be a promising simple tumor marker for the diagnosis of early HCC. In addition, a combination serum HCC diagnostic test that included AFP, DCP, and the C3a fragment had higher sensitivity than each individual marker. These results suggest that this combination test may be a useful HCC screening method, although the low specificity may pose challenges. Further examinations are needed to determine whether the C3a fragment or a combination test can be used to detect early HCC.

The results of our study demonstrated that the C3a fragment (8.1 k m/z) is a highly expressed novel tumor marker that is abundant in the sera of early HCC patients but not in the sera of healthy volunteers or HCV-CLD patients. A similar study by Lee et al. [17] used the ProteinChip SELDI system to show that C3a is a potential candidate biomarker for HCV-HCC. However, Lee et al. found that the molecular weight of C3a was represented by an approximately 8.9 k m/z peak. C3a has a very short half-life and is immediately cleaved into the more stable C3adesArg (8.9 k m/z), which is the anaphylatoxin C3a that lacks the C-terminal arginine and is stable state in the serum [23]. In our study, the 8.9 k m/z peak was also significantly different among HCV-HCC patients, HCV-CLD patients, and healthy volunteers (Fig. 3c, d). However, the discriminatory power of the 8.9 k m/z peak (ROC AUC was 0.60) was lower than the 8.1 k m/z peak (ROC AUC was 0.70) to distinguish between HCV-HCC and HCV-CLD. In addition, unlike the 8.1 k m/z peak, the levels of the 8.9 k m/z peak did not significantly increase with time as HCC progressed in 10 HCV-HCC cases (Fig. 4b). In contrast, Li et al. identified two proteins (8926 m/z and 8116 m/z) as complement component C3adesArg and a C-terminal truncated form of C3adesArg; the latter was a C-terminal truncation of C3adesArg that lacked the C-terminal sequence RASHLGLA (referred to as C3adesArgΔ8) in breast cancer patients [24]. However, these two biomarkers cannot be used to discriminate between breast cancers and benign tumors, and there were minimal differences in the peak intensities between breast cancer patients and healthy controls. Therefore, the C3a fragment with a molecular weight of 8.1 k m/z appears to be a potential diagnostic marker for HCC, although we cannot explain why the 8.1 k m/z fragment of C3a is overexpressed in HCC patients and did not confirm whether our C3a fragment (8.1 k m/z) is C3adesArgΔ8.

C3a, including C3adesArg, was also previously identified as a tumor marker for lymphoid malignancies, breast and colorectal cancers using the ProteinChip SELDI system [24–26]. Complement activation and subsequent deposition of complement components on tumor tissues has been demonstrated in cancer patients [27]. Malignant ovarian cells isolated from ascitic fluid samples had C3 activation products deposited on their cell surface [28]. Complement components are important mediators of inflammation and help regulate the immune response. C3a is biologically active and binds to mast cells and basophils, triggering the release of their vasoactive contents [29]. We investigated C3a expression by immunochemical examination of HCC tissues and Western blot analysis of proteins extracted from human HCC cell lines, including HepG2 and HuH-7. However, specific C3a expression, including the C3a fragment (8.1 k m/z), was not detected.

The complement system can be activated after exposure to tumor antigens [30]. It is speculated that small tumors can trigger a systematic reaction. Therefore, elevated C3a (8.9 k m/z) levels in the serum of HCV-HCC patients may reflect both a systematic immune response to HCV infection and non-specific tumor antigens rather than a specific immune response to HCC [24–26, 31]. In contrast, it is possible that overexpression of the C3a fragment (8.1 k m/z) is specific for HCC in addition to non-specific C3 activation.

In contrast to our results, Steel et al. [32] searched for HCC biomarkers using HCC-associated HBV-infected patient sera and found that the C-terminal fragment of complement C3 was down-regulated. Kawakami et al. [33] searched for characteristic alterations in the sera of HBV- and HCV-HCC-infected patients who had undergone curative radiofrequency ablation treatment and showed that C3 was up-regulated after treatment. In these studies, C3 was separated and identified using 2-DE of a mixture of proteins from a small number of patient sera samples, and this process identified various molecular weights for C3. In addition, we analyzed the sera of 25 patients with HCC-associated HBV infections, and the profile of several proteins was different between HCV- and HBV-infected patients. Although 35 protein peaks, including the C3a fragment, were overexpressed in the sera of both HCV-HCC and HBV-HCC patients compared to sera from healthy volunteers, the C3a fragment (8.1 k m/z) was particularly overexpressed in the sera of HCV-HCC patients and was not significantly different between HBV-HCC patients and HCV-CLD patients without HCC (data not shown). The biologic and pathogenic activities of HCV and HBV are different, and the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis may differ between HBV and HCV infections [34–36]. Although the number of samples, cause of liver disease, and method of protein identification may affect these results, we speculate that the C3a fragment with a molecular weight of 8.1 k m/z is a candidate tumor marker for HCV-HCC but not HBV-HCC.

AFP, which is a commonly used HCC tumor marker, is elevated not only during HCC, but also during hepatocyte regeneration following liver damage. Previous reports revealed that AFP was abnormally elevated in the sera of patients with acute hepatitis, chronic hepatitis, and liver

cirrhosis. This lack of specificity for HCC means that AFP has a comparatively high false-positive rate [37]. The C3a fragment may also be elevated during hepatocyte regeneration following liver damage [38], and early diagnosis of small HCC tumors may be difficult with one marker alone. Therefore, the false-positive rates for HCC must be carefully considered [39–41]. Also, a combination of markers, including AFP, DCP, and the C3a fragment, in the serum should be verified to improve the diagnostic rate.

The ProteinChip SELDI system can separate and partially characterize multiple proteins in tissue and serum samples. Our previous report used a panel of proteins to diagnose early HCC with the ProteinChip SELDI system [15]. This panel diagnosis of seven protein peaks included a discriminant peak of 4060 m/z. This 4060 m/z peak may be a double-charged 8130 m/z peak, although the C3a fragment (8130 m/z) was not used to develop this diagnostic method. These results suggest that the C3a fragment is a useful HCC biomarker, regardless of whether this fragment carries a single or double charge. In addition, the panel diagnosis method is more useful than measuring the C3a fragment alone to diagnose and predict the occurrence of HCC. However, this method must be performed using the ProteinChip SELDI system, which is expensive and does not detect putative interactions between various proteins. Identifying a specific HCC protein such as the C3a fragment will also further our understanding of the molecular mechanisms of hepatocarcinogenesis. Therefore, the C3a fragment should not only be considered a simple HCC tumor marker, but should also be evaluated for its contribution to HCC carcinogenesis.

In conclusions, serum profiling with the ProteinChip SELDI system may be used to distinguish HCC from chronic liver disease without HCC and to detect early HCC in HCV-infected patients. Because we identified the C3a fragment (8.1 k m/z) in serum samples from HCC patients, the C3a fragment is a promising marker that can be used to screen for HCV-HCC and to develop new therapeutic targets.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical significance of alanine aminotransferase levels and the effect of ursodeoxycholic acid in hemodialysis patients with chronic hepatitis C

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Abstract
Background The natural history of hepatitis C virus (HCV) carriers and the effect of ursodeoxycholic acid (UDCA) have not been fully elucidated among hemodialysis (HD) patients.
Methods Eighty-four anti-HCV antibody- and HCV RNA-positive and 154 anti-HCV antibody-negative HD patients who were retrospectively observed for at least 3 years were analyzed. We investigated the factors associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) and decreased platelet count (PLT) (more than 20% decrease during the follow-up period), which were considered to be indicators of hepatic fibrosis. In addition, another 16 HD patients with HCV who received 300 mg/day UDCA orally for at least 6 months were investigated. Changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) and PLT were assessed.
Results After the 60.3-months mean follow-up period, HCV infection was independently associated with both thrombocytopenia [odds ratio (OR) 2.589] and decreased PLT (OR 2.339) in 238 HD patients. In 84 HD patients with HCV, the average ALT levels ($\geq$ 15 IU/L) during the follow-up period was associated with thrombocytopenia (OR 3.882) and decreased PLT (OR 4.470). In addition, ALT, AST and GGT significantly decreased at 6 months after starting UDCA, but PLT did not change in 16 HD patients with HCV.
Conclusions These results indicate that HCV infection is a risk for thrombocytopenia which should be associated with hepatic fibrosis in HD patients. In addition, the clinical course of ALT levels predicts the progression of thrombocytopenia, and UDCA may effectively lower ALT levels in HD patients with HCV.

Keywords Hemodialysis · HCV · Thrombocytopenia · ALT · Ursodeoxycholic acid

Introduction

Chronic kidney disease (CKD) patients who are on hemodialysis (HD) continue to have a higher prevalence of hepatitis C virus (HCV) infection than the general population [1–4]. The prevalence of anti-HCV seropositivity among patients undergoing regular dialysis in developed countries ranges between 7 and 40% [5–8].

