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Antifibrotic effects of oriental herbs GLM001 on liver cirrhosis induced by bile duct ligation

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SUMMARY

Liver cirrhosis is characterized by hyperaccumulation of fibrous tissue components and is commonly observed in latter or terminal states of chronic hepatic diseases. In this study, the antifibrotic effects of GLM001 on liver cirrhosis were examined in bile duct ligated rats and patients with hepatic diseases. GLM001 (250 mg/kg rat weight/day) was administrated to cirrhotic rats for 4 weeks and to humans for 14 weeks. Bile duct ligated rats significantly increased liver collagen content and biochemical markers of hepatic injury. Liver histology showed collagen fiber deposition was increased and the normal architecture was lost with large zones of necrosis being observed frequently. GLM001 administrated rats showed significantly decreased liver collagen content, and accumulation of collagen fiber in histological analysis. Patients, who were treated with GLM001, showed decreases in biochemical markers of hepatic diseases. These results demonstrate the usefulness of GLM001 as an antifibrotic agent for liver cirrhosis.

Key words: Liver cirrhosis; Collagen accumulation; Bile duct ligation

Hepatic cirrhosis is an important feature of chronic liver disease. Chronic damages to the liver by a variety of causes frequently result in fibrogenesis, the increased deposition of extracellular matrix (Leveille CR *et al.*, 1967). One of the major extracellular matrix components is collagen (Schuppan D, 1990). Hepatic stellate cells, when transformed into myofibroblasts, are the major source of this newly synthesized collagen (Maher JJ *et al.*, 1990; Bissell DM *et al.*, 1996). In the long run, the pathological accumulation of collagen disrupts the organ's lobular structure and impairs hepatic function. The end stage of fibrogenesis is cirrhosis, a disorder for which no specific treatment is currently available. In spite of the complex regulatory events leading to fibrogenesis, the biosynthesis of collagen is a uniform process, making it a suitable target for therapeutic intervention (Friedman SL, 1993). Since collagen is the major component of the extracellular matrix deposited in hepatic cirrhosis, most antifibrotic therapies have been directed toward the control of collagen metabolism. A number of drugs can specifically modulate collagen biosynthesis at the transcriptional level or at various post-translational stages. These antifibrotic drugs include corticosteroid, azathioprine,

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penicilamine, colchicine, zinc, prostaglandin, cyclosporine, and interferons (Ballardini G *et al.*, 1984; Dickson ER *et al.*, 1985; Neubauer J *et al.*, 1985; Wiesner RM *et al.*, 1987; Beukers R *et al.*, 1992; Rockey DC *et al.*, 1992; Schuppan D, 1995; Peterson TC *et al.*, 1996; Yasuda H *et al.*, 1996; Batta AK *et al.*, 1998; Bickel M *et al.*, 1998; Rodriguez L *et al.*, 1998; Shimizu IY *et al.*, 1999). Many therapeutic strategies have been proposed to treat this disease, but unfortunately, neither the drugs nor the surgical procedures proposed so far have proven to be completely effective.

In this work, we have studied the antifibrotic effect of GLM001 on rats with bile duct ligation induced liver cirrhosis. And, in order to confirm the correlations between animal experiments and human trials clinical study was performed. We showed here that GLM001 administration decreased the accumulation of collagen in cirrhotic rat liver, and decrease the biochemical markers in patients with hepatic diseases.

MATERIALS AND METHODS

Preparation of GLM001

GLM001 was prepared by Green Life World Co., Ltd., seoul, Korea. Main components of GLM001 arebupleurum falcatum 10g, astragalus membranaceus 20g, phellodendron amurense 4g, angelica gigas 8g, atractylodes japonica 8g, paeonia lactiflora 8g, inula helenium 8g, glycyrrhzia uralensis 4g, salvia miltiorrhiza 10g, lingusticum chuanxing 6g, coix lachryma-jobi var 6g, lycium chinense 8g, poria cocos 8g, scutellaria baicalensis 6g. GLM001 was prepared homogeneous powder through heating, disinfecting, and extracting processes of these components.

