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Protective effect of JBP485 on concanavalin A-induced hepatocyte toxicity in primary cultured rat hepatocytes

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ABSTRACT

Cyclo-*trans*-4-L-hydroxyprolyl-L-serine (JBP485) is a dipeptide isolated from Laennec, and Laennec is a hydrolyzate of human placenta. Evidence has indicated that JBP485 exhibits potent anti-hepatitis activity. In this study, we investigated the protective effect and possible mechanisms of action of JBP485 in Concanavalin A (Con A)-induced hepatotoxicity *in vitro*. Two *in vitro* models were established. Model I: primary cultured female rat hepatocytes were only incubated with Con A (50 μg/ml); model II: co-culture system of hepatocytes and autologous splenic lymphocytes, both were stimulated with Con A (20 μg/ml). JBP485 (25 μM) was pre-incubated with the two models. Our results showed that JBP485 reduced cellular aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and tumor necrosis factor alpha (TNF-α) leakage following the application of Con A in both of the models. Potential protective mechanisms were elucidated by measuring DNA fragmentations, immunocytochemistry and RT-PCR. We showed that DNA fragmentations in hepatocytes were attenuated in the JBP485 pre-incubated groups, and at the same time, immunocytochemistry and RT-PCR indicated that expression levels of caspase-3 protein and mRNA in the JBP485 treated groups were decreased compared with those in the untreated groups. Moreover, intercellular adhesion molecule-1 (ICAM-1) was also down-regulated by this dipeptide. The results indicate that JBP485 exhibits hepatoprotective effect through inhibition of hepatocyte apoptosis and ICAM-1 expression.

1. Introduction

Hepatitis is an inflammatory liver disease induced by various causes, such as virus infection, alcohol, autoimmune response, drugs or xenobiotics injury, etc. Although many unsolved problems still remain in the related mechanisms, it is generally accepted that activated T cells are involved in both acute and chronic hepatitis (Napoli et al., 1996; Chisari, 1997; Lapierre et al., 2007). Cytokines secreted by lymphocytes dominantly modulate the immune reaction in the liver, which ultimately results in liver dysfunction, hepatic cirrhosis, and even liver failure. Accordingly, several types of drugs have been developed to treat hepatitis, e.g. interferon and purine analog for viral hepatitis, prednisone and azathioprine for the autoimmune chronic hepatitis. In addition, silymarin and glycyrrhizin have frequently been used as adjunct liver-protective drugs (Ye and Lu, 2004). However, a new compound, cyclo-*trans*-4-L-hydroxyprolyl-L-serine (JBP485, Fig. 1) has been developed. Liu et al. (2000) were the first to report that JBP485 exhibited potent anti-hepatitis activity. JBP485, a dipeptide, was first isolated from Laennec (a trade name for the hydrolyzate of human placenta), as mitogens for a baby hamster kidney cell line, and was synthesized by chemical means (Yagi et al., 1998). Laennec injection has been clinically used to treat chronic hepatic injuries for over 40 years in Japan. Earlier results showed that Laennec stimulated liver regeneration and decreased cytosolic enzyme activities in serum in α-naphthylisothiocyanate (ANIT)-intoxicated rats (Liu et al., 1995). It is noteworthy that JBP485 was well absorbed after oral administration. Liu et al. (2000) suggested that it was possible that JBP485 was recognized by the peptide transporter system in the gastrointestinal tract. Therefore, it is valuable to clarify the anti-hepatitis molecular mechanism of JBP485 to develop a new oral anti-hepatitis drug.

Con A is a powerful immunostimulant (Taniguchi et al., 1989) and inflammogen (Shier, 1976) *in vivo*, when given intravenously to mice without pre-treatment by a specific hepatotoxic agent such as D-galactosamine. Con A-induced selective liver failure, which is characterized by polyclonally activating T cells, and follows the systemic release of cytokines (Tiegs et al., 1992; Gantner et al., 1995; Küsters et al., 1996). As previously reported, Con A bound strongly to the hepatocyte plasma membrane, which correlated well with the degree of hepatotoxicity induced by Con A (Leist and Wendel, 1996), resulting from the fact that Con A-binding not only induced a direct toxic effect on primary cultured hepatocytes independent of the presence of T cells but enhanced the

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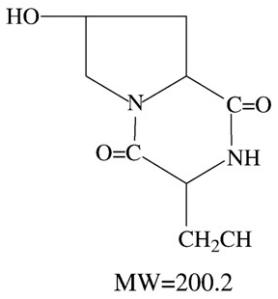

Fig. 1. Chemical structure of JBP485.

susceptibility of hepatocytes to activated autologous lymphocytes (Yoshifumi et al., 1996). Both activation of lymphocytes and Con A-binding to hepatocytes are essential for hepatic cytotoxicity. Hepatocytes are first sensitized by Con A or even killed by Con A at a high concentration, and then interact with polyclonally activated T cells, which results in cell apoptosis or even necrosis. The interaction between hepatocytes and lymphocytes is mainly mediated by ICAM-1/lymphocyte function associated antigen (LFA-1) interaction (Yoshifumi et al., 1996). To a certain extent, the pathogenesis of this hepatitis model is similar to human immune-mediated hepatitis, such as autoimmune and viral hepatitis. Therefore, the hepatitis model induced by Con A may have implications for the development of new treatment options.

