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ORIGINAL ARTICLE

BENEFICIAL EFFECT OF 4-METHYLUMBELLIFERONE AGAINST BILE DUCT LIGATION-INDUCED HEPATIC FIBROSIS IN RATS

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Abstract Background & Aims: Liver fibrosis progresses to cirrhosis as the consequence of chronic liver injury. 4-methylumbelliferone (4-MU), a derivative of coumarin, has been used as an approved drug to treat biliary spasm. Recent publications have reported that 4-MU inhibits production of hyaluronan (HA), which is a key component of extracellular matrix deposition in fibrotic and cirrhotic liver. We investigated the effect of 4-MU on fibrotic liver in rats.

Methods: Liver fibrosis was induced by bile duct ligation (BDL). Male Sprague-Dawley rats received a standard diet or the same diet containing 1% or 5% of 4-MU from 1 week before BDL. The rats were sacrificed at 3 weeks after BDL.

Results: Administration of 4-MU increased serum 4-MU concentration levels, thereby decreasing serum HA levels in a dose-dependent manner. 4-MU treatment suppressed liver fibrosis in interlobular and pericentral areas with reduced alpha-smooth muscle actin expression, which suggested hepatic stellate cell activation.

Conclusion: Treatment with 4-MU suppressed the experimental hepatic fibrosis accompanied by inhibition of HA production. Although further experimental studies are needed, 4-MU could protect against fibrogenesis and may be repurposed as a safe therapeutic application for hepatic fibrosis.

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Key words: liver fibrosis; hyaluronan; 4-methylumbelliferone; alpha-smooth muscle actin; hepatic stellate cell.

Introduction

Liver fibrosis leading to cirrhosis is one of the major health care burdens worldwide. The causes of liver fibrosis are multiple including hepatitis B and C virus infection, toxins, non-alcoholic steatohepatitis, and congenital liver disease[1)]. Regardless of the type of liver injury, chronic liver injury results in hepatocyte damage, and subsequent activation of hepatic stellate cells (HSCs) with increased production of extracellular matrix (ECM)[2)]. ECM provides the mechanical scaffolding for the normal and fibrotic liver. The components of hepatic ECM include collagens, glycoproteins, glycosaminoglycans, matrix-bound growth factors and cytokines, proteoglycans, and matricellular protein[3)]. In addition to providing a scaffold, ECM regulates cellular functions and protease activities[3, 4)]. In the liver, HSCs are resident perisinusoidal cells, and are the primary source of ECM in the normal and fibrotic liver[4)].

Hyaluronan (HA), a nonsulfated linear glycosaminoglycan, presents in many tissues as a major constituent of the ECM[5, 6)]. HA synthesis is mediated through HA synthase (HAS), which assembles UDP-glucuronic acid and UDP-N-glucosamine at the plasma membrane, and synthesized HA is secreted into the extracellular space[7)]. Due to its physiochemical properties, HA is very hydrated, providing the ECM with an

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ideal environment in which cells can move and proliferate[8, 9]. Thus, HA plays important roles in development, morphogenesis, tumorigenesis, migration, apoptosis, cell survival, and inflammation[10-13]. The liver is the primary organ responsible for both synthesis and degradation of HA[14]. HA is degraded by hepatic sinusoidal endothelial cells in a specific receptor-mediated process, and it has been found to be elevated in patients with liver fibrosis and cirrhosis because of sinusoidal capillarization[15]. Therefore, serum HA levels have been used clinically to assess the staging of liver fibrosis[16, 17]. In addition, liver fibrosis is known as a major risk factor for the development of hepatocellular carcinoma (HCC)[18]. Previous studies have showed that high serum HA levels are a poor prognostic factor in patients with HCC after hepatic resection or radiofrequency ablation[19, 20].

4-methylumbelliferone (4-MU), a coumarin derivative, inhibits HA synthesis through the suppression of gene expression and post-translational modification of HAS enzymes[21, 22]. 4-MU had been used to treat the functional and obstructive spasms of the biliary tract in clinical practice. Recent studies have reported that 4-MU has other beneficial properties such as anti-tumor, anti-inflammatory, and anti-angiogenic effects in experimental research[23]. Further, 4-MU has an anti-fibrogenic effect in the kidneys through the inhibition of HA synthesis[24]. Based on these profound phenomena, we hypothesized that 4-MU suppresses liver fibrosis by inhibiting HA synthesis. Therefore, this study was conducted to evaluate the effects of 4-MU on liver fibrosis in a bile duct-ligated rat model.

