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L-Cysteine administration prevents liver fibrosis by suppressing hepatic stellate cell proliferation and activation

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Abstract

Recent studies showed that the function of some amino acids is not only nutritional but also pharmacological. However, the effects of amino acids on liver fibrosis and hepatic stellate cell (HSC) remain unclear. In this research, as a result of screening of amino acids using liver fibrosis induced by DMN administration, L-cysteine was selected as a suppressor of liver fibrosis. Furthermore, the number of activated HSCs, which increased in the fibrotic liver after DMN administration, was decreased in L-cysteine-fed rats. Treatment of freshly isolated HSCs with L-cysteine resulted in inhibition of the increase in smooth muscle α-actin (αSMA) expression by HSCs and BrdU incorporation into the activated HSCs. These findings suggest that L-cysteine is effective against liver fibrosis. The mechanism of inhibition of fibrosis in the liver is surmized to be direct inhibition of activated HSC proliferation and HSC transformation by L-cysteine.

Keywords: L-Cysteine; Liver fibrosis; Dimethylnitrosamine; Hepatic stellate cell

The functions of some amino acids have been elucidated as being not only nutritional, but also pharmacological. For example, arginine plays roles in maintaining immunity, ammonia detoxification, and liver function improvement [1]. In liver disease, the branched-chain amino acids improve protein nutrition in liver cirrhosis [2], and *S*-adenosyl L-methionine administration improves the survival of alcoholic patients with liver cirrhosis [3].

HSCs (hepatic stellate cells), produce the matrix outside of cells and play important roles in the injury repair and fibrosis in the liver. When acute liver injury is produced in rats, mononuclear cells gather at necrotic foci, and HSC will also increase around the foci. Following the deposition of collagen and extracellular matrix (ECM), a hepatic scar is formed. Liver fibrosis is a result of chronic liver injury, and it is characterized by the composure to space of Disse of matrices outside cells, such as collagen, fibronectin, laminin, and proteoglycan. Activation of HSCs is important in this case as well [4].

If HSCs, which isolated from normal rat liver, are cultivated for several days in uncoated plastic culture dishes, change in shape and become so-called "myofibroblast-like cells, activated HSC" and gain the functions of proliferation, increased ECM synthesis, contractility, release some cytokines, matrix degradation, chemotaxis, and loss of lipid droplets [5]. αSMA is a reliable marker for identification of activated HSCs in human and rat livers [6,7]. In liver cirrhosis, quiescent HSCs decrease in number and myofibroblast-like cells (activated HSCs) increase. Regardless of the pathogenesis of animal models, proliferated activated HSCs are similarly observed in bile duct-ligated rat liver and after administration of toxic reagents (carbon tetrachloride [8], dimethylnitrosamine (DMN) [9], thioacetamide TAA [10], etc.). When HSCs are activated, the receptor for platelet-derived growth factor (PDGF) is expressed, and proliferation of activated HSCs in response to PDGF

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becomes more active. For HSCs, PDGF is one of the most potent mitogens with various proliferative factors [11].

Antifibrotic therapy requires the development of antifibrotic compounds. In the present study, we selected L-cysteine, which can attenuate liver fibrosis induced by DMN administration to rats, from amino acids. The number of activated HSCs was lower in the L-cysteine diet/DMN group than in the control diet/DMN group. Further observations showed that proliferation and activation of cultured HSCs isolated from rats were inhibited by L-cysteine. This result suggests that the antifibrotic effect of L-cysteine was exerted directly on the HSCs.

Materials and methods

Animals and experimental protocol. Male Sprague–Dawley rats (Charles River Japan, Yokohama, Japan) weighing 160–190 g were housed, with unrestricted access to food (CRF-1, Oriental Yeast, Tokyo, Japan) and water, in air-conditioned animal quarters that were lit between 07:00 and 19:00 h. L-Cysteine diet preparation as follow: L-Cysteine (Ajinomoto, Tokyo, Japan) was added to CRF-1 to 0.5%, 1.0%, and 2.0% and mixed uniformly with a mixer (DALTON, Tokyo, Japan). The L-cysteine diet (supplemented with L-cysteine) or the control diet (CRF-1) was provided ad libitum for four weeks. Hepatic fibrosis was induced by intraperitoneal injection of 0.5% DMN (Wako Pure Chemical Industries, Osaka, Japan) at 2 ml/kg body weight for three consecutive days per week for 4 weeks. After 4 weeks of DMN administration ± L-cysteine, blood samples were drawn from the inferior vena cava, and liver specimens were taken for light microscopy, immunohistochemistry, and determination of hydroxyproline. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of AJINOMOTO.

