ELSEVIER

European Journal of Pharmacology 391 (2000) 175–181

ejp

www.elsevier.nl/locate/ejphar

Protective effects of soyasapogenol A on liver injury mediated by immune response in a concanavalin A-induced hepatitis model

Hiroyuki Kuzuhara, Shoji Nishiyama *, Nobuto Minowa, Kazue Sasaki, Shoji Omoto

Drug Discovery, Pharmaceutical Research Center, Meiji Seika, 760 Morooka, Kouhoku, Yokohama 222-8567, Japan

Received 26 August 1999; received in revised form 22 December 1999; accepted 24 December 1999

Abstract

The present study was carried out to analyze the effects of soyasapogenol A on the liver injury mediated by the immune response in concanavalin A-induced hepatitis in mice. Soyasapogenol A reduced the number of infiltrating inflammatory cells in the liver and significantly lowered the elevated level of plasma tumor necrosis factor-α (TNF-α) 2 h after concanavalin A treatment, and then markedly reduced the elevated plasma alanine aminotransferase activity and decreased the number of apoptotic bodies in the liver parenchymal cells but not in the sinusoidal cells at 24 h. Since the effect of soyasapogenol A on the elevated plasma TNF-α level was not appreciable compared to the preventive effect of soyasapogenol A on the elevated plasma alanine aminotransferase level, these results suggest that soyasapogenol A directly prevents apoptosis of hepatocytes, and secondly, inhibits the elevation of plasma TNF-α, which consequently resulted in the prevention of liver damage in the concanavalin A-induced hepatitis model.

Keywords: Alanine aminotransferase; Concanavalin A; Apoptosis; Interferon-γ; Soyasapogenol A; Tumor necrosis factor-α (TNF-α)

1. Introduction

Human hepatitis infection is a health problem worldwide that can be managed pharmacologically in only a few cases. Interferon, which is widely used to treat chronic active hepatitis B and C, is only effective in approximately 30% of the cases (Blair et al., 1996) and is associated with serious side-effects such as fever, general fatigue and psychiatric complications (Hoshino et al., 1995).

Triterpenes, which occur widely in nature, are the major components of several traditional medicinal herbs and display a variety of biological effects, such as hepatoprotective, anti-inflammatory, skin tumor prevention and immuno-modulatory effects (Price et al., 1987). Liu et al. (1994) analyzed 10 triterpenes and found that hederin, ursolic acid and oleanolic acid afforded the most effective protection against chemical-induced liver injury. It was also reported that a mixture of soyasaponins I, II III, A1 and A2, extracted from *Glycine max* Merr (or Daizu in Japanese), decreased the level of plasma alanine aminotransferase in rats fed a high fat diet containing peroxidized corn oil (Ohminami et al., 1984). Thus, it is assumed that the aglycon of soyasaponins, soyasapogenols can also protect against liver injury.

The development of new drugs depends primarily on the availability of suitable animal models. The commonly used method of inducing toxic liver injury with xenobiotics hardly reflects the clinical condition in human beings since the liver damage occurring in chronic hepatitis B virus and hepatitis C virus infections appears to be mainly mediated by the immune response against the virus (Liaw et al., 1995; Marinos et al., 1995). Since in the concanavalin A-induced hepatitis model, liver injury in mice results from T-cell stimulation and the release of tumor necrosis factor-α (TNF-α), interferon-γ and interleukin-2 in vivo, the model spectrum for acute inflammatory liver disease was extended in rodents (Tiegs et al., 1992; Gantner et al., 1995; Küsters et al., 1996).

* Corresponding author. Tel.: +81-45-541-2521; fax: +81-45-543-9711.

E-mail address: shoji_nishiyama@meiji.co.jp (S. Nishiyama).

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00931-0

Therefore, the present study was carried out to analyze the effects of soyasapogenol A on concanavalin A-induced hepatitis in mice.

