Alterations of mast cells and TGF-β1 on the silymarin treatment for CCl₄-induced hepatic fibrosis

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Abstract

AIM: Silymarin is a potent antioxidant, antiinflammatory and anti-fibrogenic agent in the liver, which is mediated by alteration of hepatic Kupffer cell function, lipid peroxidation, and collagen production. Especially, in hepatic fibrogenesis, mast cells are expressed in chronic inflammatory conditions, and promote fibroblast growth and stimulate production of the extracellular matrix by hepatic stellate cells.

METHODS: We examined the inhibitory mechanism of silymarin on CCl₄-induced hepatic cirrhosis in rats. At 4, 8, and 12 wk, liver tissues were examined histopathologically for fibrotic changes produced by silymarin treatment.

RESULTS: In the silymarin with CCl₄-treated group, increase of hepatic stellate cells and TGF-β1 production were lower than in the CCl₄-treated group at early stages. Additionally, at the late fibrogenic stage, expressions of TGF-β1 were weaker and especially not expressed in hepatocytes located in peripheral areas. Moreover, the number of mast cell in portal areas gradually increased and was dependent on the fibrogenic stage, but those of CCl₄+silymarin-treated group decreased significantly.

CONCLUSION: Anti-fibrotic and antiinflammatory effects of silymarin were associated with activation of hepatic stellate cells through the expression of TGF-β1 and stabilization of mast cells. These results suggest that silymarin prevent hepatic fibrosis through suppression of inflammation and hypoxia in the hepatic fibrogenesis.

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INTRODUCTION

Silymarin, a standardized extract of the milk thistle (Silybum marianum [L.] Gaertner) has a long tradition as an herbal remedy[1]. The flavonoid silymarin was introduced as a “hepatoprotective” agent a few years ago and is used clinically in Europe and Asia for the treatment of liver diseases[2]. The protective action of silymarin is explicable in terms of its capacity for trapping free radicals and has a stabilizing effect on the cytoplasmic membranes. In experimental animals, this flavonoid has a protective action on the liver, which is particularly vulnerable to poisoning by several hepatotoxic substances such as carbon tetrachloride (CCl₄), thioacetamide, and D-galactosamine[3]. Silymarin is a potent antioxidant that inhibits lipid peroxide formation in the liver cells[4], and possesses antiinflammatory properties mediated by alteration of hepatic Kupffer cell function[5].

Hepatic fibrosis has been noted in chronic liver disease, and is characterized by increased production and deposition of collagen, glycoproteins, and proteoglycans that compose the extracellular matrix (ECM)[6]. Availability of animal models is crucial for the study of liver fibrosis and/or cirrhosis. It is well known that hepatic cirrhosis animal models for chronic liver damage induced by CCl₄ in rats produce liver fibrosis and biochemical and histological patterns that resemble human liver cirrhosis[7]. Thus, the rat model of liver cirrhosis has been useful in studying the effects of hepatoprotective drugs with therapeutic potential to be used in humans[8]. In hepatic fibrogenesis, myofibroblasts (MFBS) such as hepatic stellate cells (HSCs) are the major source of increased ECM[9]. When they are exposed to soluble factors from damaged hepatocytes and from activated Kupffer cells, MFBS will lose Vitamin A and their lipid contents and undergo activation. The activated MFBS migrate and proliferate at the site of liver injury, playing a pivotal role in the formation of fibrous tissue[10]. Among the various cytokines, TGF-β1 plays an important role as a profibrogenic factor in chronic liver disease, triggering the expression of procollagen-I and tissue inhibitor of metalloproteinases-1 (TIMP-1), key effectors of...
fibrogenesis. TGF-β1 is also the most potent mast cell chemo-attractant so far identified and induces mast cell migration at femtomolar (fM) concentrations. Various other cell types, e.g., monocytes, neutrophils, and fibroblasts also migrate towards TGF-β1[10].

