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Astilbin prevents concanavalin A-induced liver injury by reducing TNF-α production and T lymphocyte adhesion

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Abstract

The aim of this study was to evaluate the effect of astilbin on concanavalin A (Con A)-induced hepatitis, a T cell-dependent model of liver injury. Con A administration resulted in a severe liver injury in mice, with a strong increment in spleen cell adhesion and liver infiltration of T cells, as well as in tumour necrosis factor (TNF)-α production. Against this liver injury, astilbin significantly inhibited the elevation in transaminase activity, reduced the TNF-α production, and improved the histological changes, including inflammatory infiltration, hepatocyte necrosis and degeneration and Kupffer cell hyperplasia. In addition, astilbin inhibited the adhesion of spleen cells and purified T lymphocytes isolated from the liver-injured mice to fibronectin, laminin and type IV collagen. Moreover, the adhesion of human Jurkat T cells to endothelial cell line ECV-304 was also inhibited by astilbin. These results suggest that the improvement of the T cell-mediated liver injury by astilbin may be related to the reduction in TNF-α production and in T cell adhesion to extracellular matrices and endothelial cells.

Introduction

In many liver diseases, including viral hepatitis (Ferrari et al 1987; Minutello et al 1993) and autoimmune disorders (Eggink et al 1982), activated T lymphocytes play a crucial role in the progressive development of liver parenchymal damage. Although there are many unsolved problems related to the mechanisms, the elimination or dysfunction of activated T cells might contribute to the improvement of the damage in several types of acute and chronic liver disease. Among the models of liver injury in animals, the injection of plant lectin concanavalin A (Con A) into mice is known to be capable of activating T lymphocytes and to lead to a liver-selective necrotic injury (Tiegs et al 1992). By using this liver injury model, many immunosuppressants, such as FK-506 and ciclosporin (cyclosporin A), have been demonstrated to be effective (Tiegs et al 1992), which suggests that it is important to regulate the immune response occurring in the liver for the treatment of hepatic diseases. However, these immunosuppressants usually have severe side effects due to their non-selective effect pattern. For example, ciclosporin could inhibit the whole process of T lymphocyte activation and function.

Previously, we have examined the effects of various kinds of Chinese herbs either on the activation of T lymphocytes or on the function of activated T lymphocytes. As a result, some of them have been found to show an interesting activity, selectively inhibiting either the induction or the effector phase of the delayed-type hypersensitivity (DTH) reaction without affecting immune organs (Xu et al 1991, 1993, 1997; Xu & Xu 1993). Based on these studies, astilbin, a flavanone, was isolated as a main principle contained in *Rhizoma Smilacis Glabrae* (RSG) and showed a selective immunosuppressive feature when administered during the effector, but not the induction, phase of various DTH reactions, including ear contact dermatitis, footpad reaction and immunological liver injury (Xu et al 1993, 1997, 1999; Cai et al 2003a). Such characteristics are quite different from those of the immunosuppressants in current use, and may provide a new possibility for the selective treatment of various immune diseases, including hepatitis. Its preliminary mechanism was found to involve a significant induction of apoptosis in the liver-infiltrating T lymphocytes (Xu et al 1999) and the mitogen-activated Jurkat T cells (Yan & Xu 2001), and a selective suppression of activated T cell functions, such as cell

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migration with reduced matrix metalloproteinase (MMP) activity (Cai et al 2003a, b). These findings drive us to confirm the usefulness for treating immunologically related diseases by selectively inhibiting activated T lymphocytes. Considering the indispensable role of TNF-α production and T lymphocyte adhesion to extracellular matrix (ECM) for the progress of Con A-induced liver injury (Tiegs et al 1992; Mizuhara et al 1994; Gantner et al 1995; Yuan et al 2003), this study was designed to investigate the effect of astilbin on Con A-induced liver injury by focusing on the TNF-α production and T lymphocyte adhesion.

Materials and Methods

Drugs and reagents

Astilbin, 3,3′,4′,5,7-pentahydroxyfla vanone 3-(6-deoxy-(L-mannopyranoside)), with more than 92% purity, was isolated from the rhizome of *Smilax glabra* (RSG), a *Liliaceae* plant. Briefly, the dried and cut rhizome of *S. glabra* was extracted twice with methanol for each 2 h under reflux. After removal of the solvent by evaporation, the methanol extract was suspended in water and successively partitioned with petroleum ether and ethyl acetate. Astilbin was obtained from the ethyl acetate soluble fraction by chromatography using a silica gel column, as well as Cosmosil 75C_{18}-OPN, and its structure was elucidated by mass spectrometry (MS) and nuclear magnetic resonance (NMR) analysis (Chen et al 1999). The nature of astilbin is always constant for individual experiments. Other drugs and reagents were purchased as follows: ciclosporin (Sandoz Ltd, Basel, Switzerland); concanavalin A (Con A; Sigma, St Louis, MO); kits for determining serum alanine transaminase (ALT) and aspartate transaminase (AST) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); ELISA kit for tumour necrosis factor-α (TNF-α) (Jingmei Biotech Co. Ltd, Shengzhen, China); bovine serum albumin (BSA; Sigma, St Louis, MO); type IV collagen (Sigma Chemical Co.); fibronectin (Sigma Chemical Co.); laminin (CalBiochem); mouse T cell enrichment columns (R & D systems, USA) and 3-(4, 5-dimethyl-2-thia zol)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma Chemical Co.).