Animals and induction of liver cirrhosis

Male Sprague-Dawley rats weighing 250-300 g were used in the experiment. The animals had free access to food (SAMTACO) and water. Liver cirrhosis was induced by bile duct ligation and scission (BDL/S). Three groups of rats were used. Control group was sham operated. GLM001 group was bile duct ligated and administered with a dose of 250 mg/kg of GLM001 per rat through an intragastric tube. Administration of GLM001 continued for 4 weeks. Another group (BDL/S) of rats was bile duct ligated but received only saline instead of the GLM001. Each group consisted of twelve rats. Animals were observed daily specially for yellowness due to jaundice on the skins around ear, foot, and tail, and weighed in every 7 day. In 4 weeks rats were weighed before termination, blood sample collected, and weights of liver, kidney and spleen measured.

Clinical trial

Twenty three patients with intermediary hepatic disease were participated in the study (22 males, 1 female: mean age 49.6±10.4 years, range 30-70 years). Most of them admitted to receive treatment for hepatic diseases. Diagnosis of hepatic disease was made using serum enzyme activity. Patients were administrated at a dose of 9,000 mg/day for 14 weeks.

Biochemical analysis of rat serum

After 4 weeks of treatment, the rats were anaesthetized with ether and blood was obtained by cardiac puncture for biochemical analysis. Blood samples were kept at room temperature for 1 hr and centrifuged at 3,000 rpm for 15 min. to obtain sera. Sera were kept at −20°C until use. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities, levels of total-bilirubin and unconjugate-bilirubin were measured by commercial provider's procedures (EMBIEL LTD).

Collagen quantification

Collagen concentrations were determined by measuring hydroxyproline content in liver, spleen, and kidney tissues. Hydroxyproline was measured by a modification of the method of Jamall *et al.* (Jamall IS *et al.*, 1981). Briefly, remaining liver tissue after samples were taken for histology was homogenized in 6N HCl to yield a weight/volume ratio 3%, and then hydrolyzed at 110°C for 16 hr. The hydrolysate was filtered, 25 ul aliquots in triplicate were dried, and the sediment was dissolved in 1.2 ml of 50% isopropanol. Chloroamine-T solution (0.2 ml of 0.84% solution) was added. After 10 min, 1.0 ml of Ehrlich's reagent was added. And the mixture was incubated at 50°C for 90 min. Upon reaching room temperature, the samples were read at 558 nm. The concentration of hydroxyproline in

each sample was determined from a standard curve generated from known quantities of hydroxyproline which had been hydrolyzed as described above.

Histological analysis

For morphometric studies, three liver fragments (>1 cm^2 each) were randomly taken from the right, median and left lobe of each rat liver. Liver fragments were fixed in 10% buffered paraformaldehyde solution and embedded in paraffin. Liver tissues were stained with H&E (hematoxylin and eosin) and trichrome immunological staining method.

Statistical analysis

All values, expressed as the mean±S.D., were statistically analyzed through analysis of student t-test. The p value less than 0.05 was considered significant.

RESULTS

Observation of cirrhotic rats

The progression of jaundice, evaluated by observation of rat skin of ear, foot, and tail. The GLM001 group was improved in jaundice and appeared lighter in color than the BDL/S group. Hepatomegaly and splenomegaly are observed in BDL/S group. Micronodules are observed in liver surface of BDL/S group (Fig 1a). Rats of control group showed a consistent increase of body weight, and animals of GLM001 and BDL/S groups either showed slight increase or decrease during 4-weeks of observation. Liver weight increased significantly ($p<0.05$) in GLM001 and BDL/S groups than in the control group, however, the ratio between liver and body weights of GLM001 group was significantly lower than that of BDL/S group ($p<0.05$) (Table 1). Spleen weights were lowest in the control group, increased significantly in BDL/S group. For kidney weights there was little difference between the control group and GLM001 group.