HCV infection in HD patients is usually recognized as asymptomatic and cirrhosis is infrequent in this population [9]. One of the reasons for these findings is that the clinical course of chronic hepatitis C extends over decades and dialysis patients generally have higher morbidity and mortality rates than the general population, making the long-term consequences of HCV infection with HD difficult to establish [6]. However, more recently, the prognosis of HD patients has been improving, so addressing HCV infection in these patients is becoming more important [10].

The strong association between serum alanine aminotransferase (ALT) levels and the fibrosis progression rate or occurrence of hepatocellular carcinoma has been well documented in HCV carriers without HD [11–13]. HD

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patients with persistent HCV infection also have higher ALT levels than those patients without HCV, and ALT values may predict the outcome of HCV infection in patients with HD [14]. In contrast, ALT values are still typically within the normal range in HCV carriers with HD and ALT values are lower in HCV carriers with HD than those without HD. Recently, the risk of liver disease-related deaths is higher in chronic hepatitis C patients with ALT levels closer to the upper limit of the normal range (ULN) (20–29 IU/L) compared to patients with lower ALT levels (< 20 IU/L) [15, 16]. In addition, it has been proposed that the cut-off for serum ALT levels should be reduced by half to screen for hepatic damage in HCV carriers with HD [17]. However, the association between serum ALT levels in those patients with HCV and fibrosis progression has not been fully elucidated.

Platelet count (PLT) is a simple biomarker of hepatic fibrosis in HCV carriers [18]. PLT is also lower in HCV RNA-positive HD patients than in HD patients with HCV RNA-negative serum [16]. In addition, severe hepatic fibrosis is independently associated with thrombocytopenia ($< 1.3 \times 10^5/\mu L$) in HCV carriers with end-stage renal disease [19]. This study evaluated the association of ALT status over a long period and changes in PLT, which was considered an indicator of hepatic fibrosis, in HD patients.

Several trials have examined the efficacy of interferon monotherapy or interferon plus ribavirin combination therapy in HD patients with HCV, and some of these patients obtained a sustained virological response [20]. However, the virological response was limited and side effects may occur more frequently in patients with HD than in those without HD [21, 22]. Therefore, other therapies should be considered for these patients. For chronic hepatitis C patients with or without HD, ursodeoxycholic acid (UDCA) has already been used up to 150 mg/day as routine care in Japan. In addition, the effect of UDCA up to 900 mg/day in HCV carriers who are not undergoing HD was investigated [23], and the use of UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients after April 2007 in Japan. However, the effect of UDCA was not fully elucidated in HCV carriers with HD. Therefore, in this retrospective study we investigated the clinical significance of biochemical markers in the natural course of disease with particular emphasis on PLT and assessed the effect of oral UDCA on serum biomarkers in those patients with HCV.

Materials and methods

Study population

The patients in this study were retrospectively recruited. This study was approved by the Kagoshima University Graduate School of Medical and Dental Sciences. The study population consisted of patients who were on HD in August 2008 and whose data were obtained at least 3 years before August 2008 at 17 HD facilities in Kagoshima, Japan. Their alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total cholesterol (TC) and PLT were monitored once or twice each month. In 2539 patients, 243 patients were positive for anti-HCV, 143 patients were excluded because they were positive for hepatitis B virus surface (HBs) antigen, they were positive for anti-HCV antibody (anti-HCV) but were not examined for HCV RNA, they had received antiviral treatment or they had hepatocellular carcinoma (HCC). The final population enrolled in this study consisted of 100 patients. Among this cohort of 100 HD patients who were both anti-HCV- and HCV RNA-positive, 84 subjects had not received UDCA and were enrolled in study 1 (HD + HCV Group) and 16 subjects had already received 300 mg/day UDCA for at least 3 months after April 2007 when UDCA up to 900 mg/day was approved for use by chronic hepatitis C patients in Japan and were enrolled in study 2 (UDCA Group). The control subjects in study 1 were 154 HD patients who were anti-HCV-negative (HD Group), and the controls in study 2 were the 84 HD patients among the study 1 population who were both anti-HCV- and HCV RNA-positive but had not received previous treatments including UDCA and were observed until August 2008 (non-UDCA Group). Of the 84 HD patients who were controls in study 2, 2 patients died before November 2008. Blood samples were obtained before routine HD procedures and then were used to assay for ALT, AST, GGT, TC and PLT. The relationship of these markers to PLT and the percent change in PLT were examined, and the percent change in PLT was calculated according to the formula: $\Delta\%PLT = [PLT \text{ (at the end of study)} - PLT \text{ (at enrollment)}]/PLT \text{ (at enrollment)} \times 100$.

Serum HCV markers

Serum anti-HCV and HBsAg were determined using a commercially available third-generation enzyme-linked immunosorbent assay and anti-HBs assay, respectively. For anti-HCV antibody-positive patients, HCV RNA was quantified using the COBAS TaqMan HCV kit (COBAS AmpliPrep/COBAS TaqMan HCV assay, Roche Diagnostics, Tokyo, Japan) during the follow-up period. The serologically defined HCV genotype (HCV serotype) was also determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

Study 1

The HD + HCV Group, which contained 84 HD patients with HCV, was compared to the HD Group, which contained 154 HD anti-HCV-negative patients. We compared the basal characteristics at enrollment and the changes in PLT during the follow-up period between the two groups. In addition, we divided the HD + HCV patients into the following four groups according to the average ALT level of all available ALT levels during the follow-up period: Group A, ALT < 15; Group B, $15 \leq$ ALT < 20; Group C, $20 \leq$ ALT < 30; and Group D, $30 \leq$ ALT. Clinical characteristics at baseline or average ALT levels and change in PLT during the follow-up period were compared between these four groups.

Study 2

Sixteen patients with HD and HCV had been treated with 300 mg/day UDCA orally for at least 3 months after April 2007, when UDCA up to 900 mg/day was approved for chronic hepatitis C patients, until August 2008 (UDCA Group). These patients were observed every month for at least 6 months before the administration of UDCA and then monitored for the efficacy of UDCA for more than 3 months until August 2008. Then, these patients were observed for a total of at least 6 months until November 2008. We compared the basal characteristics between the UDCA Group just before UDCA treatment and the non-UDCA Group in May 2008. In addition, the changes in ALT, AST, GGT and PLT during the follow-up period were compared between the two groups. For example, the percent of ALT was calculated according to the formula: %ALT = [ALT (−6, 0, 1, 2, 3 or 6 M]/ALT[0 M] × 100).

Statistical analysis

When appropriate, χ^2 test, Fisher's exact test, Student's t test and Mann–Whitney U test were used to compare the frequencies or means. Logistic regression models were used for calculating the odds ratios (ORs), 95% confidential intervals (CIs) and P values. Statistical analyses were performed using STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A P value less than 0.05 was considered statistically significant.

Results

Demographic characteristics of study 1 subjects

As shown in Table 1, 84 HD patients among the anti-HCV-positive patients were HCV carriers (positive for HCV RNA). One hundred fifty-four HD patients were anti-HCV-negative. On average, the frequency of males, levels of AST, ALT and GGT were higher and TC and PLT were lower at baseline in patients with HCV than those in patients without HCV. The follow-up period was also shorter in patients with HCV than those in patients without HCV. In contrast, there were no significant differences between the two groups with respect to age and duration of dialysis.

Table 1 Baseline characteristics of hemodialysis patients

	HCV (+)[a]	HCV (−)[b]	*P* value
Number	84	154	
Age (year)	64.4 ± 10.3	62.2 ± 12.5	0.165
Sex (male/female)	54/30	77/77	0.034
Duration of HD (years)	13.5 ± 9.6	11.8 ± 7.4	0.669
Follow-up period (months)	56.8 ± 15.8	62.2 ± 7.9	0.039
HCV RNA (Log IU/mL)[c]	4.9 ± 1.4	–	
Serotype (I/II/undetermined)[c]	59/21/4	–	
AST (IU/L)	19.7 ± 8.5	14.9 ± 6.7	<0.001
ALT (IU/L)	18.5 ± 9.3	13.2 ± 7.1	<0.001
GGT (IU/L)	41.5 ± 43.0	30.1 ± 42.1	0.002
TC (mg/dl)	153.7 ± 41.0	167.1 ± 35.0	0.003
PLT (× 10^5/μl)	1.59 ± 0.53	1.93 ± 0.73	<0.001

Unless otherwise indicated, data are given as the mean ± SD or number of patients

HD hemodialysis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *TC* total cholesterol, *PLT* platelet count

[a] HCV (+), both anti-HCV antibody and HCV RNA positive

[b] HCV (−); anti-HCV antibody negative

[c] HCV RNA and serotype were examined during follow-up period

Predictors of thrombocytopenia in HD patients

Table 2 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT $< 1.3 \times 10^5$/μl) at the end of study 1 (August 2008) using 9 baseline characteristics in all HD patients with or without HCV. Older age, HCV viremia, elevated AST, ALT, and GGT levels were significantly associated with thrombocytopenia. In addition, a multivariate analysis revealed that HCV viremia was independently associated with thrombocytopenia (Table 2). Furthermore, after the 60.3-month mean follow-up period (mean of HD + HCV Group, 56.7 months; HD Group, 62.2 months), PLT in the HD + HCV Group had decreased (from 1.59×10^5/μL to 1.22×10^5/μL) significantly compared to that in the HD Group (from 1.93×10^5/μL to 1.77×10^5/μL) (average Δ%PLT in each patient: −22.4 vs. −5.3%, $P < 0.001$). Variables that were statistically significant by a univariate analysis were further analyzed to identify variables that

