Table 2 Univariate and multivariate analyses of variables associated with thrombocytopenia (< 1.3 × 10^5/μl) 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 × 10^5/μL) 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 × 10^5/μL) 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 ($\times 10^5/\mu l$)	1.62 ± 0.55	1.62 ± 0.61	1.46 ± 0.42	1.64 ± 0.51	0.764

Abbreviations as in Table 1

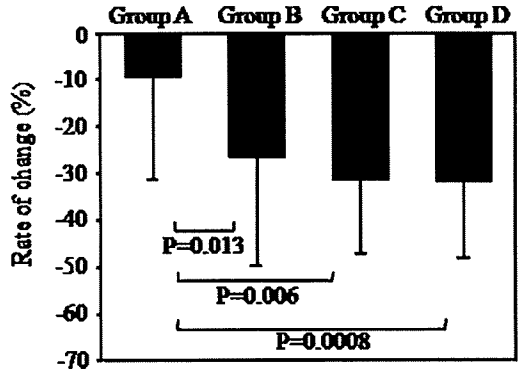

Fig. 1 Comparison of the rate of change in platelet counts by average alanine aminotransferase (ALT) levels during the follow-up period. Group A, average ALT < 15; Group B, 15 ≤ average ALT < 20; Group C, 20 ≤ average ALT < 30; Group D, 30 ≤ average ALT

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 ($\times 10^5/\mu l$)	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

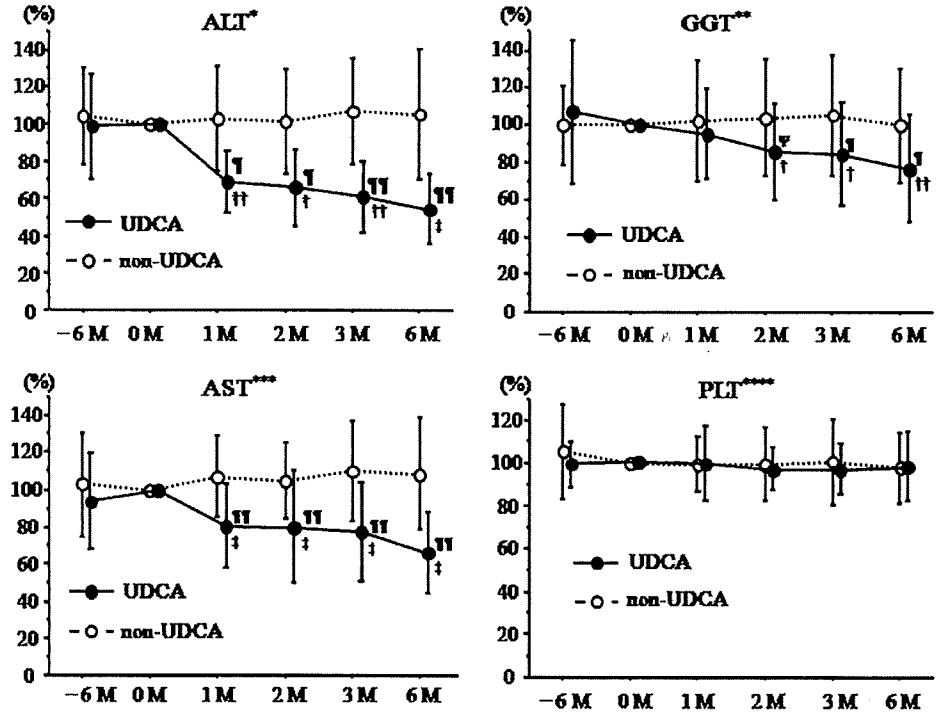

Fig. 2 Efficacy of ursodeoxycholic acid (UDCA) in hemodialysis (HD) patients with hepatitis C virus. Percent of ALT, AST, GGT and PLT in the UDCA group ($n = 16$) 6 months (−6 M) or just (0 M) before and during the treatment period [1, 2, 3 or 6 months (M)] compared to patients in the non-UDCA group ($n = 84$ 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). $^{\P}P < 0.01$ (UDCA vs. non-UDCA), $^{\P\P}P < 0.001$ (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.

