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ORIGINAL PAPER

Anti-hepatitis B activities of ganoderic acid from *Ganoderma lucidum*

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Abstract Ganoderic acid, from *Ganoderma lucidum*, at 8 μg/ml inhibited replication of hepatitis B virus (HBV) in HepG2215 cells over 8 days. Production of HBV surface antigen and HBV e antigen were 20 and 44% of controls without ganoderic acid. Male KM mice were significantly protected from liver injury, induced with carbon tetrachloride, by treatment with ganoderic acid at 10 mg and 30 mg/kg·d (by intravenous injection) 7 days. Ganoderic acid at the same dosage also significantly protected the mice from liver injury induced by *M. bovis* BCG plus lipopolysaccharide (from *Escherichia coli* 0127:B8).

Keywords Ganoderic acid · *Ganoderma lucidum* · Hepatitis · Hepatoprotection · Virus

Introduction

Ganoderma lucidum (Fr.) Krast (Polyporaceae), known as *Lingzhi* in China and *Reisi* in Japan, is a traditional Chinese medicine used to prevent and treat several diseases, including hepatitis B. It has immunity regulation properties (Zhang et al. 2002) and also has anti-tumor (Wang et al. 1997), anti-viral (Sahar et al. 1998; Kim et al. 2000) and hepato-protective activities (Back et al. 1999). The main bioactive substances in *G. lucidum* include sterols, triterpenes and polysaccharides (Min et al. 1998, 2000, 2001; Oh et al. 2000). Triterpenes of *G. lucidum*, known as ganoderic acids (GA), have anti-HIV-1 activities (Sahar et al. 1998), anti-thrombosis activity (Su et al. 1999), neuroprotective activity (Zhao et al. 2005), anti-tumor activities (Li et al. 2005). GA can selectively inhibit eukaryotic DNA polymerase activities (Mizushina et al. 1999). In this study, we have extracted GA from *G. lucidum* grown under submerged culture and investigated its anti-hepatitis B activity.

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Materials and methods

Materials

Lipopolysaccharide (LPS, from *Escherichia coli* 0127:B8) was purchased from Sigma. Bacille Calmette-Guérin (BCG, *Mycobactarium bovis*, D_2PB320) was purchased from Shanghai Institute of Bioproducts.

Cultivation of *Ganoderma lucidum*

G. lucidum CCGMC 5.616 (China General Microbiological Fermentation Collection Center, Beijing, China) was precultured in 500 ml flasks for 7 days at 30°C on a rotary shaker (150 rpm). This was inoculated at 10% (v/v) into a 5 l stirred-tank bioreactors holding 3.5 l medium (40 g glucose/l, 4 g peptone/l, 2 g yeast extract/l, 1.5 g KH_2PO_4/l, 0.75 g $MgSO_4 \cdot 7H_2O$/l and 0.01 g vitamin B_1/l) and the culture was grow at 30°C for 96 h. The culture broth was obtained by removal of the cells by centrifugation.

Extraction of ganoderic acid (GA)

GA was extracted as described by Tsujikura et al. (1992). The cell-free broth was concentrated to one fourth of the initial volume under reduced pressure, and then 3 × vol ethanol were added gradually, and the mixture was stirred for 3 days at 4°C. After removal of the precipitate by centrifugation, the supernatant was concentrated at 45°C and lyophilized. The residue was suspended in water and extracted with chloroform. The chloroform phase was extracted with 5% (w/v) $NaHCO_3$. The pH of $NaHCO_3$ phase was adjusted to 2–3 with HCl. GA was then extracted again with chloroform. After removal of chloroform, GA was dissolved in dimethylsulfoxide (DMSO) for bioactivity assay.

Cell line and antiviral assay

Cell line HepG2215 (HepG2 cell transferred with HBV DNA, Sells et al. 1987) was obtained from 302 Hospital of the Chinese People's Liberation Army. Cells were cultured in 24-well plates at 10^5 cells/well in MEM medium containing 10% (v/v) fetal bovine serum, 100 units streptomycin/ml and 100 units penicillin/ml, 1 mM glutamine/ml and 200 μg Geneticin G418/ml. After 3 days, the medium was replaced with fresh medium containing GA from 0.5 μg/ml to 8 μg/ml (the control group contained no GA). The concentrations of fetal bovine serum and Geneticin G418 in the fresh medium were amended to 2% (v/v) and 380 μg/ml, respectively. After 4 days, the cultured medium was replaced again with fresh medium containing GA. After 8 days, the cultured medium was replaced with PBS solution containing 0.5 mg MTT/ml. The cultured medium was used to detect HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) using the radioimmunoassay kits (purchased from Institute of Atomic Energy of China, Beijing, China). After 4 h incubation, PBS solution was removed and 1 ml DMSO was added into each well. The absorbancy at 490 nm was measured with a microplate reader.

