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Cytoprotective Activity of Components of Garlic, Ginseng and Ciuwjia on Hepatocyte Injury Induced by Carbon Tetrachloride *In Vitro*

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ABSTRACT

The antihepatotoxic activity of ten components related to plants were investigated using freshly isolated hepatocytes which maintained specific liver functions such as glucagon-dependent glycogenolysis and albumin producibility. Six components of garlic, i.e., S-methyl cysteine, S-ethyl cysteine, S-propyl cysteine, S-allylmercapto cysteine, alliin and S-allyl cysteine, and two syringaresinols of ciuwjia were synthesized. Two ginsenosides were purified from extract of ginseng.

Both syringaresinols and S-allyl cysteine at concentrations of 250ng/ml and 0.5μg/ml, respectively, completely suppressed cytotoxicity on hepatocytes by CCl_4, as judged from GPT level released in the culture medium and morphology of the hepatocytes in stained specimens. The same was observed with S-propyl cysteine, S-allylmercapto cysteine and two ginsenosides at concentrations of 10μg/ml or less. Alliin was also effective but suppressed only GPT leakage. Four positive control drugs used was less effective. S-methyl cysteine and S-ethyl cysteine showed no obvious effect in any concentrations.

Recently, attempts to find antihepatotoxic agents have been made using freshly isolated hepatocytes which have a variety of specific liver functions[6,14,16]. The data available demonstrate that this *in vitro* assay method is a screening system suitable for evaluating liver protective drugs, since highly reliable results have been obtained more quickly than with *in vivo* assay methods.

Garlic, ginseng and ciuwjia have been reported to have various interesting physiological activities toward animal cells such as stimulation of metabolisms[1,12], detoxification[3,5,7,17], regulation of growth or differentiation[10], etc., and have been used empirically as medicine since prehistoric times.

This paper described the protection of hepatocytes with constituents of these plants from damage induced by carbon tetrachloride (CCl_4). Culture conditions and a few biological properties of primary cultured hepatocytes were also examined.

MATERIALS AND METHODS

Materials

Components tested are listed in Chart 1. S-methyl cysteine (SMC), S-ethyl cysteine (SEC), S-propyl cysteine (SPC), S-allylmercapto cysteine (SAMC), alliin and S-allyl cysteine (SAC) are main components of *Allium sativum formapekinense* (Garlic), and (±)-and (−)-syringaresinol exist in *Eleutherococcus*

senticosus (Ciuwjia). These eight compounds were synthesized in our laboratory. Ginsenosides Rb_1 and Rg_1 were extracted from *Panax ginseng* and purified[4]. All the compound were dissolved in distilled water.

Bovine insulin, bovine glucagon and dexamethasone were purchased from Sigma, St Louis, U.S.A.; medium 199 (M199, Earle's salts) and fetal bovine serum (FBS) from GIBCO, New York, U.S.A.; collagenase, carbon tetrachloride (CCl_4), glucose C-test kit and transaminase (GPT) C-test kit from Wako Pure Chem. Indust., Osaka, Japan; ^{3}H-amino acid mixture from Amersham, Essex, England; and anti rabbit IgG serum from MILES-YEDA LTD., Rehouot, Israel.

Isolation of hepatocytes

Hepatocytes from fed male rats of the Wistar strain (Clea Japan Inc., Osaka, Japan), weighing 180 to 250g, were isolated by the collagenase perfusion technique from portal vein *in situ* according to Seglen[13] with some modifications. After perfusion, cells were suspended, passed through a #150 mesh nylon filter and washed gently three times with M199 to eliminate cell debris and non-parenchymal cells. The cells were suspended in M199 supplemented with 10% FBS and plated on ϕ35mm plastic dishes (Falcon, Oxnard U.S.A.) at a concentration of 5×10^5 cells/ml/dish. Viability of the cells was greater than 90% as judged by the trypan blue exclusion test. The cells were incubated for 3 hr in a humidified incubator at 37°C under 5% CO_2 in air for the hepatocytes to adhere to the substrate, and then washed once with M199 to remove floating cells and used for the following experiments.

