Preventive Effects of Saponins from *Puerariae Radix* (the root of *Pueraria lobata* OHWI) on *in Vitro* Immunological Injury of Rat Primary Hepatocyte Cultures¹⁾

Tomonori ARAO,^a Manabu UDAYAMA,^a Junei KINJO,^a Toshihiro NOHARA,^{*,a}
Takayuki FUNAKOSHI,^b and Shoji KOJIMA^b

Laboratory of Pharmacognosy^a and Laboratory of Hygienic Chemistry,^b Faculty of Pharmaceutical Sciences,
Kumamoto University, 5-1 Oe-Honmachi, Kumamoto 862, Japan.

Received March 25, 1997; accepted May 29, 1997

The preventive effects of saponins from *Puerariae Radix* toward *in vitro* immunological liver injury using an antiserum against the rat liver plasma membranes on primary cultured rat hepatocytes were studied. Crude saponin from *Puerariae Radix* inhibited the elevation of alanine aminotransferase (ALT) activity at the dose of 90 $\mu\text{g/ml}$. The inhibition was stronger than that of glycyrrhizin, which was a positive control drug. The representative saponins in this drug, soyasaponin I and kudzusaponin SA₃, were also more effective than glycyrrhizin, although their effects were weaker than that of crude saponin at the lower doses (90, 200 $\mu\text{g/ml}$). At 500 $\mu\text{g/ml}$, kudzusaponin SA₃ showed antihepatotoxic activity equal to that of crude saponin.

Key words hepatoprotective effect; primary cultured rat hepatocytes; triterpenoidal saponin; *Pueraria lobata*

Puerariae Radix, the root of *Pueraria lobata* (WILLD.) OHWI, is one of the most important oriental crude drugs used as a perspiration, antipyretic and antispasmodic agent. This drug, whose Japanese name is Kakkon, is also the main crude drug of Kakkon-To which has been used for treatment of the common cold since ancient times in China and Japan. During the course of our studies on the constituents of this drug, we have reported fourteen new oleanene-type triterpene saponins.²⁾

Among the pharmacological actions of *Puerariae Radix*, the antispasmodic principles are known to be attributed to isoflavones, represented as daidzein.³⁾ However, the active principles of perspiration and antipyretic activity has never been elucidated in a routine manner to date. Very recently, Shiraki *et al.* disclosed that Kakkon-To significantly suppressed the rise of IL-1 level in serum.⁴⁾ Since IL-1 is a mediator of the inflammation responsible for fever induction, the insoluble actions (perspiration and antipyretic) of this drug are assumed to be due to similar antiinflammatory action.

Meanwhile, our laboratory has clarified that the oleanene-type triterpene saponins from *Puerariae Flos* (the flowers of *Pueraria lobata*)⁵⁾ and *Abri Herba* (the whole plants of *Abrus cantoniensis*)⁶⁾ are effective for *in vivo* experimental hepatic injury. On the other hand, Higuchi *et al.* reported an assay method using *in vitro* immunological liver injury and confirmed the hepatoprotective effects of oleanene-type triterpene saponins from soybeans.⁷⁾ They used an antiserum against rat plasma membranes as a lesion model of liver cells. The mechanism of this immunological liver injury is regarded as being caused by complement mediated cell damage.⁸⁾ Since the antibody-complement system plays an important role in humoral immunity, we tried to clarify the actions of oleanene-type triterpene saponins in *Puerariae Radix* on *in vitro* immunological liver injury of rat primary hepatocyte cultures.

MATERIALS AND METHODS

Animals Male Wistar rats (6 weeks old, body weight 150—160 g) and male New Zealand white rabbit (body weight 3 kg) were used.

Materials The following reagents were purchased: Eagle's minimum essential medium (Eagle's MEM, Nissui), fetal bovine serum (GIBCO), L-glutamine (Wako), collagenase (Wako), trypsin inhibitor (Wako), insulin (Sigma), dexamethasone (Nacalai Tesque), Freund's incomplete adjuvant (Difco Laboratories) and percoll (Sigma). All other reagents used were of analytical grade. Glycyrrhizin was isolated from Glycyrrhizae Radix (Uchida Wakanyaku Co., Ltd.).