Animals

Male and female ICR mice, 5–6 weeks old, were obtained from the Experimental Animal Center of Nanjing Medical University (Nanjing, China), maintained in plastic cages at $21 \pm 2\,^{\circ}C$ with free access to pellet food and water and kept on a 12-h light–dark cycle. This study complied with current ethical regulations on animal research in our university, and all mice used in the experiments received humane care.

Cell lines

Human leukaemia Jurkat cell line (passage nos 20–30), a T-cell line (Roy et al 1998; Liu et al 1999; Yan & Xu 2001), was obtained from the Shanghai Institute of Cell Biology (Chinese Academy of Science, Shanghai, China). ECV-304 (ECV, passage nos 20–30), a spontaneously transformed immortal endothelial cell line (Roy et al 1998) established from the vein of an apparently normal human umbilical cord, were obtained from the China Center for Type Culture Collection (Wuhan, China). Both cells were kept in culture by seeding them in 25-mL culture flasks containing RPMI 1640 (GIBCO BRL) medium supplemented with 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin and 10% fetal calf serum (FCS) in a standard culture incubator with humidified air containing 5% CO_2 (v/v) at 37 °C.

Induction of liver injury with Con A

Acute liver injury was induced by injecting mice with 20 mg kg^{-1} Con A in 250 μL phosphate-buffered saline (PBS) via the tail vein. Eight hours after the injection, the mice were exsanguinated from the suborbital vein and the serum was collected for assaying ALT and AST activity by using the commercial kits as the protocols indicated. For histological examinations, mice were sacrificed, their abdomens were opened by a midline incision and portions from the right liver lobe were excised, fixed in 4% paraformaldehyde and embedded in paraffin. The tissue sections (5 μm) were stained with haematoxylin–eosin and read on a 0–3 scale (0, no change; 1, mild; 2, moderate; and 3, severe) by a pathologist who had no knowledge of the induction of liver injury or other experimental data.

Preparation of splenocyte suspensions and purification of T cells

The spleen was aseptically taken from mice, crushed gently and separated into single cells by squeezing in 5 mL D-Hank's solution (GIBCO BRL). The cells obtained were passed through eight layers of gauze and centrifuged at 1000 rev min^{-1} for 5 min at 4 °C. The pellet of cells was added to 10 mL sterile 0.17 M Tris (hydroxymethyl aminomethane)–0.75% NH_4Cl (pH 7.5), followed by centrifugation to remove erythrocytes. After washing twice with RPMI 1640 medium supplemented with 100 U mL^{-1} of penicillin, 100 U mL^{-1} of streptomycin and 10% FCS, they were re-suspended in RPMI 1640 and used for cell culture. In some cases, the prepared mouse spleen cell suspensions were loaded onto T cell Enrichment Columns. Briefly, the column was equilibrated with column wash buffer. After the wash buffer had drained down to the level of the white filter, 1 mL cell suspension (1×10^8 cells/mL) was applied to the top of the column and incubated at room temperature for 10 min. Then cells were eluted from the column with 4×2 mL of column wash buffer. The collected cells were centrifuged at 1000 rev min^{-1} for 5 min and re-suspended in RPMI 1640 medium. The amount of recovered cells from each column was about 1.5×10^7. Their viability and purity ($CD3^+$ cells) reached about 95% and 88%, respectively.

TNF-α production and assay

Spleen cells isolated from mice 2 h after Con A administration were suspended in RPMI 1640 medium supple-

mented with 100 U mL^{-1} of penicillin and 100 U mL^{-1} of streptomycin at a density of 5×10^6 mL^{-1}. Portions (0.2 mL) were seeded onto 96-well, flat-bottom microplates (Falcon) and cultured at 37 °C with 5% (v/v) CO_2 in air. After 24 h, the supernatants were aspirated and stored at −20 °C until TNF-α assay. TNF-α concentrations in the supernatant as well as in mouse serum 2 h after Con A inoculation were assayed by ELISA kit for quantification of mouse TNF-α, as described by the manufacturer. In brief, standards and samples were added to the wells coated with immobilized monoclonal antibody to TNF-α. After washing away any unbound substances, the anti-TNF-α antibodies (labelled with biotin and streptavidin conjugated to horseradish peroxidase) were added in turn with a wash after each binding. Then a substrate solution consisting of stabilized hydrogen peroxide and tetramethylbenzidine was added and colour developed in proportion to the amount of TNF-α bound in the initial step. The reaction was stopped by addition of 2 N sulfuric acid and optical density was read with an ELISA reader (Sunrise Remote/Touch Screen; TECAN, Austria) at 450 nm. The concentration of TNF-α in a sample was determined by interpolation from a standard curve, prepared with standard samples supplied by the manufacturer. The threshold of detection was 26 pg mL^{-1} and the standard curve's range was 0–2000 pg mL^{-1}.