Hydroxyproline content. To determine the content of hydroxyproline, liver homogenates were prepared from specimens in a solution containing of 3% trichloroacetic acid (Wako Pure Chemical Industries, Osaka, Japan). After centrifugation, pellets were hydrolyzed in a solution containing 6 N HCl at 110 °C for 20 h. After centrifugation, supernatants were evaporated on an FC-950 (Sakuma Seisakusho, Tokyo, Japan), and the pellet was dissolved in distilled water. The solution was filtered through a 0.22 μm Syringe Driven Filter Unit (Amicon, Beverly, MA) and Ultrafree-MC membrane (Millipore, Bedford, MA), and then injected into an amino acid analyzer (HITACHI L-8800, Japan). Hydroxyproline was detected as having the same retention time as the commercial standard amino acid mixture (AN-II Wako Pure Chemical Industries, Osaka, Japan) and the concentration was calculated.

Blood markers. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb), and NH_3 were measured using commercial kits for standard analysis (Fuji Film, Tokyo, Japan). Hyaluronic acid levels were determined using a commercial ELISA kit (Fujirebio, Tokyo, Japan).

Immunohistochemistry and azan staining. For light microscopy, liver specimens were fixed in phosphate-buffered formaldehyde and embedded in paraffin. Tissue block sections were then mounted on slides, deparaffinized in xylene, rehydrated in alcohol, and stained with Mallory's azan. To count the number of αSMA-positive cells, endogenous peroxidase was blocked with 1% hydrogen peroxide, Monoclonal smooth muscle α-actin antibody (DAKO Carpinteria, CA) was incubated for 1 h at room temperature. The antibody binding was detected with DAKO ENVISION™+ systems HRP (DAKO, Carpinteria, CA) and HRP was visualized using diaminobenzidine. The sample was examined using an Axiophoto microscope (Carl Zeiss, Thornwood, NY).

Quantification of smooth muscle actin-positive cells. The number of α-smooth muscle actin-positive cells was expressed as a percentage of the total area of the specimen, as previously described [12].

Isolation and cultivation of HSCs. HSCs were isolated from rat liver by a modification of the method described by Kawada et al. [13]. Briefly, the liver was digested by in situ portal vein perfusion of a pronase E (1.0 mg/ml) and collagenase (0.5 mg/ml) in Gey's balanced salt solution. The digested liver was separated from the rat, minced, and incubated in Gey's balanced salt solution containing 0.7 mg/ml pronase E, 0.7 mg/ml collagenase, and 20 μg/ml DNaseI. The resultant suspension of cells was centrifuged in a 7.8% Nycodenz gradient for 15 min. HSCs separated into a band just above the interface of the Nycodenz solution and the aqueous buffer. This band was harvested, and the cells were washed and then suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, NY) containing 10% fetal bovine serum (FBS, tissue Culture Biologicals, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, NY). The purity of isolated HSCs was over 90% as assessed by typical starlike configuration with droplets. Freshly isolated HSCs were cultured in uncoated plastic dishes (BD Biosciences, Franklin, NJ).

Quantification of proliferated HSCs. The culture medium of freshly isolated HSCs was changed with DMEM supplemented with 10% FBS every three days. On the 7–9th day, HSCs were trypsinized, reseeded into 24-well dishes, and cultured overnight in DMEM supplemented with 10% FBS. On the next day, the culture medium was changed to DMEM supplemented with 0.1% FBS. After two days, the medium was changed to minimum essential medium (MEM, Sigma–Aldrich, Irvin, UK) containing 0.1% FBS supplemented with 25 ng/ml rat recombinant PDGF-BB (platelet-derived growth factor-BB, Sigma, St. Louis, MO) ± 0–10 mM L-cysteine or 0–30 mM *N*-acetylcysteine (NAC). The cells were harvested after 24 h. Six hours before harvest, BrdU was added to the culture medium (final concentration: 10 μM). BrdU uptake by the cells was detected using a Cell Proliferation Assay Kit (Amersham Biosciences, England). Cells viability was detected using MTT assay [14].

