Hamaguchi et al., the Brinkman index did not differ between the NAFLD and non-NAFLD groups at baseline, which is inconsistent with our results. This may be because the mean period of 414 days in Hamaguchi et al. was not long enough to investigate the influence of smoking. In addition, the results of our analysis are very important because a significant association between cigarette smoking and the development of NAFLD was present in a population in which a strong association between the development of NAFLD and metabolic syndrome risk factors was evident.

Risk factor modification such as weight loss and medication for insulin resistance and dyslipidemia should cure or prevent NAFLD. In this study, we found that cigarette smoking is a risk factor for NAFLD. Therefore, smoking cessation is likely to decrease the risk of NAFLD among current smokers. However, in our subanalysis of three groups that included new quitters, and continuing smokers and nonsmokers, smoking cessation seemed to convey a higher risk for developing NAFLD than continuing to smoke (Table 7). It has been reported that smoking is a risk factor for diabetes mellitus, but smoking cessation is also associated with substantial weight gain and may lead to a higher short-term risk of type 2 diabetes [17, 29]. Despite the fact that we were unable to clarify the effect of smoking cessation on the treatment or development of NAFLD because (1) our study population was too small, (2) the date when smoking was stopped was not considered, and (3) metabolic syndrome risk factors that arose or were cured during the 10-year period and the effects of treatment on these diseases were not fully considered, we speculate that smoking cessation without weight gain is likely to be beneficial for patients with NAFLD.

Subjects who drank ≤20 g/day of alcohol were included in our study. In a cross-sectional study in Japanese men, low alcohol consumption (40–140 g/week) significantly reduced the incidence of fatty liver (AOR = 0.824 [95% CI 0.683–0.994]) [20]. We have also found that alcohol intake may inhibit the development of fatty liver through an association of alcohol drinking pattern with obesity [48]. In the current study, intake of a small amount of alcohol had an inhibitory effect on NAFLD development in multivariate analysis that included the number of metabolic syndrome risk factors as a variable (Table 5). In animal models, cigarette smoking and alcohol intake have been shown to contribute to the development and exacerbation of fatty liver [49]. Thus, further studies of the apparent synergistic effect of alcohol intake and cigarette smoking on NAFLD are required in humans.

There are several limitations in this study. Firstly, ultrasonography is effective for diagnosing fatty liver, but detecting fatty liver in patients with ≤30% liver fat or in obese patients is relatively difficult [4]. Moreover, as simple fatty liver and NASH cannot be distinguished by ultrasonography, an association of cigarette smoking with fatty liver severity could not be shown. Secondly, although the frequency of NAFLD development in continuing smokers was higher than that in continuing nonsmokers in the limited group of subjects, the difference was not statistically significant in multivariate analysis (Table 5). This may be due to the modest sample size. Finally, our study was a follow-up study of a 10-year interval in which the data was obtained at only two points, in 1998 and in 2008. Longitudinal studies such as those involving the evaluation of NAFLD incidence and smoking patterns over time using several points during a 10-year period may provide more convincing evidence of the contribution of cigarette smoking to NAFLD development.

In conclusion, metabolic syndrome risk factors increase the risk of NAFLD. In this retrospective study, we found that cigarette smoking, a risk factor for metabolic syndrome, was also a risk factor for NAFLD development independent of metabolic syndrome risk factors. In addition, although NAFLD development became more likely as the Brinkman index increased, smoking cessation was also a likely risk factor for NAFLD development, which is partially explainable by an increase in BMI. Therefore, having never smoked is important for the prevention of NAFLD in nonsmokers, and additional treatment or prevention of metabolic syndrome risk factors may be necessary to encourage the cessation of cigarette smoking to treat or prevent NAFLD in smokers.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Alcohol drinking patterns and the risk of fatty liver in Japanese men

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Abstract

Background Alcohol is considered to be a major cause of fatty liver (FL). In contrast, however, recent investigations have suggested that moderate alcohol consumption is protective against FL. To clarify the role of alcohol consumption in FL development, we examined the association between drinking patterns and FL prevalence.

Methods We enrolled 9,886 male participants at regular medical health checks. Each subject's history of alcohol consumption was determined by questionnaire. The subjects were classified according to alcohol consumption as non-, light, moderate, and heavy drinkers (0, <20, 20–59, and ≥60 g/day, respectively). FL was defined by ultrasonography. Independent predictors of FL were determined by logistic regression analysis.