Cell–ECM adhesion assay

Cell adhesion to extracellular matrix (ECM) glycoproteins was performed essentially as previously described (Arai et al 1999) with some modifications. In brief, 96-well, flat-bottom tissue culture plates (Falcon) were coated with 50 μL of fibronectin (10 μg mL^{-1}), laminin (10 μg mL^{-1}) or type IV collagen (50 μg mL^{-1}) in PBS overnight at 4 °C. Non-specific binding sites were blocked with 0.2% BSA for 2 h at room temperature, followed by washing three times with RPMI 1640 medium. Spleen cells or spleen T cells (5×10^5/well) suspended in RPMI 1640 were allowed to adhere in the microplate at 37 °C for 1 h. After adhesion, non-adherent cells were removed by washing three times with RPMI 1640. Then the cells remaining bound to the plates were quantified by MTT assay (Konrad et al 2000). Briefly, MTT solution (2 mg mL^{-1} in PBS, 40 μL) was added to each well and the cells were incubated at 37 °C for 4 h. The supernatant was aspirated carefully and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the precipitate and the absorbance of the colour substrate was measured with an ELISA reader (Sunrise Remote/Touch Screen; TECAN, Austria) at 540 nm. All assays were run in triplicate, and the results were expressed as percentage of bound cells. The absorbance of 5×10^5 cells added without previous washing was considered as 100% cell adhesion. The specificity of each cell adhesion assay was corroborated using BSA as substratum. In some cases, the effect of drug on the adhesion was expressed as a percent inhibition against the control without drug treatment ((bound cells$_{control}$ − bound cells$_{drug}$)/bound cells$_{control}$).

Cell–cell adhesion assay

The cell–cell adhesion assay was performed essentially as described previously (Roy et al 1998) with some modifications. Briefly, monolayers of ECV-304 were seeded at a density of 2×10^4 cells/well in 96-well tissue culture plates and cultured at 37 °C for 24 h. Before the cell–cell adhesion assay, the ECV-304 monolayers were washed three times with RPMI 1640 and Jurkat cells were treated with or without astilbin for 3 h after being activated with 10 μg mL^{-1} Con A for 24 h. After washing three times, 2×10^5 Jurkat cells were co-cultured with ECV-304 monolayers at 37 °C for 1 h. After the co-culture period, non-adherent Jurkat cells were removed from monolayers by washing each well three times with RPMI 1640 medium. Cells remaining attached to ECV-304 monolayers were quantified by MTT assay as above.

Statistical analysis

Data were expressed as mean ± s.e.m. Statistical analysis was evaluated by one-way analysis of variance, followed by Student's two-tailed t-test for multiple comparisons, which was used to evaluate the difference between two groups. $P < 0.05$ was considered significant.

Results

Effect of astilbin on Con A-induced liver injury in mice

Astilbin (20 and 40 mg kg^{-1}) and ciclosporin (10 mg kg^{-1}) were administered intraperitoneally to 8 mice in each group twice at an interval of 5 h, respectively. One hour after the final administration, 20 mg kg^{-1} of Con A was injected intravenously to induce the liver damage. Marked elevation in serum alanine transaminase (ALT) and aspartate transaminase (AST) activity was observed as compared with the normal level in control mice 8 h after Con A injection (Figure 1A, B). Compared with this group, astilbin significantly decreased the ALT and AST elevations at 40 mg kg^{-1} (20 mg kg^{-1} caused a non-significant decrease). Ciclosporin decreased the ALT and AST almost to the normal level at 10 mg kg^{-1} (Figure 1A, B).

On histological examination, marked hepatocyte necrosis, inflammatory infiltration, Kupffer cell hyperplasia and severe hepatocyte degeneration were observed in the control group without medication (Figure 2). Compared with this, a significant improvement was made by astilbin at 40 mg kg^{-1} and ciclosporin at 10 mg kg^{-1} (Figure 2, Table 1).

