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Astilbin selectively induces dysfunction of liver-infiltrating cells — novel protection from liver damage

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Abstract

The present study aimed to examine the effect of astilbin, a flavanoid, on liver injury. When administered during the effector but not induction phase, astilbin significantly decreased the liver injury induced by delayed-type hypersensitivity to picryl chloride in mice. The pretreatment of nonparenchymal cells but not hepatocytes with astilbin in vitro caused a concentration- and time-dependent inhibition against the damage. Nonparenchymal cells isolated from astilbin-administered mice also showed a significant incompetence of hepatotoxicity, correlated with the inhibition of serum transaminase elevation. However, astilbin did not protect from CCl_4-induced liver damage. Furthermore, the flavanoid markedly promoted the apoptosis of nonparenchymal cells from liver-injured mice, whereas did not influence those from naive mice. These results suggest that astilbin provides improvement against liver injury through a selective dysfunction of liver-infiltrating cells rather than by protecting the hepatocyte membrane. Such characteristics will be of significance to pave a new way for treating immunologically related liver diseases and for developing new drugs.

Keywords: Delayed-type hypersensitivity; Liver injury; Picryl chloride; Astilbin; Hepatoprotection; Apoptosis

1. Introduction

Hepatocyte damage is a key process in the pathogenesis of various liver diseases. In clinical practice, the serum content of substances whose level is changed by hepatocyte damage is usually measured for assessing liver function, and protection from the damage is considered to be important for the therapy of hepatic diseases. Assays based on protection by drugs from chemical or immunoinflammatory damage in animals or on in vitro experiments have been widely used for screening hepatoprotective drugs. For example, CCl_4-induced acute hepatic damage is one of the most popular evaluation methods for hepatic remedies. One of the possible mechanisms for the hepatoprotection by drugs is believed to be stabilization of the hepatocyte membranes through their antioxidant action when used for pretreatment (Davila et al., 1989; Pietrangelo et al., 1995). Thus, the efficacy of the drugs is preventive and passive for defending against damages. Indeed, there is frequently a relapse after drug withdrawal in the clinical treatment for hepatitis. For these reasons, it would be important to eliminate or decrease the hepatotoxic causes, which could protect more effectively from hepatocyte damage than would the means mentioned above.

One of the main causes of hepatocyte damage in a variety of hepatic diseases involves T cell-mediated cellular immunity (Barnaba et al., 1989; Peters et al., 1991;

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Chisari and Ferrari, 1995; Diepolder et al., 1996; Lohr et al., 1996). Immunotherapy has also been used for suppressing the over-activated immune reaction that causes hepatic damage. However, most of immunosuppressive agents or glucocorticoids usually show non-selective inhibition against the immune system with serious side-effects. The efficacy of immunomodulatory agents is still not clearly proven. An ideal drug, with high efficacy and low toxicity, is badly needed for hepatitis.

Previously, we have established a new model with liver injury induced by a delayed-type hypersensitivity reaction to picryl chloride for mimicking the immunopathogenesis in hepatitis, and have demonstrated the key role of $CD4^+$ T lymphocytes that mainly constitute the liver-infiltrating leukocytes (Xu et al., 1993a, 1996, 1998). Furthermore, we have evidenced the usefulness of this model for immunopharmacological evaluation by using various drugs for hepatitis (Xu et al., 1997a), as well as for showing the effectiveness of some suppressors to delayed-type hypersensitivity in regulating the damage (Xu et al., 1997b). The latter drugs included some kinds of aqueous extracts from Chinese herbs that have been evidenced to selectively inhibit delayed-type hypersensitivity reactions. One of them, the aqueous extract from *Rhizoma Smilacis Glabrae*, showed a significant improvement of this liver injury when administered after the elicitation of the injury. Our other investigations have also proved that the extract could markedly inhibit the cellular but not humoral immune responses (Xu et al., 1993b). These findings suggested the possibility of selectively inducing dysfunction of over-activated immune cells without affecting the normal immune system. The present study, therefore, examined the effects and mechanisms of astilbin, one of the main constituents from the extract for decreasing the liver damage.

2. Materials and methods

2.1. Animals

Male BALB/c and ICR mice, 6–8 weeks, were purchased from Shizuoka Laboratory Animal Center, Japan, and maintained with free access to pellet food and water in plastic cages at 21 ± 2°C, and kept on a 12-h light/dark cycle. This study complied with current ethical regulations on animal research of this institute, and all mice used in the experiment received humane care.