Results The prevalence of FL displayed a "U-shaped curve" across the categories of daily alcohol consumption (non-, 44.7%; light, 39.3%; moderate, 35.9%; heavy drinkers, 40.1%; $P < 0.001$). The prevalence of FL was associated positively with body mass index and other obesity-related diseases and inversely with alcohol consumption (light, odds ratio [OR] 0.71, 95% confidence interval [CI] 0.59–0.86; moderate, OR 0.55, CI 0.45–0.67; heavy, OR 0.44, CI 0.32–0.62) as determined by multivariate analysis after adjusting for potential confounding variables. In addition, examination of drinking patterns (frequency and volume) revealed that the prevalence of FL was inversely associated with the frequency of alcohol consumption (≥21 days/month) (OR 0.62, CI 0.53–0.71) but not with the volume of alcohol consumed.

Conclusions Our observations suggest that alcohol consumption plays a protective role against FL in men, and consistent alcohol consumption may contribute to this favorable effect.

Keywords Fatty liver · Alcohol consumption · Nonalcoholic fatty liver disease · Metabolic syndrome

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Introduction

Fatty liver (FL) disease is commonly divided into nonalcoholic (NAFLD) and alcoholic (AFLD) FL disease categories. NAFLD is an increasingly recognized condition, predominantly linked to metabolic syndrome, which, in turn, is associated with obesity and insulin resistance [1–4]. The clinical importance of NAFLD is due to its wide

spectrum of histological damage, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can lead to cirrhosis, hepatocellular carcinoma, and hepatic failure [5]. Alcohol dehydrogenase-mediated ethanol metabolism generates a reduced form of nicotinamide adenine dinucleotide (NADH), which promotes steatosis by stimulating the synthesis of fatty acids and opposing their oxidation. The hepatic lipogenic pathway is activated after the consumption of 24 g of ethanol per day [6]. Alcohol intake is a risk factor for both hypertriglyceridemia and FL [7, 8], and daily intake as low as 20–30 g of alcohol per day may be sufficient to cause alcohol-induced liver disease in some cases.

In contrast, recent investigations have reported a protective role of moderate alcohol consumption against FL [9–13]. Light to moderate alcohol consumption is associated with lower cardiovascular mortality [14–16] and a reduced risk of developing type 2 diabetes [17–19]. Mechanisms proposed to explain this observation include improved lipid profiles, especially high-density lipoprotein-cholesterol [15], and increased insulin sensitivity [19–21]. The mechanism of protection against FL, however, remains unclear.

In this study, to elucidate the relationship between the quantity and patterns of alcohol consumption and FL prevalence, we conducted a cross-sectional study of a male Japanese population.

Subjects, materials, and methods

Study population

The study subjects were Japanese men aged 30–69 years, who participated in regular health check-ups from April 2000 to March 2007. Of the initial 10,283 candidates, 204 (2.0%) hepatitis B virus surface antigen (HBsAg)-positive and 195 (1.9%) hepatitis C virus antibody (HCV Ab)-positive subjects were excluded from the analysis (two subjects were positive for both HBsAg and HCV Ab). The remaining 9,886 men were enrolled in this study. Serological testing for HBsAg and HCV Ab was performed by enzyme immunoassay and enzyme-linked immunosorbent assay, respectively. This study, conducted at Kagoshima Kouseiren Medical Health Care Center, was approved by the ethics committee of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare.

A diagnosis of FL was made, using ultrasonography (SSA-250A and SSA-700A; Toshiba, Tokyo, Japan; Logic 400; GE Healthcare Japan, Tokyo, Japan), from findings of bright liver (increased echogenicity) with liver–kidney contrast (increased echogenicity of the liver in comparison to the right kidney). Body mass index (BMI) was calculated from the equation: body weight (kg)/height^2 (m^2). Body composition was categorized according to the Western Pacific Region of WHO criteria pertaining to obesity (WPRO criteria): BMI <18.5 kg/m^2 (underweight), 18.5–22.9 kg/m^2 (normal weight), 23.0–24.9 kg/m^2 (overweight), and 25 kg/m^2 or more (obese). Venous blood samples were taken from all subjects before nine o'clock after an overnight fast and were analyzed immediately. Alanine aminotransferase (ALT), aspartate aminotransaminase (AST), and γ-glutamyl transpeptidase (γ-GTP) activities and the concentrations of total cholesterol, triglycerides, and glucose were measured by standard laboratory procedures. High-density lipoprotein cholesterol (HDL-C) levels were determined by direct homogeneous assay of serum samples using detergents (Sekisui Medical, Tokyo, Japan).