Preparation of Primary Cultured Rat Hepatocytes Liver cells were isolated according to a procedure developed by Berry and Friend.⁹⁾ The detailed procedure, except for the cell concentration (1×10^5 cells/ml) in the well plate, was described in the previous paper.^{6c)}

Preparation of Antiserum to the Rat Liver Plasma Membranes The rat liver plasma membranes were prepared according to a procedure developed by Loten *et al.*¹⁰⁾ The suspension of rat plasma membrane obtained was mixed with the same volume of Freund's incomplete adjuvant to use as an antigen. An antibody to the rat plasma membrane was raised in rabbits first by injection of 5 mg of protein followed by four injections of 2.5 mg of protein over a period of 5 weeks. The antiserum to the rat liver plasma membranes was prepared by the method of Harboe and Ingild.¹¹⁾

Effect of the Antiserum Against Rat Plasma Membranes toward Primary Cultured Rat Hepatocytes One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the medium (300 μl) containing the antiserum against rat plasma membranes (0, 10, 20, 40, 80, and 160 $\mu\text{l/ml}$). At 20, 30, 40, 50, and 60 min after the antiserum was challenged, aliquots of medium were drawn out at each time for the determination of aspartate

* To whom correspondence should be addressed.

aminotransferase (AST) and alanine aminotransferase (ALT).

Effects of Saponins toward *in Vitro* Immunological Liver Injury One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the medium (300 μ l) containing the antiserum against rat plasma membranes (40 μ l/ml) and DMSO solution (4 μ l) of the tested compound or glycyrrhizin [final concentration 0 (reference), 10, 30, 90, 200, 500 μ g/ml]. At 40 min after the antiserum was challenged, the medium was drawn out for the determination of ALT.

Instrument and Assay Method The activities of AST and ALT were assayed by an autoanalyzer, COBAS MIRA (Roche), using commercial kits based on the GOT and GPT assay method.¹²⁾

Statistical Analysis The data are shown as the mean $\pm$ S.D. ($n=4$ or 12). After analysis of variances, Tukey's test was employed to determine the significance of differences between reference and experimental samples.

RESULTS AND DISCUSSION

The activities of AST and ALT released from the hepatocytes after treatment with the antiserum are shown in Fig. 1. The extracellular AST activity significantly increased at 20, 30, 40, 50 and 60 min, respectively, after treatment with 10, 20, 40, 80 and 160 μ l/ml antiserum. This tendency didn't change at any dose until 5 h. However, at 60 min after the challenge of any dose, most of the cells were stained by trypan blue, which suggested that the cells had died almost completely. On the other hand, the ALT level reached a plateau at 80 and 160 μ l/ml after 20 min of the challenge. Since the extracellular ALT activity was increased with the treatments of 10, 20 and 40 μ l/ml antiserum and with the progress of time, the ALT value seemed to reflect the liver injury correctly. Especially at 40 μ l/ml, the ALT level increased with time and nearly

reached a plateau at 40 min. Therefore, treatment with 40 μ l/ml antiserum for 40 min was considered sufficient for injury on primary cultured rat hepatocytes. Moreover, the antihepatotoxic activities of the tested compound and glycyrrhizin (positive control) were determined by the activities of ALT released with the hepatocytes.

Since the triterpene saponins in this drug are divided into monodesmosides and bisdesmosides, we tested the representative ones, *i.e.* soyasaponin I, as the monodesmoside and kudzusaponin SA₃ as the bisdesmoside. Figure 2 shows the results of the antihepatotoxic action of glycyrrhizin (positive control). Figures 3, 4, and 5 indicate the same actions for crude saponin, soyasaponin I and kudzusaponin SA₃, respectively. After the addition of these samples, no morphological change in the hepatocytes was observed at any dose. At 500 μ g/ml, glycyrrhizin showed its efficacy toward injured liver cells, but this action is lower than the efficacy toward the CCl₄-induced liver injury demonstrated in the previous paper.^{6c)} Generally, glycyrrhizin is not so effective toward *in vitro* immunological liver injury.¹³⁾ Our result also supported those data. The preventive effect of the crude saponin from *Puerariae Radix* toward immunological liver injury was shown to