Animals

Male KM mice weighing 18–22 g were reared at 23 ± 3°C in a 12 h light/dark cycle. Animals were fed with a standard laboratory chow and water ad libitum. The animal experiments were carried out in accordance with the Regulation of the Care and Use of Medical and Laboratory Animals, Ministry of Health, P.R. China.

Assay of protection from carbon tetrachloride-induced liver injury of mice

Mice were randomly divided into five groups of ten animals each. The groups included normal control, model, standard reference and treatment with GA at two concentrations. The normal control group was given no drugs and the model group was given carbon tetrachloride (CCl_4). Standard reference group was given biphenyldicarboxylate at 20 mg/kg·d in saline (i.v.) and the GA groups were given GA at 10 mg or 30 mg/kg·d (i.v.) for seven consecutive days. On the eighth day, all groups were given olive oil with CCl_4 [0.1% (v/v), 10 ml/kg, i.p.] except that the normal control group was given 10 ml olive oil/kg only (i.p.). No food was provided immediately after CCl_4 administration but tap water was available ad libitum. Sixteen hours after CCl_4 administration, blood was withdrawn from the eye socket. All blood samples were centrifuged at 1200 × *g* and 4°C for 10 min to obtain the serum. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with Venusleon-180 Eos bravo biochemistry measuring system.

Table 1 The inhibition effects of ganoderic acid on hepatitis virus antigen and its cytotoxicity to HepG2215 cells

Dose (μg/ml)	Inhibition ratio on HBsAg		Inhibition ratio on HBeAg		Cytotoxinic rate
	4 days	8 days	4 days	8 days	
0.5	19	4	22	49	0
1	42	28	46	43	0
2	67	39	60	47	0
4	74	59	74	48	0
8	87	80	89	56	13

The HepG2215 cells (a hepatitis B virus DNA transferred HepG2 cell line) were cultured on 24-well plates in MEM medium containing ganoderic acid (no ganoderic acid for normal control group). On the 4th day and 8th day after ganoderic acid administration, the content of hepatitis B virus surface antigen (HBsAg) and e antigen (HBeAg) in the cultured medium were measured with radioimmunoassay. Each sample was tested four times. The content of HBsAg and HBeAg in the cultured medium of normal control were 2.23 μg/ml and 0.56 μg/ml on the 4th day, and 2.86 μg/ml and 0.71 μg/ml on the 8th day, respectively. The inhibition ratio was calculated with the formula as:

$$\text{Inhibition ratio} = \frac{\text{antigen content of control–antigen content of ganoderic acid group}}{\text{antigen content of control}} \times 100\%$$

On the 8th day, the cytotoxicity of ganoderic acid to the cells was tested with MTT assay. The absorbance at 490 nm was measured with an EL × 800 Microplate Reader and the cytotoxity was expressed as:

$$\text{Cytotoxitic rate} = \frac{\text{absorbance of control–absorbance of ganoderic acid group}}{\text{absorbance of control}} \times 100\%$$

Assay of protection from BCG/LPS-induced liver injury of mice

Mice were randomly divided into five groups of ten animals each. The groups included normal control, model, standard reference and treatment with GA at two concentrations. Each animal, except the animals in the normal control group, was treated with 0.2 ml BCG (containing 5×10^6 bacteria, i.v) on the first day. The standard reference group was given 20 mg cyclophosphamide/kg·d (i.v.) and GA groups were given 10 or 30 mg GA/kg·d (i.v.) from the first to ninth day. After 10 days, each animal (except normal control) was given 7.5 μg LPS (i.v.). Blood was withdrawn from the eye socket 16 h post the LPS-treatment. All blood samples were centrifuged at 1200 × g and 4°C for 10 min to obtain the serum. The activities of ALT and AST were measured as above.

Results and discussion

Antiviral activity of GA

The results in Table 1 indicate that GA inhibited the HBsAg and HBeAg secretion from HepG2215 cells, and the inhibition was GA dosage dependent. The HepG2215 cell line originated from an independent transfection of HepG2 cells with HBV DNA. The cell line can stably produce HBV virions (Sells et al. 1987). The HBsAg and HBeAg, expressed by the cells, are the structural proteins of HBV virions. Inhibition of HBsAg and HBeAg secretion into the medium by GA probably reflected inhibition effects of GA on the replication of HBV in the cells, or on the expression of HBsAg and HBeAg. GA was nontoxic to HepG2215 cells at the tested dosages.