Quantification of activated HSCs. Freshly isolated rat HSCs were seeded into uncoated-plastic dishes and cultivated in DMEM supplemented with 10% FBS. On the next day, the culture medium was changed to MEM + 0–10 mM L-cysteine or 0–10 mM NAC with 10% FBS. After 5 days, αSMA expression was detected using a cell ELISA. The cells were incubated with Monoclonal anti-smooth muscle α-actin antibody (αSMA) (DAKO, Carpinteria, CA) for 1 h at room temperature. The second antibody used was HRP-conjugated goat anti-mouse IgG (DAKO Carpinteria, CA). αSMA expression was detected with tetramethylbenzidine (ICN Biomedicals).

Statistical analysis. Data were presented as the means ± standard deviation (SD). We used the William's test (SAS ver 6.12, Sasinstitute, Cary, NC). The level of statistical significance was set at $P = 0.05$.

Results

Administration of L-cysteine improves DMN-induced liver fibrogenesis

Fig. 1A shows the representative Azan–Mallory staining of a normal liver specimen (control diet), control diet/DMN, and 2.0% L-cysteine diet/DMN group rats. Cirrhosis was detectable with the control diet/DMN and moderate liver fibrosis was seen with 2.0% L-cysteine diet/DMN. Accordingly, L-cysteine-fed rats showed a tendency to reduce accumulation of hydroxyproline in the liver and increased plasma hyaluronic acid in a

dose-dependent manner (Figs. 1B and C). The accumulation of hydroxyproline on the 1.0% and 2.0% L-cysteine diets showed statistically significant improvement. Plasma hyaluronic acid was also statistically decreased by the 1.0% and 2.0% L-cysteine diet. These results suggest that feeding of L-cysteine suppresses the development of liver fibrosis in rats. Neither the body weight nor the food intake of rats was significantly different between the L-cysteine diet/DMN groups and the control diet/DMN group (data not shown).

Blood markers of liver function

The plasma values of ALT, AST, and Alb on L-cysteine-administered rats were improved by L-cysteine in a concentration-dependent manner (Table 1). The plasma AST and ALT levels were lower in the L-cysteine diet/DMN-treated rats than control diet/DMN-treated rats in an L-cysteine dose-dependent manner. Plasma albumin was slightly improved and plasma NH_3 level was not significantly changed.