2.2. Drugs and reagents

Astilbin, 3,3′,4′,5,7-pentahydroxyflavanone 3-(6-deoxy-(L-mannopyranoside), was isolated from the rhizome of *Smilax glabra*, a Liliaceae plant, which was purchased from Nanjing Medicinal Material (Nanjing, China) and identified as *Smilacis glabra* Roxb. by Dr. Zhunan Gong (Department of Pharmacognosy, China Pharmaceutical University). Cyclophosphamide and biphenyl dimethyl dicarboxylate were purchased from Shanghai 12th Pharmaceutical Factory (Shanghai, China) and Beijing Xiehe Pharmaceutical (Beijing, China), respectively.

2.3. Liver injury induced by delayed-type hypersensitivity to picryl chloride

Liver injury was induced as in our previous reports (Xu et al., 1996, 1998). Namely, mice were sensitized twice by painting 0.1 ml of 1% picryl chloride in ethanol on the skin of their abdomens at an interval of 5 days. Five days after the second sensitization, they were injected with 10 μl of 0.2% picryl chloride in olive oil into the liver followed 18 h later by bleeding and isolating the serum. The serum alanine and aspartate transaminases were used as the parameter for indicating hepatic damage (Neuschwander-Tetri, 1995). The activities of both transaminases were determined with a commercial kit according to the guidelines provided.

2.4. Liver cell preparation

Parenchymal and nonparenchymal cells were isolated from naive or liver injured mice 12 h after the challenge with picryl chloride by a modified two-step perfusion method. In brief, mice received pentobarbital intraperitoneally at a dose of 40 mg/kg. The liver was first perfused in situ via the portal vein with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (ethylene glycol-bis-((-amino ethyl)-*N*,*N*′-tetraacetic acid) (Dojindo Chemical Inst., Kumamoto, Japan) and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) (pH 7.4) at 37°C until the blood in the liver was completely removed. Then, the solution was replaced with 0.1% collagenase (182 units/mg, Wako Pure Chemical, Osaka, Japan) (Wako) in HBSS containing 4 mM $CaCl_2 \cdot 2H_2O$ and 0.8 mM $MgSO_4 \cdot 7H_2O$. After a few minutes of perfusion, the liver was excised rapidly from the body cavity and dispersed into cold HBSS. The cell suspension generated was filtered through a 100-mesh gauze, and parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at $50 \times g$ for 2 min. The pellet containing hepatocytes was washed twice to remove dead cells and debris, and then suspended in RPMI-1640 with 2 mM L-glutamine (Gibco BRL), containing 10% (v/v) newborn-calf serum, 100 U/ml of penicillin and 100 U/ml of streptomycin (1640 medium). The supernatant obtained after the first centrifugation of the slurry generated by perfusion of the liver with collagenase was centrifuged at $300 \times g$ for 10 min to obtain nonparenchymal cells. The pellets of nonparenchymal cells were washed twice with 1640 medium. The hepatocytes and nonparenchymal cells

were found to be about 90% viable as estimated by Trypan blue exclusion and generally used immediately for culture or for apoptosis measurement.

2.5. Cell culture and transaminase-releasing assay

Hepatocytes were suspended in WE medium at a density of 1×10^5 cells/ml. Portions (0.2 ml) were seeded onto 96-well microplates (Falcon) and cultured in a humidified incubator at 37°C with 5% (v/v) CO_2 in air. After 3 h, the hepatocyte monolayers were washed twice with 1640 medium, and then 1×10^5 nonparenchymal cells suspended in 0.2 ml 1640 medium were added to the wells. After 3 h of further culture, the supernatant was collected and used for assaying alanine and aspartate transaminase activities.