Effect of astilbin on TNF-α production

The serum levels of TNF-α increased significantly 2 h after Con A inoculation. In contrast to this marked elevation, intraperitoneal administration of astilbin at 40 mg kg^{-1} caused a significant reduction (20 mg kg^{-1} produced a non-significant decrease) (Figure 3A). Similarly, the spleen cells isolated from liver-injured mice released a significantly

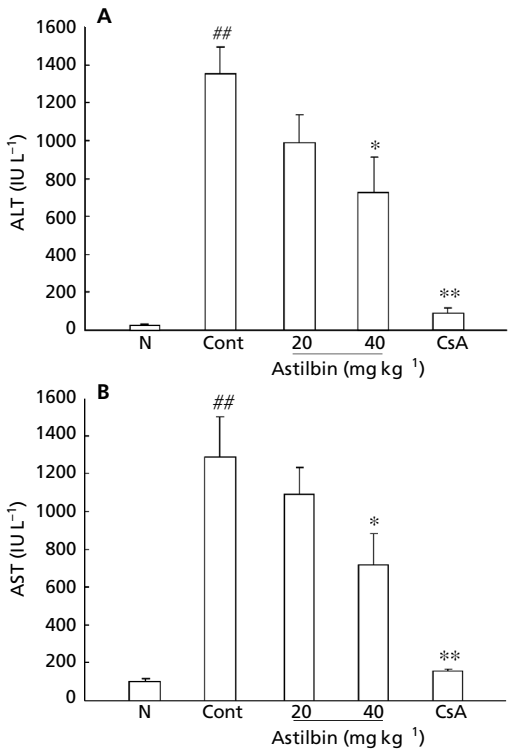

Figure 1 Effect of astilbin and ciclosporin on the elevation of serum alanine transaminase (ALT) and aspartate transaminase (AST) activity in mice with liver injury induced by Con A. Astilbin (20 or 40 mg kg^{-1}) and ciclosporin (CsA, 10 mg kg^{-1}) were administered intraperitoneally twice at an interval of 5 h. One hour after the final administration, mice were injected intravenously with 20 mg kg^{-1} of Con A, followed 8 h later by bleeding. Each column indicates the mean ± s.e.m. of results from 8 mice. N, normal mice; Cont, control mice without medication. One-way analysis of variance revealed a significant effect at $P < 0.01$. *$P < 0.05$, **$P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs normal (Student's two-tailed t-test).

increased level of TNF-α after 24 h incubation. Against this, the increased TNF-α release was significantly inhibited dose-dependently in cells from astilbin-administered mice (Figure 3B).

Effect of astilbin on the adhesion of spleen cells and purified spleen T lymphocytes to fibronectin, laminin and type IV collagen

Spleen cells and the purified T cells isolated from mice 2 h after Con A administration, which showed the highest adhesion ability (Yuan et al 2003), were allowed to adhere to fibronectin, laminin and type IV collagen for 1 h after being treated with astilbin for 3 h. The bound cells from the mice with Con A hepatitis were markedly increased as compared with those from normal mice (Figure 4A). Compared with this increase, astilbin concentration-dependently decreased the cell adhesion to fibronectin, laminin and type IV collagen. Furthermore, the purified T cells showed stronger adhesion to all three components of extracellular matrix (ECM) and astilbin also significantly inhibited the ability of the cells to adhere to each of them (Figure 4B). Its inhibitory rates at 10^{-6} g mL^{-1} were 38.8% for spleen cells and 43.6% for T lymphocytes to fibronectin, 23.0% for spleen cells and 29.1% for T lymphocytes to laminin and 45.7% for spleen cells and 47.0% for T lymphocytes to type IV collagen, respectively.

Figure 5 shows the in-vivo effect of astilbin on the adhesion potential of spleen cells and purified T lymphocytes. The intraperitoneal administration of astilbin dose-dependently decreased the ability of the cells to adhere to fibronectin, laminin and type IV collagen when tested in-vitro. Its inhibitory rates at 40 mg kg^{-1} were 27.4% for spleen cells and 36.6% for T lymphocytes to fibronectin, 18.5% for spleen cells and 28.5% for T lymphocytes to laminin and 37.4% for spleen cells and 43.6% for T lymphocytes to type IV collagen, respectively.

Effect of astilbin on the adhesion of Jurkat T-lymphocytes to ECV-304 endothelial cells

Jurkat T-cells were treated with various concentrations of astilbin for 3 h at 37 °C after being activated with 10 μg mL^{-1} Con A for 24 h. After washing three times, they were applied for a 1-h adhesion to the ECV-304 cells. Activation with Con A resulted in a distinct increase in bound cell numbers as compared with non-activated cells (Figure 6). Pre-treatment with astilbin caused a significant decrease in the cell adhesion to ECV-304 cells in a concentration-dependent manner.