Table 2 Univariate and multivariate analyses of variables associated with thrombocytopenia (< $1.3 \times 10^5/\mu l$) in HD patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	1.994	1.141–3.484	0.015
Sex			
Female	1.0		
Male	1.494	0.868–2.571	0.147
Duration of dialysis (years)			
<10	1.0		
≥10	1.065	0.624–1.818	0.816
Follow-up period (months)			
<55	1.0		
≥55	0.727	0.4–1.321	0.296
HCV			
(−)	1.0		
(+)	4.533	2.555–8.043	<0.0001
AST (IU/L)			
<30	1.0		
≥30	7.741	2.095–28.603	0.002
ALT (IU/L)			
<20	1.0		
≥20	3.793	2.017–7.133	<0.0001
GGT (IU/L)			
<50	1.0		
≥50	2.836	1.396–5.758	0.004
TC (mg/dl)			
<150	1.0		
≥150	0.58	0.296–1.135	0.112
Multivariate analysis			
Age (years)			
<60	1.0		
≥60	1.783	0.937–3.394	0.078
HCV			
(−)	1.0		
(+)	2.589	1.317–5.091	0.006
AST (IU/L)			
<30	1.0		
≥30	5.123	0.996–26.339	0.050
ALT (IU/L)			
<20	1.0		
≥20	1.75	0.786–3.896	0.171
GGT (IU/L)			
<50	1.0		
≥50	1.743	0.783–3.88	0.174

Abbreviations as in Table 1

were independently associated with a more than 20% decrease in PLT. As a result, male sex (OR 2.375; 95% CI, 1.319–4.278; $P = 0.004$) and HCV viremia (OR 2.339; 95% CI, 1.295–4.224; $P = 0.005$) were factors that were independently associated with more than a 20% decrease in PLT.

Predictors of thrombocytopenia in HD patients with HCV

Table 3 summarizes the results of a univariate analysis of factors associated with thrombocytopenia (PLT < $1.3 \times 10^5/\mu L$) at the end of study 1 (August 2008) using 10 baseline characteristics in HD patients with HCV. The patients with HCV and thrombocytopenia had significantly higher frequencies of elevated ALT and GGT levels at baseline. However, age, sex, duration of HD, follow-up period, history of diabetes mellitus (DM), and elevated AST and TC levels were not significantly different between patients with and without thrombocytopenia. In addition, elevated ALT and GGT levels at baseline were not significantly associated with thrombocytopenia in patients with HCV by a multivariate analysis.

On the other hand, a univariate analysis that compared a decrease in PLT of more than 20% with a decrease less than 20% revealed that male sex and elevated ALT levels at baseline were associated with decreased PLT in patients with HCV. A multivariate analysis of two variables that were statistically significant by a univariate analysis also revealed that high ALT levels (ALT ≥ 20 IU/L) at baseline were independently associated with decreased PLT in patients with HCV (OR 3.318; 95% CI, 1.256-8.764; $P = 0.016$).

Furthermore, we divided patients with HCV into four groups according to average ALT levels during the follow-up period. As Table 4 shows, 30, 19, 18 and 17 patients were in Groups A, B, C and D, respectively. Age, duration of dialysis, follow-up period, HCV RNA levels, distribution of HCV serotype, frequency of diabetes mellitus, TC levels and PLT were not significantly different between the four groups. However, serum AST levels and ALT levels at baseline were significantly different, and these levels gradually increased from Group A to D. The distribution of sex was also significantly different and the frequency of males was higher in Groups B, C and D than in Group A. The decreasing rate of change in PLT was significantly higher in Groups B, C, and D compared to Group A (Fig. 1). In addition, the average ALT levels (≥ 15 IU/L) during the follow-up period were independently associated with thrombocytopenia (OR 3.882; 95% CI, 1.257–11.987;

Table 3 Univariate and multivariate analyses of variables associated with thrombocytopenia (PLT < 1.3×10^5/ul) in HD + HCV patients

Variables	Odds ratio	95% CI	*P* value
Univariate analysis			
Age (years)			
<60	1.0		
≥60	0.616	0.247–1.534	0.298
Sex			
Female	1.0		
Male	1.273	0.518–3.129	0.599
Duration of HD (years)			
<10	1.0		
≥10	1.321	0.555–3.141	0.529
Follow-up period (months)			
<55	1.0		
≥55	1.057	0.445–2.515	0.899
History of diabetes mellitus			
−	1.0		
+	1.426	0.557–3.646	0.459
Serotype			
I	1.0		
II	1.051	0.384–2.871	0.923
AST (IU/L)			
<30	1.0		
≥30	3.4	0.676–17.103	0.138
ALT (IU/L)			
<20	1.0		
≥20	2.686	1.083–6.662	0.033
GGT (IU/L)			
<50	1.0		
≥50	4.333	1.235–15.206	0.022
TC (mg/dl)			
<150	1.0		
≥150	0.727	0.27–1.958	0.528
Multivariate analysis			
ALT (IU/L)			
<20	1.0		
≥20	1.972	0.665–5.847	0.221
GGT (IU/L)			
<50	1.0		
≥50	3.305	0.876–12.467	0.078

Abbreviations as in Table 1

$P = 0.018$) by multivariate analysis using two variables including average ALT levels and GGT at baseline. The average ALT levels were also associated with decreased PLT (OR 4.470; 95% CI, 1.571–12.719; $P = 0.005$) by multivariate analysis using average ALT levels and sex. These results indicate that the clinical course of ALT levels is associated with thrombocytopenia and a decrease in PLT in patients with HCV.

Demographics of HD patients with HCV who were treated with UDCA

We enrolled 16 HD patients with HCV who were treated with 300 mg/day UDCA orally for more than 3 months in August 2008, and compared these patients (UDCA group) to 84 HD patients with HCV who were not treated with UDCA (non-UDCA group). The UDCA group and non-UDCA group showed similar demographics in regard to age, sex, HCV RNA levels, distribution of HCV serotype, GGT and PLT. The UDCA group, however, had a shorter duration of dialysis and higher AST and ALT levels just before UDCA administration compared to those in the non-UDCA group in May 2008 (Table 5).

Efficacy of UDCA in HD patients with HCV

After administering UDCA, percent of ALT and AST significantly decreased after one month and remained constant up to 6 months compared to the non-UDCA group (Fig. 2). Percent of GGT also significantly decreased after 2 months of UDCA treatment compared to the non-UDCA group. In addition, ALT, AST and GGT levels significantly decreased after UDCA treatment compared to levels before treatment, but PLT did not change during the 6 months of UDCA treatment (Fig. 2). In contrast, serum AST, ALT, GGT and PLT in the non-UDCA group did not change during the 6-month period from May 2008 to November 2008.

Discussion

Our study indicated that HD patients persistently infected with HCV are at risk for thrombocytopenia (less than $1.3 \times 10^5/\mu L$) and a decrease in PLT (more than 20%), although the exact dates of HCV infection were not clear in our study population. In addition, the basal or clinical course of ALT levels appears to predict decreased PLT or thrombocytopenia in patients with HCV. In this study population, the prevalence [243 anti-HCV positive among 2539 HD patients (9.6%)] and age distribution (average age was 63 years old) of anti-HCV antibody-positive subjects and the frequency of the HCV serotype I (74%) were similar to previous reports on HD patients with HCV in Japan [24–26], suggesting that the clinical course of anti-HCV-positive subjects in this study reflects those in Japan as a whole.