The subjects were investigated for the presence of concomitant metabolic abnormalities. Hypertension was defined as being present in patients on medication for hypertension, those with systolic blood pressure >130 mmHg, and/or those with diastolic blood pressure >85 mmHg. Dyslipidemia was defined as being present in patients on medication for dyslipidemia, those with total cholesterol >220 mg/dl, triglycerides >150 mg/dl, and/or HDL-C <40 mg/dl. Diabetes mellitus (DM) was considered to be present in patients on medication for DM and/or in those with fasting blood glucose >125 mg/dl. ALT or AST elevations were defined as levels over 30 IU/L.

Using a common questionnaire, a history of alcohol intake was determined by a public health nurse without knowledge of the status of FL. Each subject reported their current frequency of alcohol consumption (A, 0; B, 1–5; C, 6–10; D, 11–20; E, ≥21 days/month) and volume of alcohol drunk per day (a, <20; b, 20–39; c, 40–59; d, 60–99; e, ≥100 g/day). All participants except former drinkers were divided into four groups according to the frequency (A–E) and volume (a–e) of alcohol consumption, as follows: non-drinkers, A; light drinkers, B-a–e, C-a–c, D-a or -b, E-a; moderate drinkers, C-d or -e, D-c or -d, E-b or -c; and heavy drinkers, D-e, E-d or -e (daily volumes consumed by non-, light, moderate, and heavy drinkers were approximately 0, <20, 20–59, and ≥60 g/day, respectively).

Statistical analysis

The distributions of each variable were compared between FL subjects and non-FL subjects. Continuous variables, including age, BMI, ALT, AST, γ-GTP, triglycerides, and HDL-C, were analyzed by *t*-test, and categorical variables were examined by the χ^2 test. In addition, associations of alcohol consumption (non-, light, moderate, and heavy drinkers) with clinical factors and smoking status were

examined by analysis of variance (ANOVA) or the χ^2 test. Maximum likelihood odds ratios (ORs) for FL risk and their 95% confidence intervals (95% CIs) were calculated using logistic regression models. Statistical analyses were performed using STATA version 9.2 (StataCorp, TX, USA). All *P* values presented are two-sided.

Results

Subject description

Among the 9,886 subjects, 3,816 men (38.6%) met the criteria for FL (Table 1). The subjects' characteristic

Table 1 Characteristic features of study subjects

	All	Fatty liver		*P* value
		(−)	(+)	
Number (%)	9886 (100%)	6070 (100%)	3816 (100%)	
Age (years)	50.7 [50.5, 50.9]	51.4 [51.2, 51.7]	49.5 [49.2, 49.8]	<0.001*
30–39	12.0%	11.4%	12.8%	<0.001**
40–49	31.2%	28.2%	36.0%	
50–59	32.5%	32.6%	32.5%	
60–69	24.3%	27.8%	18.6%	
BMI	23.7 [23.6, 23.7]	22.5 [22.5, 22.6]	25.6 [25.5, 25.7]	<0.001*
<18.5	2.4%	3.9%	0.1%	<0.001**
18.5–22.9	37.7%	51.9%	15.2%	
23.0–24.9	26.9%	27.3%	26.4%	
25–	33.0%	17.0%	58.4%	
Laboratory data				
ALT (IU/L)	25.7 [25.5, 36.7]	21.6 [21.4, 21.8]	33.9 [33.3, 34.4]	<0.001*
AST (IU/L)	24.7 [24.5, 24.9]	23.4 [23.2, 23.6]	27.0 [26.7, 27.3]	<0.001*
γ-GTP (IU/L)	36.2 [35.6, 36.7]	30.7 [30.1, 31.3]	47.0 [45.9, 48.1]	<0.001*
Triglycerides (mg/dl)	120 [119, 122]	102 [100, 103]	156 [153, 159]	<0.001*
HDL-C (mg/dl)	53.9 [53.6, 54.2]	57.0 [56.7, 57.4]	49.3 [48.9, 49.6]	<0.001*
Presence of clinical manifestation				
Fatty liver	38.6%			
ALT elevation	32.7%	18.8%	54.8%	<0.001***
AST elevation	21.2%	15.3%	30.5%	<0.001***
Hypertension	46.5%	42.1%	53.5%	<0.001***
Dyslipidemia	55.9%	45.9%	71.8%	<0.001***
Diabetes mellitus	11.4%	7.9%	16.8%	<0.001***
Smoking status				
Never smoker	29.4%	29.7%	28.8%	0.001***
Former smoker	31.2%	29.9%	33.3%	
Current smoker	39.4%	40.4%	37.9%	
Alcohol consumption				
Never drinker	8.6%	7.7%	10.0%	<0.001***
Former drinker	2.9%	2.6%	3.3%	
Light drinker	45.9%	45.4%	46.7%	
Moderate drinker	39.1%	40.8%	36.4%	
Heavy drinker	3.5%	3.4%	3.6%	