Here we studied the effect of JBP485 on Con A-induced hepatic cytotoxicity and its possible mechanism in established *in vitro* models. Our findings demonstrated that JBP485 showed a potent hepatoprotective effect through inhibition of hepatocyte apoptosis and ICAM-1 expression.

2. Materials and methods

2.1. Animals

Wistar rats (from the Experimental Animal Center of Dalian Medical University, Dalian, China) were treated in accordance with local institutional guidelines for the care and use of laboratory animals.

2.2. Reagents

Con A and collagenase were purchased from Wako (Japan); JBP485 was provided by Japan Bioproducts Industry Co. Ltd (Tokyo, Japan). Before use, Con A and JBP485 were dissolved and adjusted to the required concentration with William's E medium (Hyclon, USA) free of serum. Anti-rat caspase-3 antibody and anti-rat ICAM-1 antibody were purchased from Boster Biological Technology Co. Ltd (Wuhan, China). Other reagents will be further specified when mentioned.

2.3. Cell culture

2.3.1. Hepatocyte cultures

Hepatocytes were isolated from female Wistar rats weighing 130 to 160 g by a two-step *in situ* collagenase perfusion method (Kato et al., 1994). Briefly, the liver was perfused *in situ* through the portal vein with 0.05% collagenase solution. After the liver had been excised, the cells were dispersed in ice-cold phosphate-buffered saline (PBS) (pH 7.4). Hepatocytes were then filtered through a 200 μM pore mesh and centrifuged twice for 1 min at 50 *g* to remove non-parenchymal cells. Hepatocytes were cultured in William's E medium, supplemented with 10% fetal bovine serum (FBS, Tianjin Haoyang Bio Co. Ltd, China), 10^{-6} M dexamethasone, 10^{-9} M insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. Then 500 μl aliquots of hepatocytes at a density of 2.5×10^5 10^5 cells/ml were plated in 24-well type I collagen-coated plates with a viability exceeding 85% according to the Trypan Blue exclusion method, and incubated at 37 °C, in a 5% CO_2 and 95% air-humidified atmosphere for 3 h of cell attachment, and then the medium was changed. After 24 h incubation, the medium was exchanged for William's E medium without serum before the monolayer cells were used in the experiments.

2.3.2. Splenic lymphocyte preparation

The spleen was removed from the same rat. A single cell suspension was obtained by forcing the spleen through a 200-gauge stainless steel mesh and prepared in PBS, and then 4 ml of the spleen cell suspension was gently overlaid on 2 ml of Lymphocyte Separation solution (Lymphocyte-Rat; Tianjin Haoyang Bio Co. Ltd, China) in a centrifuge tube and centrifuged at 375 *g* for 20 min. Lymphocytes were harvested from the interface of the PBS-Lymphocyte Separation solution, while erythrocytes and dead cells were discarded in the pellet. The lymphocytes were gently pipetted and washed twice with PBS before being used in the experiments.

2.3.3. Co-culture

Mixed lymphocytes were incubated for 3 h of cell attachment, and then the lymphocytes in the suspension were collected and re-cultured, and the attached cells discarded. Hepatocytes were pre-treated with Con A (20 μg/ml). 24 h later, the autologous lymphocytes (1.25×10^7 10^7 cells/ml) activated with Con A (20 μg/ml) for 48 h were then added to the hepatocytes, and co-incubated at a lymphocyte/hepatocyte ratio of 10:1 for 8 h.

2.3.4. ICAM-1 induction/inhibition experiment

In the ICAM-1 induction/inhibition experiment, the pooled supernatant from Con A (20 μg/ml)-treated lymphocytes was added to the hepatocytes and incubated for 8 h, JBP485 (25 μM) was pre-treated to hepatocytes 1 h before the medium of lymphocytes was added.

2.4. MTT assay

The viability of cells was evaluated by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, at the end of the incubation period, the cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37 °C prior to medium removal, 500 μl of acidic isopropyl alcohol was then added to each well, and the plate was stirred thoroughly for 15 s on a shaker. Next, 100 μl of this solution was transferred to 96-well microtiter plates. The absorbance of each well at 570/620 nm was measured using a microplate reader (Thermo Multiskan Ascent, Finland). The cell survival rate was calculated using the following formula (Eq. (1)), and IC_{50} values were calculated from the concentration–response curve as the concentration of Con A resulting in an absorbance equivalent to 50% of the control value.

$$\text{Cell survival rate}(\%) = \frac{\text{OD}_{\text{ConA}}}{\text{OD}_{\text{Blank}}} \times 100 \quad (1)$$