It is known that patients on HD often have thrombocytopenia [27], and there is a negative correlation between the dialysis period and PLTs [27, 28]. It was also reported that megakaryocytes are produced at lower levels in the bone marrow [28], platelets are destroyed due to the

Table 4 Baseline characteristics of four groups of HD patients with HCV according to the clinical course of average ALT levels

Average ALT	A; ALT < 15	B; 15 ≤ ALT < 20	C; 20 ≤ ALT < 30	D; 30 < ALT	*P* value
Number	30	19	18	17	
Age (years)	67.8 ± 10.8	60.8 ± 10.6	64.0 ± 9.7	63.1 ± 8.7	0.105
Sex male/female	11/19	15/4	14/4	14/3	0.001
Duration of dialysis (years)	14.4 ± 10.7	14.2 ± 9.2	12.8 ± 8.8	11.7 ± 9.1	0.945
Follow-up period (months)	53.2 ± 14.3	55.4 ± 16.4	64.2 ± 16.0	57.5 ± 16.3	0.290
HCV-RNA (Log IU/mL)	4.9 ± 1.6	4.8 ± 1.3	5.2 ± 1.2	4.8 ± 1.4	0.774
HCV Serotype (I/II/undetermined)	21/7/2	13/6/0	13/4/1	12/4/1	0.949
History of diabetes mellitus (-)/(+)	23/7	12/7	12/6	10/7	0.592
AST (IU/L)	15.0 ± 4.7	19.8 ± 8.6	22.8 ± 9.8	24.9 ± 8.0	<0.001
ALT (IU/L)	10.4 ± 4.1	19.3 ± 6.8	22.3 ± 8.0	27.8 ± 7.9	<0.001
GGT (IU/L)	21.3 ± 15.2	34.8 ± 22.1	81.2 ± 71.2	48.5 ± 35.2	<0.001
TC (mg/dl)	149.7 ± 31.4	152.3 ± 46.1	154.9 ± 37.0	161.2 ± 57.2	0.970
PLT (× 10^5/μl)	1.62 ± 0.55	1.62 ± 0.61	1.46 ± 0.42	1.64 ± 0.51	0.764

Abbreviations as in Table 1

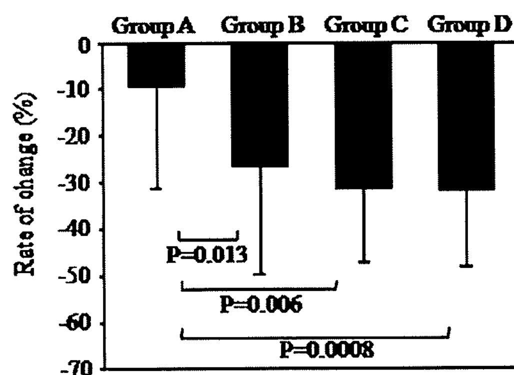

Fig. 1 Comparison of the rate of change in platelet counts by average alanine aminotransferase (ALT) levels during the follow-up period. Group A, average ALT < 15; Group B, 15 ≤ average ALT < 20; Group C, 20 ≤ average ALT < 30; Group D, 30 ≤ average ALT

Table 5 Demographics of HD patients with HCV who were treated with UDCA

	UDCA[a]	Non-UDCA[b]	*P* value
Number	16	84	
Age (years)	66.4 ± 8.6	69.2 ± 10.2	0.261
Sex male/female	9/7	54/30	0.743
Duration of dialysis (years)	6.5 ± 6.6	18.2 ± 9.9	<0.001
HCV-RNA	4.1 ± 2.6	4.9 ± 1.4	0.918
Serotype (I/II/undetermined)	12/4/0	59/21/4	0.669
AST (IU/L)	30.2 ± 24.2	19.2 ± 10.2	0.008
ALT (IU/L)	25.3 ± 16.9	17.1 ± 9.9	0.004
GGT (IU/L)	32.3 ± 23.4	41.4 ± 39.1	0.793
PLT (× 10^5/μl)	1.55 ± 0.56	1.39 ± 0.56	0.577

Abbreviations as in Table 1

[a] Data was obtained at just before the treatment period

[b] Data was obtained in May 2008

appearance of the anti-platelet antibodies [28, 29] and uremic materials reduce the effects of hemopoietic cells [30]. In our study, PLT in HD patients without HCV was significantly decreased after 62.2 months (−5.3%). However, PLT decreased even more dramatically in HD patients with HCV after 56.7 months (−22.4%) compared to patients without HCV. In addition, persistent HCV infection was independently associated with thrombocytopenia and a decrease in PLT in HD patients by a multivariate analysis, but dialysis period was not associated with those. Although the data regarding liver histology and serum markers of hepatic fibrosis were lacking in our study, it has also been reported that severe hepatic fibrosis is associated with thrombocytopenia in HCV carriers with end-stage renal disease [19]. These results suggest that thrombocytopenia is more associated with HCV viremia than with the HD procedure or dialysis period in HD patients.

Hepatocellular carcinoma (HCC) and hepatic failure are critical complications in HCV patients, even in those undergoing HD [10, 31]. These complications occur more frequently in patients with advanced hepatic fibrosis [32, 33]. It has been reported that hepatic fibrosis can be predicted by thrombocytopenia in chronic hepatitis C patients with or without HD [19, 34]. In addition, hepatitis is usually assessed by ALT levels, and changes in ALT levels have been shown to be the most important factor that affects hepatic fibrosis in chronic hepatitis C patients without HD [11, 12]. In this study, we showed that basal ALT levels are associated with thrombocytopenia by a univariate analysis and with decreased PLT by a multivariate analysis. The clinical

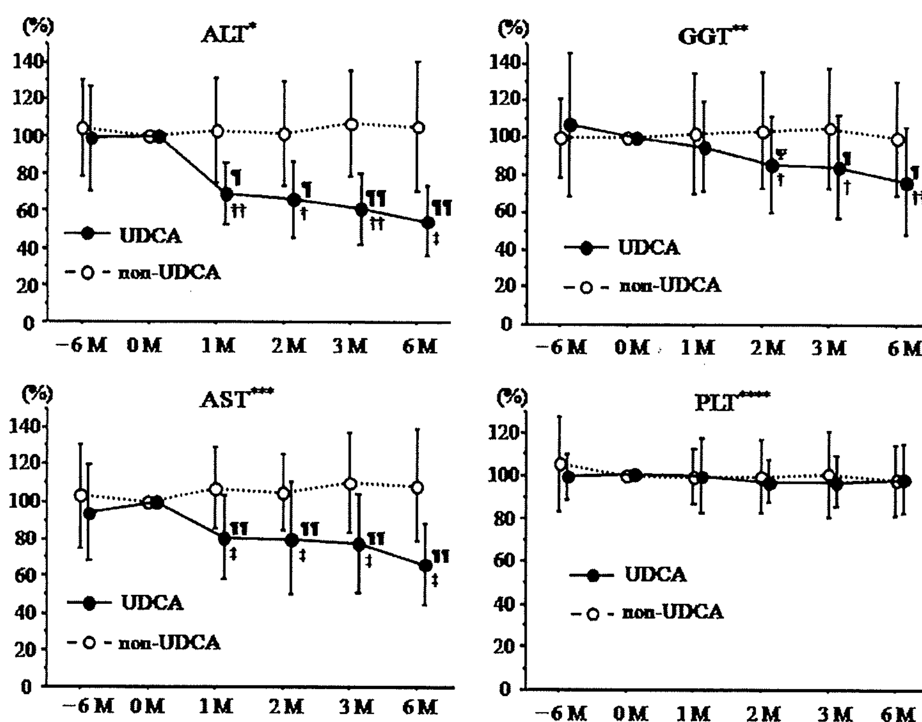

Fig. 2 Efficacy of ursodeoxycholic acid (UDCA) in hemodialysis (HD) patients with hepatitis C virus. Percent of ALT, AST, GGT and PLT in the UDCA group ($n = 16$) 6 months (−6 M) or just (0 M) before and during the treatment period [1, 2, 3 or 6 months (M)] compared to patients in the non-UDCA group ($n = 84$ excluding 6 M) in December 2007 (−6 M), May 2008 (0 M), June (1 M), July (2 M), August (3 M) or November 2008 (6 M, $n = 82$; two patients died before November 2008). Data are expressed as mean ± standard deviation. Closed (*black*) and open circles indicate the UDCA group and non-UDCA group, respectively. The percent of ALT was calculated according to the formula: %ALT = (ALT[−6 M, 0 M, 1 M, 2 M, 3 M or 6 M]/ALT[0 M]) × 100. *ALT* alanine aminotransferase, *AST* asparate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *PLT* platelet count. $^{\Psi}P < 0.05$ (UDCA vs. non-UDCA). $^{¶}P < 0.01$ (UDCA vs. non-UDCA), $^{¶¶}P < 0.001$ (UDCA vs. non-UDCA), $^{\dagger}P < 0.05$ (vs. 0 M), $^{\dagger\dagger}P < 0.01$ (vs. 0 M), $^{\ddagger}P < 0.001$ (vs. 0 M)

course of ALT is also associated with these clinical changes. These results indicate that ALT is an important predictor of thrombocytopenia which should be associated with hepatic fibrosis in HD patients with HCV. In contrast, serum ALT levels are significantly lower in chronic hepatitis C patients on HD than in chronic hepatitis C patients with normal renal function [19]. It was reported that a vitamin B6 deficiency [35], uremic toxins [36], or ultraviolet-absorbing materials [37] are associated with low ALT levels in HD patients. Furthermore, ALT levels have been reported to predict liver disease-related deaths in HD patients, even when ALT levels are in the normal range [38, 39]. Our study also revealed that both patients with abnormal ALT levels (Group D) and normal ALT levels close to the ULN (Groups B and C) had a significant decrease in PLT compared to patients with low ALT levels (Group A). These findings suggest that ALT levels can be used to assess liver damage in HD patients with HCV, although the normal range of ALT should be determined in those patients with HCV in a large cohort study or by liver biopsy.