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

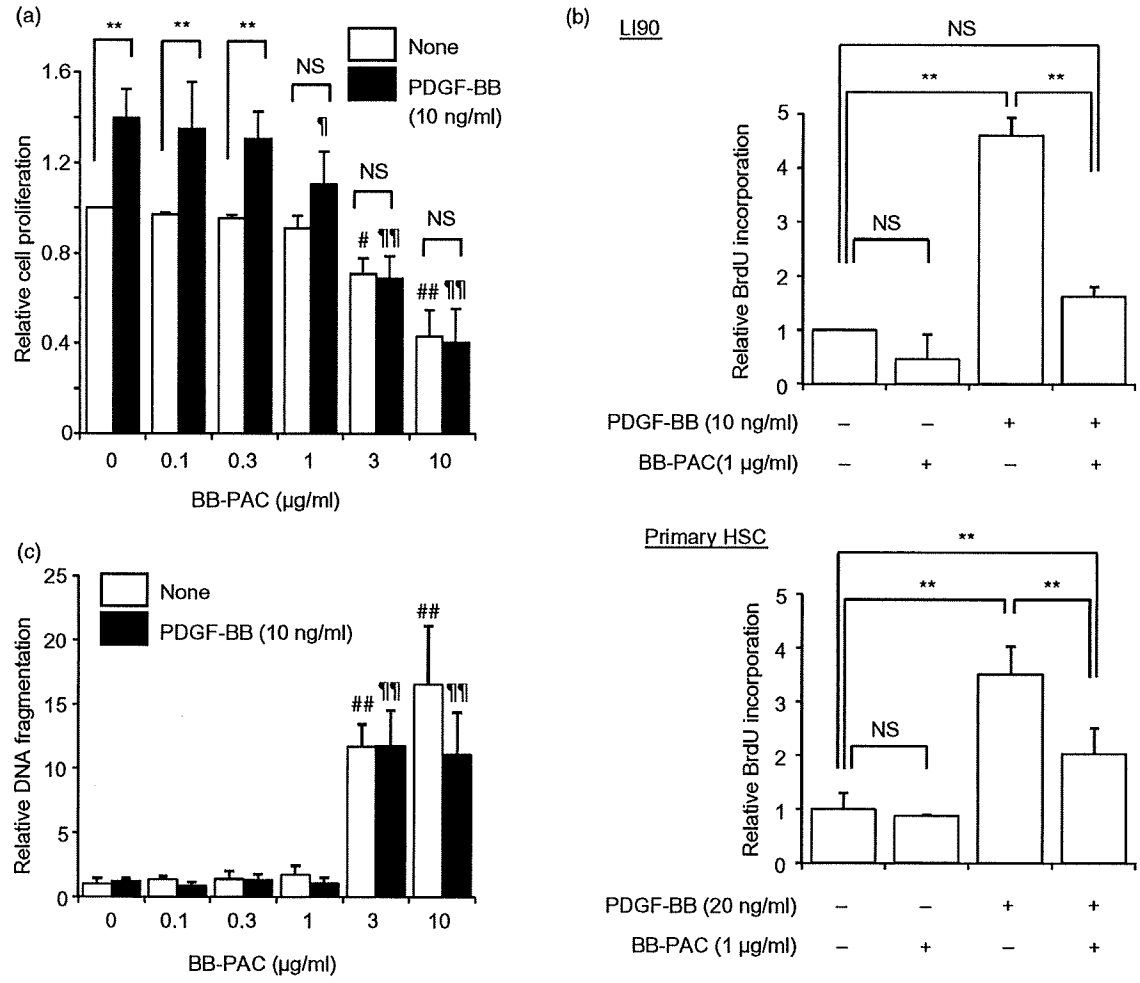

Figure 1 Suppressive effects of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-induced proliferation and DNA synthesis in LI90 hepatic stellate cells. (a) LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed 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 $¶P < 0.05$ and $¶¶P < 0.01$, 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-