Data are presented as geometric means [corresponding 95% confidence intervals] or proportions

BMI body mass index, *ALT* alanine aminotransferase, *AST* aspartate aminotransaminase, *γ-GTP* γ-glutamyl transpeptidase, *HDL-C* high-density lipoprotein cholesterol

* *P* values were obtained by *t* test

** *P* values for trend were obtained by likelihood ratio test using a logistic regression model

*** *P* values were obtained by χ^2 test

features and the differences in biological parameters in relation to FL are summarized in Table 1. There were 1,131 (11.4%) non-drinkers, including 284 former drinkers; 4,540 (45.9%) light drinkers, 3,868 (39.1%) moderate drinkers; and 347 (3.5%) heavy drinkers.

The subjects with FL were significantly younger than the the subjects without FL ($P < 0.001$). BMI was significantly higher in FL (+) subjects in comparison to FL (−) subjects ($P < 0.001$). ALT, AST, and two parameters typically increased in association with alcohol consumption, γ-GTP and triglycerides, were significantly higher in FL (+) subjects in comparison to those who were FL (−) (all $P < 0.001$). In contrast, HDL-C, another parameter correlated with alcohol consumption, was lower in the FL (+) subjects than in FL (−) subjects ($P < 0.001$).

There was a U-shaped association between the prevalence of FL and categories of alcohol consumption ($P < 0.001$; Table 2). The prevalence of FL was highest in never drinkers among the five categories of alcohol consumption. AST, γ-GTP, triglycerides, and HDL-C, which

Table 2 Comparison of characteristic features across the categories of alcohol consumption