2. Materials and methods

2.1. Animals

Male BALB/c mice, weighing an average of 21–25 g were purchased from Charles River Laboratory (Yokohama, Japan) and maintained on a commercial diet (Oriental Yeast, Tokyo, NMF). They received water ad libitum. The animals were housed in a temperature- and light-controlled room as previously reported (Nishiyama et al., 1988). All the experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

2.2. Concanavalin A-induced hepatitis

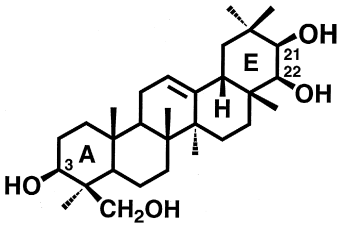
Control mice were given solvent (100% polyethylene glycol #400 (PEG-400): 100% dimethyl sulfoxide (DMSO): 0.5% carboxymethyl cellulose sodium salt (CMC) = 1:1:2) administered subcutaneously (s.c.) into the back at 2 and 14 h prior to an intravenous injection of sterilized phosphate-buffered saline (control group). Concanavalin A at a dose of 20 mg/kg body weight was intravenously injected (concanavalin A alone group). A suspended solution containing soyasapogenol A at the doses shown in the legend was injected s.c. into the back of the animals at 2 and 14 h prior to an intravenous injection of concanavalin A (concanavalin A + soyasapogenol A group). Prednisolone was suspended in 0.5% CMC and administered p.o. at 2 and 14 h before the injection of concanavalin A (concanavalin A + prednisolone group). The animals were not starved overnight before being killed, and were quickly decapitated under ether anesthesia between 0900 and 1000 h.

2.3. Plasma transferase assay

Plasma and the liver from a mouse were obtained at various intervals after concanavalin A injection. Alanine aminotransferase level was determined at 340 nm according to the standard method, using an automatic analyzer.

2.4. Histological examination

Liver specimens were routinely fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin. Tissue sections (4 μm thick) were stained with hematoxylin–eosin for routine examination.



soyasapogenol A

Fig.1. Structure of soyasapogenol A.

2.5. Assay of apoptosis

Apoptotic bodies were detected with the digoxigenin-peroxidase method, using a commercial kit from Oncor (MD 20877, USA).

2.6. Assay for cytokine levels in plasma

The animals were not starved overnight before being killed, and were quickly killed under ether anesthesia between 0900 and 1000 h. Blood was withdrawn by heart puncture into a heparinized syringe. Plasma TNF-α, interferon-γ and interleukin-2 levels were assayed using a commercial kit (Factor-Test-X, Intertest-γ and Intertest-2X, Genzyme, Cambridge, USA).

2.7. Chemicals

Soyasapogenol A (Fig. 1) was extracted from *G. max* Merr (Daizu in Japanese) in our laboratory. Concanavalin A, prednisolone, PEG-400, DMSO and CMC were purchased from Sigma (St. Louis, USA).

2.8. Statistical analysis

The data were evaluated based on Student's *t*-test, a non-parametric Dunnett test or Mann–Whitney *U*-test.

3. Results

The alanine aminotransferase level increased markedly in the concanavalin A alone group on day 1 and returned to the control level on day 7 (Fig. 2). Prior to the increase of the alanine aminotransferase level in the concanavalin A alone group, a marked increase in the plasma TNF-α and interferon-γ levels was observed after 2 and 4 h, respectively (Fig. 3) and portal areas and hepatic lobuli were infiltrated with mononuclear and poly-morphonuclear cells after 2 and 4 h (Fig. 4a and b). Although the level of plasma TNF-α decreased markedly 24 h after the concana-

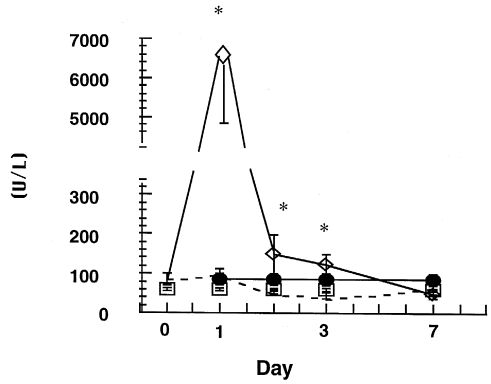


Fig. 2. Time course of alanine aminotransferase activity in the concanavalin A alone group and the concanavalin A + soyasapogenol A group. Each value is expressed as U/L and $\chi \pm$S.E.M. for three mice treated with concanavalin A alone (◇) or concanavalin A + soyasapogenol A (●) at each time. Significantly different from the concanavalin A alone group at $P < 0.05$(*), based on Student's *t*-test. One group of mice ($n = 12$) received an injection of concanavalin A at a dose of 20 mg/kg body weight intravenously and another group of mice ($n = 12$) was given s.c. 2 mg/mouse of soyasapogenol A at 2 and 14 h prior to an injection of concanavalin A. Control mice ($n = 15$) were s.c. given the solvent at 2 and 14 h prior to the injection of a saline solution (□). All the animals were killed at the time shown after the treatment with concanavalin A.