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,

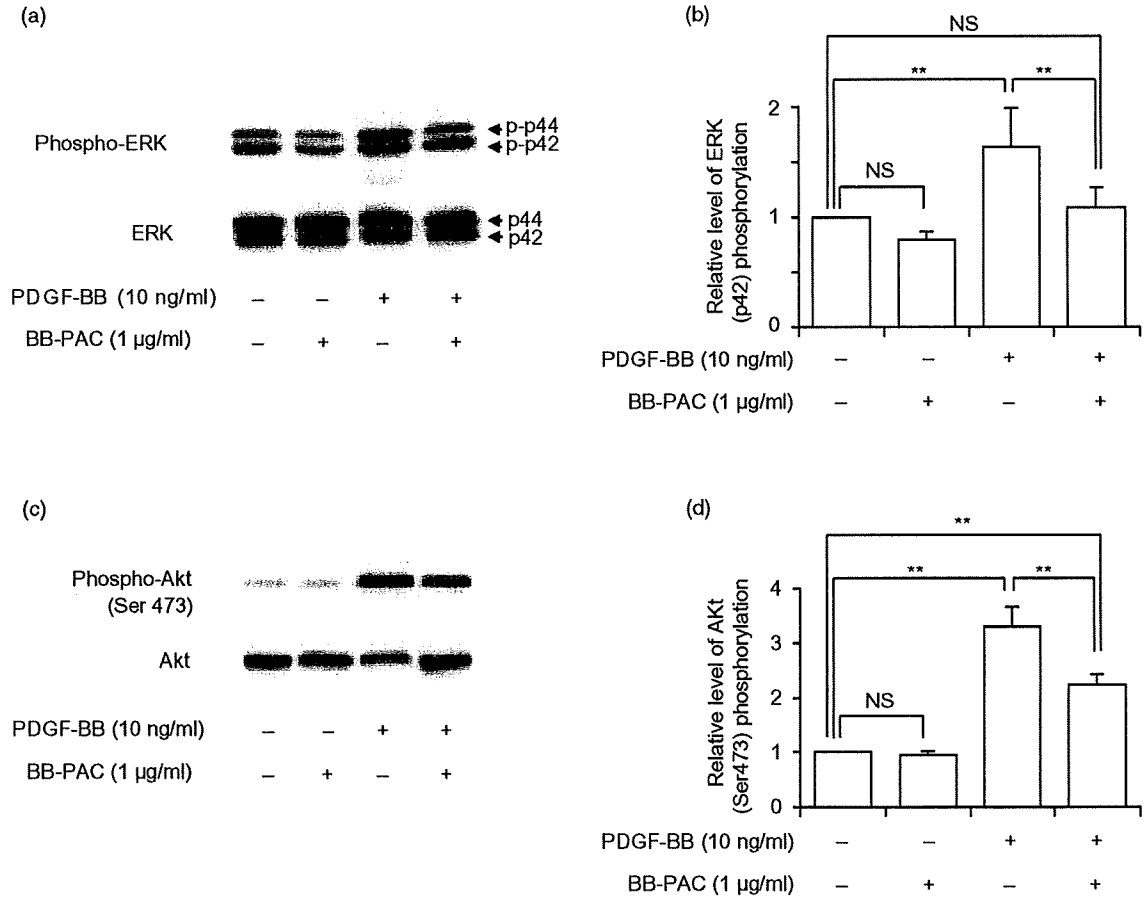

Figure 2 Effects of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-mediated phosphorylation of extracellular signal-regulated kinase (ERK) and Akt. LI90 cells were pre-incubated for 1 h with 1 μg/mL BB-PAC, followed by a 15-min incubation with or 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.

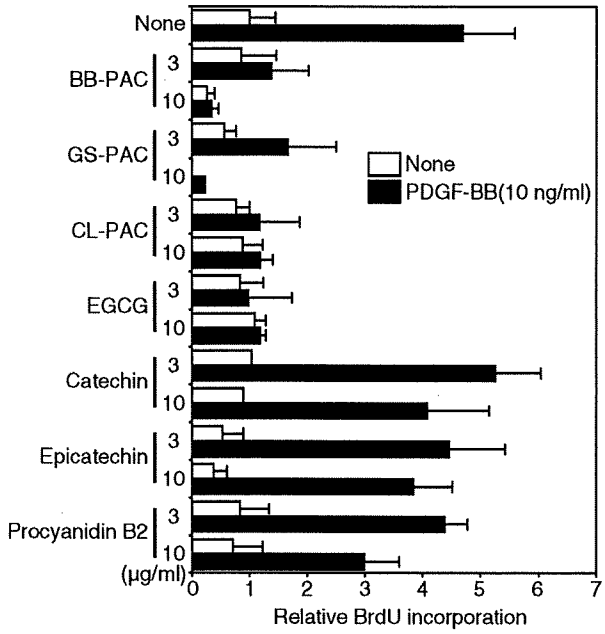

Figure 3 Comparison of the inhibitory effects of proanthocyanidin extracted from blueberry leaves (BB-PAC), PAC from grape seeds (GS-PAC) and *Croton lechleri* (CL-PAC) and monomeric and dimeric PAC. LI90 cells were pre-incubated for 1 h with 3 or 10 μg/mL BB-PAC, GS-PAC, CL-PAC, epigallocatechin-3-gallate (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