HCV carriers with persistently normal ALT (PNALT) are more often females than chronic hepatitis C patients with abnormal ALT [40]. This distinction is likely due to lifestyle differences such as alcohol consumption [40], hormonal factors [41] or lower serum iron levels [42]. Although the normal range of ALT in HD patients with HCV may be different compared to the range in HCV carriers with normal renal function, our study demonstrated that females are more likely to have lower ALT levels, even in HD patients (Table 4). This difference in sex may also affect the decrease in PLT. In contrast, the frequency of serotype II, which is reportedly higher in PNALT patients than in chronic hepatitis C patients with abnormal ALT [43], was not different between the four groups in this study (Table 4). A further analysis of the factors associated with elevated ALT levels in those patients with HCV is required.

Interferon therapy has been shown to improve hepatic fibrosis [44] and to reduce the occurrence of HCC in chronic hepatitis C patients with normal renal function. Compared to untreated patients, the risk of HCC after interferon treatment in patients who did not achieve a virological response was shown to be 0.20, 0.36 and 0.91 in chronic hepatitis C patients whose ALT levels were

normal, moderately elevated (less than twice the upper normal limit) and highly elevated, respectively [45]. These results indicate that ALT might predict the mortality of patients with liver-related diseases who have or have not received interferon treatment. Although lower serum ALT levels decreased the risk of HCC, biochemical and virological responses were limited [20, 46] and HD was one of the factors associated with patients who did not respond to interferon and ribavirin treatment [21, 22]. Other therapies that lower serum ALT levels but do not involve interferon-based treatment need to be investigated. Recently, it has been established that UDCA up to 900 mg/day dose-dependently improves biochemical indices such as serum ALT, GGT and bilirubin [23]. Although UDCA seems to lower serum ALT levels, the risk of liver fibrosis, and possibly the incidence of hepatocellular carcinoma, liver histology, serum hepatic fibrosis markers and prognosis (including the incidence of HCC) should also be evaluated over a long time period in HCV carriers with or without HD.

Our study had several limitations; a small number of patients was simply treated with UDCA as routine care, selection of patients depended on each physician and then the data collected retrospectively after a specified duration of therapy. However, this study showed that UDCA effectively had reduced serum ALT, AST and GGT levels in HD patients with HCV. Interestingly, UDCA decreased ALT levels even in patients with normal ALT levels less than 30 IU/L (data not shown). Therefore, HCV patients with normal ALT levels should also be considered for the indication of treatment.

Although the patients in this study were treated with 300 mg/day UDCA, it has also been reported that a 600 mg/day dose of UDCA more effectively decreases ALT and AST levels than a 150 mg/day dose in chronic hepatitis C patients with normal renal function [23]. In addition, PLT did not change during UDCA treatment. Future studies need to investigate the dose-dependent effects of UDCA on ALT levels and prospective double-blind UDCA treatment over a long period in HD patients with HCV.

In conclusion, HCV viremia and ALT levels at basal conditions and during the clinical course of disease were associated with thrombocytopenia and decreased PLT in HD patients. We recommend that HCV carriers on HD who have ALT levels greater than 15 IU/mL be considered for treatment. In addition, UDCA should be considered for HD patients who have chronic hepatitis due to HCV infection but cannot receive interferon-based therapy.

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Original Article

Proanthocyanidin derived from the leaves of *Vaccinium virgatum* suppresses platelet-derived growth factor-induced proliferation of the human hepatic stellate cell line LI90

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Aim: Hepatic stellate cell (HSC) proliferation plays a pivotal role in liver fibrogenesis, and agents that suppress HSC activation, including platelet-derived growth factor (PDGF)-induced HSC proliferation, are good candidates for antifibrogenic therapies. In this report, we use the LI90 HSC line to elucidate the antifibrogenic effects of proanthocyanidin derived from the leaves of *Vaccinium virgatum*.

Methods: Proanthocyanidin (PAC) was extracted from the leaves of blueberry *V. virgatum* (BB-PAC), grape seeds (GS-PAC) and *Croton lechleri* (CL-PAC). These extracts were examined for their effects on PDGF-BB-induced LI90 cell proliferation and DNA synthesis. Extracellular signal-regulated kinase (ERK) and Akt phosphorylation and PDGF receptor-β (PDGFR-β) expression were evaluated by western blot analysis.

Results: BB-PAC potently suppressed PDGF-BB-induced proliferation and DNA synthesis of LI90 cells. BB-PAC also suppressed PDGF-BB-induced DNA synthesis in primary cultured rat HSC. Moreover, GS-PAC and CL-PAC suppressed PDGF-BB-induced DNA synthesis in LI90 cells. In contrast, the monomeric PAC catechin and epicatechin and dimeric PAC procyanidin B2 only slightly suppressed PDGF-BB-induced DNA synthesis. Western blot analysis showed that BB-PAC completely or partially inhibited PDGF-BB-induced ERK and Akt phosphorylation, respectively. In addition, BB-PAC partially inhibited the PDGF-BB-induced degradation of PDGFR-β.

Conclusion: Our results suggest that BB-PAC suppresses activated HSC by inhibiting the PDGF signaling pathway. In addition, these results provide novel findings that may facilitate the development of antifibrogenic agents.

Key words: Akt, extracellular signal-regulated kinase, hepatic stellate cell, platelet-derived growth factor-β, platelet-derived growth factor, proanthocyanidin.

INTRODUCTION

HEPATIC STELLATE CELLS (HSC) play a pivotal role during liver fibrogenesis. After hepatic damage from viral infection, cholestasis, metabolic diseases, persistent alcohol abuse or autoimmune liver diseases and others, HSC proliferate and transform from quiescent HSC into activated myofibroblasts. These cells produce excessive amounts of extracellular matrix compounds and matrix degradation inhibitors, which can result in hepatic fibrosis and ultimately cirrhosis, the end stage of fibrosis.[1,2] The functions of HSC are modulated by several cytokines and growth factors including platelet-derived growth factor (PDGF), which is a potent mitogen for HSC that is primarily produced by specialized liver macrophages known as Kupffer cells.[3]

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PDGF-induced HSC proliferation plays a critical role in hepatic fibrogenesis. Therefore, agents that suppress PDGF-induced HSC proliferation are potential candidates for antifibrogenic therapies.

Recent research has focused on identifying naturally occurring antifibrotic compounds that target PDGF-induced HSC proliferation or the production of collagen, transforming growth factor (TGF)-β and matrix metalloproteinases. A diverse range of natural products obtained from foods, including polyphenols, alkaloids and terpenoids, have been suggested to have an inhibitory effect on HSC,[4–9] and these products may provide novel therapeutic agents for hepatic fibrosis without side-effects. Proanthocyanidins (PAC) are naturally occurring polyphenols that are comprised of complicated mixtures, consisting primarily of polymers of flavan-3-ols such as catechin, epicatechin, gallocatechin, epigallocatechin, epigallocatechin-3-gallate (EGCG) and their dimeric and trimeric compounds. These PAC are derived from common foods such as tea, grapes, cranberries, almonds, chocolate and cacao beans.[10–14] Furthermore, it has been reported that drinking tea and coffee decreases the risk of clinically significant chronic liver disease.[15] In addition, EGCG, one of the green tea flavan-3-ols and a component of PAC, was previously shown to have a potent inhibitory effect on HSC proliferation.[3,16] However, the effect of other PAC components on HSC proliferation has not been fully elucidated.

The fruits and leaves of *Vaccinium virgatum* (blueberry), a member of the Ericaceae family, contain abundant levels of PAC that has a high ratio of polymerized PAC.[17,18] Therefore, patients with liver diseases such as hepatic fibrosis can easily consume these PAC by eating a diet rich in blueberries and other PAC-containing foods. However, it has not been reported whether the polymerized PAC found in natural foods such as blueberries also effectively prevent hepatic fibrosis and HSC proliferation.[19,20] Therefore, we extracted polymerized PAC from blueberry leaves (BB-PAC) and examined its effects on HSC proliferation and the DNA synthesis induced by PDGF-BB using the LI90 human HSC line and primary cultured rat HSC. Furthermore, we investigated the mechanism by which BB-PAC inhibits LI90 cell proliferation and DNA synthesis.

METHODS

PAC fractionation

A LYOPHILIZED POWDER of fresh *V. virgatum* Aiton leaves was kindly supplied by Unkai Shuzo (Miyazaki, Japan). The lyophilized powder (10 g) was sequentially extracted three times with *n*-hexane, ethyl acetate, and 100% methanol (100 mL, 30 min). The methanol extract was concentrated under reduced pressure to yield 3.5 g of extract. The extract (500 mg) was dissolved in 60% methanol, applied to a Sephadex LH-20 column, and successively separated with 60% methanol, 100% methanol and 70% acetone. The 70% acetone extract yielded approximately 100 mg of BB-PAC.[12] Grape seeds (Gravinol from Kikkoman, Chiba, Japan) and *Croton lechleri* (Sangre de Drago from Raintree Nutrition, Carson City, NV, USA) were extracted three times with 100% methanol (100 mL, 30 min), and then prepared as described above to yield GS-PAC and CL-PAC, respectively. Thiolysis was performed to characterize the polymerization states of these three PAC, including the mean degree of polymerization and the catechin composition.[21] EGCG and catechin were purchased from Kurita Analysis Service (Ibaragi, Japan). Epicatechin was purchased from Sigma (St Louis, MO, USA), and procyanidin B2 was purchased from Bio Chemika (Buchs, Switzerland).