Fig. 1. Dose and Time Dependence of the Antiserum against Rat Plasma Membranes on the Activity of AST and ALT Released from Primary Cultured Rat Hepatocytes

One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the medium containing the antiserum against rat plasma membranes (0, 10, 20, 40, 80, and 160 μ l/ml). At 20, 30, 40, 50, and 60 min after the antiserum was challenged, aliquots of the medium were drawn out at each time for the determination of AST and ALT. The data are the mean $\pm$ S.D. for 4 independent cell preparations.

Fig. 2. Effect of Glycyrrhizin toward *in Vitro* Immunological Liver Injury on Primary Cultured Rat Hepatocytes

One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to a medium containing the antiserum against rat plasma membranes (40 µl/ml) and the DMSO solution (4 µl) of the tested compound or glycyrrhizin. At 40 min after the antiserum was challenged, the medium was drawn out for the determination of ALT. The data are the mean $\pm$ S.D. for 12 independent cell preparations. Reference (Ref.) values were challenged with the antiserum and not treated with glycyrrhizin. Significantly different from Ref., effective ** p < 0.01.

Fig. 3. Effect of Crude Saponin toward *in Vitro* Immunological Liver Injury on Primary Cultured Rat Hepatocytes

Conditions were the same as those given in the legend to Fig. 2 except that crude saponin was measured instead of glycyrrhizin. The data are the mean $\pm$ S.D. for 12 independent cell preparations. Reference (Ref.) values were challenged with the antiserum and not treated with crude saponin. Significantly different from Ref., effective ** p < 0.01.

be much more effective than that of glycyrrhizin. It is significantly effective even at 90 µg/ml, about 1/5 concentration of required glycyrrhizin. Furthermore, it almost completely depressed the ALT release over 200 µg/ml. Soyasaponin I and kudzusaponin SA₃ also showed antihepatotoxic action greater than that of glycyrrhizin. Although kudzusaponin SA₃ was more effective than soyasaponin I, both saponins depressed the ALT release almost completely at 500 µg/ml.

In the meantime, very recently, we disclosed the antihepatotoxic actions of soyasaponins I—IV which were isolated from *Glycine soya* using the same model.¹⁴⁾ The order of potency was soyasaponins III > IV > I > II. Especially, soyasaponin III was quite effective at less than

Fig. 4. Effect of Soyasaponin I toward *in Vitro* Immunological Liver Injury on Primary Cultured Rat Hepatocytes.

Conditions were the same as those given in the legend to Fig. 2 except that soyasaponin I was measured instead of glycyrrhizin. The data are the mean $\pm$ S.D. for 12 independent cell preparations. Reference (Ref.) values were challenged with the antiserum and not treated with soyasaponin I. Significantly different from Ref., effective * p < 0.05, ** p < 0.01.

Fig. 5. Effect of Kudzusaponin SA₃ toward *in Vitro* Immunological Liver Injury on Primary Cultured Rat Hepatocytes

Conditions were the same as those given in the legend to Fig. 2 except that kudzusaponin SA₃ was measured instead of glycyrrhizin. The data are the mean $\pm$ S.D. for 12 independent cell preparations. Reference (Ref.) values were challenged with the antiserum and not treated with kudzusaponin SA₃. Significantly different from Ref., effective * p < 0.05, ** p < 0.01.

50 µg/ml. Since soyasaponins I—IV have the same aglycone (soyasapogenol B), the differences of the actions arose from the differences of the sugar units linked at C-3. That is to say, the saponins having a disaccharide group (soyasaponins III and IV) showed greater action than those with a trisaccharide group (soyasaponins I and II). Therefore, the strong depressing action for the crude saponin of *Puerariae Radix* toward the ALT release might be attributed to the saponin mixture having a disaccharide group (kudzusaponins SA₁, SA₂, SA₄, A₂ and A₄). A survey of other antihepatotoxic saponins in this drug, using the presently devised method, is now in progress.