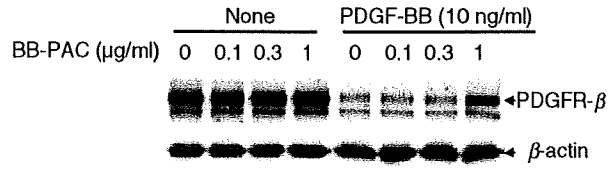

Figure 4 Effect of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-induced PDGF receptor-β (PDGFR-β) degradation. LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed by a 24-h incubation with or without 10 ng/mL 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.

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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Increased Rate of Death Related to Presence of Viremia Among Hepatitis C Virus Antibody–Positive Subjects in a Community-Based Cohort Study

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The overall mortality of patients infected with hepatitis C virus (HCV) has not been fully elucidated. This study analyzed mortality in subjects positive for antibody to HCV (anti-HCV) in a community-based, prospective cohort study conducted in an HCV hyperendemic area of Japan. During a 10-year period beginning in 1995, 1125 anti–HCV-seropositive residents of Town C were enrolled into the study and were followed for mortality through 2005. Cause of death was assessed by death certificates. Subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered as having hepatitis C viremia and were classified as HCV carriers; subjects who were negative for both HCVcAg and HCV RNA (i.e., viremia-negative) were considered as having had a prior HCV infection and were classified as HCV noncarriers. Among the anti–HCV-positive subjects included in the analysis, 758 (67.4%) were HCV carriers, and 367 were noncarriers. A total of 231 deaths occurred in these subjects over a mean follow-up of 8.2 years: 176 deaths in the HCV carrier group and 55 in the noncarrier group. The overall mortality rate was higher in HCV carriers than in noncarriers, adjusted for age and sex (hazard ratio, 1.53; 95% confidence interval, 1.13-2.07). Although liver-related deaths occurred more frequently among the HCV carriers (hazard ratio, 5.94; 95% confidence interval, 2.58-13.7), the rates of other causes of death did not differ between HCV carriers and noncarriers. Among HCV carriers, a higher level of HCVcAg (≥100 pg/mL) and persistently elevated alanine aminotransferase levels were important predictors of liver-related mortality. *Conclusion:* The presence of viremia increases the rate of mortality, primarily due to liver-related death, among anti–HCV-seropositive persons in Japan. (HEPATOLOGY 2009;50:393-399.)

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Hepatitis C virus (HCV) was identified 20 years ago. It is now known that between 50% and 85% of acute HCV infections become chronic[1-3]; after developing chronic infection, spontaneous HCV clearance is very rare. Approximately 170 million people worldwide are infected with HCV, and chronic HCV infection is a major health problem. HCV is a common cause of fatal liver disease, including liver cirrhosis and hepatocellular carcinoma (HCC). However, the liver-related mortality rate associated with chronic HCV infection is highly variable across different populations. In

Abbreviations: ALT, alanine aminotransferase; anti-HCV, antibody to HCV; CI, confidence interval; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcAg, hepatitis C virus core antigen; HR, hazard ratio; IFN, interferon.

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patients that have been infected for more than 20 years, the occurrence of liver cirrhosis, HCC, and liver disease–related mortality are reported to be 10%-50%, 1%-23%, and 4%-15%, respectively.[4-6]

The range in published HCV-related mortality rates is due in part to the variability in the natural history of HCV infection as well as in the subjects studied. Some HCV-positive individuals have persistently normal alanine aminotransferase (ALT) levels and exhibit no clinical symptoms. Persons with this phenotype were often not included in previous hospital-based studies that focused on liver-related mortality in patients with HCV-associated liver disease/cirrhosis.[4,5] A few studies have systematically examined the risk of causes of death after HCV infection in a community-based setting[7-9]; however, the status of HCV viremia was not clear in these studies. In addition, the age range of HCV-infected subjects followed for mortality can vary considerably, with some cohort studies conducted in subjects whose average age was younger than 45 years[1,10-13] and others among older individuals.[9,14,15] To overcome some of these limitations, we analyzed mortality in 1125 subjects positive for antibody to HCV (anti-HCV) with data on viremia status, who were enrolled in a population-based cohort study in an HCV hyperendemic area of Japan between 1995 and 2005. These subjects were followed prospectively until death or until the end of the study in December 2005.