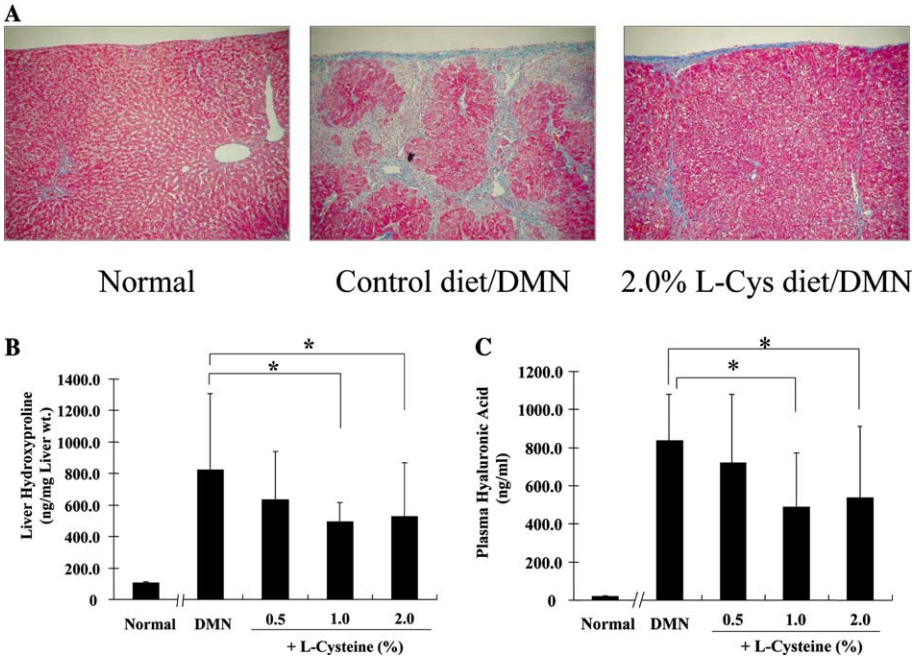

Fig. 1. Liver fibrosis of DMN ± L-cysteine-administered rats. Hepatic fibrosis was induced by intraperitoneal injection ($n = 12$ in each group) of 0.5% DMN at 2 ml/kg body weight for three consecutive days per week for 4 weeks. The number of normal group rats was started with 4. None, 0.5%, 1.0%, and 2.0% L-cysteine-containing diets were supplied ad libitum during this period. Rats were killed 4 days after final injection of DMN. Seven rats were dead until four weeks caused by liver failure. The number of survivable rats for four weeks were 4 in normal group, 9 in control diet/DMN, 10 in 0.5% L-cysteine diet/DMN group, 10 in 1.0% L-cysteine/DMN group, and 12 in 2.0% L-cysteine diet/DMN group. (A) Azan–Mallory-stained sections of representative lesions of livers of untreated rats and rats treated with DMN ± L-cysteine. The liver samples were obtained 4 weeks after starting treatment (left panel) untreated, (middle panel) control diet/DMN, and (right panel) 2.0% L-cysteine diet/DMN (original magnification 100×). (B) Hydroxyproline contents in liver on dose dependent L-cysteine administration. (C) Hyaluronic acid levels of rats for blood drawn from the inferior vena cava. Data represent the means ± SD. *$P < 0.05$ vs. control diet/DMN.

Table 1
Effect of L-cysteine on serum markers

Treatment	AST (IU/L)	ALT (IU/L)	ALB (g/dl)	NH_3 (μg/dl)
Control diet	74.5 ± 11.1	34.5 ± 4.2	3.6 ± 0.1	45.3 ± 7.8
DMN + control diet	254.5 ± 328.0	121.7 ± 258.3	2.5 ± 0.4	165.6 ± 89.0
DMN + 0.5% L-cysteine diet	224.1 ± 90.1	120.1 ± 22.4	2.6 ± 0.3	145.6 ± 25.8
DMN + 1.0% L-cysteine diet	175.7 ± 113.3*	107.2 ± 38.2	2.8 ± 0.6*	141.5 ± 136.2
DMN + 2.0% L-cysteine diet	184.8 ± 79.6*	106.4 ± 32.7	2.8 ± 0.4*	144.9 ± 61.8

Value are means ± SD.
* $P < 0.05$ vs. control diet/DMN.

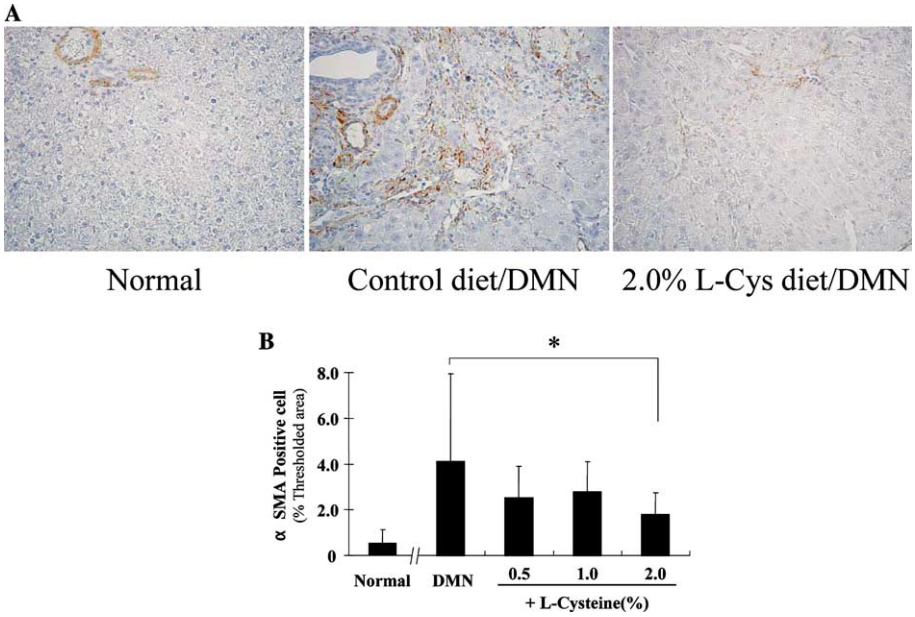

Fig. 2. (A) Immunohistochemical staining for αSMA in liver sections at 4 weeks after DMN administration. αSMA-positive cells were observed. (left panel) normal, (middle panel) control diet/DMN, and (right panel) 2.0% L-cysteine diet/DMN (original magnification 100×). (B) Quantification of the number of αSMA-positive cells. The mean value of αSMA-positive cells in six ocular fields (magnification 40×) per one specimen was assessed as percent area. Liver specimens were the same as used in Fig. 1. Data represent the means ± SD. *$P < 0.05$ vs. control diet/DMN.