2.5. Determination of biochemical marker leakage

At the end of different incubation periods, the supernatants were collected and stored at −80 °C until use for determination of cytokine levels and enzymes. The activities of AST and LDH in the supernatant were measured with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the content of TNF-α was determined using a radioimmunoassay kit (China PLA General Hospital, Military Postgraduate Medical College). The assays were performed exactly as described in the manufacturer's instructions. The inhibition rate (I) was calculated using the following formula (Eq. (2)), where V represents the measured values of AST, LDH or TNF-α in the model group, treated group or blank/control group, respectively.

$$I(\%) = \frac{V_{\text{Model}} - V_{\text{Treated group}}}{V_{\text{Model}} - V_{\text{Blank/Control}}} \times 100 \quad (2)$$

Table 1
Rat primer sequences used for semi quantitative RT-PCR

Gene	Accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product
Caspase-3	NM 012922	atggactctggaatatatctg	cttttgtgagcattgacaca	736 bp
ICAM-1	NM 012967	cagcagaccactgtgctttga	gtcgagcttcaggaccctagt	406 bp
β-actin	NM_031144	attgaacacggcattgtcac	gtcgagcttcaggaccctagt	560 bp

2.6. DNA gel electrophoresis assay

Following incubation of hepatocytes in the presence or absence of drugs for the indicated times, the monolayer was washed with PBS, and then 0.3 ml hypotonic lysis buffer (50 μg/ml proteinase K in 1 mM EDTA, 100 mM Tris–HCl pH 8.0, and 1% SDS) was added to each well. After incubation for 2 h at 50 °C (or 37 °C overnight), the cell lysis was collected, and the supernatant was extracted with a mixture of hydroxybenzene/chloroform/isoamyl alcohol (25:24:1). DNA was deposited with dehydrated alcohol and sodium acetate, and dissolved in TE buffer (1 mM EDTA, 10 mM Tris–HCl pH 8.0). Electrophoresis was performed in 1.5% agarose gel (containing 0.5 μg/ml ethidium bromide) for 1 h, and the bands were visualized and photographed under transmitted ultraviolet light.

2.7. Two-step RT-PCR

Total RNA of cultured rat hepatocytes was extracted using the TRIZOL reagent (Invitrogen). RT-PCR requires 500 ng of total RNA each time. Specific designed primers are shown in Table 1. Two-step RT-PCR was performed according to the protocol of the kit (Takara, Dalian, China), and amplified in a GeneAmp PCR system (Techne TC512, UK). RNA samples were first reverse transcribed and then immediately amplified by PCR. Quantity One (version 4.40) software was used to analyze the OD values of the electrophoresis bands. To exclude variations due to RNA quantity and quality, the data for caspase-3 and ICAM-1 were adjusted to β-actin expression (OD of caspase-3 or ICAM-1 mRNA versus OD of β-actin mRNA).

2.8. Immunocytochemistry

Immunocytochemistry staining for caspase-3 and ICAM-1 was performed *in situ* on the 24-well collagen-coated plates using the streptavidin–biotin–peroxidase complex. After the medium was removed at different time points, the cells were fixed with 70% alcohol and pre-treated with 0.3% H_2O_2–methanol solution and 3% normal goat serum, respectively. The cells were then incubated at 4 °C overnight with rabbit anti-rat caspase-3 or ICAM-1 antibody (1:500 dilution), followed by incubation in biotinylated goat anti-rabbit IgG and then SABC, both for 20 min at 37 °C. 3,3-Diaminobenzidine–H_2O_2 was used in color development, and the cells were counterstained with hematoxylin and visualized by inverted digital image light microscopy (Nikon ECLIPSE, Japan). The cells stained brown were considered as positive cells, and the green levels were scanned using SimplePCI 6.2 software (Compix Inc, USA).

2.9. Statistical analysis

Data were expressed as means ± S.D., and analyzed by analysis of variance (ANOVA) and Student's *t*-test using the statistical software SPSS 11.5. Differences were considered significant when $P<0.05$.

3. Results

3.1. Concentration-dependent hepatocyte toxicity induced by Con A

To examine whether cytotoxicity induced by Con A in primary cultured rat hepatocytes showed a concentration-dependent effect, the cell survival rate was measured by MTT assay after 24 h exposure to Con A. Con A in itself did not have any cytotoxic effect at a concentration of 20 mg/ml within 24 h (Fig. 2A). The threshold concentration of Con A-

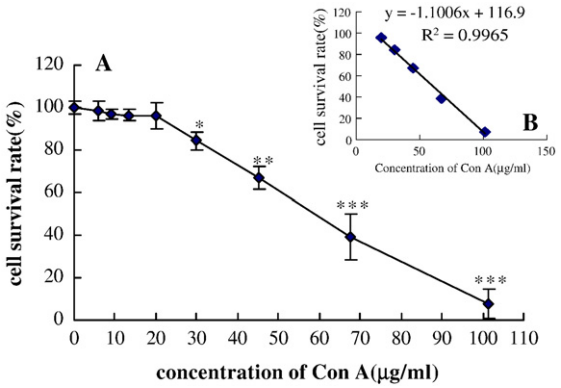

Fig. 2. Dose-dependent cytotoxicity directly induced by Con A. Hepatocytes (1.25×10^5 cells per well) were treated with Con A at various concentrations for 24 h. At the end of the incubation time, the viability of cells was evaluated by MTT assays (A), and cell survival rate was calculated as described (Eq. (1)). Standard curve of concentration–response (B). * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Each value represents the mean of three replicates; error bars represent S.D.