Materials and Methods

Animals and reagents

Male six-week-old Sprague-Dawley rats were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). They were housed in cages in a temperature- and humidity-controlled room with a 12-hour light/dark cycle and given free access to water and diet. All animals received humane care, and the experiment was performed in accordance with Hirosaki University's Guidelines for Animal Experimentation. 4-MU was purchased from Sigma-Aldrich (St. Louis, MO, USA). The diet containing 4-MU (1% or 5%) was pelleted by Oriental Yeast Co., Ltd. (Tokyo, Japan).

Animal treatments

Liver fibrosis was induced by bile duct ligation (BDL). In the BDL model, the surgical procedure was performed under isoflurane anesthesia, as previously described[25]. Briefly, the common extrahepatic bile duct was exposed after midsection laparotomy, and the bile duct was double ligated with 3-0 silk sutures and dissected. In the sham-operated rats, the bile duct was exposed but not ligated.

Rats were divided into four groups: (1) sham operation (n=4), (2) BDL alone (n=4), (3) BDL + 1% 4-MU (n=5), and (4) BDL + 5% 4-MU (n=5). The sham operation group and BDL alone group consisted of sham-operated rats and BDL rats given a control diet, respectively. In the BDL + 1% 4-MU group and BDL + 5% 4-MU group, the rats received a diet containing 1% 4-MU and 5% 4-MU, respectively. Rats were fed the control diet or diet containing 4-MU starting 1 week before BDL, and they were sacrificed 3 weeks after BDL or sham operation. Liver and blood samples were collected for analysis.

Measurement of serum 4-MU concentration

The concentration of serum 4-MU was assayed by using high-performance liquid chromatography (HPLC)[26]. Briefly, the internal standard (30 μl of a 50 μg/ml solution of umbelliferone in methanol) was added to 100 μl of a whole blood sample. After mixing the blood sample with 1.0 ml of ethyl acetate, the mixture was centrifuged at 2,000 *g* for 10 min. The ethyl acetate layer was removed, evaporated

to dryness under air, and reconstituted in 500 μl of methanol. The methanol solution was centrifuged at 4000 *g* for 10 min and 200 μl of injection sample was obtained. The HPLC system consisted of an L-6200 liquid chromatograph, L-7200 autosampler, F-1050 fluorescence spectrophotometer, and D-7500 integrator (Hitachi Co., Tokyo, Japan). Reverse phase chromatography was carried out on a TSK-GEL ODS-120T column (0.46 cm × 25.0 cm; Tosoh Co., Tokyo, Japan) with a mobile phase of 40% methanol in water at a flow rate of 0.5 ml/min. The column temperature for this HPLC procedure was 35°C, and detection was performed at excitation and emission wavelengths of 350 and 450 nm, respectively.

Serum biochemical analysis

Serum HA was measured by ELISA (R&D Systems, Inc., Minneapolis, MN, USA). Serum total bilirubin (T.Bil), alkaline phosphatase (ALP), and alanine transaminase (ALT) were measured by an automated analyzer (Spotchem EZ SP-4430, Arkrey Inc., Kyoto, Japan). Serum Type Ⅳ collagen 7S (Ⅳ-c-7S) was determined by RIA2 antibody assay.

Histological and immunohistochemical analysis

The right biggest lobe was removed and kept in 10% formaldehyde neutral buffer solution. Liver sections were stained with hematoxylin and eosin (H&E) or Azan for the observation of inflammation and fibrosis. Immunohistochemical staining of alpha-smooth muscle actin (α-SMA) was performed using the standard avidin-biotin-peroxidase complex (ABC) method. Heat-induced antigen retrieval was performed. Sections were incubated with primary anti-α-SMA antibody (Dako, Denmark) and biotinylated secondary antibody, followed by the avidin–biotin–peroxidase complex. The immunoreactive signal was developed by the peroxidase substrates, 3,3-diaminobenzidine tetrahydrochloride.