Mast cells, which are derived from hematopoietic progenitors, leave the bone marrow and migrate to areas of inflammation. A number of factors responsible for this directional migration and tissue maturation of mast cells have been identified. These include the CXC family of chemokines, stem cell factor (also known as kit-ligand, steel factor, and mast cell growth factor), and TGF-β1[11]. Thus, the activation of mast cells and the subsequent exocytosis of granules are followed by production and secretion of cytokines and other factors that lead to leukocyte infiltration and local inflammation. Mast cell hyperplasia in the liver has also been observed in a variety of experimental models of rat liver fibrosis, such as that induced by CCl₄, diethylnitrosamine, radiation, porcine serum, and bile duct resection[12]. In addition, silymarin acts to stabilize hepatocyte membranes and block receptor binding of various toxins and drugs. Antioxidant activity is also hepatoprotective in vivo and in vitro studies, showing that silymarin has free radical scavenging activity and enhances superoxide dismutase activity in erythrocytes and lymphocytes[13]. Silymarin also protects against glutathione depletion and increases protein synthesis by hepatocytes when there is damage to parenchymatous tissue[14].

In this study, we examined the inhibitory mechanism of silymarin on CCl₄-induced hepatic cirrhosis in rats. The objective of the present study was to observe the alteration of MFBs, TGF-β1, and mast cells histopathologically on the silymarin treatment and to elucidate the correlation between these changes and the antifibrotic effect of silymarin.

**MATERIALS AND METHODS**

**Animals and treatments**

Studies were performed on thirty male Wistar rats weighing 130-150 g. They were housed in a room at 22±2 ℃ and a 12-h light-dark cycle. Feed (PMI Nutrition International, USA) and water were supplied ad libitum.

Hepatic fibrosis was induced experimentally by intraperitoneal injection (IP) of 1.0 mL/kg body weight of 10% CCl₄ (Sigma, USA) dissolved in olive oil (Sigma, USA), three times a week for 12 wk. Two groups of fifteen animals each were used. These groups are schematically shown in Table 1. The first (CCl₄) group received CCl₄, 1.0 mL/kg IP three times a week and 0.25% carboxymethylcellulose (CMC, Sigma, USA), 1.0 mL/kg per oral, 5 d a week for 12 wk. The second (CCl₄+Sily) group received CCl₄, 1.0 mg/kg/IP and a daily oral dose of 50 mg/kg silymarin 5 times a week. Silymarin was given as a suspension in 0.25% CMC. Five rats of each group were sacrificed at wk 4, 8, and 12 respectively.

**Serum biochemical measurements**

Serum was collected and assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using standard enzymatic assay kits. Each assay is a colorimetric assay with detection of a highly colored end product measured at 490-520 nm using autoanalyzer - UV/V is spectrophotometer (Hitachi 736-10, Hitachi, Japan). The absorbance of each end product is proportional to the enzyme’s activity.

**Determination of hepatic hydroxyproline content**

Hydroxyproline (HYP) was determined colorimetrically in duplicates from 0.2 g of liver tissue using a modified method of Jamall et al[15]. Briefly, the frozen tissue was homogenized in 4 mL of 6N HCl and hydrolyzed at 110 ℃ for 16 h. The hydrolysate was filtered, and then 30 μL aliquot of these samples was evaporated under vacuum. The sediment was dissolved in 1.2 mL of isopropanol and incubated with 0.2 mL of 0.84% chloramines-T in acetate-citrate buffer (pH 6.0) for 10 min at room temperature. Then, 1.0 mL of Ehrlich’s reagent was added and the mixture was incubated at 60 ℃ for 25 min. The absorbance of the sample solution was measured at 560 nm wavelength (Hitachi 736-10, Hitachi, Japan). Next, the hydroxyproline content in 100 mg of liver was calculated from the standard curve of 4-hydroxy-L-proline (Sigma, USA) (μg/100 mg liver weight).

**Histopathological analysis**

Liver tissues from each rat were rapidly removed, fixed in 10% neutral-buffered formalin, and processed routinely. Paraffin-embedded sections were cut into 4 μm thick sections. The sections were stained with hematoxylin and eosin (HE) and with special Azan stain, for collagen fibers. In there experiments, the degree of fibrosis in each section of liver was classified as a grade 0-4[16].

