions given by the Institute of Laboratory Animal Resources [32].

Western blotting

Frozen tissues were resuspended in lysis buffer containing Tris-HCl 50 mmol/l (pH 7.4), 1% Triton X-100, EDTA 10 mmol/l, EGTA 2 mmol/l, NaF 10 mmol/l, protease inhibitors, $Na_4P_2O_7$ 1 mmol/l, and Na_2VO_4 2 mmol/l for 1 h. After incubation for 30 min at 4 °C, liver tissue homogenates were centrifuged at 9000 *g* for 15 min. The supernatant was collected and centrifuged at 105,000 g for 60 min. This supernatant was decanted and the bottom phase was collected. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as the standard. For Western blot analysis, each lane contained either 30 μg cytosolic protein. Electrophoresis was performed using a 13.5% running and 4% stacking polyacrylamide gel. Protein was electrotransferred to nitrocellulose filters. The blot was incubated for 1 h in Tween-PBS with 5% nonfat dried milk and incubated overnight at 4 °C with anti-GST-P antibody diluted 1:1000 in PBS. The protein was identified using alkaline phosphatase-labeled anti-rabbit lgG antibody [33]. The intensity of each band was determined by scanning with a computing densitometer.

Northern blotting

Total RNA was extracted from individual liver pieces by the guanidine isothiocyanate-phenol-chloroform procedure [34]. The concentration of RNA was determined by obtaining the absorbance at 260 and 280 nm, and 20 μg of RNA was loaded into each well of the agarose gel. Total RNA was separated using formaldehyde 1% agarose gels, and transferred overnight to a nylon membrane by capillary blotting. Blots were baked for 60 min at 85 °C, prehybridized for 1 h, and then hybridized with the ^{32}P-labeled GST-P probe in hybridization solution at 45 °C for 25 h [26]. Membranes were washed twice for 5 min with 6×SSPE (NaCl 0.15 mol/l, NaH_2PO_4 0.01 mol/l, EDTA 1 mmol/l) plus 0.1% SDS at 37 °C, twice for 15 min with 1×SSPE plus 0.1% SDS at 37 °C, and twice at high stringency at 45 °C with 1×SSPE plus 0.1% SDS for 5 min. The equivalence of the amounts of the total RNA loaded per gel lane was assessed by stripping the membranes, reprobing for GAPDH, and also by monitoring 18S and 28S RNA. Blots were then exposed to Kodak X-OMAT AR-5 film at –70 °C with an intensifying screen. The densities of the autoradiogram bands in digitized images were measured using the public domain National Institutes of Health IMAGE program and a personal computer. For all RNA samples, the density of individual mRNA bands was divided by that of the GAPDH mRNA band to correct for differences in RNA loading.

Statistical methods

The results are expressed as means ± SD, and the data obtained were evaluated by ANOVA as appropriate. The level of significance was set at 5% for each analysis.

Results

Body and liver weights

The effects of AM administration on liver weights are shown in Table 1, Growth curves are shown in Fig. 2.

Table 1 Mean changes in body weight and relative liver weights in rats

Group	Change in body weight	Liver weight as percentage of body weight
M	42.8 ± 8.4**	2.0 ± 0.1**
C	95.0 ± 10.1	1.3 ± 0.2
T	72.3 ± 8.4*	1.5 ± 0.2*
H	83.8 ± 14.0*	1.4 ± 0.2*

*$P < 0.05$ vs the model group (group M)
**$P < 0.05$ vs the control group (group C)

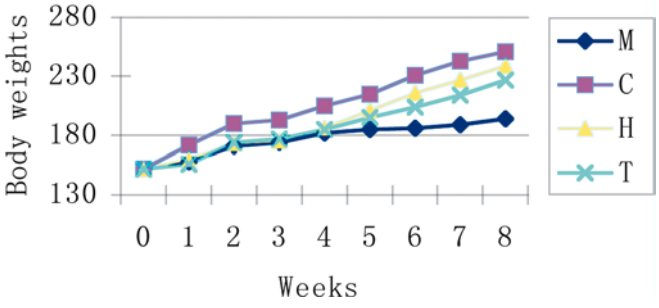

Fig. 2 Growth curves

Because of the variability in both final body weight, liver sizes were compared using the relative liver weights (the liver weight divided by the body weight) to adjust for body size difference. DEN-PH-AAF caused a significant decrease in body weight and increase in relative liver weight. AM essentially prevented this DEN-PH-AAF body weight loss and liver weight gain. This effect was greater in the H group than in the T group. No mortality or major adverse effects were observed in either the H or the T group.