2.6. Apoptosis measurements

Apoptosis was evaluated by detecting the cells with fragmented or condensed nucleus after Hoechst 33342 (Molecular Probes, USA) staining, and cleaved DNA fragments. The Hoechst 33342 staining was carried out according to the method previously reported (Darzynkiewicz et al., 1994) with a modification. Briefly, cells in RPMI 1640 medium were stained with 1 mM Hoechst 33342 for 1 min at room temperature. After being washed 3 times, they were fixed in 2% formaldehyde in phosphate-buffered saline and visualized in a fluorescence microscope. The apoptotic cells among the total population were determined by counting a total of 200 cells in five different fields. The results were expressed as a percentage of apoptotic cells. DNA fragmentation was measured as described by Yamada (1995). Briefly, portions of 10^6 cells with or without drug treatment were washed and spun down. Pellets were resuspended in 0.6 ml of lysing buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.2% Triton X-100 (Wako)] and incubated for 10 min on ice. After centrifugation at 20 000 $\times g$, supernatants were mixed gently for 2–3 min with an equal volume of TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA)-saturated phenol (Wako), followed by centrifuging and mixing supernatants with chloroform:isoamylalcohol (24:1). Then, after centrifugation at 20 000 $\times g$, the supernatants containing DNA were precipitated overnight at −20°C in 0.3 M NaCl and 70% ethanol. The loading buffer (Wako) containing 0.02% bromophenol blue, 0.02% xylene cyanol FF, 50% glycerol and 0.1% sodium dodecyl sulfate was then added to the samples in a 1:10 (v/v) ratio. Electrophoresis was carried out in 2% agarose for 120 min at 50 V and DNA was visualized with ethidium bromide.

2.7. Statistics

The data for serum alanine transaminase level and apoptotic cell counts were expressed as means ± S.E.M. and statistically evaluated using Student's *t*-test.

3. Results

3.1. Administration of astilbin, during the effector but not induction phase, significantly decreased the liver injury induced by delayed-type hypersensitivity to picryl chloride in mice

Astilbin (50 and 100 mg/kg) was administered p.o. and cyclophosphamide (10 mg/kg) was given i.p. for 10 days from the 1st sensitization with picryl chloride (induction phase), or 3 times, 0, 5 and 10 h, after the challenge with picryl chloride (effector phase). As shown in Fig. 1, a marked elevation in serum alanine transaminase activity was observed in control mice as compared with the normal level. Compared to the control, the high dose of astilbin, given in the effector phase, produced a significant decrease and the low dose produced a tendency to a decrease. However, when the drug was given in the induction phase, the liver injury was not reduced. Cyclophosphamide produced a significant decrease when given in either induction or effector phase. On histopathological examination, the

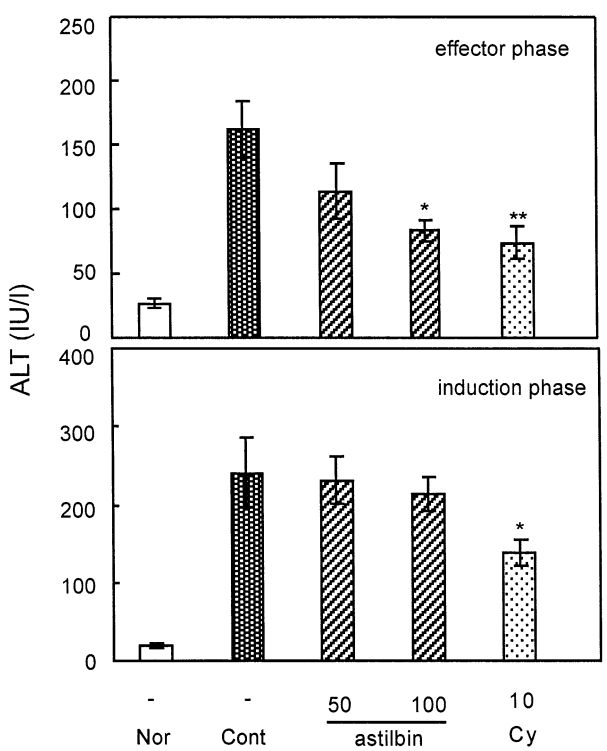

Fig. 1. Effect of astilbin and cyclophosphamide on the elevation of serum alanine transaminase activity in mice with liver injury induced by delayed-type hypersensitivity. Mice were sensitized twice by painting 0.1 ml of 1% picryl chloride in ethanol on the skin of their abdomens at an interval of 5 days. Five days after the second sensitization, they were injected with 10 μl of 0.2% picryl chloride in olive oil into the liver followed 18 h later by bleeding. Astilbin (p.o.) and cyclophosphamide (i.p.) were given for 10 days from the first sensitization (induction phase) or 3 times 0, 5 and 10 h after the challenge (effector phase) with picryl chloride. Each column represents the mean ± S.E.M. ($n = 7$–9). $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

main changes in the control were the inflammatory infiltration and hepatocellular coagulation necrosis as reported previously (Xu et al., 1993a, 1996, 1997a,b, 1998). These changes were markedly reduced in most mice by astilbin administered in the effector but not in the induction phase.