	Never drinker	Former drinker	Light drinker	Moderate drinker	Heavy drinker	*P* value
Number	847 (100%)	284 (100%)	4,540 [100%)	3,868 (100%)	347 (100%)	
Age (years)	51.6 [50.9, 52.3]	54.0 [52.8, 55.2]	50.4 [50.1, 50.7]	50.7 [50.4, 51.0]	48.1 [47.3, 48.9]	<0.001*
30–39	11.5%	7.8%	14.0%	10.0%	11.8%	<0.001**
40–49	28.7%	23.9%	30.6%	32.4%	39.2%	
50–59	30.0%	27.8%	29.4%	36.4%	40.6%	
60–	29.9%	40.5%	26.0%	21.2%	8.4%	
BMI (kg/m^2)	23.7 [23.5, 24.0]	23.6 [23.2, 24.0]	23.7 [23.6, 23.8]	23.6 [23.5, 23.7]	23.9[23.5, 24.2]	0.031*
<18.5	2.6%	4.2%	2.5%	2.0%	2.9%	0.048**
18.5–22.9	37.4%	38.4%	37.0%	38.9%	34.0%	
23.0–24.9	24.6%	25.0%	26.6%	27.9%	28.2%	
25.0–	35.4%	32.4%	33.9%	31.2%	34.9%	
Laboratory data						
ALT (IU/L)	25.4 [24.6, 26.8]	28.0 [26.2, 29.8]	25.4 [25.0, 25.7]	25.6 [25.2, 26.0]	30.5 [28.9, 32.3]	<0.001**
AST (IU/L)	22.7 [22.2, 23.1]	24.3 [23.3, 25.4]	23.7 [23.5, 23.9]	26.0 [25.7, 26.3]	30.5 [29.1, 32.0]	<0.001**
Fatty liver (−)	21.1 [20.6, 21.6]	22.4 [21.3, 23.6]	22.4 [22.1, 22.6]	24.7 [24.4, 25.0]	28.5 [27.1, 30.0]	<0.001***
Fatty liver (+)	24.7 [23.9, 25.6]	27.0 [25.2, 28.9]	25.8 [25.5, 26.2]	28.5 [28.0, 29.1]	33.8 [31.1, 36.8]	<0.001***
γ-GTP (IU/L)	22.5 [21.6, 23.3]	25.8 [23.9, 27.9]	30.2 [29.6, 30.8]	48.1 [46.9, 49.2]	67.1 [61.4, 73.4]	<0.001**
Fatty liver (−)	18.1 [17.3, 18.9]	21.2 [19.3, 23.3]	25.3 [24.8, 26.0]	40.8 [39.6, 42.0]	57.0 [50.9, 63.8]	<0.001***
Fatty liver (+)	29.2 [27.6, 31.0]	33.4 [29.9, 37.3]	39.7 [38.5, 40.9]	64.5 [62.1, 67.0]	85.8 [74.9, 98.3]	<0.001***
Triglycerides (mg/dl)	116 [111, 120]	107 [100, 113]	115 [113, 116]	127 [124, 130]	147 [137, 158]	<0.001**
HDL-C (mg/dl)	48.2 [47.5, 48.9]	49.8 [48.3, 51.2]	52.4 [52.0, 52.7]	57.1 [56.6, 57.5]	58.2 [56.6, 59.7]	<0.001**
Presence of clinical manifestation						
ALT elevation	32.4%	37.3%	31.2%	33.0%	45.5%	<0.001**
AST elevation	13.8%	20.4%	17.1%	26.3%	37.2%	<0.001**
Dyslipidemia	60.3%	56.3%	53.8%	56.8%	62.5%	<0.001**
Fatty liver	45.1%	43.7%	39.3%	35.9%	40.1%	<0.001**
Hypertension	37.4%	44.7%	42.6%	52.2%	58.5%	<0.001**
Diabetes mellitus	11.3%	18.0%	10.2%	12.3%	11.5%	<0.001**
Smoking status						
Never smoker	37.5%	36.6%	36.8%	19.6%	15.6%	<0.001**
Former smoker	24.1%	39.8%	30.0%	33.6%	30.3%	
Current smoker	38.4%	23.6%	33.2%	46.8%	54.2%	

Data are presented as geometric means [corresponding 95% confidence intervals] or proportions

BMI body mass index, *ALT* alanine aminotransferase, *γ-GTP* γ-glutamyl transpeptidase, *AST* aspartate aminotransaminase, *HDL-C* high-density lipoprotein cholesterol

* *P* values obtained by analysis of variance (ANOVA) after logarithmic transformation of each data

** *P* values obtained by χ^2 test

*** *P* values obtained by likelihood ratio test

are known factors associated with alcohol consumption, were significantly increased across the categories with increasing alcohol consumption (all $P < 0.001$). In addition, AST and γ-GTP were significantly higher in FL (+) subjects in comparison with FL (−) subjects in each category of alcohol consumption, and these parameters increased across the categories with increasing alcohol consumption in subjects both with and without FL.

Independent predictors of fatty liver

Independent predictors significantly affecting the prevalence of FL were identified by logistic regression analysis (Table 3). FL risk tended to decrease with age and increase with BMI. A significant elevation of FL risk was observed in subjects with ALT elevation, hypertension, dyslipidemia, and DM. Multivariate analysis revealed that alcohol