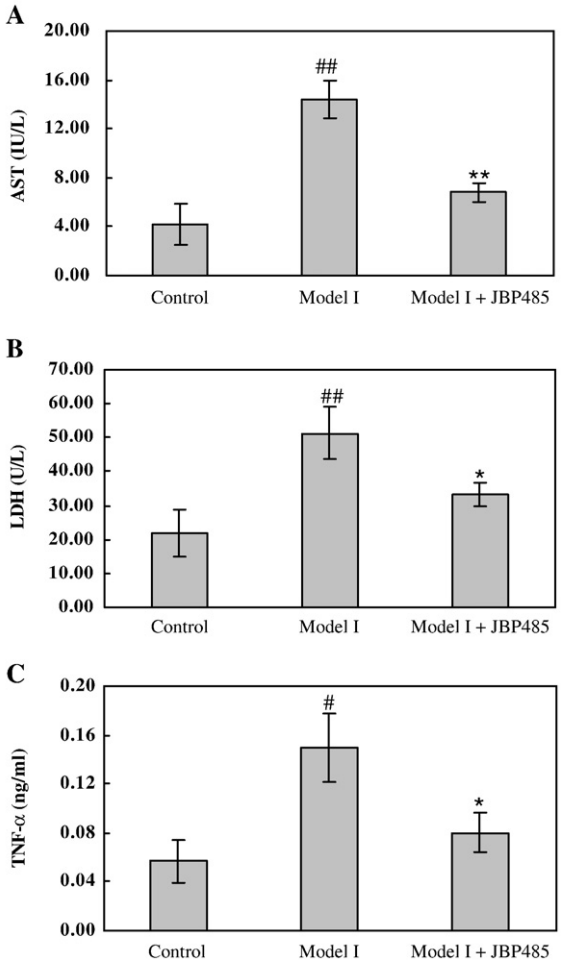

Fig. 3. Effect of JBP485 on hepatic cytotoxicity directly induced by Con A. Changes in the supernatant levels of AST (A), LDH (B), and TNF-α (C) were detected in triplicate at the time points indicated in the control group, model I (Con A 50 μg/ml alone) group, and JBP485 treated groups. JBP485 (25 μM) was added to hepatocytes 1 h before Con A treatment. 24 h later, levels of enzyme and cytokine were measured. # $P<0.05$, ## $P<0.01$ vs. control group; * $P<0.05$, ** $P<0.01$ vs. model I group. Each value represents the mean of three replicates; error bars represent S.D.

induced toxicity was 25 μg/ml (≈0.1 μM) (Fig. 2A). The IC_{50} value of Con A was 60.79 μg/ml, and the concentration of Con A was 50 μg/ml when the cell survival rate was 60% (majority of cells survived) (Fig. 2B). We choose the concentration of 50 μg/ml and 20 μg/ml as the direct intoxicant concentration and pre-stimulated concentration *in vitro*, respectively.

3.2. Effect of JBP485 on hepatocyte toxicity directly induced by Con A

To examine whether JBP485 lessens the hepatocyte toxicity directly induced by Con A (Model I), we determined the effect of JBP485 on the biochemical marker leakage from primary cultured hepatocytes. The increase in liver cytosolic enzyme activities caused by Con A (50 μg/ml) was inhibited by addition of JBP485. A high inhibition rate was found in both AST value (74.9±16.9%) and LDH value (60.6±9.6%) (Fig. 3A, B). Intriguingly, a similar result was obtained with inflammatory cytokine release (Fig. 3C). A remarkable TNF-α increase was found in the supernatant after Con A treatment, which was reduced in the presence of JBP485 with an inhibition rate of 74.5±8.8% (Fig. 3C).