Patients and Methods

Study Population. Since 1993, we have been following anti–HCV-seropositive residents in a hyperendemic area (Town C) of Japan. The overall prevalence of anti-HCV positivity is higher (20.6%) in this region than in the surrounding area.[16] Town C is a small town in mid-western Miyazaki Prefecture, Japan, and the Town C HCV Study is a cohort study examining the natural course of HCV infection.[17-20] A general health examination program, begun in 1993, has been conducted annually for residents over 20 years of age. An ultrasonography-based liver disease screening program was initiated in 1994 to detect HCC in Town C residents who were identified as positive for anti-HCV. A total of 1321 anti–HCV-positive residents were enrolled into the cohort from 1994 through the last liver disease screening in 2006. Informed consent was obtained from subjects at the time of enrollment. The study was approved by the human subjects committees of the Harvard School of Public Health, the University of Miyazaki Faculty of Medicine, the Boston University School of Public Health, and the Kagoshima University Graduate School of Medical and Dental Sciences.

Our analysis focuses on the 1125 subjects with hepatitis C viremia data between 1995 and February of 2005, who were followed for mortality from the beginning of 1996 through the end of 2005. Anti–HCV-seropositive subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered to be persistently infected with HCV and were classified as HCV carriers. Anti–HCV-positive subjects who were negative for HCVcAg and HCV RNA were assumed to have had a prior HCV infection and were classified as noncarriers. Subjects who underwent oral or intravenous administration of medical herbs or other palliative therapies or who had received interferon therapy were not excluded from the analyses. A subgroup analysis was conducted on HCV carrier subjects with at least three independent ALT measurements obtained at an annual general health examination or liver disease screening; ALT levels $\geq$35 were considered abnormal.

Follow-Up. For this analysis, follow-up started at the date of first HCV viremia measurement (baseline) and ended at date of death or December 31, 2005. During the course of the study, 12 residents moved to other areas, and their follow-up time was censored at that point; no other subjects were lost to follow-up. Cause of death was based on the information from the death certificate and was classified into one of seven categories: HCC, liver disease excluding HCC, neoplasms excluding HCC (i.e., other neoplasms), stroke, heart disease, pulmonary disease excluding lung cancer, and other/unknown causes.

Laboratory Methods. Serum anti-HCV antibodies were detected using second-generation enzyme immunoassay testing (Immunocheck F-HCV antibody; International Reagents Co., Kobe, Japan) or third-generation chemiluminescence enzyme immunoassays (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan). In the anti–HCV-positive residents, serum levels of HCVcAg were tested with a fluorescence enzyme immunoassay (Immunocheck F-HCV Ag Core; International Reagents Co., Kobe, Japan),[21] with a detection threshold of 8 pg/mL. The presence of HCV RNA was determined by reverse transcription polymerase chain reaction (Amplicor HCV Monitor, version 1.0 [Nippon Roche, Tokyo, Japan] or version 2.0 [Nippon Roche or Roche Diagnostics K.K., Tokyo, Japan]) in study subjects whose HCVcAg levels were below the detection threshold.

Serologically defined HCV genotype (HCV serotype) was determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was examined (HCV Core Genotype; SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b

Table 1. Baseline Characteristics of Anti-HCV Antibody-Positive Subjects in Town C HCV Study