valin A treatment, the plasma interferon-γ level tended to remain higher than that of the control for 24 h. A number of apoptotic bodies and diffuse cloudy swelling of hepatocytes in the central zone were observed in the concanavalin A alone group 24 h after the treatment. This hepatocellular apoptosis was accompanied by minimal to mild multifocal hepatocellular necrosis (Fig. 5a).

At 2 and 4 h after the concanavalin A treatment, the number of mononuclear and poly-morphonuclear cells in the portal areas and hepatic lobuli of the concanavalin A + soyasapogenol A group generally decreased compared to that in the concanavalin A alone group (Fig. 4b and c). After 2, 8 and 24 h, the plasma TNF-α level in the concanavalin A + soyasapogenol A group was significantly lower than that of the concanavalin A alone group (Fig. 3). The plasma interferon-γ level in the concanavalin A + soyasapogenol A group tended to be lower than that in the concanavalin A alone group from 2 to 8 h. A 50% decrease in the level of plasma interferon-γ in the concanavalin A + soyasapogenol A group, compared to the level in the concanavalin A alone group was observed 24 h after the concanavalin A treatment (Fig. 3). The plasma interleukin-2 level, like the TNF-α level, peaked 2 h after the concanavalin A treatment but there was no significant difference between the concanavalin A alone group and the concanavalin A + soyasapogenol A group (the data are not shown). Marked alleviation of the multifocal necrosis of hepatocytes and a decrease in the number of apoptotic bodies in the concanavalin A + soyasapogenol A group were observed 24 h after the concanavalin A treatment (Fig. 5a and b) and the plasma alanine aminotransferase level in the concanavalin A + soyasapogenol A group was markedly lower than that in the concanavalin A alone group (Fig. 2 and Table 1). Compared with the effects of prednisolone, soyasapogenol A together with prednisolone at a dose of 0.2 mg/mouse also significantly lowered the elevated plasma alanine aminotransferase level 24 h after the concanavalin A treatment (Table 1). Although no significant difference in the number of apoptotic bodies in the sinusoids of the concanavalin A alone group and the concanavalin A + soyasapogenol A group was observed, the number in the parenchyma in the concanavalin A + soyasapogenol A group was markedly lower than that in the concanavalin A alone group 24 h after the concanavalin A treatment (Fig. 6). The sinusoids are lined by endothelial cells. Associated with the sinusoids are the Kupffer cells, the hepatic stellate cells and the pit cells. Digoxigenin-peroxidase staining positive cells in these four types of cells (endothelial cells, Kupffer cells, hepatic stellate cells and pit cells) could not be clearly identified.