3.2. Treatment with astilbin, of nonparenchymal cells but not hepatocytes, blocked the ability of nonparenchymal cells to release transaminases from hepatocytes

Nonparenchymal cells and hepatocytes were isolated from mice with liver injury induced by delayed-type hypersensitivity 12 h after the challenge with picryl chloride (Xu et al., 1998). Regarding the spontaneous release of alanine and aspartate transaminases from hepatocytes, the co-culture for 3 h with nonparenchymal cells resulted in a marked elevation of the transaminases released in the culture supernatant. The treatment of nonparenchymal cells with astilbin at various concentrations, before their addition to hepatocytes, dose dependently blocked the release of both enzymes (Fig. 2). When the nonparenchymal cells were centrifuged in 25, 50 and 80% Percoll, the fraction in the interface of 50/80% Percoll caused a strong release of alanine transaminase (35.0 ± 3.1 against 12.6 ± 2.2 IU/l in spontaneous release). Astilbin at the concentration of 1×10^{-5} g/ml produced a significant decrease (20.5 ± 2.1 at $P < 0.05$) of the elevated release. However, the cells in the 25/50% interface did not show any hepatotoxic potential, and no change was observed with astilbin.

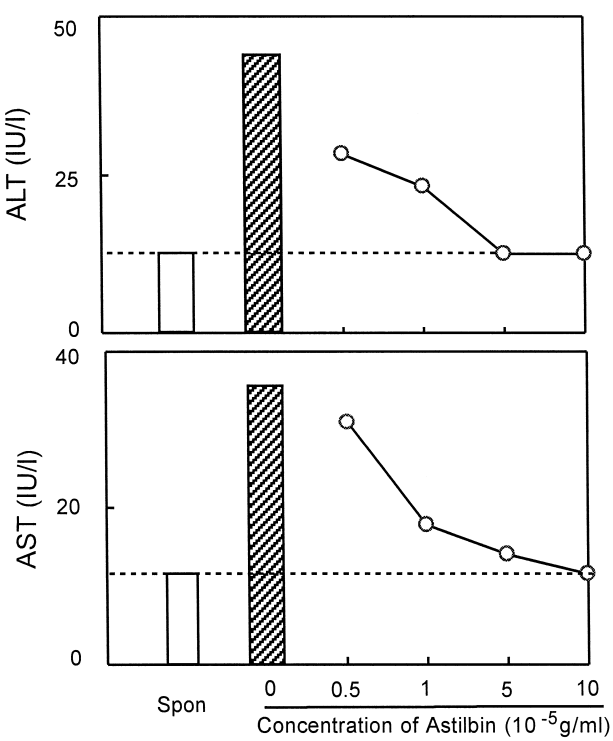

Fig. 2. Effect of astilbin on the potential of nonparenchymal cells to cause the release of alanine and aspartate transaminases from hepatocytes in vitro. Hepatocytes (20,000 in 0.2 ml WE medium) were pre-cultured in a 96-well microplate for 5 h. After two washings, they were co-cultured with 8×10^4 nonparenchymal cells at 37°C for a further 3 h, followed by collecting the supernatant and assaying alanine and aspartate transaminase activities. Drug treatment was performed for nonparenchymal cells at various concentrations for 1 h before the co-culture. Each point represents the mean of three experiments and each experiment included triplicate sets.