Discussion

In our previous studies, we have documented evidence that astilbin, given orally, could dose-dependently cause the selective inhibition of delayed-type hypersensitivity in the effector phase but not in the induction phase, and could suppress collagen-induced arthritis by causing the apoptosis and dysfunction of activated T lymphocytes (Xu et al 1999; Cai et al 2003a, b). This study provided further experimental evidence to confirm the in-vivo therapeutic role for astilbin in a murine model of T-cell-mediated fulminant liver injury. As a result, intraperitoneal administration of astilbin significantly inhibited the elevation of serum alanine and aspartate transaminases (Figure 1). Pathological observation also showed that the liver injury induced by Con A was improved by astilbin, including the inhibition of inflammatory infiltration, Kupffer cell hyperplasia and hepatocyte degeneration (Figure 2, Table 1). Since T cell activation and function play a crucial role in the process of Con A-induced hepatitis (Tiegs et al 1992; Mizuhara et al 1994), the improvement of Con A-induced liver injury may also be related to the selective inhibition of activated T lymphocytes by astilbin, as demonstrated previously (Xu et al 1999; Cai et al 2003a, b).

In an attempt to elucidate how astilbin exerts its protective effect, we examined the effect of astilbin on TNF-α

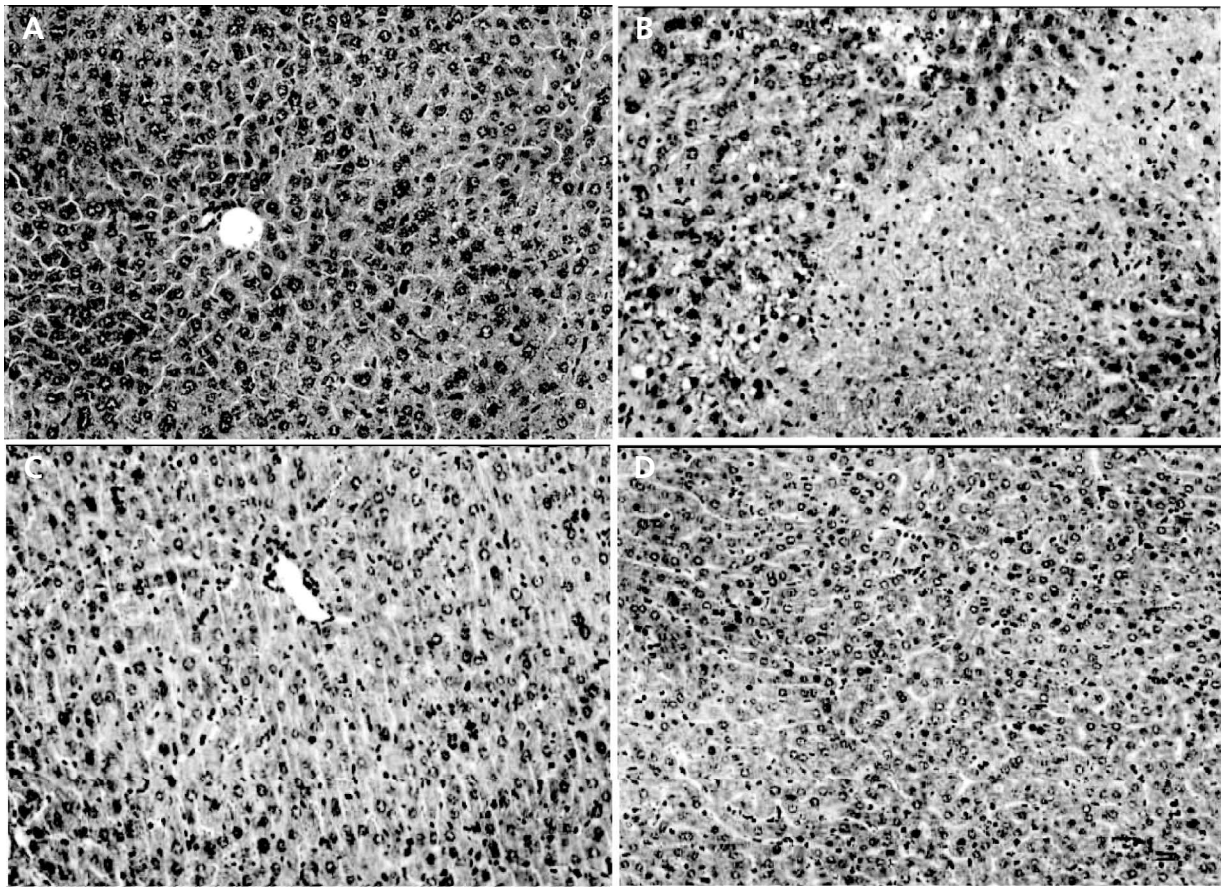

Figure 2 Effect of astilbin on histopathological changes in the Con A-injured livers. A. Normal tissue structure from naive mouse. B. A section of mouse liver from a control group 8 h after inoculation with 20 mg kg^{-1} Con A. C. A section of liver from mouse pre-treated with 40 mg kg^{-1} astilbin twice at an interval of 5 h before Con A injection. D. A section of liver from mouse pre-treated with 10 mg kg^{-1} ciclosporin twice at an interval of 5 h before Con A injection (original magnification $\times$100).

levels, which reached a peak in serum at 2 h after Con A injection and decreased rapidly after that time as reported by Santucci et al (2000). As a result, pre-treating mice with this flavanone significantly decreased the TNF-α level in plasma (Figure 3A). We also found that ciclosporin could significantly reduce the TNF-α level in serum 2 h after Con A injection (from 1666 to 134 pg mL^{-1}, data not shown). This effect of astilbin may contribute to the improvement of Con A-induced liver injury because earlier studies have found that TNF-α immunoneutralization

Table 1 Histopathological changes of livers in mice with Con A-induced liver injury.