Characteristics	All Patients (n = 1125)	HCV Carriers (n = 758)	HCV Noncarriers (n = 367)	*P* Value
Age (years)				
Mean (±SD)	64.2 (±11.1)	64.9 (±10.6)	62.6 (±11.9)	0.007
Range	28-97	32-97	28-90	
Sex				
Male	456 (40.5%)	313 (41.3%)	143 (39%)	0.46
Female	669 (59.5%)	445 (58.7%)	224 (61%)	
ALT (IU/L)	40 ± 42.8 (1062)	47 ± 47.5 (719)	25.3 ± 25 (343)	<0.001
GGT (IU/L)	35.8 ± 46 (912)	39.1 ± 50.7 (612)	29.2 ± 33.6 (300)	<0.001
HCV core antigen level (pg/mL)				
Mean (±SD)		207.5 (±208.4)	-	
Median		140	-	
Range		20-1445	-	
HCV serotype				
I		463 (64.5%)	-	
II		220 (30.6%)	-	
Indeterminate		35 (4.9%)	-	
HBs antigen				
Positive	6 (0.6%)	4 (0.6%)	2 (0.6%)	0.99
Negative	948 (99.4%)	638 (99.4%)	310 (99.4%)	
History of alcohol intake				
Daily	365 (34.3%)	236 (32.9%)	129 (37.2%)	
Occasionally	206 (19.4%)	140 (19.5%)	66 (19.0%)	0.37
None	493 (46.3%)	341 (47.6%)	152 (43.8%)	
History of blood transfusion				
Yes	165 (15.7%)	101 (14.3%)	64 (18.6%)	0.07
No	885 (84.3%)	605 (85.7%)	280 (81.4%)	

Abbreviations: ALT, alanine aminotransferase; GGT, gamma-glutamyltranspeptidase; HBs antigen, hepatitis B surface antigen; HCV, hepatitis C virus.

with serotype II. No other HCV genotype was detected in this study population.

Statistical Analysis. One-factor analysis of variance, χ^2 tests, Fisher's exact tests, and the Mann-Whitney U tests were used, when appropriate, for statistical comparisons of the baseline characteristics of the HCV carrier and noncarrier groups of subjects. Cox proportional hazards regression was used to obtain hazard ratios (HRs) and 95% confidence intervals (CIs) that were adjusted for age and sex; for the analyses of cause-specific mortality, subjects who died from a different cause were censored at the time of death. The cumulative incidence of death was analyzed by the Kaplan-Meier method, and differences in the survival curves were evaluated by the log-rank test. Statistical analyses were performed using Statistical Analysis System (SAS, version 9.1; SAS Institute, Cary, NC), 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 to be statistically significant.

Results

Demographic Characteristics of Study Subjects. As shown in Table 1, 758 (67.4%) of the anti–HCV-positive subjects were HCV carriers (i.e., positive for HCVcAg or HCV RNA), with a mean age at enrollment of 64.9 years. The HCV noncarrier group, who were considered to have had a prior HCV infection, included 367 subjects whose mean age at enrollment was 62.6 years. On average, the HCV carriers were older and had higher levels of ALT and gamma-glutamyltransferase (GGT) than the noncarriers, at baseline. In contrast, there were no significant differences between the two groups with respect to sex, alcohol intake, or history of blood transfusions. The number of subjects positive for hepatitis B surface antigen was small and not significantly different between the two groups. Sixty-seven subjects reported that they had previously received interferon (IFN) therapy, all of whom were categorized as HCV carriers when they entered the study. Fifteen of these subjects were treated prior to entering the study, five were treated during the study, and one was treated both prior to and during the study; for the other 46 subjects, the timing of IFN treatment was unknown. Although the results of IFN therapy could not be fully determined for these 67 subjects, 41 of 44 with available data in 2005 were positive for HCV RNA at that time and only three (7%) were negative for HCV RNA.

Overall and Cause-Specific Mortality. Over an average of 8.2 years of follow-up, 231 deaths occurred among the 1125 subjects (Table 2). The overall mortality

Table 2. Cause of Death in Subjects Positive for Anti-HCV Antibody

Cause of Death	All Patients	HCV Carriers	HCV Noncarriers
All causes	231	176	55
1. All liver-related deaths	76	70	6
a. HCC	45	41	4
b. Non-HCC	31	29	2
2. Neoplasms excluding HCC	41	28	13
3. Stroke	30	20	10
4. Heart disease	22	13	9
5. Pulmonary disease excluding lung cancer	32	22	10
6. Other/unknown	30	23	7

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

rate was 25.0 per 1000 person-years in this study population. Most deaths were liver-related, with 45 due to HCC and 31 to other liver diseases including cirrhosis, hepatic failure, and ruptured esophageal varix. The next most frequent cause of death was other neoplasms (n = 41), followed by pulmonary disease excluding lung cancer (n = 32), stroke (n = 30), other/unknown causes (n = 30), and heart disease (n = 22).