Protection from CCl_4-induced liver injur\stop

CCl_4 induced a significant increase in activities of alanine and aspartate transaminases (ALT and AST) in serum of the model group, as compared to the control (Table 2). Significantly ($P < 0.01$) lower ALT and AST levels were observed in GA-treated groups than the model group, indicating that GA offered a protection from CCl_4-induced liver injury in the animal of the treated groups. Interestingly, the GA at 10 and 30 mg/kg was even more effective for AST activity than the standard reference biphenyldicarboxylate at 20 mg/kg.

Table 2 The preventive effect of ganoderic acid on hepatotoxicity induced by carbon tetrachloride

Group	Dosage (mg/kg·d)	ALT (U/l)	AST (U/l)
Normal control		49 ± 10	155 ± 21
Model		592 ± 71[a]	556 ± 143[a]
Biphenyldicarboxylate	20	233 ± 69[b]	327 ± 74[b]
Ganoderic acid	10	305 ± 68[b]	318 ± 74[b]
	30	265 ± 91[b]	277 ± 82[b]

Male KM mice were randomly divided into five group of ten in each group. Animals (except normal group) were given CCl_4 [10 ml/kg of a solution of olive oil containing 0.1% (v/v) CCl_4, i.p.] after administration with ganoderic acid or standard reference biphenyldicarboxylate (the model group did not receive ganoderic acid and biphenyldicarboxylate) for seven consecutive days. Sixteen hours after giving CCl4 the blood of each animal was withdrawn from the eye socket, and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. Values are mean ± S.D. ($n = 10$)

[a]Statistically significant when compared with the normal data ($P < 0.01$)

[b]Statistically significant when compared with the model data in the same columns ($P < 0.01$)

Table 3 The preventive effect of ganoderic acid on liver injury induced by *M. bovis* Bacille Calmette-Guérin and lipopolysaccharide

Group	Dosage (mg/kg·d)	ALT (U/l)	AST (U/l)
Control		68 ± 27	251 ± 34
Model		275 ± 43[a]	509 ± 52[a]
Cyclophosphamide	20	98 ± 28[b]	386 ± 97[b]
Ganoderic acid	10	181 ± 57[b]	415 ± 80[b]
	30	149 ± 51[b]	377 ± 85[b]

Male KM mice were randomly divided into five group of ten in each group. Each animal (except normal group) was given 0.2 ml Bacille Calmette-Guérin (containing 5×10^6 bacteria, i.v) on the first day, and 7.5 μg lipopolysaccharide (i.v.) after administration with ganoderic acid or standard reference cyclophosphamide (the model group did not receive ganoderic acid and cyclophosphamide) for 10 consecutive days. Sixteen hours after giving lipopolysaccharide, the blood of each animal was withdrawn from the eye socket, and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. Values are mean ± S.D. ($n = 10$)

[a]Statistically significant when compared with the normal data ($P < 0.01$)

[b]Statistically significant when compared with the model data in the same columns ($P < 0.01$)

Protection from BCG/LPS-induced liver injury

The levels of ALT and AST were significantly ($P < 0.01$) higher in the model group than in the normal control group (Table 3). This indicated that BCG + LPS induced liver injury. Cyclophosphamide could suppress the activities of ALT and AST as expected. GA reduced liver injury because the ALT and AST levels in the GA treated groups were significantly ($P < 0.01$) lower than those in the model group. However, GA at the treated dosages appeared to be not more effective than cyclophosphamide for ALT.

The models of CCl_4-induced hepatotoxicity and BCG + LPS-induced immunity liver injury models are conventionally used to investigate novel liver protective agents. Administration of CCl_4 and BCG + LPS caused a rapid increase in ALT and AST levels, which reflect liver injury. Serum transaminase elevation has been reported to be associated with a number of inflammatory disorders (Hoder and Wilkinson 1980) and hepatocellular damages (Sinha and Saran 1972). Leakage of large quantities of enzymes into the blood stream is often associated with massive necrosis of liver (Rees and Spector 1961). CCl_4-induced acute liver injury is similar to the damage of acute hepatitis (Recknagel 1967). The present study indicated that GA could reduce ALT and AST levels in the treated mice, implying that GA had a hepatoprotective effect on CCl_4- and BCG + LPS-induced liver injuries.

The GA roles in antiviral activity and reduction of liver injury illustrated in this study suggested that GA had potential to be a anti-hepatitis medicine, though further investigation on the mechanism of GA actions is necessary.

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