Western blotting

Each liver tissue was homogenized with 500 μl of ice-cold 25mM Tris-Cl buffer (pH 7.5) containing a protease inhibitor cocktail tablet (1 protease inhibitor cocktail tablet per 10 ml of buffer, Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 14,000 g 4℃ for 10 minutes, and the supernatants were collected. The protein concentration was determined by the method of bicinchoninic acid assay. The 20-μg protein was electrophoresed on 4-15% gradient sodium dodecyl sulfate (SDS) polyacrylamide gels, transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking, the membranes were incubated for 1 hour at room temperature with anti-α-SMA monoclonal antibody (Sigma-Aldrich). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody and developed using Clarity ECL Western Blotting Substrate (Bio-Rad). The density of bands was analyzed by a scanning densitometer. Each signal intensity was corrected by the values obtained from the immunodetection of anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (abcam, Cambridge, MA, USA). The relative intensity of bands was expressed as the content in the sham group.

Statistical analysis

Quantitative values are expressed as means ± standard deviation (SD). Statistical evaluations were performed using two-tailed Student's t-test. Differences were considered to indicate a significant result with p-values <0.05.

Results

Effect on organ weight and gross appearance

The body weights of BDL animals fed with the control diet or diet containing 4-MU were significantly lower than the body weight of the sham-operated rats (Table 1). Moreover,

Table 1 Body weight and relative weight of liver and spleen after treatment

	Sham (N=4)	BDL (N=4)	1% 4-MU (N=5)	5% 4-MU (N=5)
Body weight (g)	392.7±16.8	330.8±14.3*	334.4±25.0	214.1±9.2*
Liver/body weight (%)	3.81±0.07	7.47±0.43*	7.92±0.57*	7.84±0.22*
Spleen/body weight (%)	0.19±0.01	0.86±0.10*	0.88±0.05*	0.71±0.14*

*P<0.05 versus Sham group

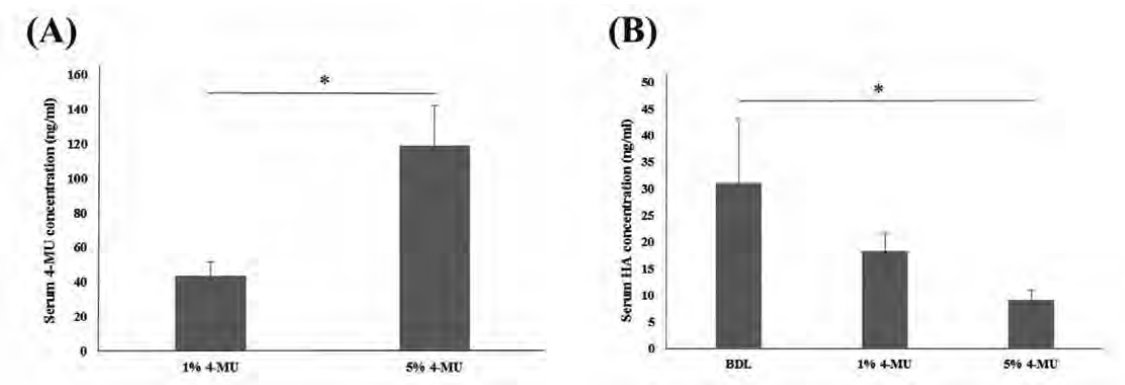

Figure 1 (A) Oral administration of 4-MU increased serum 4-MU concentration levels in BDL rats (*P<0.05). (B) Serum HA concentration levels decreased in a dose-dependent manner (*P<0.05).

the body weight in the 5% 4-MU group was the smallest among BDL animals (Table 1). The weights of the livers and spleens of BDL animals were significantly higher than these of the sham-operated rats (Table 1). Though the spleen weight in the 5% 4-MU group was slightly less, it was not significantly different from that in the BDL group (Table 1).

Serum 4-MU and HA concentrations

Oral administration of 4-MU increased serum 4-MU concentration levels in BDL rats (1% 4-MU group: 43.3 ± 8.0 ng/ml, 5% 4-MU group: 119.0 ± 23.4 ng/ml). Accordingly, serum HA concentration levels decreased in a dose-dependent manner (BDL group: 30.9 ± 12.1 μg/ml, 1% 4-MU group: 18.3 ± 3.5 μg/ml, 5% 4-MU group: 9.0 ± 1.9 μg/ml) (Fig. 1).

Effects on serum biochemistry

Serum levels of T.Bil, ALP, ALT, and Ⅳ-c-7S in the BDL group were higher than those in the sham group. Although serum levels of T.bil, ALP, and Ⅳ-c-7S in the 5% 4-MU group were less, there were no statistically significant differences between the BDL group and the 5% 4-MU group (Fig. 2).