**Immunohistochemistry**

Liver sections were deparaffinized in xylene, dehydrated in graded alcohol series, and for the block of endogenous peroxidase, sections were incubated in a solution of 3% hydrogen peroxide (H₂O₂) in methanol for 10 min. Tissue sections were washed with PBS containing 0.03% non-fat milk and 0.01% Tween 20, and then immunostained with primary antibodies for alpha-smooth muscle actin (α-SMA) and TGF-β1. The antigen-antibody complex was visualized by a labeled streptavidin-biotin method using a Histostain-PLUS Bulk Kit (Zymed Laboratories Inc., USA) and followed by diaminobenzidine (DAB) as a chromogen. After washing, slides were counter-stained with Meyer’s hematoxylin and washed with tap water. The primary antibodies used were monoclonal anti α-SMA at a dilution of 1:800 (clone 1A4, Sigma, USA) and polygonal rabbit LCI[30] antibody to mature TGF-β1 (kindly provided by Dr. Seong-Jin Kim) at a dilution of 1:100 respectively. Non-

| Table 1 Experimental designs used in this study |
|-----------------------------------------------|
| **Group ID** | **Number of animal** | **Treatment** | **Sacrificed time** |
| CCl₄ 15 | CCl₄ 1.0 mg/(kg·d), IP 3 times a wk | 4, 8, 12 wk |
| | 0.25% CMC, 1 mL/(kg·d), PO 5 times a wk |
| CCl₄ + Sily 15 | CCl₄ 1.0 mg/(kg·d), IP 3 times a wk | 4, 8, 12 wk |
| | Silymarin, 50 mg/(kg·d), PO 5 times a wk |
immunized goat sera, which were used instead of the primary antibody, served as the negative control.

**Number of mast cells in liver tissue**

Toluidine blue staining for mast cells was performed by immersion of liver sections in 0.1% toluidine blue (Sigma, USA) for 1 min at room temperature. The number of mast cells was quantified in 25 randomly selected, non-overlapping fields and expressed as the number of mast cells/mm².

**Statistical analysis**

All results were expressed as mean and standard deviation (SD). Statistical analysis of the data was done using InStat program (GraphPad Software Inc.). A Mann-Whitney U-test was conducted and the data that were considered to be significantly different were reported at probability levels of P<0.05 or P<0.005, as indicated.

**RESULTS**

**Serum biochemistry**

Serum ALT and AST levels of all CCl₄-tested animals increased in a time-dependent fashion during the whole of experimental period (Figure 1). These changes indicated that liver damage and inflammation were induced successfully. However, in the CCl₄+silymarin treatment group, increases of serum ALT and AST levels were lower than those of the CCl₄ control group. The reduced ALT and AST activities of the silymarin-treated group were 30%, 72% and 33% compared to increases of 8%, 55% and 34% in the CCl₄-treated groups, at 4, 8, and 12 wk.

**Collagen accumulation**

Collagen contents of liver tissue were quantified by determination of the hydroxyproline content (Figure 2). In the CCl₄-treated group, hydroxyproline contents at 4, 8, and 12 wk increased 21.8±3.37, 24.31±0.91, and 43.26±1.41 μg/100 mg liver weight respectively. However, in the CCl₄+silymarin-treated group, the HYP contents at 4, 8, and 12 wk were 18.05±1.89, 20.5±1.92, and 27.5±0.69 μg/100 mg liver weight respectively. At 4, 8, and 12 wk, these HYP contents of CCl₄+silymarin-treated group were reduced by 17%, 16%, and 36% respectively compared to those of control group. Reduced HYP contents of 8 and 12 wk in the CCl₄+silymarin-treated group were statistically significant, P<0.05 and <0.005 respectively.