Liver histopathological quantification

In the M group, as expected, there were a variety of advancing altered cell foci of various morphologies, portal tract inflammation and biliary hyperplasia. Proliferation of oval cells was also induced around the portal triads and in the periportal zones. In addition, several animals (3 of 12) had developed several hepatic tumors. When 2-AAF was followed by treatment with AM, there was a reduction in the number of large foci, but there were still considerable numbers of small mixed clear/vacuolated cell foci, some of which were positive for GST-P. No macroscopic tumors were visible. Table 2 presents the results for hepatocellular GST-P-positive foci. Both the numbers and diameters were less in the H and T groups than in the M group. There was a significant difference between T and H groups: the H group showed a greater reduction. GST-P-positive foci are shown in Fig. 3.

Table 2 Mean number and of hepatic GST-P-positive foci in rats per centimeter squared

Group	Diameter class (mm)				Total
	0.2–0.5	0.5–1.0	1.0–1.5	>1.5	
M	54.3 ± 9.3	28.4 ± 6.9	8.5 ± 2.9	1.7 ± 1.2	91.2 ± 13.0**
C	0.6 ± 1.5	0.0	0.0	0.0	0.6 ± 1.5
T	27.9 ± 8.2	17.0 ± 4.69	3.7 ± 3.0	0.6 ± 0.5	49.2 ± 11.3*
H	14.75 ± 4.5	8.1 ± 2.7	2.1 ± 2.2	0.4 ± 0.5	25.3 ± 5.1*

*$P < 0.05$ vs the model group (group M)
**$P < 0.05$ vs the control group (group C)

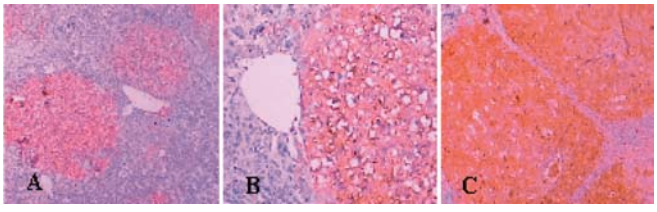

Fig. 3A–C Immunohistochemical staining for GST-P foci in rat livers. **A** Treatment (group T), ×100; **B** treatment (group T), ×200; **C** treatment with DEN-PH-AAF alone (group M), ×100

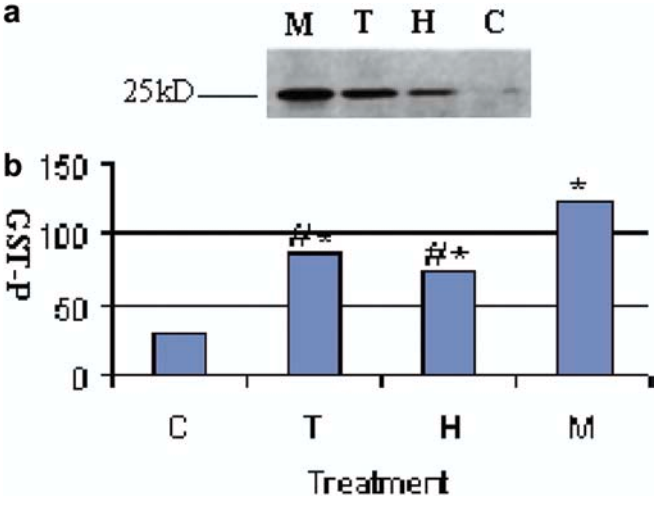

Fig. 4A, B Effect of AM on DEN-PH-AAF-induced rat liver GST-P protein expression. **A** Visualization of the amplified fragments corresponding to GST-P from representatives of each group of rats. **B** Densitometric analysis of GST-P protein expression. Data are presented as means ± SD ($n = 12$). *$P < 0.05$ vs control (group C), #$P < 0.05$ vs treatment with DEN-PH-AAF alone (group M); one-way ANOVA with Scheffe's test

Western blot hybridization of GST-P in the liver

GST-P was detectable at its expected molecular weight by Western blotting. A marked increase was observed in the M group in comparison to the C group (Fig. 4). Marked decreases were observed in the H and T groups in comparison to the M group, The decrease in GST-P expression in the H group was more marked than in the T group (Fig. 4).