Of the 231 deaths, 176 were in the HCV carrier group and 55 were in the noncarrier group (Table 2). After adjusting for age and sex, HCV carriers had a significantly higher overall mortality rate (HR, 1.53; 95% CI, 1.13-2.07), compared to HCV noncarriers (Table 3). The elevated mortality rate among the subjects with evidence of HCV viremia was due to a much higher occurrence of liver-related deaths (HR, 5.94; 95% CI, 2.58-13.7). In contrast, HCV viremia was not significantly associated with death from other malignancies, stroke, heart disease, or pulmonary disease. The cumulative risk of death, based on Kaplan-Meier estimates, was 28.0% for the HCV carrier group and 17.9% for the HCV noncarrier group over 10.3 years (Fig. 1), a statistically significant difference ($P < 0.001$).

Table 3. The Association of HCV Viremia with Causes of Mortality Among Anti-HCV Antibody-Positive Subjects in Town C HCV Study

Cause of Death	HR	95% CI
All causes	1.53	(1.13, 2.07)
1. All liver-related deaths	5.94	(2.58, 13.7)
a. HCC	4.85	(1.73, 13.5)
b. Non-HCC	8.11	(1.94, 33.8)
2. Neoplasms excluding HCC	1.04	(0.54, 2.02)
3. Stroke	0.89	(0.41, 1.90)
4. Heart disease	0.68	(0.29, 1.60)
5. Pulmonary disease excluding lung cancer	1.05	(0.50, 2.22)
6. Other/unknown	1.59	(0.68, 3.71)

Abbreviations: CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio.

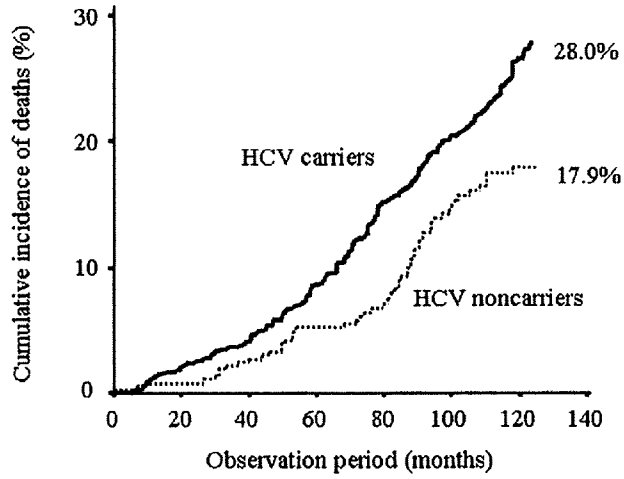

Fig. 1. Cumulative incidence of all-cause deaths in HCV carriers and noncarriers.

Predictors of Mortality Among HCV Carriers. The age-adjusted and sex-adjusted association between HCV serotype and HCVcAg level and mortality was examined among the subjects with HCV viremia. Compared to HCV serotype II, those with serotype I infection did not have a higher rate of overall (HR, 1.04) or liver-related mortality (HR, 1.12); however, having an indeterminate HCV serotype was related to both overall (HR, 3.59; 95% CI, 2.1-6.1) and liver-related death (HR, 2.12; 95% CI, 0.78-5.75). Of note, both serotype I infection (HR, 2.21; 95% CI, 0.91-5.33) and indeterminate HCV infection (HR, 3.89; 95% CI, 0.97-15.7) appeared to increase HCC mortality. In addition, a significantly increased rate of liver-related death was associated with a higher level (≥100 pg/mL) of HCVcAg (HR, 1.81; 95% CI, 1.08-3.06); the effect of higher HCVcAg level was stronger with respect to other liver-related death (HR, 2.58; 95% CI, 1.04-6.41) than to HCC death (HR, 1.48; 95% CI, 0.77-2.82). HCVcAg level had no effect on overall mortality among the HCV carriers (HR, 1.06).

In a subgroup analysis of 719 HCV carrier subjects who had data for at least three separate ALT measurements, 173 had persistently normal ALT levels while 141 had persistently abnormal levels. Subjects whose ALT levels fluctuated were not included in the analysis. Adjusting for age and sex, overall mortality (HR, 2.23; 95% CI, 1.37-3.61) and liver-related death (HR, 11.0; 95% CI, 4.35-27.9) were significantly higher for HCV carriers with persistently elevated ALT than for those with persistently normal ALT. The strongly elevated rate of liver-related mortality was evident for death due to both HCC (HR, 11.1) as well as other liver-related disease (HR, 14.5).