**Histopathological observations**

During the process of cirrhosis, the grade of hepatic fibrosis changed from grade 0 to grade 4 (Table 2). At 4 wk, centrilobular necrosis and moderate fatty change of liver were found in the CCl₄-only-treated group. In this group, there was collagen accumulation around the blood vessels (Figure 3A, G). At 8 wk, fatty change was rarely observed, but collagen fibers were more abundant in the centrilobular area and neighboring central veins were bridged by fibrous septa. Then, pseudolobuli were formed by thin fibrous septa (Figure 3B, H). At 12 wk, pseudolobuli were formed and macrovesicular lipid droplets were detected. The collagenous septa were much thicker, and pseudolobuli were subdivided into smaller lobuli (Figure 3C, I).

In the silymarin with CCl₄ injection group, at 4 wk, the presence of connective tissue was almost normal around the central veins (Figure 3D, J). At wk 8, moderate to severe fatty change was detected around the periportal area and central vein. A slight accumulation and spread of collagen fibers around central veins was observed too (Figure 3E, K). At wk 12, large lipid accumulation was detected more than...
that of the CCl₄-treated group and pseudolobuli were formed by thin collagenous septa (Figure 3F, L). The presence of collagenous fibers, as demonstrated by Azan stain, was confirmed too.

**Immunohistochemical analysis**

Normal expression of myofibroblasts (MFBs) (e.g., hepatic stellate cells, HSCs, etc.) was identified by α-SMA-positive staining and was limited to the central veins and portal triad. As liver damage, progressed α-SMA-positive cells markedly increased around blood vessels and fibrotic tissue in the CCl₄-only-treated group at wk 4, 8, and 12 (Figure 4A-C). In the silymarin with CCl₄-treated group, at 4 wk, α-SMA-positive cells were slightly detected around central and portal veins, the same as in the control group (Figure 4D), and then at wk 8 increasing α-SMA-positive MFBs exhibited the same pattern of collagen fiber spread from the central veins (Figure 4E). However, the numbers of α-SMA-positive cells in the silymarin with CCl₄-treated group was lower than in the CCl₄-only-treated group at wk 8 (Figure 4B, E).

### Table 2 Histopathological findings of liver tissue in the CCl₄- or CCl₄ with silymarin-treated groups

| Group       | 4 wk          | 8 wk          | 12 wk         |
|-------------|---------------|---------------|---------------|
|             | Grade¹ | Lesion            | Grade | Lesion                        | Grade | Lesion                        |
| CCl₄        | 2      | Mild fibrosis     | 3   | Severe fibrosis                | 4     | Cirrhosis                     |
| CCl₄+Sily    | 0-1    | Mild fatty change | 1-2  | Mild fatty change and fibrosis | 2-3   | Severe fatty change and fibrosis |

¹Fibrotic grade classified by Fujiwara et al., Grade 0: none, Grade 1: short collagen extended from central veins, Grade 2: slender septa link the central veins but lobular architecture is preserved, Grade 3: pseudolobuli are formed thin septa, Grade 4: parenchyma is subdivided into pseudolobuli by thin septa.

**Figure 3** Hepatic fibrosis and accumulation of collagen in only CCl₄- or CCl₄ with silymarin-treated groups. In only CCl₄-treated group - A and G: At 4 wk, collagen (arrowhead) accumulation around the blood vessels; B and H: At 8 wk, collagen fiber (arrowhead) was more abundant in the centrilobular area and neighboring central veins were bridged by fibrous septa; C and I: At 12 wk, pseudolobuli were formed actively and macrovesicular lipid droplets were detected. The collagenous septa (arrowhead) were much thicker, and pseudolobuli were subdivided into smaller lobuli. In CCl₄ with silymarin-treated groups - D and J: At 4 wk, connective tissue was almost normal around the areas of central veins; E and K: At 8 wk, the slight accumulation and spread of collagen fibers around central veins was observed; F and L: At 12 wk, large lipid accumulation was detected more than that of CCl₄-treated group and pseudolobuli were formed by thin collagenous septa. HE stain, A-F; Azan stain, G-L. Original magnification: ×33, A-L.
wk 12, the CCl\(_4\)-treated group developed into a cirrhotic stage, and \(\alpha\)-SMA-positive MFBs were observed mild along to the thick collagenous septa (Figure 4C), compared to 8 wk. This indicated expression of the collagen matrix in the hepatocytes against activation of myofibroblasts, which disappeared at the stage of cirrhosis. However, at 12 wk, MFBs of the CCl\(_4\)+silymarin-treated group showed positive expression of \(\alpha\)-SMA identical to the pattern in fibrosis (Figure 4F).