Effect on hepatic fibrosis

Hematoxylin and eosin staining confirmed diffuse severe bile duct proliferation, inflammation, and necrosis in BDL rats. There were no significant histological differences in these findings between the BDL group and 4-MU groups (data not shown). In addition, liver fibrosis was assessed by Azan staining, which showed that the 4-MU groups had less bridging formation of fibrosis than the BDL group (Fig. 3).

Effects on myofibroblast activation

Hepatic fibrogenic reactions involve α-SMA-positive portal myofibroblasts and activated hepatic stellate cells. Immunohistochemical staining showed an enhancement of α-SMA-positive signals mainly in portal and periportal areas spreading to interlobular and pericentral areas in BDL rats. In the 5% 4-MU group,

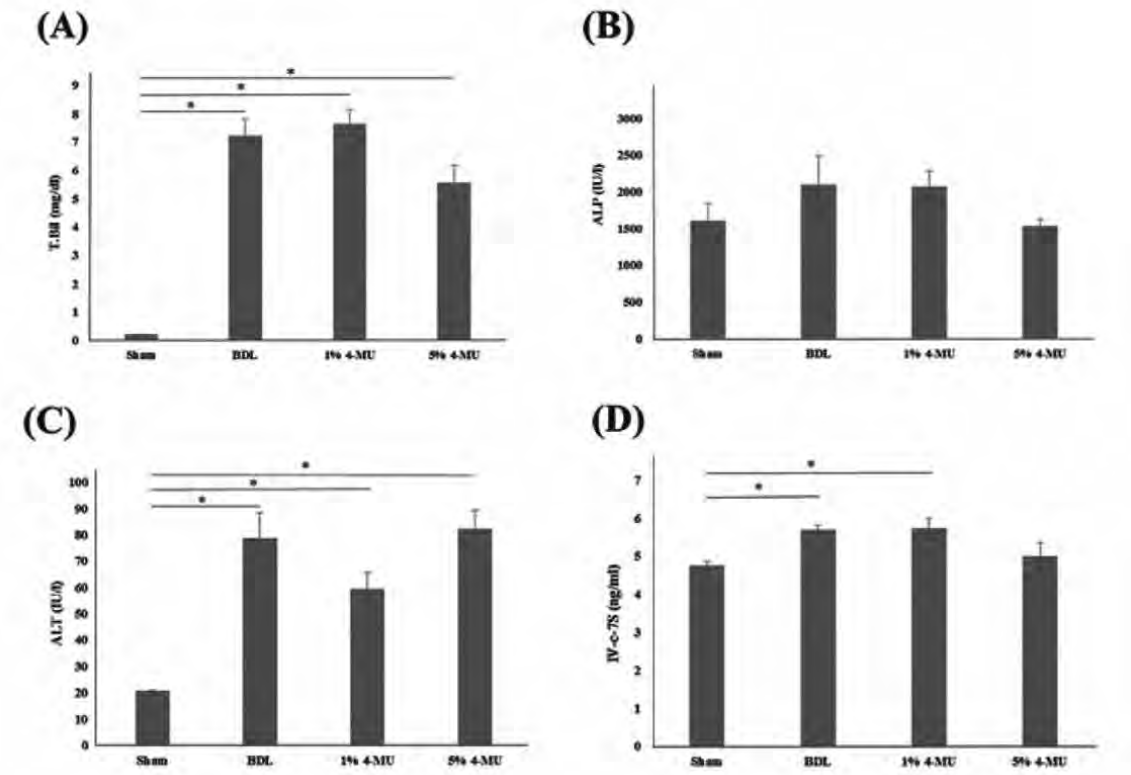

Figure 2 (A) Serum levels of T.Bil in the BDL group were higher than those in the sham group (*P<0.05). (B) There was no statistical difference in serum levels of ALP between any groups. (C) Serum levels of ALT in the BDL group were higher than those in the sham group (*P<0.05). (D) Serum levels of Ⅳ-c-7S in the BDL group and 1% 4-MU group were higher than those in the sham group (*P<0.05).

α-SMA-positive signals were diminished in interlobular and pericentral areas (Fig. 4A). Liver fibrosis varied widely in severity even in the BDL group; thus, quantitative analysis with an image processing program was not performed. Western blot analysis showed that expression levels of α-SMA protein were similar between the BDL group and the 4-MU groups (Fig. 4B).