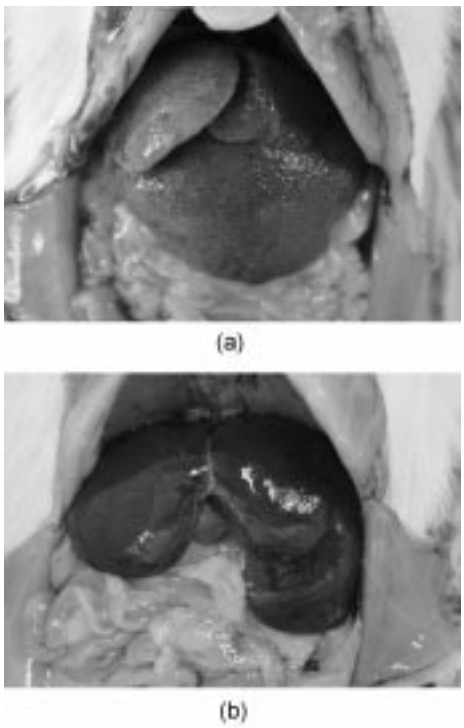

Fig. 1. Livers of a untreated BDL/S rat(a) and a GLM001 treated BDL/S rat(b). In the untreated BDL/S rat the liver became enlarged, and looks rough due to nodule developments throughout the surface. In GLM001 rat, smooth surface of liver, comparing to untreated BDL/S rats mildly hydropsed bile duct indicated by an arrow, and slightly adhesive gastrointestinal tracts are shown.

Biochemical analysis on liver function

To analyze liver function of rats, enzyme activities in serum samples were determined. Activities of ALT, AST, ALP, and the total bilirubin and unconjugated

Table 1. Organ weights and ratios of organ/body weight in the rats operated by BDL/S

	Liver (g)	Kidney (g)	Spleen (g)
Sham	15.14±1.01 (3.41±0.12%)	3.41±0.19 (0.782±0.034%)	1.222±0.29 (0.278±0.007%)
BDL/S	29.16±3.28[a] (7.02±0.57%)	4.32±0.23[a] (1.036±0.051%)	3.57±0.46[a] (0.87±0.148%)
GLM001	19.83±2.83[a,b] (4.97±0.79%)	3.538±0.20[b] (0.904±0.133%)	1.92±0.68[a,b] (0.492±0.169%)

The data show M±SD, [a]$P<0.05$: significantly different from sham operated group (control), [b]$P<0.05$: significantly different from BDL/S group, and ( ): the ratio of organ weight/body weight.

Table 2. The value of biochemical serum activity of Sham, BDL/S, and GLM001 group

	Sham	BDL/S	GLM001
ALT	38.87±0.92	58.97±5.17	45.09±2.67
AST	58.67±4.57	90.23±7.30	70.10±3.08
ALP	32.42±2.39	86.73±8.0 [a]8	58.16±6.99[a]
T-bilirubin	1.49±0.35	7.390±.5[a],2[a]	3.79±1.02[a, b]
unconjugated bilirubin	0.34±0.11	2.58±0.45[a, b]	1.02±0.24[a, b]

The data show M±SD, [a]*P*<0.05: significantly different from sham operated group (control), and [b]*P*<0.05: significantly different from BDL/S group.

bilirubin were detected higher in the BDL/S groups than the control group as in Table 2 ($p<0.05$). And, these biochemical analysis data were lower in the GLM001 group than in the BDL/S group as in Table 2.

Patients were administered with GLM001 for about 81 days and diagnosed on the basis of AST, and ALT test results in every 10 days period of administration. Among 23 treated patients 13, which is 56.52% of total number of patients, showed decreases in AST and ALT activities, where as no change in the other 10 patients. The decreased levels of AST and ALT activities were approximately 31.37% and 43.42% respectively in 13 GLM001 effective patients who showed less variation; the AST activity was 70.48±34.53 IU/L before the treatment, and decreased to 47.8327.04 IU/L after the treatment; the ALT activity was 117.15±30.27 IU/L before the treatment and reduced to 62.38±15.06 IU/L at the end of treatment (Fig 2).

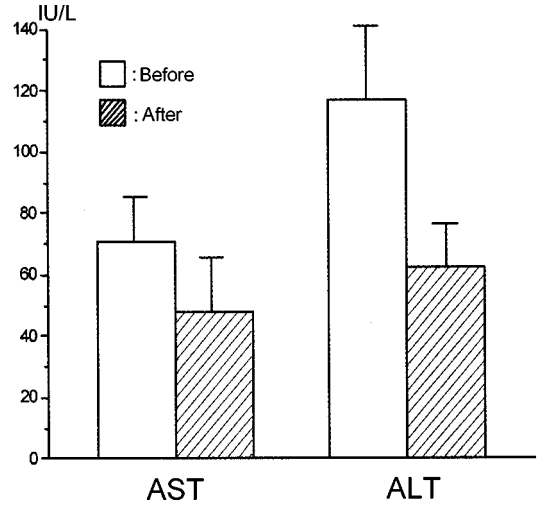

Fig. 2. Decreased rates of examined test results after GLM001 treatment on 13 patients with hepatic diseases. Aspartate transaminase activity and alanine transaminase activity were detected as described in materials and methods.