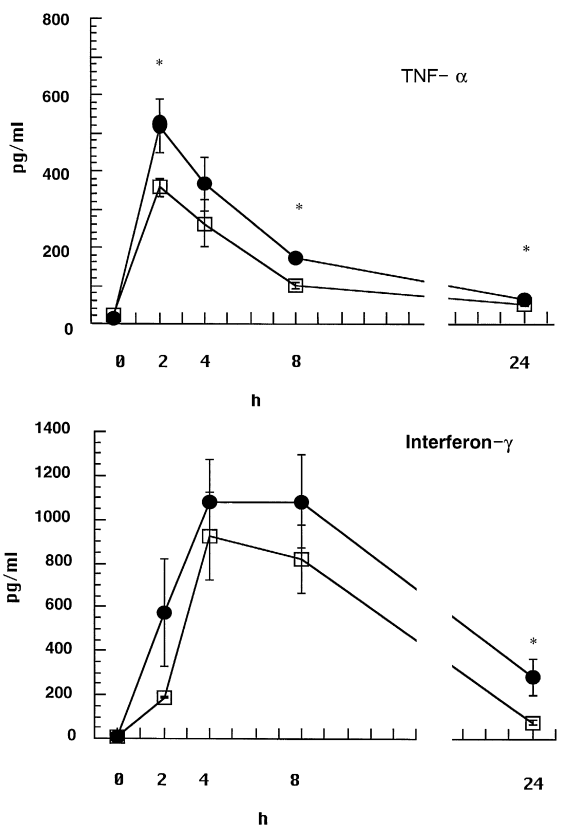
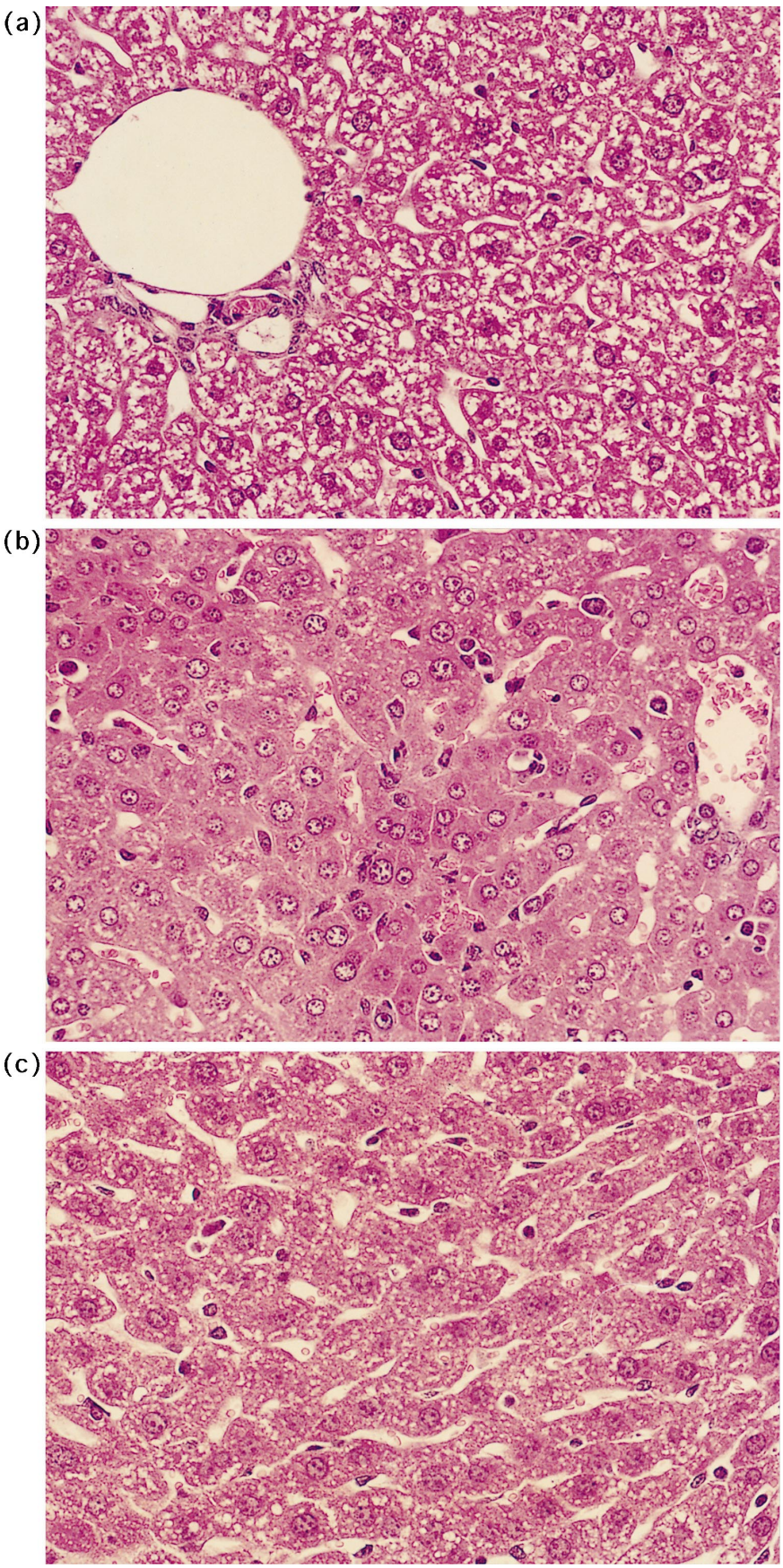