Properties of primary cultured hepatocytes

To check the response to glucagon, the hepatocytes adhered onto the dishes were incubated in M199 containing 10% FBS, 10^{-6}M dexamethasone and 10^{-9}M insulin for 5 or 56 hr. At the outset of the last 5 hr of incubation, the medium was replaced with one supplemented with 2mg/ml glucose. After the end of incubation, the cells were washed thoroughly with glucose-free Hanks' balanced salt solution (HBSS) and incubated further in HBSS in the presence of 0, 10^{-9} or 10^{-7}M glucagon. Reaction was terminated by adding ice-cold buffer. The quantity of glucose released from the hepatocytes into the medium was measured with a glucose test kit.

S-Allyl cysteine $CH_2=CH-CH_2-S-CH_2-CH(NH_2)-COOH$

S-Propyl cysteine $C_3H_7-S-CH_2-CH(NH_2)-COOH$

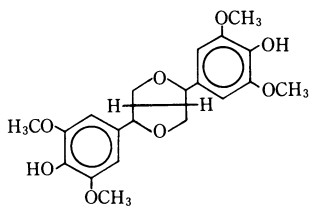
S-Allylmercapto cysteine $CH_2=CH-CH_2-S-S-CH_2-CH(NH_2)-COOH$

Alliin $CH_2=CH-CH_2-S(=O)-CH_2-CH(NH_2)-COOH$

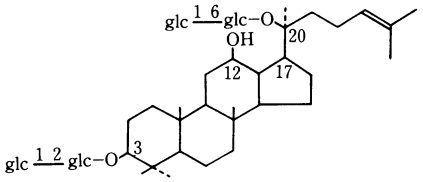
S-Ethyl cysteine $C_2H_5-S-CH_2-CH(NH_2)-COOH$

S-Methyl cysteine $CH_3-S-CH_2-CH(NH_2)-COOH$

Syringaresinol



Ginsenoside Rb_1



Ginsenoside Rg_1

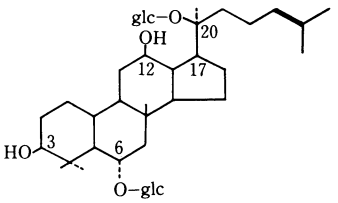


Chart 1. Chemicals and their constitutional formulae

For measuring the amount of secreted albumin into the medium, the hepatocytes were incubated in M199 containing 10% FBS, 10^{-6}M dexamethasone, 10^{-9}M insulin, and additional 5μCi/ml of ^{3}H-amino acid mixture for 24 hr, and the rate of albumin production in the medium was determined by the indirect radioimmunoprecipitate method. For this assay, albumin (Fraction V) from pooled serum of rats was purified with a column chromatography of TOYOPEARL HW-50 gel (TOYO SODA MFG CO., LTD., Tokyo, Japan). Antialbumin serum was prepared by immunizing Japanese white strain rabbits with the albumin emulsified into Freund's adjuvant. Control serum was collected from the same rabbits before immunization. The amount of total protein secreted into the medium was determined by measuring the radioactivity incorporated into trichloroacetic acid-insoluble fraction.

Determination of antihepatotoxic activity

The hepatocytes attached on plastic dishes were precultured in M199 supplemented with 10% FBS, 10^{-6}M dexamethasone, 10^{-9}M insulin and 10^{-7}M glucagon for 20 hr in order to facilitate recovery from cell damage during the process of dispersion. The cells were then washed in HBSS and incubated in the same medium in the presence of 10mM CCl_4 and various concentrations of test compounds for 60 min at 37°C. Optimum culture condition and concentration of CCl_4 were determined beforehand. Following the treatment, the medium was collected and measured for GPT level released from the hepatocytes with a transaminase kit.

Structural features of the cells were observed under an optical microscope (NIKON, Model XUW-31), using the cells fixed in 10% buffered formalin or methanol followed by May-Grünwald and periodic acid Schiff (PAS) stainings.

Vitamin E (VE, Daiichi Chem. Pharm. Co., Ltd., Tokyo, Japan), piperonyl butoxide (PB, Tokyo Kasei, Tokyo, Japan), glycyrrhizin (Tokyo Kasei, Tokyo, Japan) and glutathione (Sigma, St Louis, U.S.A.) were used as positive control drugs against hepatocyte injury induced by CCl_4. These drugs were suspended in ethanol except for glutathione which was dissolved in distilled water. Final concentration of ethanol was less than 0.1% and did not affect the results of the experiments.