It has become clear that the total crude saponin from *Puerariae Radix* possesses preventive effects toward *in vitro* immunological liver injury on primary cultured rat hepatocytes. In connection with this view, Shinohara *et*

al. elucidated the anticomplement actions of soyasaponin I analogs. They also revealed antinephritic actions of those analogs on an experimental *in vivo* model.¹⁵⁾ Since some inflammation, including hepatitis and nephritis, are caused by excessive immunoreaction, it could be understood that this result is one clue for analyzing the anti-inflammatory actions of *Puerariae Radix* responsible for its perspiration and antipyretic effects.

Acknowledgements This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (05671758).

REFERENCES AND NOTES

- 1) Part 2 in a series of studies on the hepatoprotective drugs; part 55 in a series of studies on the constituents of leguminous plants.
- 2) Kinjo J., Miyamoto I., Murakami K., Kida K., Tomimatsu T., Yamasaki M., Nohara T., *Chem. Pharm. Bull.*, **33**, 1293—1296 (1985); Arao T., Kinjo J., Nohara T., Isobe R., *ibid.*, **43**, 1176—1179 (1995); Arao T., Idzu T., Kinjo J., Nohara T., Isobe R., *ibid.*, **44**, 1970—1972 (1996); Arao T., Kinjo J., Nohara T., Isobe R., *ibid.*, **45**, 362—366 (1997).
- 3) Shibata S., Harada M., Murakami T., *Yakugaku Zasshi*, **79**, 863—868 (1959); Shibata S., *Shoyakugaku Zasshi*, **40**, 1—18 (1986).
- 4) Kurokawa M., Iwakita M., Kumeda C., Yukawa T., Shiraki K., *J. Trad. Med.*, **13**, 201—209 (1996).
- 5) Nohara T., Takeshita T., Kinjo J., Ito H., Niiho Y., Yamasaki T., *Wakan-Iyaku Gakkaishi*, **5**, 408—409 (1988); Niiho Y., Yamasaki T., Nakajima Y., Itoh H., Takeshita T., Kinjo J., Nohara T., *Yakugaku Zasshi*, **109**, 424—431 (1989); *Idem, ibid.*, **110**, 604—611 (1990).
- 6) a) Takeshita T., Ito Y., Sakai Y., Nohara T., Yasuhara M., Saito H., Kitagawa I., Ariga T., Irino N., Takaoka T., *J. Pharmacobio-Dyn.*, **13**, s-54 (1990); b) Kinjo J., *Natural Medicines*, **50**, 79—85 (1996); c) Miyao H., Arao T., Udayama M., Kinjo J., Nohara T., *Planta Med.*, accepted.
- 7) Higuchi T., Nishida K., Nagamura Y., Ito M., Ishiguro I., Sumita S., Saito S., *Igaku To Seibutugaku*, **124**, 57—61 (1992).
- 8) Shiki Y., Shirai K., Saito Y., Yoshida S., Wakashin M., Kumagai A., *Wakan-Iyaku Gakkaishi*, **1**, 11—14 (1984); Kiso Y., Kawakami Y., Kikuchi K., Hikino H., *Planta Med.*, **57**, 241—247 (1987).
- 9) Berry M. N., Friend D. S., *J. Cell. Biol.*, **43**, 506—520 (1969).
- 10) Loten E. G., Redshaw-Loten J. C., *Anal. Biochem.*, **154**, 183—185 (1986).
- 11) Harboe N., Ingild A., *Scand. J. Immunol.*, **2** (Suppl. I), 161—164 (1973).
- 12) Heerspink W., Hafkenscheid J. C., Siepel H., van der Ven-Jongekryg J., Dijt C. C., *Enzyme*, **25**, 333—341 (1980).
- 13) Shibayama Y., *Experimental and Molecular Pathology*, **51**, 48—55 (1989).
- 14) Kinjo J., Imagire M., Udayama M., Arao T., Nohara T., *Planta Med.*, submitted.
- 15) Shinohara M., Kaise Y., Nakano Y., Izawa T., Miyazaki K., Japan. Patent Sho 54-151136 (1979) [*Chem. Abstr.*, **92**, 153139B (1980)].