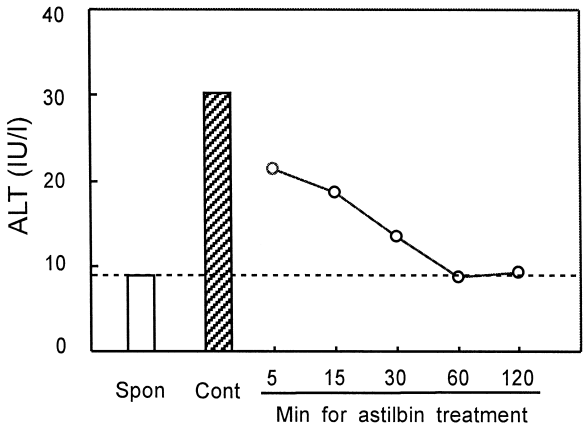

Fig. 3. Time-dependent inhibition of the alanine transaminase release from hepatocytes by the treatment of nonparenchymal cells with astilbin. Nonparenchymal cells were incubated at 37°C with 5×10^{-5} g/ml of astilbin for 5, 15, 30, 60 and 120 min. For other details, see legend to Fig. 2.

Fig. 3 shows the kinetics for the time-dependent inhibition by these agents of the hepatotoxic potential of nonparenchymal cells. The incubation with astilbin for more than 30 min completely blocked the effect of nonparenchymal cells to release alanine transaminase from hepatocytes. However, no effect was observed on pretreatment of hepatocytes with the flavanoid, even at the high dose of 10^{-4} g/ml for more than 2 h (the left panel of Table 2).

To confirm the dysfunction of nonparenchymal cells produced by astilbin in mice with liver injury, 100 mg/kg of astilbin was administered p.o. 3 times 0, 5 and 10 h after the challenge with picryl chloride. Two h after the last administration, the mice were killed to collect serum and to isolate nonparenchymal cells and hepatocytes. As compared with the control, nonparenchymal cells obtained from either astilbin-administered mice showed a significantly lower hepatotoxicity, consistent with the significant inhibition of the serum alanine transaminase elevation (Fig. 4).

3.3. Astilbin did not provide any hepatoprotection against CCl_4-induced hepatic damage either in vivo or in vitro

Astilbin (50 and 100 mg/kg) was administered p.o. for 6 days. One hour after the final administration, the mice were injected i.p. with 0.2% of CCl_4 in olive oil (0.2 ml/20 g body weight). The mice were killed 20 h later for assay of serum alanine and aspartate transaminase activities. No effect against the marked enzymatic elevation in control was observed in either of drug groups (Table 1).

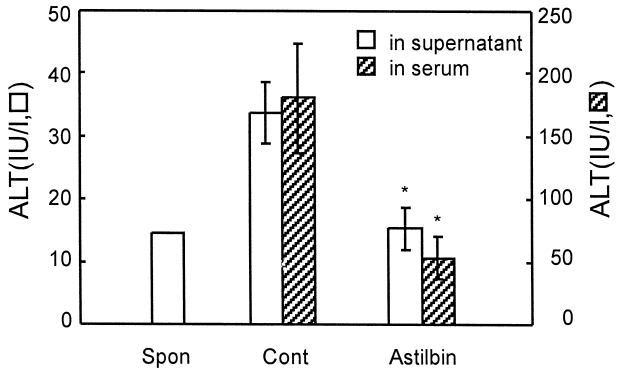

Fig. 4. Effects of p.o. administration of astilbin on serum alanine transaminase activity and the potential of isolated nonparenchymal cells to cause transaminase release from hepatocytes in vitro. Liver injury was induced by delayed-type hypersensitivity to picryl chloride. Astilbin was given p.o. 3 times 0, 5 and 10 h after the challenge with picryl chloride in liver. Two hours after the last administration, serum was collected and liver nonparenchymal cells and hepatocytes were isolated. Each column represents the mean ± S.E.M. for three to four mice. $^{*}P < 0.05$ vs. control. For other details, see legend to Fig. 2.

Similarly, hepatocytes isolated from naive mice were treated for 2 h with astilbin. After two washings with the medium, 0.1 ml of 0.2% of CCl_4 was added for a further 1 h incubation followed by determination of alanine transaminase in the supernatant. Astilbin did not affect the CCl_4-induced alanine transaminase release from hepatocytes at the concentrations of 5×10^{-6} to 1×10^{-4} g/ml (the right panel of Table 2).