Cell culture

The LI90 cell line was obtained from the Human Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's minimal essential medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (Medical & Biological Laboratories, Nagoya, Japan), 100 IU/mL penicillin and 50 mg/mL streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2, and the medium was changed weekly. We used LI90 cells with passage numbers between 20 and 26 for all experiments.

Isolation and culture of primary rat HSC

Hepatic stellate cells were isolated from male Sprague–Dawley rats (bodyweight, ~500 g) using collagenase and pronase as described previously.[22] HSC were identified by their typical star-like morphology under a light microscope, vitamin A-specific autofluorescence, and cellular expression of α-smooth muscle actin (α-SMA) detected using western blotting with α-SMA-specific antibodies. HSC were incubated in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 50 mg/mL streptomycin. The medium was changed after 3 days and every 48 hours thereafter. Differentiated myofibroblasts generated after reseeding 14- to 18-day-old primary HSC were used in the experiments.

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Measurement of cell proliferation, DNA synthesis and apoptosis

The 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously.[23] Briefly, 5 mg/mL MTT was added to the culture in 1/10 the media volume. After a 2-h incubation, extraction buffer (12.8% sodium dodecylsulfate (SDS), 0.41 M acetate buffer at pH 4.5, and 32% *N,N*-dimethylformamide) was added, and the samples were incubated overnight at 37°C. The optical densities of the samples were measured at 570 nm using a plate reader. To evaluate DNA synthesis in LI90 cells, a 5-bromodeoxyuridine (BrdU)-specific enzyme-linked immunosorbent assay (ELISA) was performed using a 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit III (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. Briefly, LI90 cells that had been cultured under various conditions were incubated for 4–6 h with BrdU to allow incorporation into cellular DNA. Next, the cells were fixed in chilled 0.5 M ethanol/HCl fixative. Cellular BrdU incorporation was detected with peroxidase-conjugated anti-BrdU antibodies and quantified using a plate reader following the manufacturer's instructions. To evaluate DNA fragmentation in LI90 cells, DNA- and histone-specific ELISA were performed using Cell Death Detection ELISA^{PLUS} kits (Roche Diagnostics) according to the manufacturer's instructions. Briefly, cellular lysates were transferred into streptavidin-coated plates. Cellular histone and fragmented DNA were detected with biotin-conjugated anti-histone antibodies and peroxidase-conjugated anti-DNA antibodies, and quantified using a plate reader.

Western blot analysis

LI90 and primary rat HSC lysates were quantitatively examined using the Lowry method with bovine serum albumin as a standard. Equal amounts of cell lysates (5–10 μg) were separated on 8% or 10% SDS polyacrylamide gels (SDS-PAGE) and electroblotted onto polyvinylidine fluoride membranes. The blots were probed with antibodies specific for phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Thr183/Tyr185), p44/42 MAPK, phospho-Akt (Ser473), Akt, or PDGF receptor-β (Cell Signaling Technology, Danvers, MA, USA), α-SMA (DAKO, Carpinteria, CA, USA) or β-actin (Sigma). After incubating the membrane with horseradish peroxidase-conjugated secondary antibodies, reactivity was visualized using a Chemi Doc XRS-J digital densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and electrogenerated chemiluminescence western blotting detection reagents (GE Healthcare Bio-sciences, Tokyo, Japan). Densitometric analysis was performed using Quantity One Software (Bio-Rad Laboratories).

Statistical analysis

All results are expressed as the means ± standard deviation (SD) of at least three independent experiments. A one-way ANOVA followed by Tukey's multiple comparison test was used to evaluate differences between groups.

RESULTS

PAC polymerization states

PROANTHOCYANIDINS FROM THREE different sources, BB-PAC, GS-PAC and CL-PAC, were prepared, and the polymerization states were analyzed by thiolysis. The results showed that the mean degree of polymerization for BB-PAC, GS-PAC and CL-PAC was 8.4, 14.4 and 8.3, respectively (data not shown). BB-PAC contained more epicatechin than GS-PAC and CL-PAC. On the other hand, GS-PAC and CL-PAC had higher levels of catechin than BB-PAC (data not shown). In addition, the BB-PAC sample contained approximately 10–30% dimeric and trimeric PAC, whereas these PAC forms were undetected in GS-PAC and CL-PAC (data not shown).

BB-PAC inhibits PDGF-BB-induced HSC proliferation

To determine the effect of BB-PAC on LI90 cell proliferation, LI90 cells were incubated for 96 h with 0.1–10 μg/mL BB-PAC, and viable cells were counted using the MTT method. BB-PAC decreased the viability of LI90 cells at concentrations greater than 3 μg/mL (Fig. 1a). LI90 cells were also incubated for 96 h with varying concentrations of BB-PAC, ranging 0.1–10 μg/mL, in the presence or absence of 10 ng/mL PDGF-BB. BB-PAC completely blocked PDGF-BB-induced cell proliferation at a concentration of 1 μg/mL (Fig. 1a). A BrdU-specific ELISA was used to determine whether 1 μg/mL BB-PAC inhibited the PDGF-BB-mediated enhancement in DNA synthesis. BB-PAC at 1 μg/mL completely inhibited PDGF-BB-induced DNA synthesis, whereas it did not affect DNA synthesis in the absence of PDGF-BB (Fig. 1b). In addition, 1 μg/mL BB-PAC significantly inhibited PDGF-BB-induced DNA synthesis in primary

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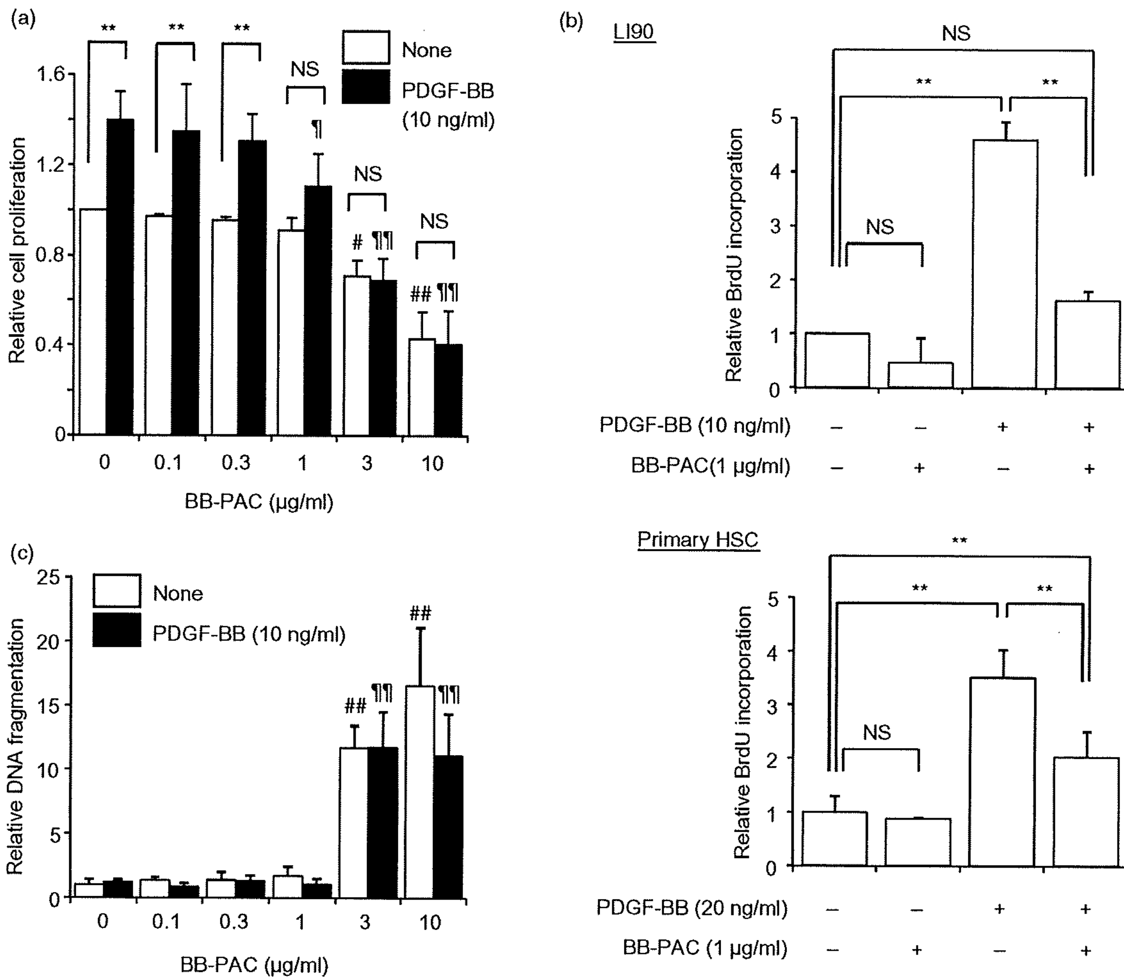