Discussion

HA is a major component of ECM in liver fibrosis, and serum HA levels are elevated during the progression of liver fibrosis. In this study, we have shown that administration of 4-MU, a HA synthesis inhibitor, reduces serum HA levels with the increment of serum 4-MU concentration, and suppresses liver fibrosis in interlobular and pericentral areas in a rat BDL model.

4-MU has been used for the treatment of biliary spasm. In this study, oral administration of 4-MU increased serum 4-MU concentration levels in BDL rats. In the BDL model, bile acids do not drain into the intestine due to bile duct obstruction. Although bile acids influence gastrointestinal absorption of many compounds, our data confirm that 4-MU was absorbed regardless of the absence of bile acids (Fig. 1A). In addition, 4-MU suppressed serum HA concentration levels in a dose-dependent manner (Fig. 1B). On the other hand, body weight in rats given a high dose of 4-MU was much lower than that of control diet-fed BDL rats (Table 1). However, we cannot explain why

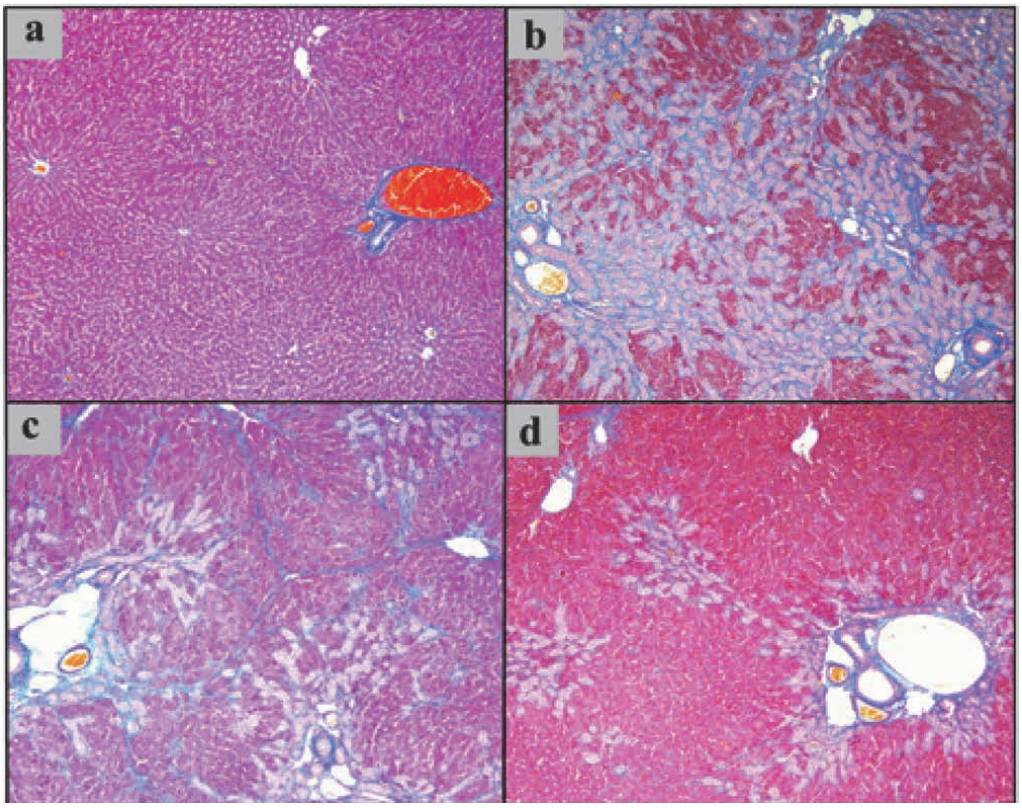

Figure 3 Azan staining showed fibrosis of the liver only around the blood vessels in sham-operated rats (a). The 1% 4-MU group (c) and the 5% 4-MU group (d) had less bridging formation of fibrosis than the BDL group (b).