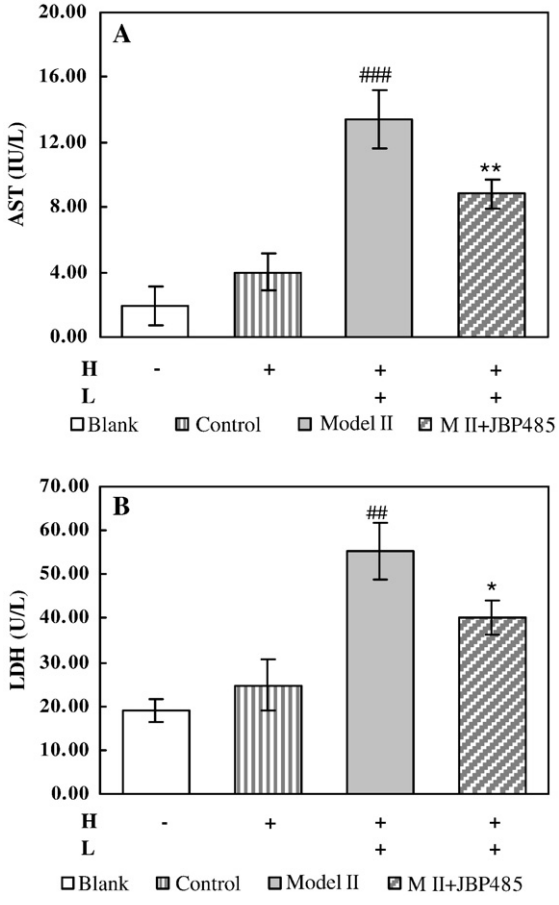

Fig. 4. Effect of JBP485 on hepatic cytotoxicity induced by co-culture of Con A-treated hepatocytes with activated autologous lymphocytes. Model II was established by Con A (20 μg/ml) pre-treated hepatocytes co-cultured with Con A (20 μg/ml) stimulated lymphocytes. Cells were co-incubated for 8 h, and then levels of AST (A) and LDH (B) in the supernatant were determined. Hepatocytes were pre-treated with JBP485 (25μM) for 1 h before Con A was added. 24 h later, hepatocytes were then washed before the interaction was performed in the presence of the same tested compound. Blank group (□), Control group: hepatocytes treated with Con A (20 μg/ml) alone (□). Model II group (□), JBP485 treated group (□). H+: hepatocytes stimulated by Con A, H−: hepatocytes without any treatment; L+: lymphocytes activated with Con A, L−: lymphocytes without any treatment. $^{\#\#}$ $P<0.01$, $^{\#\#\#}$ $P<0.001$ vs. blank or control group; * $P<0.05$, ** $P<0.01$ vs. the model II group. Each value represents the mean of three replicates; error bars represent S.D.

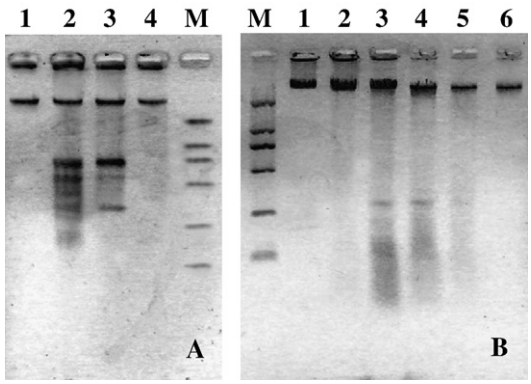

Fig. 5. Agarose gel electrophoresis assay for DNA fragmentations. Lane 1: blank; Lanes 2 and 3: model I; Lane 4: JBP485 (25 μM) treated group (A). Lane 1: blank; Lane 2: control; Lanes 3 and 4: model II; Lanes 5 and 6: JBP485 treated group (B). Lane M: DL2000 marker, from top to bottom: 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, and 100 bp.

3.3. Effect of JBP485 on hepatocyte toxicity induced by interaction with lymphocytes

To examine whether JBP485 relieves the hepatocyte toxicity induced by the interaction between hepatocytes and lymphocytes (Model II), the change in the leakage of liver cytosolic enzyme was also determined. Con A itself did not have any cytotoxicity at a concentration of 20 μg/ml. A significant increase was seen in AST (Fig. 4A) and LDH (Fig. 4B) induced by the co-culture, compared to the untreated group or to the Con A (20 μg/ml) treated alone group. JBP485 decreased the enzyme levels with the inhibition rate of I_{AST} 49.4±5.4% and I_{LDH} 51.4±9.8% (Fig. 4).

3.4. Inhibition of hepatocyte apoptosis by JBP485 pre-treatment

To examine whether the reduction in biochemical leakage correlated with apoptosis inhibition, the inhibitory effects on DNA fragmentation and caspase-3 expression were investigated. DNA electrophoresis experiments showed that no DNA fragmentations were found in the blank or the control group. Relatively high molecular weight fragmentation was found in the model I group; in contrast, relatively low molecular weight fragmentation was detected in the model II group. Pre-treatment with JBP485 reduced fragmentation (Fig. 5). We also examined caspase-3 expression at the gene level (Fig. 6) and at the protein level (Fig. 7). First, no significant change of caspase-3 mRNA expression occurred in the Con A (50 μg/ml) group

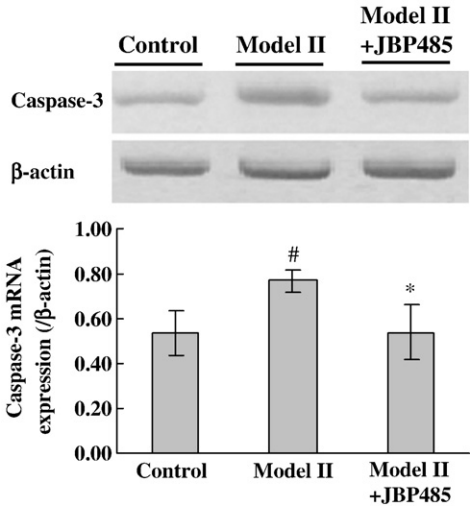

Fig. 6. Effect of JBP485 on the expression of Caspase-3 mRNA in hepatocytes co-cultured with autologous lymphocytes. $^{\#}$ $P<0.05$ vs. control group; * $P<0.05$ vs. model II group. Each value represents the mean of three replicates; error bars represent S.D.