Determination of the total collagen content in the liver, kidney, and spleen

Collagen qunantity was determined by measuring hydroxyproline content in liver, spleen, and kidney tissues. Hydroxyproline content was appeared as significantly higher in liver tissues of the GLM001 and the BDL/S rats comparing to those of rats in the control group ($p<0.05$). In the GLM001 group hydroxyproline content in liver tissue was lower than in the BDL/S group by approximately 41% as in Table 3 ($p<0.05$). And, in the GLM001 group hydroxyproline contents in kidney and spleen tissues were lower than in the BDL/S group.

Histopathological studies on cirrhotic liver

To study the effect of GLM001 on liver cirrhosis, liver tissues were stained with Hematoxylin-Eosin and trichrome. Histological analysis of liver sections revealed that prolonged biliary obstruction is

Table 3. Hydroxyproline content in organ tissues of Sham, BDL/S, and GLM001 group

	Liver (ug/0.1g)	Kidney (ug/0.1g)	Spleen (ug/0.1g)
Sham	51.54±5.65	78.24±9.48	104.08±11.65
BDL/S	121.57±13.15[a]	152.73±16.78[a]	237.10±27.34[a]
GLM001	72.15±16.25[a, b]	93.52±15.6[a, b]	148.86±19.23[a, b]

The data show M±SD, [a]*P*<0.05: significantly different from sham operated group (control), and [b]*P*<0.05: significantly different from BDL/S group.

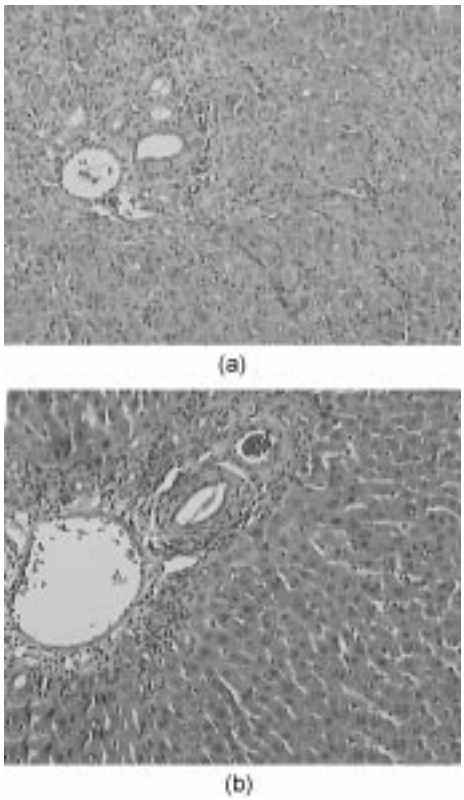

Fig. 3. H&E stained liver sections of cirrhotic rats (a: BDL/S, b: GLM001). In the BDL/S group, the normal architecture was lost, and large zones of necrosis and newly proliferating bile ducts were observed. GLM001 group restored the normal architecture of the liver.

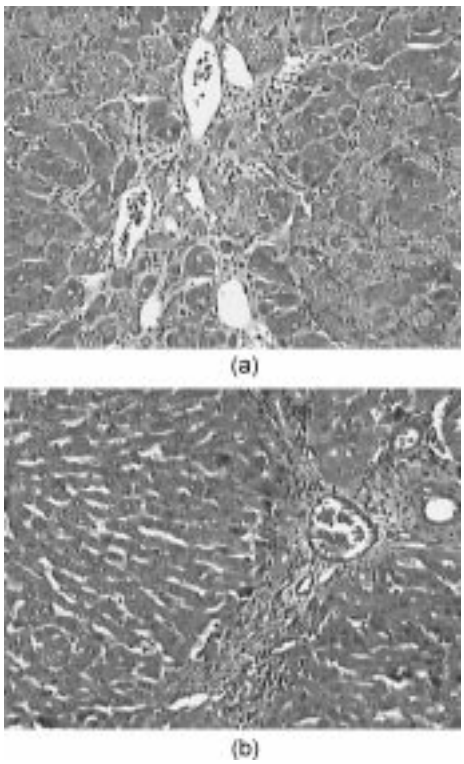

Fig. 4. Trichrome stained liver sections of cirrhotic rats (a: BDL/S, b: GLM001). Stained collagen fibers were built up around portal triad and newly proliferating bile ducts in BDL/S group. In contrast to BDL/S rats collagen development is confined in the limited area at the liver section of a GLM001 treated rat in b.

accompanied by an increase in collagen deposition around the portal triad. In the BDL/S group, the normal architecture was lost, and large zones of necrosis were observed frequently (Figs 3a, and 4a). GLM001 administration to BDL rats restored the normal architecture of the liver, although slight collagen accumulation and necrosis were also observed (Figs 3b, and 4b).