RESULTS

Properties of cultured hepatocytes

Characteristics of cells *in vitro* is so changeable through cultivation that we examined whether or not the hepatocytes cultured maintained specific liver functions; the ability of responding to glucagon and of producing albumin. As shown in Fig. 1, glucagon stimulated glycogenolysis of the cells in a dose dependent fashion. Glucose level released from 5 hr cultured cells was 2.6μg/10^6 cells/min in the medium without adding glucagon, whereas it was 2.9μg/10^6 cells/min with 10^{-9}M and 4.6μg/10^6 cells/min with 10^{-7}M glucagon, respectively. The dose dependent response was also observed with

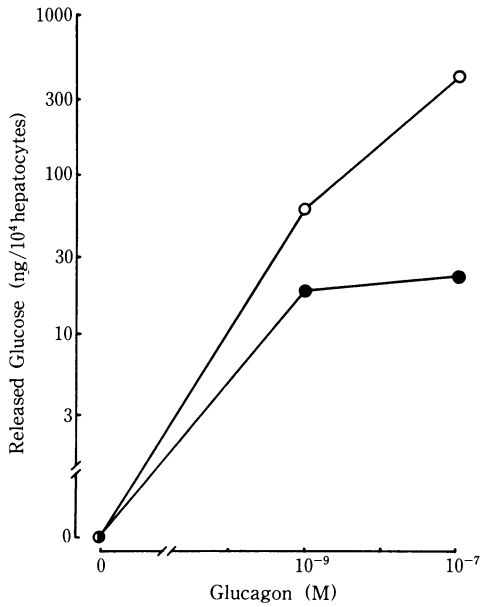


Fig. 1. Effect of glucagon on glycogenolysis in cultured hepatocytes.

Hepatocytes (2.0 — 2.5 × 10^4 cells/cm^2) adhered on plastic dishes were cultured for 5 hr (—○—) or 56 hr (—●—). In the last 5 hr of culture, cells were cultured in the medium supplimented with 2mg/ml glucose. After that each dish was washed with HBSS, and incubated in the indicated concentration of glucagon for 40 min at 37°C, and the amounts of glucose released from hepatocytes into the medium was measured. Each point plotted is the mean of triplicate values.

the cells cultured for 56 hr, but the glucagon stimulation was less than mentioned above. Similar results were reported by Brass and his coworkers[2]. A small number of glycogen granules were observed within cytoplasm of the cells up to day 4 culture.

Albumin secretion in the medium from the cultured hepatocytes continued to increase up to day 6 culture (Fig. 2). Since the amount of total protein decreased gradually, the ratio of albumin to total protein in the medium altered from one-sixth in day 2 culture to one-forth in day 6 culture.

Asymptotic decreased number of cells is seen in the culture (data not shown) as reported by others[8,11].

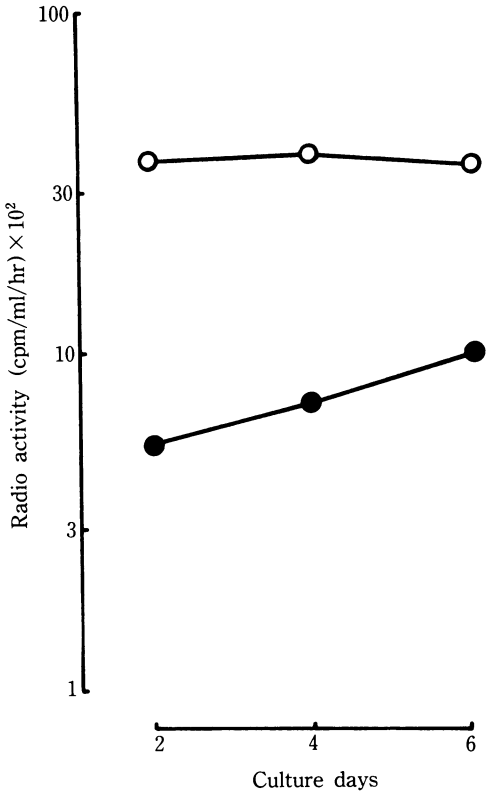


Fig. 2. Time course study on albumin producibility in cultured hepatocytes.

Hepatocytes ($2.0 - 2.5 \times 10^4$ cells/cm^2) were cultured up to day 6 culture, and 5μCi/ml of ^{3}H-amino acid mixture was added into the medium before 24 hr at indicated days, respectively. The rate of radio-labeled albumin excreted (—●—) into the medium was measured by indirect immunoprecipitate method. TCA-insoluble materials in the medium was measured for determination of total protein (—○—). Each point plotted is the mean of triplicate values.