3.4. Astilbin significantly induced apoptosis of nonparenchymal cells from liver injured mice, while it did not affect nonparenchymal cells and spleen cells from naive mice

Using Hoechst 33342-labeling, we first examined the apoptotic changes of nonparenchymal cells and hepatocytes at 12 h of liver injury when nonparenchymal cells showed peak cytotoxic activity against hepatocytes as reported previously by us (Xu et al., 1998). As indicated in Fig. 5, the ratios of apoptotic cells in drug-untreated hepatocytes and total nonparenchymal cells were about 10% and 5%, respectively. Astilbin at 5×10^{-5} g/ml significantly increased the number of apoptotic cells in total nonparenchymal cells but not in hepatocytes. When the nonparenchymal cells were separated into adherent and non-adherent fractions, the ratio of apoptotic cells in the non-adherent fraction (including 63.9% $CD4^+$ and 30.9% $CD8^+$ cells) was increased by astilbin while no influence was observed in the adherent fraction.

A DNA fragmenting assay was also performed using normal nonparenchymal cells and spleen cells, and non-

Table 1
No protective activity of astilbin from CCl_4-induced hepatic damage in mice[a]

Groups	Dose (mg/kg)	Alanine transaminase (IU/l)	Aspartate transaminase (IU/l)
Normal	–	117.6 ± 211.3	103.6 ± 11.2
Control	–	456.7 ± 13.3	322.7 ± 21.9
Astilbin	50	434.6 ± 17.4	323.1 ± 10.2
	100	444.2 ± 19.4	331.7 ± 22.0
Biphenyl dimethyl dicarboxylane	150	246.2 ± 13.0[b]	325.6 ± 39.0

[a]Drugs were given p.o. to ICR mice for 6 days. One hour after the final administration, mice were injected i.p. with 0.2% of CCl_4 in olive oil at the dose of 0.2 ml/20 g body weight. They were killed 20 h later for assaying the serum alanine and aspartate transaminase activities. Each value indicates the mean ± S.E.M. for eight animals.
[b]$P < 0.01$ vs. control.

Table 2
No protective activity of astilbin from nonparenchymal cell- or CCl_4-induced hepatocyte damage in vitro when they were used to pretreat hepatocytes[a]

Concentrations (g/ml)	Nonparenchymal cells	CCl_4
Spontaneous	11.2 ± 3.8	11.2 ± 3.7
Control	33.2 ± 6.6	64.3 ± 4.7
5×10^{-6}	37.9 ± 7.4	64.1 ± 7.9
1×10^{-5}	34.1 ± 5.3	65.7 ± 8.4
5×10^{-5}	31.6 ± 4.5	68.2 ± 3.6
1×10^{-4}	33.4 ± 3.3	66.7 ± 5.4

[a]Hepatocytes (20,000 in 0.2 ml WE medium) were pre-cultured in a 96-well microplate for 3 h. After two washings, they were pretreated with the various concentrations of astilbin for 2 h. After two further washings, the hepatocytes were exposed to 8×10^4 nonparenchymal cells or 0.2% CCl_4 for 3 h followed by collection of the supernatant to assay alanine transaminase (IU/l).

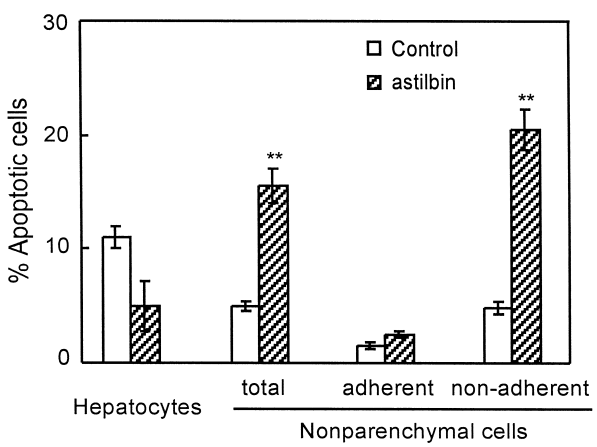

Fig. 5. Effect of astilbin on the apoptosis of nonparenchymal cells and hepatocytes from liver-injured mice. Both nonparenchymal cells and hepatocytes from liver-injured mice at 12 h were treated with 5×10^{-5} g/ml of astilbin for 1 h. After two washings, the cells were stained with Hoechst 33342 for 1 min. In the case of nonparenchymal cells, the cells were also allowed to adhere on a dish for 3 h and then the adherent and non-adherent fractions were used for Hoechst 33342 staining. Each column represents the mean ± S.E.M. for three to four mice. $^{**}P < 0.01$ vs. control.