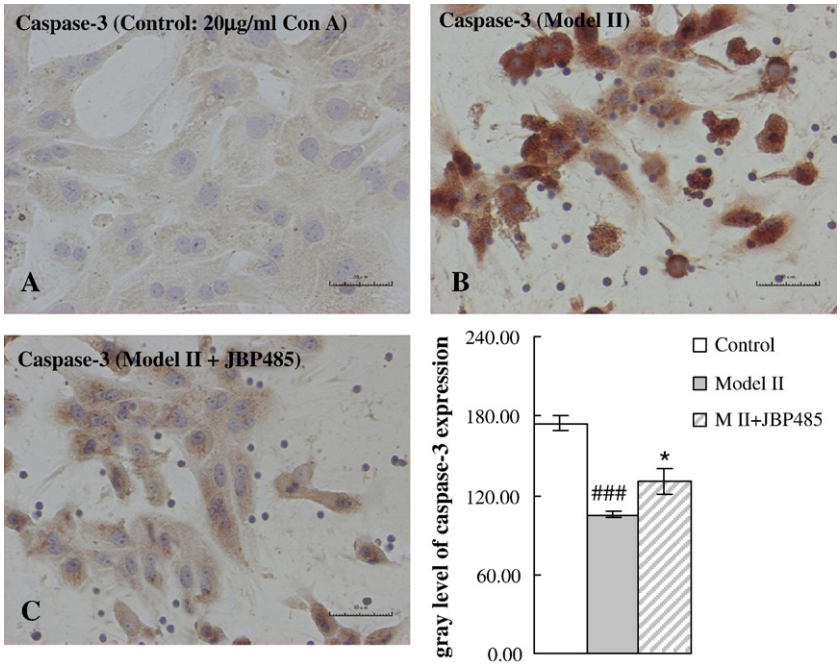

Fig. 7. Immunocytochemical staining of caspase-3 in hepatocytes. The cells stained brown were considered as positive cells. Gray levels reflect the extent of caspase-3 expression and exhibit an inverse proportional relationship between them. Photos from A to C ×200 magnification, bar = 50 μM. ### $P<0.001$ vs. control group; * $P<0.05$ vs. model II group. Each value represents the mean of three replicates; error bars represent S.D.

compared to the control group, whereas, caspase-3 mRNA was strongly expressed in model II, which was decreased almost comparable with the control group by JBP485 pre-treatment (Fig. 6). Second, immunostaining for caspase-3 expression showed no significant difference between the control and the model I group, while hepatocytes were almost all caspase-3-positive with strongly brown-stained cytoplasm resulting from the interaction with lymphocytes compared with the control group. The expression of caspase-3 significantly decreased in the JBP485 treated group compared with the model II group with an inhibition rate of 76.4 ± 16.9%. To a great extent, the change in caspase-3 protein expression is consistent with those of mRNA expression (Fig. 7). These findings indicated that JBP485 exhibited an anti-apoptosis effect.

3.5. Reduction of ICAM-1 expression by JBP485 pre-treatment

Evidence of JBP485 showing a protective effect on cytotoxicity induced by interaction between hepatocytes and lymphocytes prompted us to determine whether it regulated ICAM-1 expression. In our experiments, the expression of ICAM-1 was induced by addition of the pooled supernatant from Con A (20 μg/ml)-treated lymphocytes to hepatocytes, then we determined the effects of JBP485 on it. As predicted, after incubation with JBP485 for 24 h, the expression of ICAM-1 mRNA was reduced with an inhibition rate of 45.1 ± 17.4% (Fig. 8). Similarly, albeit to a lesser extent, the expression of ICAM-1 protein by immunocytochemical staining in hepatocytes was also reduced by JBP485 (Fig. 9). The results indicated that JBP485 down-regulated the expression of ICAM-1, which plays a critical role in the interaction between hepatocytes and lymphocytes.

4. Discussion

We examined the effect of JBP485 on hepatic cytotoxicity and the possible molecular mechanisms. Two cytotoxicity models were used in our experiments in order to evaluate their hepatoprotective activities *in vitro*. When cultured hepatocytes were pre-treated with JBP485, both inflammatory reaction and apoptosis-associated events were significantly suppressed. Furthermore, our results also indicated that JBP485 inhibits ICAM-1 expression in hepatocytes, which provided a hint as to the involvement of the compound in regulating immune-associated molecules.