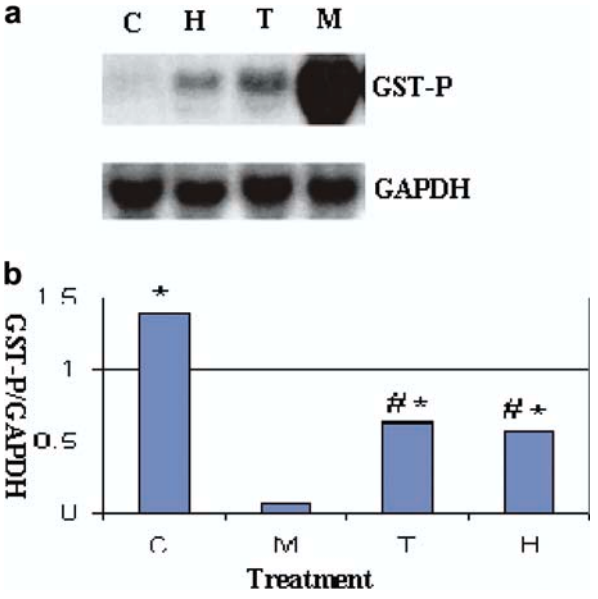

Fig. 5A, B Effect of AM on DEN-PH-AAF-induced expression of rat GST-P mRNA. **A** Visualization of the amplified fragments corresponding to GST-P and GAPDH from representatives of each group of rats. **B** Densitometric analysis of the ratio of GST-P to GAPDH mRNA expression. Data are presented as means ± SD ($n = 12$). *$P < 0.05$ vs control (group C), #$P < 0.05$ vs treatment with DEN-PH-AAF alone (group M); one-way ANOVA with Scheffe's test

Northern blot hybridization of GST-P mRNA in the liver

A 3.2-kb segment of GST-P transcript was clearly demonstrated by Northern blot analysis of total RNA isolated from the livers of all rats (Fig. 5). The levels of GST-P mRNA expression were markedly increased in the livers of models in comparison to controls, in which the expression level was very low. The levels of GST-P mRNA expression in both the H and T groups (Fig. 5) were markedly lower than in the M group, especially in the H group (Fig. 5).

Discussion

Data from this study show that AM had a significant preventive potential and is capable of retarding tumor formation in the DEN-PH-AAF rat model. We used GST-P, a classical marker employed in animal models for monitoring the carcinogenic process by quantifying altered cell foci [6, 7, 8, 9, 10, 11], to evaluate the effect of AM in hepatocarcinogenesis. AM administration resulted in a significant decrease in the number and area of GST-P-positive liver foci, and GST-P protein and mRNA expression. The development of GST-P-positive foci was only delayed and not prevented. All rats in the T, H and M groups had developed hepatocellular carcinoma by week 52.. However, more rats in the M group (9/15) were dead than in the T group (3/15) and H group

(3/15). Tumors were larger in models than in treatment and high-dose rats. Most rats (1/3, 9/12 and 10/12 in the M, H and T groups, respectively) had squamous cell carcinoma of the neck (data not show). This effect was dose related although only two doses were evaluated. Further study of the relationship between dose and effect should is needed.

GST-P expression in hepatocytes is believed to be a biomarker of carcinogenesis in the rodent liver [35, 36]. GSTs are a family of dimeric proteins that play important roles in both the intracellular transport of hydrophobic molecular compounds and the metabolism of toxic compounds. Rat hepatic GSTs have been studied most extensively, and can be grouped into at least seven different families termed alpha (α), mu (μ), pi (π), theta (θ), sigma (σ), kappa (κ) and zeta (ζ) depending on N-terminal sequence, substrate specificity and affinity for non-substrate ligands [37]. GST-P was first found in placenta, but later also in kidney, lung, and testis [7]. GST-P protein, which is hardly detectable in normal rat liver, is expressed at high levels in hyperplastic nodules and hepatocellular carcinomas [7, 38, 39, 40, 41]. The DEN-PH-AAF model is a resistant hepatocyte model. 2-AAF is known to inhibit regeneration of hepatocytes following damage by DEN treatment and PH [19]. In the resistant hepatocyte model, 99–100% of the generated hepatocyte nodules are GST-P-positive, while 90–95% of animals demonstrate γ-glutamyltransferase (GGT) activity during 2-AAF feeding [5, 42, 43, 44, 45]. Phenotypic expression of GGT has been considered to be less stable than that of GST-P because a number of studies have shown subsequent loss of compression and phenotypic reversion of GGT activity in hepatocyte nodules [8, 46, 47]. GST-P is a stable marker for persistent preneoplastic and neoplastic cells not only at the protein level but also at the mRNA level throughout hepatocarcinogenesis in the rats [20].

The mechanism by which AM provides significant protection against hepatocarcinogenesis is not completely clear. The prevention of preneoplastic lesions by AM may be attributable to the prevention of liver fibrosis and hepatoprotection [48, 49]. In addition, the inhibition of carcinogenesis by AM could be attributed to its ability to modulate mutagenesis, DNA binding and metabolism of carcinogens [50]. AM has a number of effects on the immune system [51, 52, 53], and its effects on preneoplastic lesions could be the result of its activity as a biological response modifier.

In our study, Northern blot analysis showed that the content of GST-P mRNA was proportional to protein content. This result is in agreement with those of studies concerning the relationship between protein and mRNA of GST-P [20, 22]. This result suggests that the effect of AM nay be at least partly controlled at the transcriptional level.

In conclusion, the herb, AM, delayed hepatocarcinogenesis in rat livers containing DEN-PH-AAF-induced preneoplastic foci.

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