Assay of antihepatotoxic activity of test compounds

As the hepatocytes were proved to maintain specific liver functions through a culture period, the optimum concentration of CCl_4 was determined to cause damage to hepatocytes. Increase in GPT leakage from the cells was found in the presence of 7.5mM CCl_4, and maximum GPT level in the medium was observed by the addition of 10mM CCl_4 (Fig. 3). Insulin at a concentration as high as 10^{-6}M added in the reaction medium significantly reduced the GPT leakage (Fig. 4).

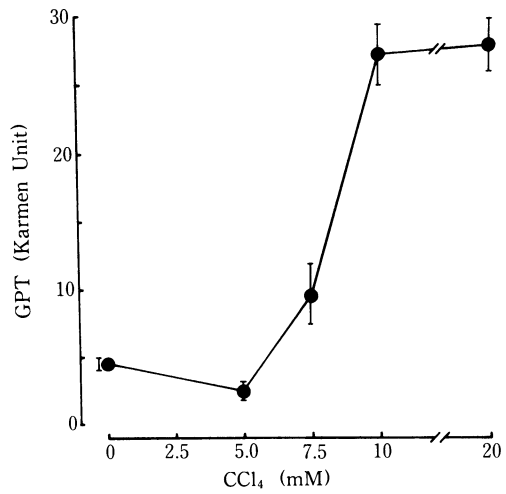


Fig. 3. Dose dependence of CCl_4 on leaked GPT level in cultured hepatocytes.

After adhersion, hepatocytes were cultured for 24 hr, and incubated in the presence of various concentrations of CCl_4 for 60 min. And then leaked GPT activity in the medium was measured by transaminase test kit. Each point is mean ± SD for five separate determinations.

The CCl_4 induced cell damage was remarkably prevented at a concentration as low as 250ng/ml of both syringaresinols as shown in Fig. 5. With a wide range of doses from 0.5 to 100μg/ml, SAC was also effective against CCl_4 induced hepatotoxity. The hepatocytes thus treated were morphologically the same as the intact cells. SPC, SAMC, ginsenoside Rb_1 and Rg_1 at concentrations of 10μg/ml or less protected the hepatocytes from CCl_4 injury, the doses were lower than that of VE.

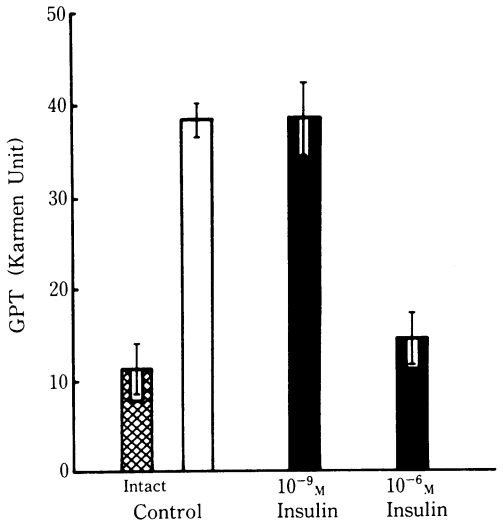


Fig. 4. Inhibitory effect of insulin on GPT leakage from hepatocytes.

Precultured hepatocytes for 3 hr were incubated in the medium containing 10mM CCl_4 with (closed column) or without (open column) insulin. GPT level leaked into the medium was determined by transaminase test kit. Latticed column indicates leaked GPT level from intact cells. Each column is the mean of five separated determinations, and the bar represents the SD at each condition.

The cell protective effect was also observed in the presence of 10 to 50μg/ml leucine (Fig. 5). Alliin and cysteine protected GPT leakage from the hepatocytes, but cell damage was slightly observed.

DISCUSSION

Is it well-known that garlic, ginseng and ciuwjia have various physiological activities and information is available about their liver protective[3,5,7,17] and other related activities[1,12]. However, major components of these plants responsible for the activities have not been elucidated as yet. Six synthesized constituents of garlic, two of ciuwjia and two of ginsenosides were investigated for their efficacy to protect cultured hepatocytes from damage induced by carbon tetrachloride.

Extremely strong protective effect from CCl_4 injury was observed when the hepatocytes were cultured in the presence of very low concentrations of syringaresinols contained in ciuwjia, showing that ciuwjia was the most promising compound tested in the present study. Although the mechanism involved is not clear, stimulation of protein synthesis of ciuwjia as reported by Ro et al[12] might be concerned directly or indirectly with the antihepatotoxicity of syringaresinols.