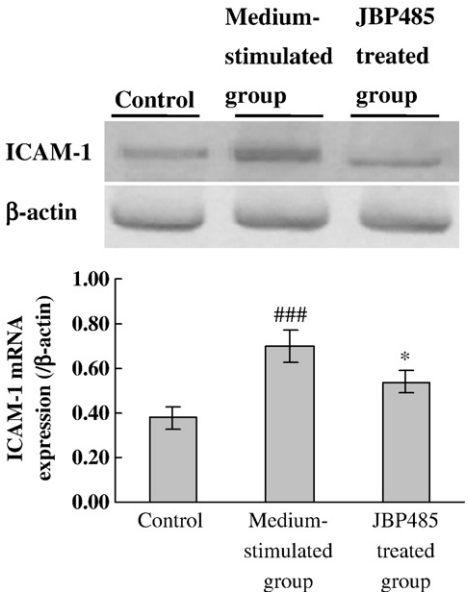

Fig. 8. Effect of JBP485 on the expression of ICAM-1 mRNA in hepatocytes. Fresh isolated hepatocytes (2.5×10^5) were seeded in 24-well plates, 24 h later, the culture medium was carefully replaced with pooled supernatant from Con A (20μg/ml)-treated lymphocytes (control group). JBP485 (25 μM) was pre-treated to hepatocytes 1 h before the medium of lymphocytes was added. ### $P<0.001$ vs. control group; * $P<0.05$ vs. medium-stimulated group. Each value represents the mean of three replicates; error bars represent S.D.

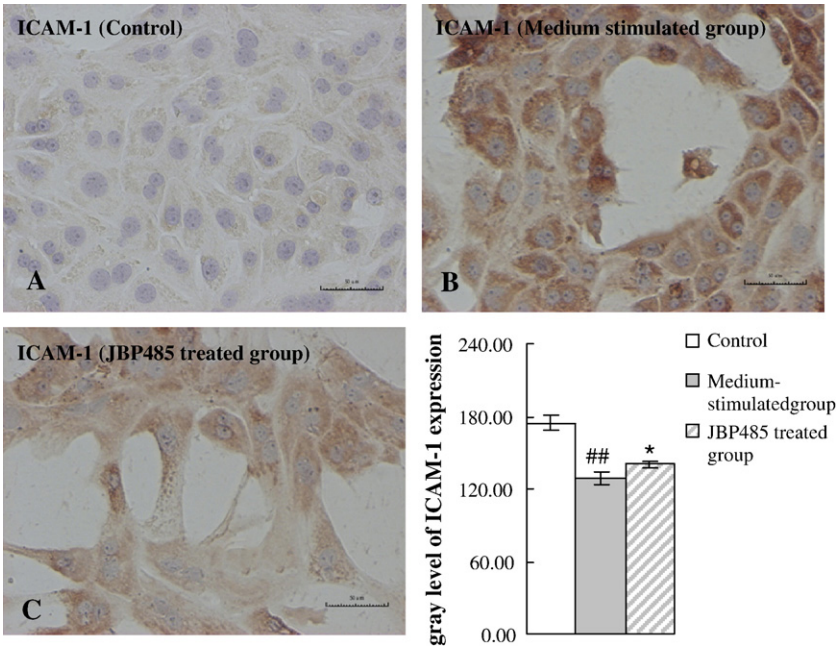

Fig. 9. Immunocytochemical staining of ICAM-1 in hepatocytes. The cells stained brown were considered as positive cells. Gray levels reflect the extent of ICAM-1 expression and exhibit an inverse proportional relationship between them. Photos from A to C ×200 magnification, bar = 50 μM. $^{\#\#}$ $P<0.01$ vs. control group; * $P<0.05$ vs. medium-stimulated group. Each value represents the mean of three replicates; error bars represent S.D.

The hepatotoxicity directly induced by Con A reflects a general toxicity due to inappropriate excessive stimulation of membrane receptors. With the advent of morphological alteration that was previously described as "feathery protrusions" (Leist and Wendel, 1996) in cultured rat hepatocytes, a metabolic response then occurred after Con A-binding to hepatocytes, which resulted in biochemical changes (Katzen et al., 1981; Shechter, 1983). Model I—cytotoxicity directly induced by Con A (50 μg/ml) was first established; JBP485 caused a remarkable decrease in activities of AST, LDH and release of TNF-α induced by Con A (Fig. 3). However, the inhibitory effect of JBP485 did not show a concentration-dependent relationship (data not shown). This finding as well as the fact that JBP485 did not reverse the morphological alteration (data not shown), indicated that this protective effect was not mediated by membrane receptors but was associated with intracellular biochemical changes. DNA fragmentation was involved in the hepatic injury directly induced by Con A, and JBP485 reduced the fragmentation (Fig. 5A). Small amounts of TNF-α derived from Con A-stimulated hepatocytes (Fig. 3C) alone are not sufficient to induce apoptosis, because transcriptional arrest simultaneously is indispensable (Leist et al., 1994), however, Con A increases transcriptional activity *in vitro* as reported (Leist and Wendel, 1996). Thus, other molecules may be involved in eliciting DNA fragmentation.