Group	Dose (mg kg^{-1})	No. of mice	Hepatocyte necrosis	Hepatocyte degeneration	Inflammatory infiltration	Kupffer cell hyperplasia
Control	—	8	1.13 ± 0.23	2.25 ± 0.25	1.5 ± 0.19	1.38 ± 0.18
Astilbin	20	8	0.75 ± 0.31	1.5 ± 0.42	1.13 ± 0.13	1
	40	8	0.63 ± 0.32	0.88 ± 0.3**	0.75 ± 0.16**	0.75 ± 0.16*
Ciclosporin	10	8	0**	0.25 ± 0.16**	0.88 ± 0.13*	0.75 ± 0.16*

Mice were administered intraperitoneally with astilbin at a dose of 20 or 40 mg kg^{-1} or ciclosporin at 10 mg kg^{-1}, respectively, twice at an interval of 5 h. One hour after the final administration, mice were injected intravenously with 20 mg kg^{-1} of Con A, followed 8 h later by bleeding. The liver tissue sections were stained with haematoxylin–eosin. The histological changes were read on a scale of 0–3 (0, no change; 1, mild; 2, moderate; 3, severe) and expressed as an average score. Each figure indicates the mean ± s.e.m. of results from 8 mice. One-way analysis of variance revealed a significant effect at $P < 0.01$. *$P < 0.05$, **$P < 0.01$ vs control (Student's two-tailed t-test).

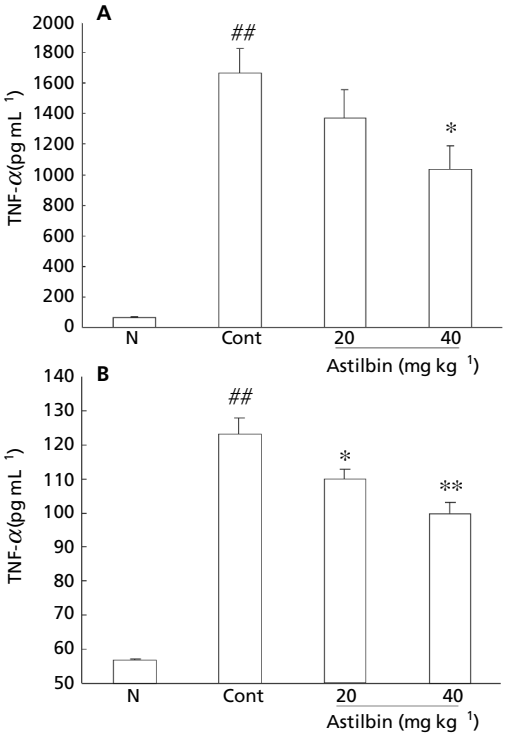

Figure 3 Effect of astilbin on the TNF-α level in serum and in culture supernatant of spleen cells from mice with Con A-induced liver damage. Mice were intraperitoneally administered with 20 or 40 mg kg^{-1} of astilbin twice at an interval of 5 h. One hour after the final administration, mice were injected intravenously with 20 mg kg^{-1} of Con A. Two hours later, mice were bled and spleen cells were isolated and cultured at 37 °C for 24 h. TNF-α levels in serum (A) and in the culture supernatant of spleen cells (B) were measured by ELISA. Each column represents the mean ± s.e.m. of results from 8 mice (A) and three experiments using 3 mice with triplicate sets in each assay (B), respectively. N, normal mice; Cont, control mice without medication. One-way analysis of variance revealed a significant effect at $P < 0.01$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs normal (Student's two-tailed t-test).