Decrease in number of HSCs by L-cysteine administration

Quantification of activated HSCs was performed by assaying the level of αSMA expression, which is a marker of activated HSCs. We observed that αSMA-positive cells were mainly associated with fibrous septa and basement membrane-like structures of the portal vein in DMN-administered rats fed the control diet (Fig. 2A). The number of αSMA-positive cells detected decrease in 2.0% L-cysteine containing diet compared with control diet group (Fig. 2B). The difference between 2.0% L-cysteine and control diet groups was significant.

Proliferation of activated HSC and HSC activation in the presence of L-cysteine

To evaluate whether L-cysteine inhibits the proliferation of activated HSCs in vitro, we monitored BrdU incorporation into the nucleus of HSCs. PDGF-BB-dependent DNA synthesis was inhibited in an L-cysteine concentration-dependent manner (Fig. 3). Inhibition of BrdU incorporation was first seen at 0.33 mM L-cysteine and 30 mM NAC.

Fig. 4 shows that αSMA expression, which was accompanied by activation of HSC, was decreased by

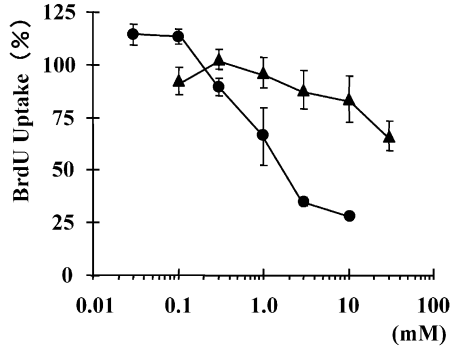

Fig. 3. Effects of L-cysteine and NAC on PDGF-BB-dependent DNA synthesis. Activated HSC was stimulated for 24 h with 25 ng/ml rat recombinant PDGF-BB + 0–10 mM L-cysteine (●) or 0–30 mM NAC (▲) contained in culture medium. DNA synthesis was detected by BrdU incorporation during the final 6 h. Data represent the means ± SD of three different experiments.

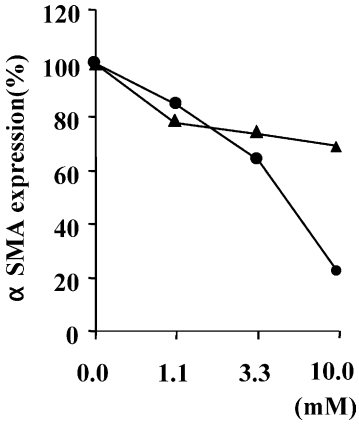

Fig. 4. Effect of L-cysteine and NAC on activation of freshly isolated HSCs. After culturing HSCs isolated from a rat for 24 h, the medium was changed to MEM + 0–10 mM L-cysteine (●) or 0–10 mM NAC (▲). HSCs were cultured for 5 days under the above conditions and αSMA expression by HSCs was measured with Cell ELISA.

L-cysteine and NAC. Especially αSMA expression was significantly inhibited by 10 mM L-cysteine, but not by 10 mM NAC. Since there was no change in the viability of HSCs using MTT assay over the tested L-cysteine concentration range, inhibition of cell proliferation and activation was not caused by death of the cells (data not shown).

Discussion

Therapies for liver fibrosis known to date are considered to reduce HSC activation, remove injurious stimuli, suppress hepatic inflammation, promote matrix degradation, and promote specific apoptosis of HSC [4]. Actually, several compounds that have those effects have been developed as antifibrotic agents; they include interferon-γ [15], interferon-α [16], hepatocyte growth factor [HGF] [17], TJ-9 [18,19], HOE077 [20], some antioxidants [21–23], and so on.