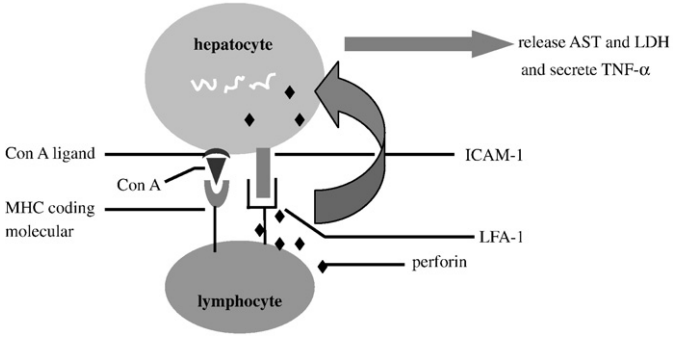

Fig. 10. Diagrammatic representation of *in vitro* model II.

The results of caspase-3 expression showed no significant difference between the Con A (50 μg/ml) alone treated group and the blank group (data not shown). This finding indicates that a certain specific mechanism may contribute to the course of fragmentation. Accordingly, we concluded that DNA fragmentation was a consequence rather than a course or upstream event of Con A toxicity; JBP485 seemed to affect the upstream event in the Con A-intoxicated course.

The mechanism of Con A-induced hepatitis *in vivo* is complex. The central molecular event – interaction between Con A-treated hepatocytes and Con A-activated lymphocytes – serves as the target of our investigation. ICAM-1/LFA-1 is thought to be critical in inducing further apoptosis via the Fas/perforin-mediated pathway in T-cell-mediated cytotoxicity (Kagi et al., 1994; Lowin et al., 1994). The presence of anti-ICAM-1/LFA-1 antibodies almost completely inhibits liver injury *in vivo,* while it partially inhibits hepatic cytotoxicity (approximately 50%) *in vitro* (Yoshifumi et al., 1996), which indicates that there are still other molecules involved in the interaction. Based on early reports, Con A bound to murine major histocompatibility complex (MHC) molecules to mimic their antigenic properties (Berke et al., 1981), which explains the fact that activated T cells only recognized the Con A-stimulated hepatocytes (Yoshifumi et al., 1996). Therefore, both Con A-binding to hepatocytes and ICAM-1/LFA-1 interaction are essential for hepatic cytotoxicity induced activated lymphocytes, which is the primary theoretical basis of Model II—cytotoxicity induced by the interaction between Con A-treated hepatocytes and Con A-activated lymphocytes (Fig. 10). Cytotoxicity induced by interaction between hepatocytes and lymphocytes was markedly mitigated by JBP485 treatment as predicted, although the inhibition rate was no more than 50% (Fig. 4). As our preliminary presumption from Model I that JBP485 did not inhibit Con A-binding to membrane receptors of hepatocytes, JBP485 is thought to disturb the ICAM-1/LFA-1 interaction rather than the recognition of T cells by Con A. Further experiments have been planned to elucidate whether the dipeptide regulates the ICAM-1 expression in hepatocytes. Inflammatory cytokines up-regulated ICAM-1 expression in cultured hepatocytes and T-lymphocyte adhesion (Volopes et al., 1990; Essani et al., 1995; Morita et al., 1994; Sano et al., 1999). It is inevitable that hepatocytes will be affected by cytokines derived from activated

lymphocytes when co-cultured with Con A-pre-treated lymphocytes, based on which, we used pooled supernatant from Con A (20 μg/ml)-treated lymphocytes to induce ICAM-1 expression in hepatocytes. It was rather surprising that JBP485 down-regulated the ICAM-1 expression either at the gene level (Fig. 8) or at the protein level (Fig. 9), then further reduced the DNA fragmentations induced by the interaction (Fig. 5B).

Although the interaction between lymphocytes and hepatocytes has been poorly investigated, compared with interactions between lymphocytes and endothelial cells, it is possible that lymphocytes easily interact with hepatocytes *in vivo*. This statement is based on the following two independent arguments. First, hepatocytes contain no basement membrane around the sinusoid; the only relevant interactions are those with sinusoidal endothelial cells and hepatocytes (Yoshifumi et al., 1996). Second, Con A has high affinity toward the hepatic sinus which results in the activation of T cells in liver tissue (Trautwein et al., 1997). The activation of T cells in liver results in secretion of several cytokines involved in cell–cell interaction and in mediating inflammation. Thus, inhibition of the interaction will contribute greatly to the therapy of immune-mediated hepatitis.

Short-term cultured rat hepatocytes are applied in our experiments, relative to other *in vitro* models. This significantly reduces the number of animals used, moreover, the suitability of isolated hepatocytes as a screening procedure early in drug development is widely accepted (Fautrel et al., 1991; Castell et al., 1997; Li et al., 2006). Evaluation of the tested compound *in vitro* may give an indication of its effect *in vivo*, although such a correlation must take into account the pharmacokinetics of the drug. The ultimate goal of *in vitro* experiments is to generate scientific information that is helpful in rational species extrapolation of drug effects from animal to human.

We conclude that JBP485 exhibited a protective effect on hepatic cytotoxicity induced *in vitro* by Con A. Not only did it suppress apoptosis, but it inhibited the expression of immune-associated adhesion molecules. Further investigations in this dipeptide may provide additional experimental foundation for its clinical application.

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