Fig. 3. Time course of plasma TNF-α and interferon-γ levels in the concanavalin A alone group and the concanavalin A + soyasapogenol A group. Each value is expressed as pg/ml and $\chi \pm$S.E.M. for three mice treated with concanavalin A alone (●) (20 mg/kg, i.v.) or concanavalin A + soyasapogenol A (□) (2 mg/mouse, s.c. 14 h and 2 h before treatment with concanavalin A). All the animals were killed at the time shown after the treatment with concanavalin A. Significantly different from the concanavalin A alone group at $P < 0.05$(*), based on Student's *t*-test. TNF-α and interferon-γ were not detected in the plasma of the control mice.


(a)
(b)
(c)

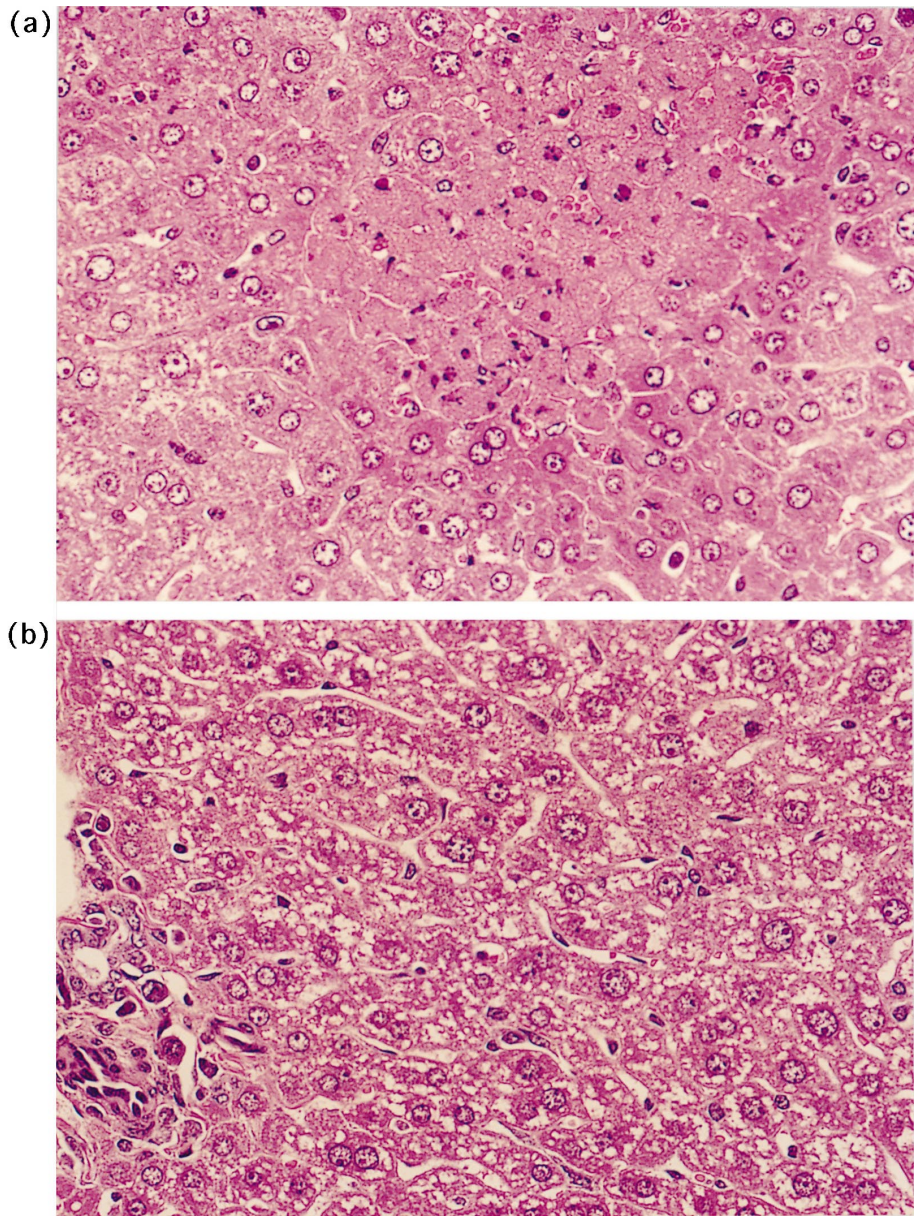


Fig. 5. Histopathological changes in the concanavalin A alone group and the concanavalin A + soyasapogenol A group at 24 h. (H&E staining for (a) and (b): ×400). (a) Concanavalin A alone group. Arrangement of acidophilic and small hepatocytes is disorganized. Apoptotic bodies are visible in the necrotic area, in which inflammatory cells were also detected. (b) Concanavalin A + soyasapogenol A group. Hepatic cells cords are regular and no marked hepatocellular degenerative changes nor apoptotic bodies were seen.

However, the mononuclear and poly-morphonuclear cells showed clearly no positive staining in the sections examined.

4. Discussion

Hepatitis B virus and hepatitis C virus, which induce inflammatory and necrotic changes in the liver, are the main causal agents of chronic viral hepatitis in humans. The liver damage observed in chronic hepatitis B virus and hepatitis C virus infections appears to be mainly mediated by the immune response against the virus, such as effects of virus-specific cytotoxic T lymphocytes and cytokines produced by immune cells (González-Amaro et al., 1994).