Figure 1 Suppressive effects of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-induced proliferation and DNA synthesis in LI90 hepatic stellate cells. (a) LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed by a 96-h incubation with or without 10 ng/mL PDGF-BB. The number of viable cells was measured by the MTT method. Data are means ± standard deviations (SD) from three independent experiments. (b) LI90 cells and primary rat hepatic stellate cells (HSC) were pre-incubated for 1 h with 1 μg/mL BB-PAC and then incubated for 24 h with or without 10 or 20 ng/mL PDGF-BB. The relative level of bromodeoxyuridine (BrdU) incorporation was measured using a BrdU-specific enzyme-linked immunosorbent assay (ELISA). (c) LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed by a 48-h incubation with or without 10 ng/mL PDGF-BB. The relative amount of fragmented DNA was measured using DNA- and histone-specific ELISA. The data are means ± SD from three independent experiments. One-way ANOVA and Tukey's multiple comparison test were used to evaluate differences between groups. $**P < 0.01$, between the indicated groups; $\#P < 0.05$ and $\#\#P < 0.01$, compared to values obtained with medium alone; and $\P P < 0.05$ and $\P\P P < 0.01$, compared to samples treated with PDGF-BB alone. NS, not significant.

rat HSC, although this effect was not as robust as that observed in LI90 cells (Fig. 1b). At concentrations of 3 and 10 μg/mL, BB-PAC enhanced DNA fragmentation in both PDGF-BB-treated and untreated LI90 cells, as measured using an ELISA (Fig. 1c). This result indicates that high BB-PAC concentrations induce apoptosis in activated HSC. In addition, we evaluated the effects of 1 μg/mL BB-PAC on TGF-β-induced α-SMA protein expression and PDGF-BB-induced collagen mRNA expression, a marker of HSC activation.[3] BB-PAC had no effect on TGF-β-induced α-SMA expression or PDGF-BB-induced collagen expression (data not shown). There-

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fore, subsequent experiments analyzed the mechanism by which BB-PAC inhibits PDGF-BB-induced LI90 cell proliferation.

Effects of BB-PAC on PDGF-BB-mediated phosphorylation of MAPK and Akt

Platelet-derived growth factor-BB is known to enhance the phosphorylation of various protein kinases. Therefore, we examined the effects of BB-PAC on PDGF-BB-induced MAPK and Akt activation by western blot analysis. LI90 cells were pre-incubated for 1 h with 1 μg/mL BB-PAC and then incubated for 15 min with or without 10 ng/mL PDGF-BB. BB-PAC at 1 μg/mL completely inhibited PDGF-BB-induced ERK phosphorylation (p44/42) (Fig. 2a,b). Phosphorylation of JNK, a MAPK family member, was also inhibited completely by pretreating with 1 μg/mL BB-PAC (data not shown). On the other hand, Akt phosphorylation (Ser473) was significantly but not completely inhibited by pretreating with 1 μg/mL BB-PAC (Fig. 2c,d).

Comparison of the inhibitory effects of BB-PAC, GS-PAC, CL-PAC, and monomeric and dimeric PAC

To examine whether the ability of BB-PAC to inhibit LI90 cell activation depended on the source of PAC,

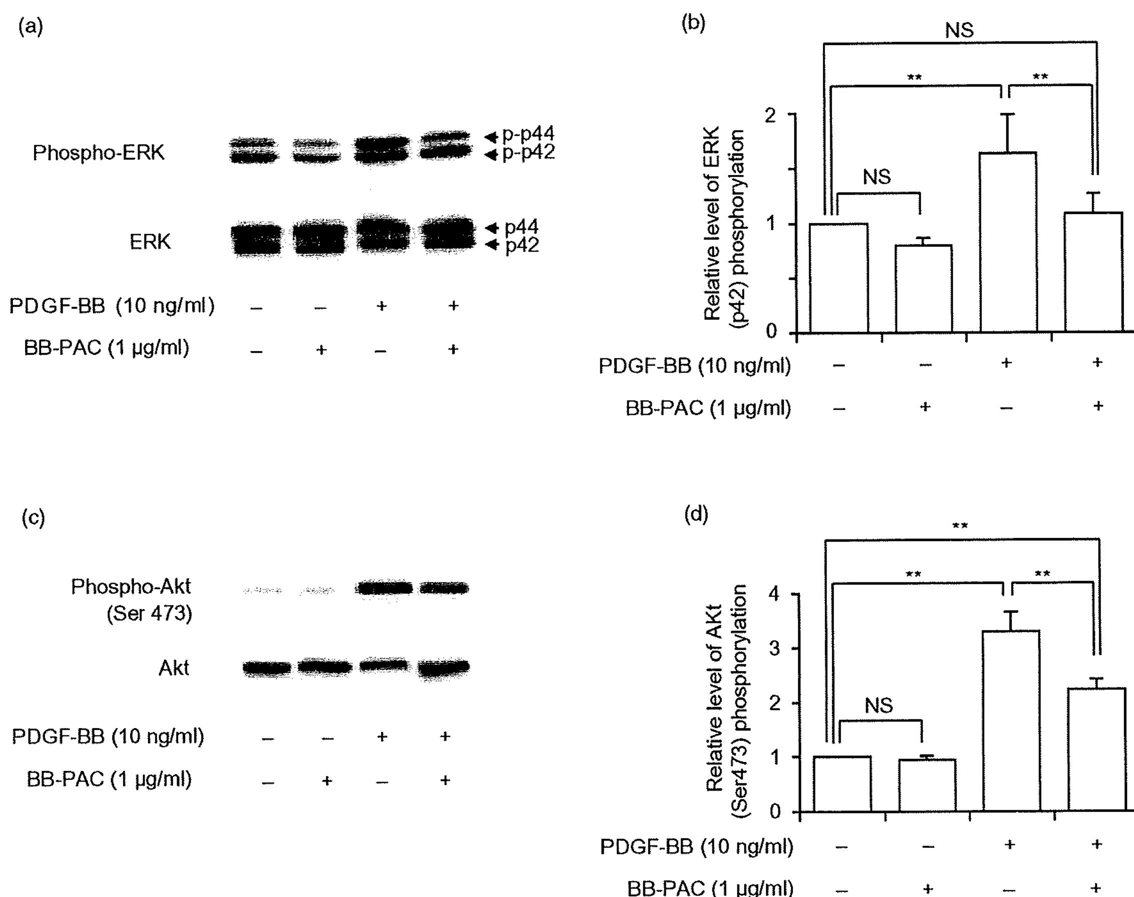

Figure 2 Effects of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-mediated phosphorylation of extracellular signal-regulated kinase (ERK) and Akt. LI90 cells were pre-incubated for 1 h with 1 μg/mL BB-PAC, followed by a 15-min incubation with or without 10 ng/mL PDGF-BB. Equal amounts of cell extracts (10 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for phosphorylated ERK, total ERK and Akt (a,c). Results are expressed as the levels of phosphor-ERK and phospho-Akt relative to the respective total protein levels (b,d). The data are means ± standard deviations from three independent experiments. One-way ANOVA and Tukey's multiple comparison test were used to evaluate differences between groups. $^{**}P < 0.01$. NS, not significant.

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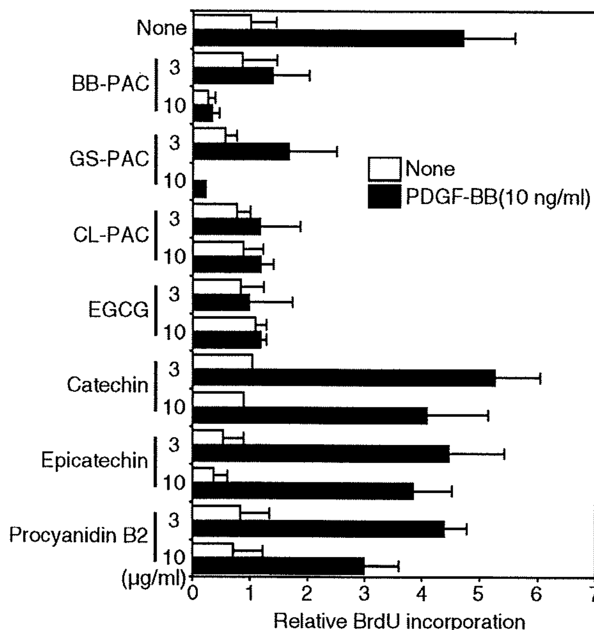