4-MU administration affects body weight, since the effect of 4-MU on organ development and growth is unclear at this point. Biliary fibrosis develops from the periportal area through the interlobular and pericentral areas, accumulating ECM around proliferating bile duct structures in cholestatic liver. In our study, although fibrosis in the periportal area was similar between control diet-fed BDL rats and BDL rats fed a diet containing 4-MU, fibrosis in interlobular and pericentral areas was milder in the rats fed 4-MU compared with rats given the control diet (Fig. 3). While the progression of liver fibrosis in the periportal area is affected preferentially by bile duct proliferation in the BDL model, fibrosis in interlobular and pericentral areas is insulated from the influence of bile duct proliferation. Therefore, in the BDL model, evaluation in the interlobular and pericentral areas is suitable for assessing the anti-fibrogenic effect of 4-MU. Blood liver function test and histology findings were not improved by 4-MU in our experiment. For that reason, though 4-MU has been used for the treatment of functional and obstructive spasms of the biliary tract, 4-MU also has a choleretic action. Thus, it is possible that 4-MU as well as ursodeoxycholic acid may be harmful in obstructive cholestasis by increasing biliary pressure and liver necrosis in the BDL model[27]. In addition, a recent study showed that histological analysis of liver fibrosis in the BDL model represents heterogeneity and varies widely in severity of fibrosis[28]. Therefore, to elucidate the effect of 4-MU against liver fibrosis in detail, other animal models such as a model of parenchymal fibrosis induced by carbon tetrachloride injury may be required. Additionally, other quantifying methods like

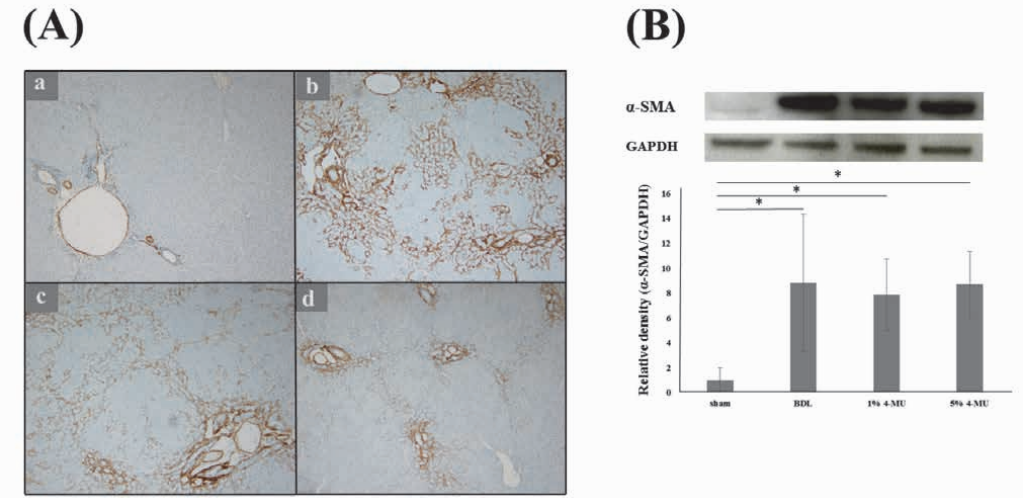

Figure 4 (A) Immunohistochemical staining showed enhancement of α-SMA-positive signals mainly in the portal and periportal areas spreading to the interlobular and pericentral areas in BDL rats (b). In the 1% 4-MU group (c) and the 5% 4-MU group (d), α-SMA-positive signals were diminished in the interlobular and pericentral areas in a dose-dependent manner. In the sham group (a), α-SMA-positive signals were rarely observed. (B) Western blot analysis revealed that expression levels of α-SMA protein in the BDL group were higher than those in the sham group (*P<0.05). Those for α-SMA protein were similar between the BDL group and 4-MU groups.

magnetic resonance imaging (MRI) are needed to evaluate liver fibrosis to avoid sampling error[28].

The hepatic stellate cell (HSC) is a key player in fibrogenesis in the liver. Activated HSCs, which express α-SMA, secrete ECM such as HA and collagen. Piccioni et al. reported that 4-MU suppressed their proliferation and induced apoptosis in human and rat HSC lines[29]. Therefore, it is speculated that 4-MU inhibits hepatic fibrogenesis through HSC inactivation. In our experiments, 4-MU decreased α-SMA expression in the interlobular and pericentral areas in BDL rats (Fig. 4A). On the other hand, the portal fibroblast (PF) is distinct from HSC, and the PF plays an important role in fibrogenesis, more so than HSC, in biliary fibrosis induced by BDL[30]. However, it has not yet been elucidated how 4-MU affects ECM secretion from PFs; thus, there is a possibility that 4-MU has a different effect on HSCs and PFs.

Taken together, we conclude that 4-MU administered orally is absorbed even in cholestatic liver disease, and it may suppress liver fibrosis. Although further studies are needed, 4-MU has been used in clinical practice safely; it might serve as an anti-fibrotic agent for chronic liver disease.

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