Since current liver disease models in experimental animals are either based on the adverse effects of hepatotox-

Fig. 4. Histopathological changes in the concanavalin A alone group and the concanavalin A + soyasapogenol A group 2 h after concanavalin A treatment (H&E staining for (a), (b) and (c): ×400). (a) Solvent group. No histological abnormalities. (b) Concanavalin A alone group. Acidophilic cells are evident and many mononuclear and polymorphonuclear cells were observed in the sinusoids. (c) Concanavalin A + soyasapogenol A group Hepatic cell cords are regular and infiltration of inflammatory cells was not observed.

Table 1
Effect of soyasapogenol A treatment on plasma alanine aminotransferase activity 24 h after the concanavalin A treatment
Doses administered were the same as those described in Fig. 2. All animals were killed 24 h after treatment with concanavalin A (solvent or soyasapogenol A alone; $n = 3$, concanavalin A alone; $n = 7$, concanavalin A + soyasapogenol A; $n = 22$, concanavalin A + prednisolone; $n = 6$. Another group of mice was given p.o. 0.2 mg/mouse of prednisolone at 2 and 14 h prior to an injection of concanavalin A.
Significantly different from the concanavalin A alone group at $P < 0.01$(**), based on non-parametric Dunnet test (for concanavalin A + soyasapogenol A group) or Mann–Whitney test (for concanavalin A + prednisolone group).

		n	Plasma alanine aminotransferase (ave. ± S.E.)
Solvent		3	63.3 ± 4.7 (IU/L)
Soyasapogenol A		3	50.3 ± 3.2
Concanavalin A		7	2068 ± 437
Concanavalin A + Soyasapogenol A	0.02 mg	7	2062 ± 518
	0.2 mg	7	478 ± 308**
	2 mg	8	55 ± 5.7**
Prednisolone		6	215 ± 92**