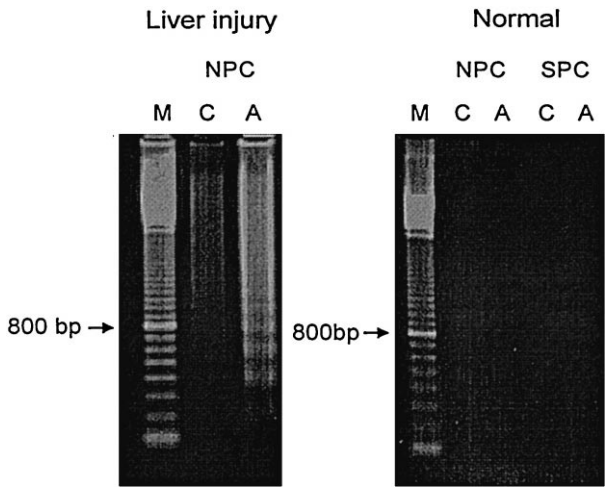

Fig. 6. Gel agarose electrophoresis of DNA isolated from normal (right) and liver injured mice (left) and effect of astilbin on it. Nonparenchymal cells from naive or liver-injured mice at 12 h were cultured without or with 5×10^{-5} g/ml of astilbin for 1 h. The results are representative of three separate experiments. NPC: nonparenchymal cells, SPC: spleen cells, M: DNA marker, C: control, A: astilbin-treated. For other details, see the text and legend to Fig. 5.

parenchymal cells at 12 h of liver injury. Compared with the drug-untreated nonparenchymal cells (control), the death of nonparenchymal cells isolated at 12 h was characterized as apoptotic as judged by the appearance of the ''DNA ladder'' pattern due to the treatment with astilbin (Fig. 6, left). However, the culture of nonparenchymal cells and spleen cells from normal mice with the flavanoid did not induce such DNA fragmentation (Fig. 6, right).

4. Discussion

The present study first examined the improving activity of astilbin on the liver injury induced in mice by delayed-type hypersensitivity to picryl chloride. Astilbin significantly inhibited the elevation of serum alanine and aspartate transaminases when administered during the effector but not the induction phase of delayed-type hypersensitivity (Fig. 1). The selective inhibition by astilbin in the effector phase may imply the suppression of the function rather than the activation of effector cells associated with the delayed-type hypersensitivity reactions in liver. This characteristic is quite different from that of the potent immunosuppressor, cyclophosphamide, which inhibits immune responses in both induction and effector phases (Fig. 1), and may be very important for the treatment of immunologically related diseases including hepatitis.

For confirming and elucidating such selectivity, we further investigated the effect of these agents on the cytotoxic activity of liver-infiltrating cells against hepatocytes by using a co-culture assay with nonparenchymal cells and hepatocytes. This assay has been described in our previous paper (Xu et al., 1998), where we evidenced that both spleen cells and nonparenchymal cells from liver-injured mice could induce a marked release of alanine and aspartate transaminases from hepatocytes in vitro and the peak release was caused by the nonparenchymal cells 12 h after the elicitation of liver injury. Therefore, in the present study, nonparenchymal cells and hepatocytes isolated at 12 h of liver injury were used. A dose-dependent inhibition of the enzyme release was observed when the nonparenchymal cells were pretreated with astilbin (Fig. 2). Also, the inhibition by astilbin was confirmed by the complete dysfunction of nonparenchymal cells after more than 30-min treatment at the concentration of 5×10^{-5} g/ml (Fig. 3). When the flavanoid was used to treat hepatocytes before co-culture, however, astilbin did not block the hepatotoxic potential of nonparenchymal cells or CCl_4. Similarly, a 6-day preventive administration of astilbin failed to reduce the CCl_4-induced liver injury in mice, while biphenyl dimethyl dicarboxylate, as a positive control in this experiment, inhibited the elevation of alanine transaminase levels significantly (Table 1). These results suggest that the improvement of delayed-type hypersensitivity- but not CCl_4-induced liver injury by astilbin may be due to the selective dysfunction of activated immune cells rather than to the protection for hepatocyte membranes. Our unpublished data also showed that astilbin inhibited Con A-induced lymphocyte transformation only at the high concentration of 1×10^{-4} g/ml. Taken together, the results suggest that astilbin may display selective inhibition of cellular immune responses based on functional suppression of the activated populations rather than on activation of immune system.