Figure 3 Comparison of the inhibitory effects of proanthocyanidin extracted from blueberry leaves (BB-PAC), PAC from grape seeds (GS-PAC) and *Croton lechleri* (CL-PAC) and monomeric and dimeric PAC. LI90 cells were pre-incubated for 1 h with 3 or 10 μg/mL BB-PAC, GS-PAC, CL-PAC, epigallocatechin-3-gallate (EGCG), catechin, epicatechin or procyanidin B2. Samples were then incubated for 24 h with or without 10 ng/mL platelet-derived growth factor (PDGF)-BB. The relative 5-bromodeoxyuridine (BrdU) incorporation levels were measured using a BrdU-specific enzyme-linked immunosorbent assay (ELISA). The data are means ± standard deviations of cultures performed in triplicate.

we prepared GS-PAC and CL-PAC, and then compared their effects to those of BB-PAC. Both GS-PAC and CL-PAC strongly suppressed PDGF-BB-induced DNA synthesis in LI90 cells and were comparable to BB-PAC (Fig. 3). Furthermore, we compared the effects of catechin, epicatechin (monomeric PAC) and procyanidin B2 (dimeric PAC) to those of BB-PAC. However, the effects observed with BB-PAC were not found with catechin, epicatechin or procyanidin B2, excluding a slight suppressive effect with 10 μg/mL procyanidin B2 (Fig. 3). In addition, 1 μg/mL GS-PAC and CL-PAC completely inhibited ERK phosphorylation and partially inhibited Akt phosphorylation (Ser473) (data not shown), similar to the inhibition with BB-PAC. The effect of the EGCG, which is a known potent inhibitor of HSC activation,[4,5,16] was also verified (Fig. 3).

Effect of BB-PAC on PDGF-BB-induced PDGF receptor-β degradation

To explore the mechanism by which BB-PAC inhibits PDGF-BB-induced LI90 cell activation, we examined whether BB-PAC affected the expression of the PDGF receptor (PDGFR). PDGFR-α and PDGFR-β are two receptor tyrosine kinases that can form homodimeric or heterodimeric receptor complexes. PDGFR-αα (referred to as PDGFR-α or PDGF-αR) is activated by PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC; PDGFR-αβ is activated by PDGF-AB, PDGF-BB and PDGF-CC; and PDGFR-ββ (PDGFR-β or PDGF-βR) is activated by PDGF-BB and PDGF-DD.[24–26] We focused on the effect of BB-PAC on PDGFR-β expression. LI90 cells were pre-incubated for 1 h with the indicated non-apoptotic concentrations of BB-PAC and then incubated for 24 h with or without 10 ng/mL PDGF-BB. Under these conditions, PDGF-BB markedly induced PDGFR-β degradation, which was partially reversed by pretreatment with 1 μg/mL BB-PAC (Fig. 4).

DISCUSSION

IN THIS STUDY, we demonstrated that BB-PAC potently inhibits PDGF-BB-induced LI90 cell proliferation. For BB-PAC to be used clinically, the specificity of its inhibitory effects on HSC must be clarified. We examined the effects of BB-PAC on basal and PDGF-BB-induced ERK phosphorylation in primary cultured rat hepatocytes. PDGF-BB did not affect ERK phosphorylation, and 0.3–10 μg/mL BB-PAC had no effect on ERK phosphorylation in untreated and PDGF-BB-treated primary cultured rat hepatocytes (data not shown). In addition, 0.01–1 μg/mL BB-PAC was not toxic for

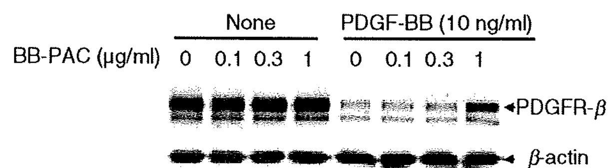

Figure 4 Effect of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-induced PDGF receptor-β (PDGFR-β) degradation. LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed by a 24-h incubation with or without 10 ng/mL PDGF-BB. Equal amounts of cell extracts (10 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and then immunoblotted with PDGFR-β- or β-actin-specific antibodies.

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Huh-7 cells, a liver parenchymal cell line (data not shown). Thus, 1 μg/mL BB-PAC, non-toxic concentration for HSC specifically suppressed PDGF-BB-induced HSC activation without affecting the viability of HSC and parenchymal cells.

We also demonstrated that GS-PAC and CL-PAC inhibited PDGF-BB-induced DNA synthesis in LI90 cells, which was similar to results obtained with BB-PAC (Fig. 3). Thiolysis analysis showed that the mean degree of polymerization of BB-PAC, GS-PAC and CL-PAC was greater than eight. In contrast, catechin, epicatechin and procyanidin B2, which are monomeric or dimeric PAC that are contained in BB-PAC, did not markedly affect PDGF-BB-induced DNA synthesis at high concentrations of 3 or 10 μg/mL. In addition, the molecular weights of catechin, epicatechin and procyanidin B2 are 290.27, 290.27 and 578.72, respectively, while that of BB-PAC, GS-PAC and CL-PAC are estimated to be at least 2300. These findings suggest that BB-PAC inhibits PDGF-BB-induced DNA synthesis at a much lower molar concentration than monomeric and dimeric PAC, and that a high degree of polymerization is one of the important structures that contributes to the inhibition of PDGF-BB-induced LI90 cell proliferation and DNA synthesis. However, EGCG has been reported to inhibit PDGF-BB-induced DNA synthesis in LI90 cells despite being a monomeric PAC. EGCG is known to bind specifically to the 67-kDa laminin receptor, which results in the anti-allergic effects of EGCG.[27,28] However, the relationship between ability of EGCG to bind the 67-kDa laminin receptor and HSC activation has not yet been reported. In addition, it is not known where polymerized PAC binds on the cellular membrane or how the associated signals are transduced. Future studies should examine the differences between the structure, activity and membrane binding of each PAC, and the relationship between their characteristics and ability to inhibit HSC proliferation.

To evaluate the inhibitory mechanism of BB-PAC on PDGF-BB-induced activation of LI90 cells, we examined the MAPK and Akt phosphorylation cascades. BB-PAC completely inhibited PDGF-BB-induced ERK phosphorylation, and slightly inhibited Akt phosphorylation. Several reports have shown that ERK and Akt phosphorylation are closely related to HSC survival. Saxena *et al.* showed that leptin-induced profibrogenic responses in HSC depended on both ERK and Akt phosphorylation.[29] More recently, Wang *et al.* showed that LY294002, an inhibitor of the PI3K/Akt pathway, induced apoptosis in rat HSC.[30] Our data showed that BB-PAC partially inhibited PDGF-BB-induced Akt phosphorylation (Fig. 2c,d). Therefore, this partial inhibition of Akt phosphorylation may induce apoptotic signaling in PDGF-BB-stimulated LI90 cells.

PDGFR, which is located upstream of MAPK and Akt, dimerizes and autophosphorylates at intrinsic tyrosine residues in response to ligand binding.[31] A previous report showed that PDGFR-β expressed in HSC was degraded in response to PDGF-BB; the degradation and subsequent expression of this receptor are thought to be important in the regulatory cycle of the HSC fibrogenic cascade.[32] On the other hand, we found that 1 μg/mL BB-PAC partially inhibited PDGF-BB-induced degradation of PDGFR-β (Fig. 4). Lechuga *et al.* showed that PDGFR-β was completely degraded in HSC following PDGF-BB stimulation for 6 h but reappeared after 48 h.[32] Furthermore, this reappearance of the receptor was inhibited by treating with LY294002, suggesting that the PI3K/Akt pathway is involved in this process.[32] Our findings showed that BB-PAC partially inhibits Akt phosphorylation in response to PDGF-BB (Fig. 2). Thus, BB-PAC may inhibit HSC activation through PI3K/Akt by interrupting both PDGFR-β degradation and its subsequent expression.

Proanthocyanidin is a potent antioxidant and this antioxidative activity may contribute to the ability of BB-PAC to inhibit PDGF-BB-induced LI90 cell proliferation. Adachi *et al.* showed that PDGF-BB-induced cell proliferation is related to the generation of reactive oxygen species (ROS) through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation.[33] The authors also reported that Mn-TBAP, an intracellular ROS scavenger, suppressed PDGF-BB-mediated LI90 cell proliferation but not PDGF-BB-induced ERK phosphorylation (p44/42).[33] The differences in the intracellular mechanisms governed by BB-PAC and Mn-TBAP are unknown. Furthermore, EGCG attenuates oxidative stress in passaged HSC by scavenging ROS and reducing lipid peroxidation.[34] Thus, it is necessary to examine the relationship between HSC activation and the antioxidative activities of BB-PAC.

Taken together, our results showed that BB-PAC potently inhibited PDGF-BB-induced proliferation and DNA synthesis of LI90 cells. This inhibitory effect may be associated with the inhibition of ERK and Akt phosphorylation and the regulation of PDGFR-β expression. Although *in vivo* studies are necessary to confirm these findings, our study provides novel insight into the potential antifibrogenic mechanisms of BB-PAC and further indicates that BB-PAC is a potential therapeutic agent for hepatic fibrosis.

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