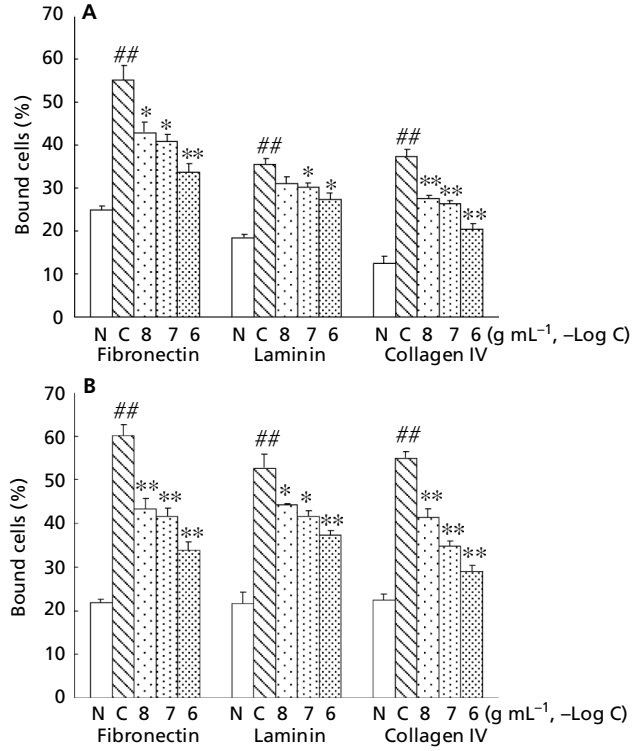

Figure 4 In-vitro effect of astilbin on the increased adhesion to fibronectin, laminin and collagen IV of spleen cells and purified T lymphocytes isolated from mice with Con A-induced liver injury. Spleen cells were isolated from naive mice or mice 2 h after Con A injection (20 mg kg^{-1} i.v.). They were treated with various concentrations (10^{-8}, 10^{-7} and 10^{-6} g mL^{-1}) of astilbin for 3 h at 37 °C and washed twice with RPMI 1640 medium. Then the spleen cells (5×10^5) (A) and the purified spleen T lymphocytes (5×10^5) (B) were applied to the adhesion assay for 1 h in 96-well microplates that had been coated with various extracellular matrices. Each column represents the mean ± s.e.m. of three experiments using 3 mice and each experiment was performed in triplicate. N, cells from normal mice; C, control (cells from Con A-treated mice without astilbin treatment). One-way analysis of variance revealed a significant effect at $P < 0.01$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs normal (Student's two-tailed t-test).

or gene ablation (Mizuhara et al 1994; Gantner et al 1995; Shirin et al 1998) and Fas or FasL gene deficiency (Kondo et al 1997; Seino et al 1997) confer protection against Con A-induced liver damage. Furthermore, previous studies have provided evidence that the inhibition of TNF-α levels in serum 2 h after Con A inoculation contributed to the alleviation of this liver damage (Shirin et al 1998; Santucci et al 2000). To investigate the relationship between the serum TNF-α level and lymphocytes, we next examined the TNF-α release from spleen cells isolated from Con A-injected mice and found that the in-vivo administration of astilbin markedly inhibited the potential of spleen cells to release TNF-α (Figure 3B). This indicates that the inhibition of serum TNF-α by astilbin may result mainly from inhibiting the ability of lymphocytes, including spleen cells. This result is also in accordance with those on the activated T cells previously reported by us (Xu et al 1999; Yan & Xu 2001; Cai et al 2003a, b).

Adhesion of cells to extracellular matrix (ECM) glycoproteins is a prerequisite for their subsequent homing to the inflammatory sites. The cell–ECM interactions dictate the subsequent recognition/activation, proliferation, differentiation and secretion of cytokines by the immune cells, in addition to affecting recruitment of additional immune cells to inflammatory loci (Nathan et al 1989; Shimizu & Shaw 1990). Considering the facts that TNF-α can enhance the adhesiveness of activated human CD4$^+$ T cells to ECM proteins (Hershkoviz et al 1994), inhibition of the adhesion of T cells to ECM can improve Con A-induced liver injury (Shirin et al 1998; Yuan et al 2003), and that astilbin could induce the apoptosis and dysfunction of activated T cells (Xu et al 1999; Yan & Xu 2001; Cai et al 2003a, b), we further examined the effect of