To confirm the role of astilbin in causing the dysfunction of nonparenchymal cells in vivo, we further examined the hepatotoxicity of nonparenchymal cells isolated from drug-administered mice with liver injury. Correlated to the inhibition of the serum alanine transaminase elevation by astilbin, there was a significant decrease in the hepatotoxic potential in the isolated nonparenchymal cells (Fig. 4). This finding indicates that the inhibition of serum transaminase activities by astilbin is due to the dysfunction of liver nonparenchymal cells, and the in vitro co-culture assay could well reflect the in vivo mechanisms involved. In the previous study (Xu et al., 1998), we analyzed the cell populations contained in nonparenchymal cells by flow cytometric analysis, and a great change in cell composition of nonparenchymal cells was found from 32–36% at 0 h to 72.6–78% LFA-1^+ cells at 12 h after the challenge with picryl chloride in liver. The latter included 47.9% of $CD4^+$ and 23.2% of $CD8^+$ T cells. This change was due to the liver infiltration and localization of lymphocytes from the periphery, and consequently led to the liver injury since nonparenchymal cells at 0 h showed no hepatotoxicity. The treatment of nonparenchymal cells at 12 h, which showed peak hepatotoxicity, with anti-CD4 or anti-CD8 monoclonal antibody plus complement abolished the release of alanine transaminase from hepatocytes completely

or slightly, respectively. These findings suggested that the hepatotoxicity is exhibited by the infiltrating lymphocytes in nonparenchymal cells rather than by resident liver populations. This was supported by the finding that the Kupffer cell-enriched cells in nonparenchymal cells did not have a hepatotoxic potential. The next question is, therefore, how does the flavanoid affect the function of liver-infiltrating cells?

In a previous paper (Xu et al., 1998), we have evidenced that the interaction between lymphocyte function associated antigen 1 (LFA-1) on nonparenchymal cells and intercellular adhesion molecule 1 (ICAM-1) on hepatocytes is the key step for causing the hepatocyte damage. However, the treatment with astilbin did not mask the LFA-1 expression (data not shown), suggesting a mechanism other than an influence on the LFA-1/ICAM-1 interaction. On the other hand, apoptosis is known to be a physiological process regulating normal tissue homeostasis (Steller, 1995). Several studies have shown the importance of apoptosis in regulating the development and maturation of the immune response (Singer and Abbas, 1994; Surh and Sprent, 1994). Considering these findings, we next carried out the study on the apoptosis of nonparenchymal cells. As seen from Hoechst 33242 staining, astilbin significantly promoted the apoptosis of nonparenchymal cells showing a condensed nuclear and a blebbing membrane. This was also confirmed by the DNA fragmentation assay (Fig. 6). These findings imply that the induction of apoptosis for nonparenchymal cells by the flavanoid may be one of the effective mechanisms to cause the dysfunction of nonparenchymal cells. In addition, when the nonparenchymal cells were centrifuged in Percoll, non-adherent cells in the interface of 50/80% Percoll caused a strong release of alanine transaminase. On the other hand, adherent cells in the interface of 25/50% Percoll showed no hepatotoxic potential. According to our preliminary experiments, the non-adherent fraction had mostly $CD4^+$ and $CD8^+$ T lymphocytes, while the adherent fraction mainly included Kupffer cells and sinusal endothelial cells. Since the induction of apoptosis by astilbin appeared in the non-adherent but not in the adherent fraction of nonparenchymal cells in the present study (Fig. 5), T lymphocyte populations are probably the main target of astilbin. It should be emphasized that the drugs did not induce the apoptosis of hepatocytes and normal nonparenchymal cells and spleen cells. These results further confirm the selective inhibition exerted on activated liver-infiltrating cells mainly including T lymphocytes.

Overall, astilbin is effective for improving the immunoinflammatory liver injury through selectively producing dysfunction of liver-infiltrating cells. The increased apoptosis of the lymphocytes may be one of the main mechanisms. Because no report on such protective manner in the remedies for hepatitis has appeared so far, the present results will be of significance for the development of new drugs as well as for the therapy of immunologically related liver diseases. Further investigations are in progress on the detailed mechanisms for the apoptosis of activated T lymphocytes by astilbin.

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