Discussion

Our study indicated that liver-related mortality is strongly associated with the presence of HCV viremia among persons who are seropositive for anti-HCV antibodies and that HCVcAg and ALT levels were predictors of liver-related mortality in HCV carriers. In this study population, the age distribution of anti–HCV-positive subjects, the prevalence of viremia, and the frequency of HCV serotype I were similar to previously reported data in Japan.[22-25] Japan has the highest incidence rate of HCC attributed to HCV infection among developed countries. Tanaka et al. estimated that HCV infection was spread in Japan during the 1920s, whereas HCV was widely disseminated in the United States in the 1960s.[26] The authors suggested that the HCC burden in the United States will likely increase in the next two or three decades, possibly to a level equal to that currently experienced in Japan.

Several studies have examined mortality in patients with HCV. Seeff et al. provided mortality data for 222 transfusion-associated hepatitis C cases and 377 control patients after approximately 25 years of follow-up.[27] Kamitsukasa et al. also reported mortality data for 302 HCV-infected patients with tuberculosis sequelae who had received a blood transfusion.[15] Although both studies showed that liver-related mortality was significantly higher in the disease groups than in the control groups, liver-related mortality was not the main cause of death. Kamitsukasa et al. reported that the main cause of death for approximately 45% of the patients in their study was tuberculosis sequelae.[15] Similar results were obtained in patients with inherited bleeding disorders and hepatitis C, where the main cause of death was human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS).[28] Moreover, there was no significant difference between patients with and without hepatitis C in the overall mortality rates in the study by Seeff et al. In contrast, our study showed that all-cause mortality and liver-related mortality with or without HCC were significantly higher in the HCV carrier group than in the noncarrier group. The incidence of HCC in Caucasian patients with HCV-related cirrhosis has been reported to be 1.2% in the United States,[29] whereas the incidence in Japanese patients is reportedly between 6% and 7%.[30] Furthermore, HCV-related cases in some studies included subjects with previous HCV infections.[15,27] Ethnic-dependent and racial-dependent variation in the rates of HCC, the composition of the comparisons groups, and/or complications unrelated to liver disease, such as tuberculosis sequelae or HIV/AIDS, may have resulted in differences in the patient prognoses between our study and previous studies.

It has been reported that HCC was the main cause of liver-related death in patients with compensated cirrhosis due to HCV infection.[31,32] Kasahara et al. found that 74% of liver-related death in patients with chronic hepatitis C who had not received IFN therapy was due to HCC.[33] Although HCC was more frequently observed than other liver-related deaths in our study, the proportion of HCC among all liver disease deaths (59% in the HCV carrier group) was relatively low compared to that study.[33] This occurrence may have been because the causes of death were obtained from death certificates in our study and cases of severe hepatic failure due to HCC may have been classified as liver disease excluding HCC.

A large community-based linkage study that included 78,438 individuals with hepatitis C indicated that the risk of dying from drug-related causes was significantly greater than from liver-related causes; however, the incidence of liver-related deaths was greater than that of drug-related deaths in patients older than 45 years.[7] In addition, other studies have shown that age appears to be an important risk factor that affects HCC development[14] and that the risk of cirrhosis is related to the patient's age at the time of infection and to disease activity.[34,35] These reports, which focused on patients with transfusion-associated chronic hepatitis C, suggest that the younger the patients are at the time of infection, the lower the rate of progression. Although the exact dates of infection and HCC diagnosis were not clear in our study population, the median age at enrollment was older than 60 years. Thus, the incidence of liver-related deaths might be expected to be greater than deaths from other causes.

In our study, HCV serotype I, which included HCV genotypes 1a and 1b, was found in 64.5% of the HCV carrier subjects in whom serotype was measured, whereas serotype 2, which included genotypes 2a and 2b, was detected in 30.6% of patients. These results agree with the overall distributions of HCV genotypes and serotypes in the entire Japanese population, which show that genotype 1b is the most prevalent genotype at 70%.[36] Several studies have demonstrated that genotype 1b is associated with severe liver disease, including cirrhosis and HCC.[37,38] In this study, there was an apparent association between HCV serotype I infection and mortality due to HCC. Other studies, however, have not found an effect of HCV genotype on liver disease development.[39,40] In addition, although an association of indeterminate serotype with mortality was observed (HR = 3.6), the reason for this finding is not clear. A larger study is needed to elucidate the role of genotype in the prognosis of HCV infection.