In the present study, we screened amino acids using a DMN-induced rat liver fibrosis model with the purpose of identifying amino acids having antifibrotic ability. As a result, we found that L-cysteine had the ability to decrease the contents of hydroxyproline in the liver and hyaluronic acid in the blood in DMN-treated rats. Others showed that L-cysteine attenuated the increase in hydroxyproline in the liver in a TAA-induced rat liver fibrosis model [24], but the mechanism of reduction of liver fibrosis was unclear. In our study of DMN-treated rats, the body weight gain was suppressed compared with that of untreated rats, but there was no difference between the L-cysteine diet/DMN group and the control diet/DMN group in terms of the mean value of body weight at the end of the experiment (data not shown). It seemed unlikely that these effects of L-cysteine were due to detoxification of DMN.

We also found that L-cysteine inhibits HSC proliferation and activation in vitro (Figs. 3 and 4), and treatment of the experimental animals with L-cysteine suppressed the increase in α-SMA-positive cells (activated HSC) (Fig. 2).

With regard to the mechanism by which L-cysteine blocks proliferation of HSCs, reports published by two groups suggested that NAC blocks the proliferation of activated HSCs in vitro. One report found that NAC caused proteolysis of the extra cellular domain of PDGF receptor β, and NAC might disturb PDGF signal transduction and DNA synthesis in activated HSCs [25]. In that paper, reducing reagents, including L-cysteine, showed the potential to degrade PDGF receptor β the same as NAC. The other paper found that NAC was associated with sustained activation of ERK kinase and induction of p21 expression [26].

L-Cysteine is more effective than NAC in causing cell cycle arrest and reducing of HSC activation (Figs. 3 and 4). These phenomena (L-cysteine > NAC) may be caused by differences in the (1) potential for reactivity with the target molecules, (2) kinetics of uptake into the cell, (3) metabolism to GSH, taurine, or other components, and (4) intra- or extracellular localization. Studying hepatocytes, Mark et al. showed that the initial rate of uptake of NAC was lower than that of L-cysteine using ^{35}S-labeled substrates [27]. In keratinocytes, David et al., showed that NAC exerted the same effect as L-cysteine on intracellular thiol levels [28]. A comparative study of the uptake of L-cysteine and NAC into HSCs is needed.

Antioxidant activity is effective for antifibrogenesis. Oxidative stress is an important stimulus of HSC activation. Also, some antioxidants, e.g., α-tocopherol [21], resveratrol, quercetin, NAC [22], and silymarin [23], suppressed the proliferation of HSC in culture models. In the case of NAC, the administration of NAC reduced hydroxyproline and lipid peroxidation in DMN-induced liver fibrosis through its antioxidant activity [16]. Lipid peroxidation has been associated with liver fibrosis caused by ethanol and hepatitis C virus, etc. [29]. If the main mechanism of antifibrosis by L-cysteine or L-cysteine derivatives is antioxidant activity, oral supplementation with L-cysteine may be an effective medical treatment for liver diseases caused by ethanol and hepatitis C. Interestingly, deactivation of singlet oxygen by L-cysteine is more effective than that by NAC [30].

L-Cysteine is utilized in the synthesis of proteins, nonprotein compounds including taurine, reduced inorganic sulfur, sulfate, and glutathione (GSH). The liver plays a major role in L-cysteine metabolism although L-cysteine is metabolized to some extent by many tissues, and some previous papers reported a relationship between liver diseases and L-cysteine metabolites (GSH and taurine). L-Cysteine is mainly incorporated into GSH in hepatocytes. GSH treatment increased the viability of hepatocytes under toxic conditions [31]. In HSCs, intracellular GSH supported by GSH–ethyl ester had an inhibitory effect on collagen synthesis in vitro [32]. Administration of glutathione in animal models inhibited in vivo TNFα production and showed a beneficial effect in an experimental model of endotoxic shock [33]. Also, taurine has cytoprotective properties [34], and administration of taurine inhibits deposition of extracellular matrix in experimental liver fibrosis in rats [35,36].

Antifibrotic agents are promising candidates for chemoprevention of hepatocelluar carcinoma (HCC) [37]. In order to control HCC caused by hepatitis C, normalization of ALT is beneficial [38,39]. Since administration of L-cysteine inhibited liver fibrosis and lowered plasma AST in rats (Table 1), we hope that dietary supplementation of L-cysteine might serve as a prophylactic or retarding therapy for HCC.

In summary, L-cysteine has an antifibrotic effect by inhibiting the activation and proliferation of HSCs. L-Cysteine administration seems to have potential to contribute significantly to decreasing liver cirrhosis and inhibiting the development of HCC.

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