Recruitment of mast cells in liver tissue

In toluidine blue stained liver sections from the CCl\(_4\)-treated group mast cells were oval in shape with metachromatic granules in portal areas (Figure 5). At 4, 8, and 12 wk, the number of mast cells of the CCl\(_4\)-treated group increased gradually: 12.43±2.38, 18.17±2.12 and 21.8±4.92 cells/mm\(^2\) respectively. However, in the CCl\(_4\)+silymarin-treated group, the number of mast cell were 10±3.33, 12.42±2.35, and 12.17±3.07 cells/mm\(^2\) respectively (Figure 6). These numbers for the CCl\(_4\)+silymarin-treated group decreased significantly by 19%, 32%, and 44% compared to those of CCl\(_4\)-treated group, indicating a silymarin-mediated protective mechanism.
DISCUSSION

Silymarin is well known to be a protective agent against various hepatotoxins, such as acetaminophen, alcohol, carbon tetrachloride, tetrachloromethane, and toluene[17]. Pretreating rats and mice with silymarin before exposure to these chemical hepatotoxins significantly reduced lipid peroxidation and hepatotoxicity[3]. Additionally, the pharmacological effects of silymarin include regulation of cell membrane permeability, leukotriene inhibition, reactive oxygen species scavenging, and suppression of NF-κappaB DNA binding activity[18].

In animal studies, silymarin is as effective as colchicine in reversing hepatic fibrosis due to CCl4-induced damage[8]. A CCl4-induced hepatic cirrhosis rat model has been useful in studying the effects of hepatoprotective drugs with therapeutic potential to be used in humans[8]. In the current study, hepatic fibrosis/cirrhosis was successfully induced by CCl4 injection in rats and favorable results were obtained similar to previous studies. Total hepatic collagen contents determined by hydroxyproline content increased gradually during the experimental period and histopathological findings of fibrosis/cirrhosis were observed in H&E and the Azan-stained section. In the present study, the hepatoprotective effect of silymarin was determined in CCl4-induced liver cirrhosis of rats. In the CCl4 with silymarin-treated group, total hepatic collagen contents were significantly lower than in the CCl4-treated group especially at 8 and 12 wk. Additionally histopathological findings of fibrosis/cirrhosis were revealed a significant reduction in the CCl4-treated group. Similar experiments by Favari et al reported the reduction of lipid peroxidation, Na+, K+, and Ca2+-ATPase levels and increases of collagen content[3,19].

In hepatic fibrogenesis, myofibroblasts (MFBs) are the major source of increased ECM. The activated MFBs migrate and proliferate at the site of liver injury and play a pivotal role in the formation of fibrous tissue. Therefore, activated MFBs are considered the major cellular target to prevent the progression of liver fibrosis during the new drug development[9]. In addition, transforming growth factor beta (TGF-β) is a potent fibrogenic cytokine produced by Kupffer cells and HSCs. There is a prolonged increase of TGF-β1 expression during hepatic fibrosis in CCl4- and diethylnitrosamine-induced models[20] and in patients with cirrhosis induced by alcohol or viral hepatitis[21].