ins or on inherited metabolic disorders affecting the liver, all these models are prone to the limitations inherent to the experimental approach. Recently, this model spectrum has been extended to include acute inflammatory liver disease induced by concanavalin A in BALB/c mice, which is likely to be based on the production and biological action of endogenous mediators (Tiegs et al., 1992). In our present study, intravenous administration of concanavalin A to mice resulted in highly specific liver injury and in the release of TNF-α, interferon-γ and interleukin-2 as reported by Gantner et al. (1995). In the concanavalin A-induced T-cell activation hepatitis model, soyasapogenol A completely prevented a marked elevation of the alanine aminotransferase level and hepatic necrosis. Soyasapogenol A also completely inhibited hepatocyte apoptosis. Since, in the concanavalin A-induced hepatitis model, both TNF-α and interferon-γ are critical mediators of liver injury (Küsters et al., 1996; Mizuhara et al., 1996), it is postulated that the mechanism whereby soyasapogenol A prevents hepatocyte apoptosis and necrosis induced by concanavalin A involves at least two components, i.e., the inhibition of elevated plasma TNF-α and interferon-γ levels. Prior to a soyasapogenol A-induced 50% decrease in the elevated plasma interferon-γ level after 24 h, soyasapogenol A also lowered the elevated plasma TNF-α level associated with the concanavalin A treatment from 2 to 8 h. At these times, soyasapogenol A reduced the increase in the number of infiltrating inflammatory cells associated with concanavalin A in the liver. Based on reports stating that TNF-α is involved in neutrophil migration and adhesion (Crawford, 1997), it is suggested that the decrease of the elevated plasma TNF-α level may be associated with reduction of the increase in the number of infiltrating inflammatory cells. However, since the effect of soyasapogenol A on the elevated plasma TNF-α and interferon-γ levels was not conspicuous compared to the preventive effect of soyasapogenol A on the elevated plasma alanine aminotransferase level, the effect may not be critical for the decrease in the alanine aminotransferase level and alleviation of necrosis. As described above, the percent decrease in the plasma TNF-α level due to soyasapogenol A, while only 25–40% was nevertheless significant. Therefore, the mechanisms of action of soyasapogenol A in apoptosis may be dependent not only on the decreased plasma TNF-α level but also on an effect on the 55-kDa TNF receptor (TNF receptor 1) or CD95 (Fas/APO1). The major condition associated with hepatic apoptosis induced by endogenous mediators may be viral infection. Many workers who used the concanavalin A-induced hepatitis model reported that apoptosis always preceded the membrane disintegration and that apoptotic mechanisms were likely to be involved in the early events of liver injury induced by concanavalin A (Gantner et al., 1995). In the present study, the decrease in the number of apoptotic bodies that was induced by treatment with soyasapogenol A was evident in the parenchyma but not in the sinusoids after 24 h, suggesting that soyasapogenol A directly prevents apoptosis of hepatocytes and secondly inhibits the increase in the plasma TNF-α and interferon-γ levels which eventually resulted in the prevention of liver damage in the concanavalin A-induced hepatitis model.

Treatment with soyasapogenol A did not inhibit the mixed lymphocyte reaction in vitro and the increase in the

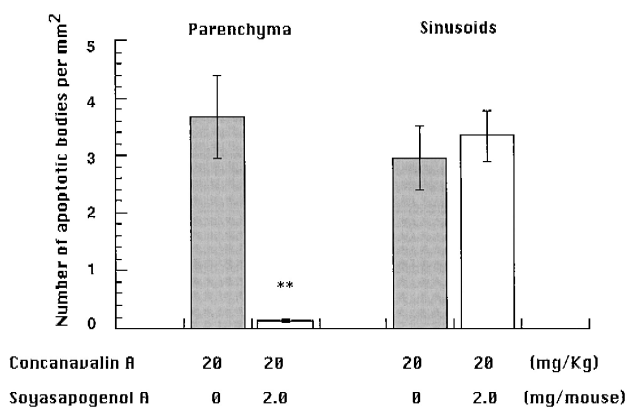


Fig. 6. Number of apoptotic bodies in the parenchyma and sinusoids of the liver from the concanavalin A alone group and the concanavalin A + soyasapogenol A group. Sixteen liver specimens from the control group ($n = 2$), the concanavalin A alone group (20 mg/kg, i.v., $n = 7$) and the concanavalin A + soyasapogenol A group (2 mg/mouse, s.c. 14 h and 2 h before treatment with concanavalin A, $n = 7$) were examined. All the animals were killed 24 h after the treatment with concanavalin A. Liver specimens were fixed in buffered formalin, stained by the digoxigenin method and the number of positive cells was counted. Each value is expressed as the number of positive cells per mm^2 ± S.E.M. in seven liver specimens from the concanavalin A alone group or the concanavalin A + soyasapogenol A group. Significantly different from the concanavalin A alone group at $P < 0.01$(**), based on Student's *t*-test.

interleukin-2 level in the concanavalin A-induced hepatitis model (data not shown). Together, the present findings suggest that soyasapogenol A induces immuno-modulatory effects but not immunosuppressive effects although the percent alleviation of liver injury by soyasapogenol A was similar to that seen with prednisolone. This effect of soyasapogenol A may be useful in the treatment of chronic immunoinflammatory liver disease in human beings.