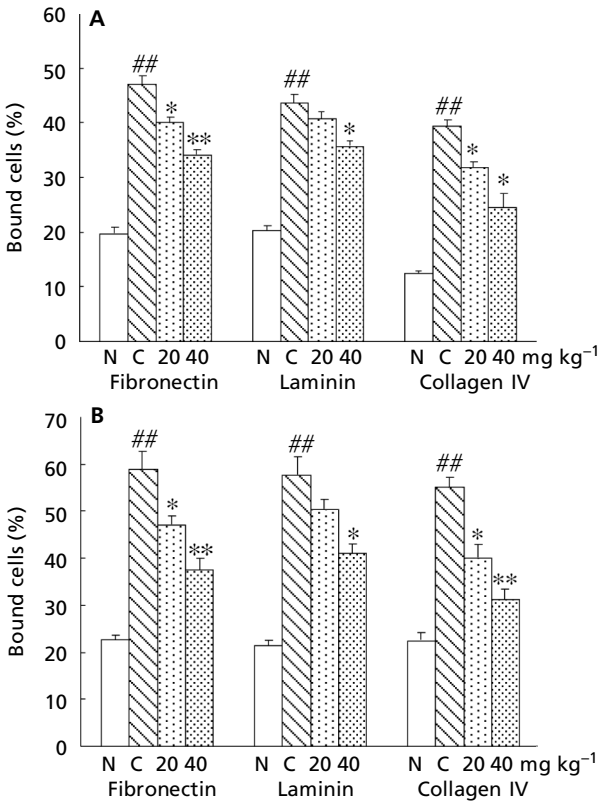

Figure 5 In-vivo effect of astilbin on the adhesion of isolated spleen cells and spleen T lymphocytes to fibronectin, laminin and collagen IV. Mice were administered intraperitoneally with 20 or 40 mg kg^{-1} of astilbin twice at an interval of 5 h before Con A injection (20 mg kg^{-1} i.v.). Spleen cells were isolated from naive mice or mice 2 h after Con A injection. Then 5×10^5 of the spleen cells (A) and the purified spleen T lymphocytes (B) were applied to the adhesion assay for 1 h. Each column represents the mean ± s.e.m. of three experiments using 3 mice and each experiment was performed in triplicate. N, cells from normal mice; C, control (cells from Con A-treated mice without medication). One-way analysis of variance revealed a significant effect at $P < 0.01$. $^*P < 0.05$, $^{**}P < 0.01$ vs control; $P < 0.01$ vs normal (Student's two-tailed t-test).

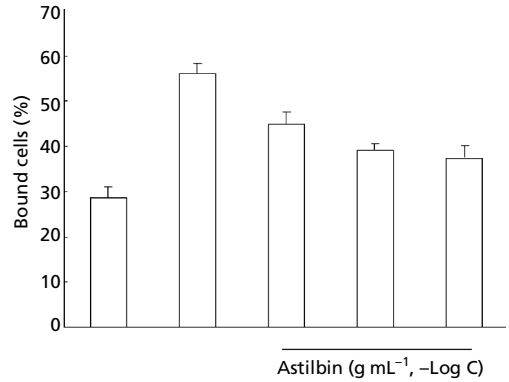

Figure 6 Effect of astilbin on the adhesion of Jurkat T-lymphocytes to ECV-304 endothelial cells. Jurkat T-cells were treated with or without astilbin for 3 h at 37 °C after being activated with 10 μg mL^{-1} Con A for 24 h. After washing three times, 2×10^5 cells were co-cultured with ECV-304 monolayers for 1 h at 37 °C. Cell adhesion was determined as described in Materials and Methods. Data were expressed as the mean ± s.e.m. of three separate experiments and each assay was performed in triplicate. N, non-activated Jurkat cells; Cont, control (Jurkat cells activated with Con A for 24 h without astilbin treatment). One-way analysis of variance revealed a significant effect at $P < 0.01$. $^*P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs normal (Student's two-tailed t-test).

astilbin on the adhesion ability of activated spleen cells from mice with Con A-induced hepatitis. The injection of Con A into mice remarkably increased the ability of spleen cells to adhere to fibronectin, laminin and type IV collagen, which are known to be the important components of ECM localized in the normal liver (Martinez-Hernandez 1984). By using the spleen cells isolated 2 h after Con A injection, which showed the highest adhesion ability (Yuan et al 2003), we found that astilbin could concentration-dependently inhibit the increased adhesion of spleen cells and purified T lymphocytes to all of the three components of ECM in-vitro (Figure 4). To confirm that astilbin had the same effect in-vivo, which may contribute to the improvement of this liver injury, we then examined the effect of astilbin on the adhesion ability in-vivo and found that this was indeed the case (Figure 5). It should be noted that astilbin showed a higher inhibitory rate on the adhesion of pure T lymphocytes than that of total spleen cells along with the remarkable increase in their adhesion percentage after purification. This finding suggests that T cells are the main population activated by Con A and the activated T lymphocytes are the main target of astilbin in this immunological liver injury in accordance with our previous suggestions (Xu et al 1999; Yan & Xu 2001). Moreover, at the concentrations used in this study, our previous data also showed that astilbin could inhibit MMP-2 and MMP-9 production, and induce the apoptosis of activated T cells (Xu et al 1999; Yan & Xu 2001; Cai et al 2003a, b).

Besides cell adhesion to ECM, cell–cell adhesion is also critical in the generation of effective immune responses. By using the adhesion assay of human Jurkat cells to the human endothelial cell line ECV-304 (Roy et al 1998), we further examined the effect of astilbin on the adhesion of Jurkat cells to ECV-304 in-vitro, and found that astilbin significantly inhibited the enhanced adhesion induced by Con A in a concentration-dependent manner (Figure 6). The inhibition of the adhesion between lymphocytes and endothelial cells may further contribute to the anti-hepatitis effect of astilbin and may be related to its apoptosis-inducing activity. An investigation of the detailed mechanisms is now in progress.

Conclusions

Our results indicate that astilbin protects mice against Con A-induced liver injury through reducing the pro-inflammatory cytokine TNF-α production and the

increased adhesion capacity of T lymphocytes that occurs after Con A activation.

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