In general, sulfur-containing amino acids act as liver protective agents[9,15]. All the six compounds contained in garlic carry cysteine molecule in their structure. Among them SAC showed the most potent antihepatotoxic activity. SAMC and SPC were also effective. Effect of alliin was much the same as cysteine but partial. SMC and SEC were not effective independent of cysteine molecule.

Recently, antioxidant and detoxifying activities of ginseng were reported by Hong et al[3] and Lee et al[7]. In our study, strong antihepatotoxic activity was observed on two ginseng saponins, ginsenoside Rb_1 and Rg_1 at fairly low concentrations. These ginsenosides may play a major role in hepato-protection of ginseng.

Our results indicate that the *in vitro* assay system for screening antihepatotoxic agents is very useful. The active compounds evaluated in our study could be expected for medicine against liver damage.

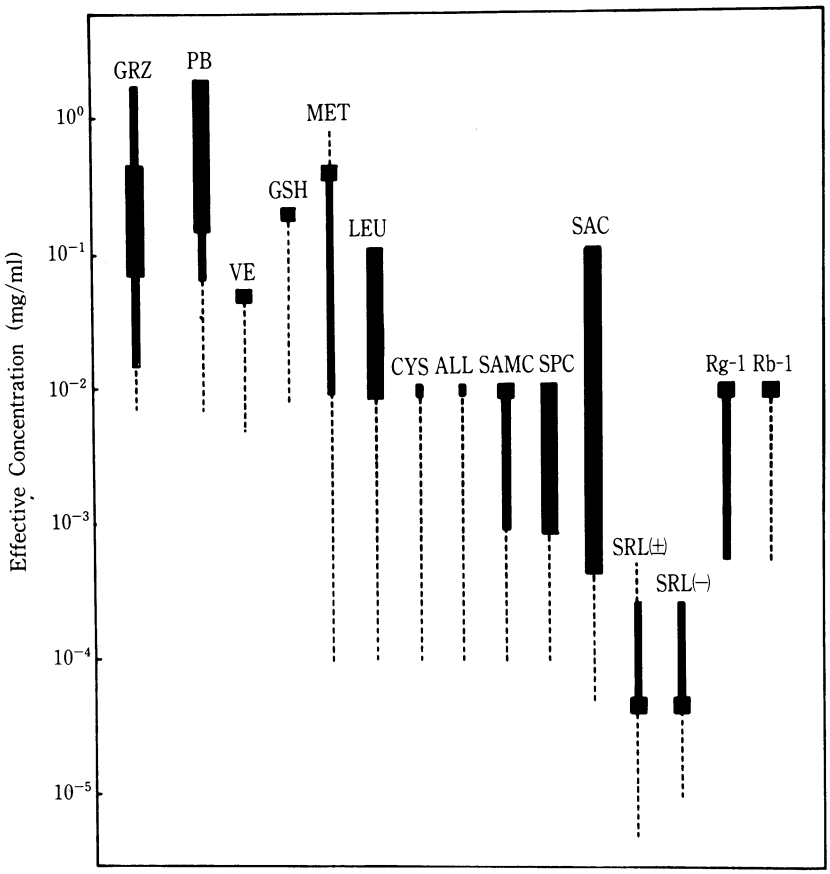


Fig. 5. Relative antihepatotoxic activity on various compounds from plants in primary cultured cells.

Hepatocytes precultured for 20 hr were incubated in the medium containing both various concentrations of compounds and 10mM CCl_4 for 60 min. Antihepatotoxic activity of these compounds were determined from the results in the amounts of GPT leakage and in the morphological observation.

dotted line: examined but no effect
solid line: dose range suppressed only GPT leakage
broad line: dose range obtained antihepatotoxic activity both in suppression of GPT leakage and in morphological observation

Abbreviations used were; GRZ: glycyrrhizin, PB: piperonyl butoxide, VE: vitamin E, GSH: glutathione, MET: methionine, LEU: leucine, CYS: cysteine, ALL: alliin, SAMC: S-allylmercapto cysteine, SPC: S-propyl cysteine, SAC: S-allyl cysteine, SRL(±): (±)-syringaresinol, SRL(−): (−)-syringaresinol, Rb_1: ginsenoside Rb_1, Rg_1: ginsenoside Rg_1.

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