Acknowledgements

We thank Prof. Hiroshi Yamamoto of Osaka University, Prof. Saburo Ohnishi, Dr. Toshiji Saibara of Kochi Medical School and Dr. Katsumi Yamauchi of Tokyo Women's Medical College for their advice with preparation of the manuscript. We also thank Dr. Jacqueline Bartman of the Tropical Agriculture Research Center for correcting the manuscript.

References

Blair, C.S., Haydon, G.H., Hayes, P.C., 1996. Current perspectives on the treatment and prevention of hepatitis C infection. Exp. Opin. Invest. Drugs 5, 1657–1671.

Crawford, J.M., 1997. Cellular and molecular biology of the inflamed liver. Curr. Opin. Gastroenterol. 13, 175–185.

Gantner, F., Leist, M., Lohse, A.W., Germann, P.G., Tiegs, G., 1995. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. Hepatology 21, 190–198.

González-Amaro, R., García-Monzón, C., García-Buey, L., Moreno-Otero, R., Alonso, J.L., Yagüe, E., Pivel, J.P., López-Cabrera, M., Fernández-Ruiz, E., Sánchez-Madrid, F., 1994. Induction of tumor necrosis factor α production by human hepatocytes in chronic viral hepatitis. J. Exp. Med. 179, 841–848.

Hoshino, U., Suou, T., Kawasaki, K., 1995. Evaluation of the side effects of interferon therapy by questionnaire in chronic hepatitis C. Acta Hepatol. Jpn. 36, 506–510.

Küsters, S., Gantner, F., Künstle, G., Tiegs, G., 1996. Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A. Gastroenterology 111, 462–471.

Liaw, Y.-F., Lee, C.-S., Tsai, S.-L., Liaw, B.-W., Chen, T.-C., Sheen, I.-S., Chu, C.-M., 1995. T-cell-mediated autologous hepatocytotoxicity in patients with chronic hepatitis C virus infection. Hepatology 22, 1368–1373.

Liu, J., Liu, Y., Mao, Q., Klaassen, C.D., 1994. The effects of 10 triterpenoid compounds on experimental liver injury in mice. Fundam. Appl. Toxicol. 22, 34–40.

Marinos, G., Torre, F., Chokshi, S., Hussain, M., Clarke, B.E., Rowlands, D.J., Eddleston, A.L.W.F., Naoumov, N.V., Williams, R., 1995. Induction of T-helper cell response to hepatitis B core antigen in chronic hepatitis B: a major factor in activation of the host immune response to the hepatitis B virus. Hepatology 22, 1040–1049.

Mizuhara, H., Uno, M., Seki, N., Yamashita, M., Yamaoka, M., Ogawa, T., Kaneda, K., Fujii, T., Senoh, H., Fujiwara, H., 1996. Critical involvement of interferon gamma in the pathogenesis of T-cell activation-associated hepatitis and regulatory mechanisms of interleukin-6 for the manifestations of hepatitis. Hepatology 23, 1608–1615.

Nishiyama, S., Onosaka, S., Taguchi, T., Konishi, Y., Tanaka, K., Kinebuchi, H., 1988. Stimulation of cadmium uptake by estradiol in the kidney of male rats treated with cadmium. Biochem. Pharmacol. 37, 3091–3096.

Ohminami, H., Kimura, Y., Okuda, H., Arichi, S., Yoshikawa, M., Kitagawa, I., 1984. Effects of soyasaponins on liver injury induced by highly peroxidized fat in rats. Planta Med. 50, 440–441.

Price, K.R., Johnson, I.T., Fenwick, G.R., 1987. The chemistry and biological significance of saponins in foods and feedingstuffs. CRC Crit. Rev. Food. Sci. Nutr. 26, 27–135.

Tiegs, G., Hentschel, J., Wendel, A., 1992. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J.Clin. Invest. 90, 196–203.