DISCUSSION

The most prevailing causes of hepatic cirrhosis are excessive consumption of alcohol and virus induced chronic hepatic diseases. Regardless of causes, extracellular matrix deposition is a constant feature in liver cirrhosis. Proteolytic enzymes are thought to play a primary role for degradation of the connective tissue generated in process such as fibrosis (Kountaras J *et al.*, 1984; Rodriguez-Fragoso L *et al.*, 1995). Different enzymatic systems may contribute to the overall process of extracellular matrix degradation, including plasminogen activators, matrix metalloproteinases (i.e. collagenase), and it is likely that these enzymatic systems can act either independently or in a concerted manner (Friedman SL *et al.*, 1990; Vassalli JD *et al.*, 1991; Vassalli JD *et al.*, 1994). Hepatic fibrogenesis ultimately results in cirrhosis, a condition marked by diffuse lobular fibrosis and conversion of normal liver architecture into structurally abnormal nodules. One of approaches for treating liver cirrhosis is to inhibit the progression of collagen synthesis, and to reverse the collagen production process to remove the excessively produced extracellular matrix in liver tissues.

In order to investigate the antifibrotic potential of a given agent, selection of appropriate animal models that most closely reproduce human chronic liver diseases is important. In some cases, there

were different effects among several experimental hepatic fibrosis models. For example, interferon-prevented the development of liver fibrosis in carbon tetrachloride model but not in the biliary model, and biphenyl dicarboxylate had less effect on fibrotic rats induced by bile duct ligation and scission, but significant effects on chemically-induced liver injury (Fort JC *et al*, 1998; Kim SM *et al.*, 1999; Nan JX *et al.*, 2000). The common human chronic liver diseases are often characterized by moderate or even absent inflammation and necrosis in spite of progression to cirrhosis. However, carbon tetrachloride model is induced by free radicals with severe inflammation and necrosis. In contrast, bile duct ligation and scission has been used to produce a reliable experimental model due to high yield of liver fibrosis, low mortality and morphological compatibility to that of human etc (Kountouras J *et al.*, 1984). Especially, this obstructive model may prove to be a useful tool for studying human cholestasis. It is also a good model for evaluating drugs that are beneficial to the liver (Muriel P *et al.*, 1994). Therefore, we chose bile duct ligation and scission in rats as a model of liver cirrhosis in this work.

This study was an investigation of natural substances that regulate synthesis and accumulation of collagen in liver tissue. Our results demonstrate that GLM001 administration could reverse liver fibrosis and liver damage induced by biliary obstruction in rats. GLM001 reduced the degree of hepatocellular injury as determined by lower serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and morphological aspects of the fibrotic liver. Since collagen is the major component of the extracellular matrix deposited in hepatic cirrhosis, most antifibrotic therapies have been directed toward the control of collagen metabolism. We also found that GLM001 inhibited the collagen deposition in liver by administrating a dose of 250 mg / kg body weight / day. And from the histological appearances, GLM001 reduced accumulation of collagen fiber around the portal triad. In human patients the drug also showed a decreased level of enzyme activities in serum suggesting that the drug functions for the liver disorders both in rats and humans, and the animal model used in this experiment is suitable for determining hepatic pathogenesis and its therapy.

GLM001 showed antifibrotic effects of reducing hepatocellular damage and of restorating liver homeostasis in bile duct ligated rats. And administration of GLM001 decreased biochemical markers in patients with hepatic diseases. However, antifibrotic effects of GLM001 can not be clearly explained with these data but GLM001 may be useful for the treatment of liver cirrhosis. The mechanism by which GLM001 inhibits the collagen deposition on cirrhotic liver is still not clear, and more investigations in the field of toxicology and pharmacology are required.

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