In the current study, as liver damage progressed in the CCl4-only-treated group, α-SMA-positive cells markedly increased around fibrous septa. The number of these cells increased from wk 4 to 8, but slightly decreased along the
thick collagenous septa at 12 wk, developed into the cirrhotic stage. Characteristically, in immunohistochemical analysis for TGF-β1, positive reactions were mainly expressed by HSC and macrophages around the portal region, at early (4 wk) and middle (8 wk) stages of the fibrotic processes, but they were predominantly observed in hepatocytes located in pseudolobules peripherally, at the cirrhosis-occurred stage (12 wk). The mechanism of TGF-β1 expression in hepatocytes has been studied by several researchers, but is still unclear. Furthermore, these alterations of TGF-β1 expression were already reported by our laboratory and we suggested that hypoxia might be associated with fibrogenesis in the liver[22]. However, in the silymarin+CCl4-treated group of our study, there was increase of α-SMA-positive cells such as MFBs, lower than that of CCl4-only-treated group, at 4, 8, and 12 wk. Additionally, expressions of TGF-β1 were weaker than those of CCl4-treated group, during all experimental periods, especially at 12 wk not expressed in hepatocyte located in peripheral areas of pseudolobules. Thus, based on the results of the current study, it is concluded that silymarin has protective effect of proliferation and TGF-β1 production in MFBs.

Fuchs et al[23] reported on the basis of in vitro studies that the potential antifibrotic properties of silymarin might be the inhibition of hepatic stellate cell proliferation and transformation. Jia et al[24] observed that silymarin suppresses expression of profibrogenic procollagen alpha1 (I) and TIMP-1 most likely via down-regulation of TGF-β1 mRNA in rats with biliary fibrosis. However, in vivo studies of the anti-fibrotic activities of silymarin have not yet elucidated histopathologically the preventive mechanism of activation or proliferation of MFBs by silymarin during the CCl4-induced hepatic fibrogenesis. These results suggest that alterations of the numbers of MFBs and TGF-β1 expression in the liver may be involved in the hepatoprotective effects of silymarin observed in other studies. Another study of silymarin explained the antifibrotic action through the effects on TGF-β1 expression[25]. Silymarin has been noted to regenerate cells and enhance RNA synthesis in the rat liver[25].

In liver fibrosis and/or cirrhosis, several studies have reported a relationship between mast cell density, hepatocellular damage, mRNA encoding TGF-β1, hepatic stellate cell activation, and collagen levels[26-28]. Mast cells have been implicated in chronic inflammatory conditions resulting in fibrosis, such as Crohn’s disease, and have been identified in human liver. The number of mast cells are reported to increase in chronic liver diseases associated with fibrosis[22]. Armbrust et al[29] demonstrated that in the late stage of liver fibrogenesis, mast cells may be involved by displaying protease inhibitory activity in the fibrotic septa. In our previous study, the chronic injection of CCl4 induced rat liver cirrhosis concomitant with a marked increase of mast cells[30]. In this study, the number of mast cells in portal areas gradually increased in the fibrogenic stage, but the number of mast cells in the CCl4+silymarin-treated group decreased significantly compared to those of CCl4-treated group. In the study of Fantozzi et al, there was inhibition of neutrophil-mediated histamine release dose-dependently. These results further stress the concept of a neutrophil-mast cell interaction, which may be involved in inflammatory processes[31]. Moreover, mast cells secrete various mediators, which promote fibroblast growth, stimulate production of the extracellular matrix by fibroblasts of hepatic stellate cells, and produce components of the extracellular matrix themselves[32,33]. However, it is unclear whether they play a central role in its development.

The anti-inflammatory effects of silymarin are also based on multiple activities including mast cell stabilization, inhibition of neutrophil migration, Kupffer cell inhibition, inhibition of leukotrienes, and prostaglandin formation. However, results of earlier studies have not histopathologically shown expression of mast cells in fibrotic liver tissue after silymarin treatment. Thus, silymarin has been histopathologically shown to have significant antiinflammatory effect on hepatic tissue, including mast cell stabilization. In addition, it is likely that the hepatoprotective effect of silymarin is related to prevention of hypoxia in hepatic fibrogenesis.

In conclusion, the anti-fibrotic and antiinflammatory effects of silymarin were histopathologically observed in the hepatic fibrogenesis of chronic liver damage induced by CCl4 treatment. Furthermore, these effects were associated with activation of MFBs, expression of TGF-β1, and stabilization of mast cells. These results suggest that silymarin prevents hepatic fibrosis through the suppression of inflammation and hypoxia in CCl4-induced rat liver cirrhosis.

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