Table 3 Predictive factors of fatty liver by logistic regression analysis

	All subjects		Limited subjects[c]
	Univariate OR [95% CI]	Multivariate OR [95% CI][a]	Multivariate OR [95% CI][b]
Age (years)			
30–39	1.0 (referent)	1.0 (referent)	1.0 (referent)
40–49	1.14 [0.99, 1.30]	1.27 [1.07, 1.52]	1.41 [0.82, 2.41]
50–59	0.89 [0.78, 1.02]	1.14 [0.95, 1.36]	0.92 [0.52, 1.64]
60–69	0.59 [0.51, 0.69]	0.84 [0.69, 1.02]	0.94 [0.50, 1.76]
P for trend	<0.001	<0.001	0.333
BMI (kg/m^2)			
<18.5	0.05 [0.01, 0.19]	0.06 [0.01, 0.25]	–[d]
18.5–22.9	1.0 (referent)	1.0 (referent)	1.0 (referent)
23.0–24.9	3.30 [2.93, 3.72]	2.42 [2.12, 2.75]	3.15 [2.04, 4.86]
>25	11.8 [10.5, 13.2]	6.01 [5.27, 6.84]	5.50 [3.40, 8.89]
P for trend	<0.001	<0.001	<0.001
Presence of clinical manifestation			
ALT elevation	5.23 [4.78, 5.73]	2.46 [2.19, 2.77][b]	–
Hypertension	1.59 [1.46, 1.72]	1.18 [1.06, 1.32]	–
Dyslipidemia	3.00 [2.75, 3.27]	1.19 [1.05, 1.35]	–
Diabetes mellitus	2.34 [2.06, 2.65]	1.88 [1.61, 2.20]	–
Smoking status			
Never smoker	1.0 (referent)	1.0 (referent)	1.0 (referent)
Former smoker	1.15 [1.04, 1.28]	1.07 [0.94, 1.22]	–
Current smoker	0.97 [0.88, 1.07]	0.83 [0.73, 0.95]	0.58 [0.39, 0.87]
P for heterogeneity	0.001	<0.001	
Alcohol consumption			
Never drinker	1.0 (referent)	1.0 (referent)	1.0 (referent)
Former drinker	0.91 [0.72, 1.24]	0.89 [0.63, 1.25]	–
Light drinker	0.79 [0.68, 0.91]	0.71 [0.59, 0.86]	0.71 [0.38, 1.34]
Moderate drinker	0.68 [0.59, 0.79]	0.55 [0.45, 0.67]	0.81 [0.40, 1.62]
Heavy drinker	0.81 [0.63, 1.05]	0.44 [0.32, 0.62]	0.54 [0.15, 2.03]
P for trend	<0.001	<0.001	0.525

P values were obtained by likelihood ratio test

ALT alanine aminotransferase, *BMI* body mass index

[a] Odds ratios (ORs) and corresponding 95% confidence intervals [95% CIs] were obtained by logistic regression models using variables in this table and serum levels of alanine aminotransferase, γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

[b] ORs and corresponding 95% CIs were obtained by logistic regression models using variables in this table and serum levels of γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

[c] Subjects were limited to those who had no history of ALT elevation, hypertension, dyslipidemia, and diabetes mellitus. Subjects who stopped drinking or who were ex-smokers were also excluded from the analysis ($n = 1,481$)

[d] There was no subject with fatty liver in this BMI category

consumption was inversely associated with FL risk, after adjusting for the effects of all variables in Table 3, and serum levels of ALT, γ-GTP, triglycerides, and HDL-C (P for trend <0.001). This association was still present when the study subjects were limited to those who were not former smokers or drinkers, and who had no history of ALT elevation, hypertension, dyslipidemia, and DM, although the ORs for alcohol consumption were not statistically significant.

Influence of drinking patterns on FL prevalence

The prevalence of FL was examined in 10 subgroups classified by the frequency of alcohol consumption (0, 1–10, 11–20, and >20 days/month) and volume of alcohol consumed on days subjects drank (0, <20, 20–59, and ≥60 g/day) (Fig. 1). The prevalence of FL in non-drinkers was 44.7%. The prevalences of FL in those who consumed alcohol on 1–10, 11–20, and ≥21 days/month were 41.8, 39.1, and 30.7% (<20 g/day); 52.8, 48.5, and 35.0% (20–59 g/day); and 54.9, 53.6, and 38.4% (≥60 g/day), respectively. The prevalence of FL decreased with increased frequency of alcohol consumption and increased with increasing volumes of alcohol consumed per day (Fig. 1, Table 4).

For current alcohol drinkers, the FL risk was examined based on alcohol drinking patterns, the frequency of alcohol consumption, and the daily volume of alcohol (Table 4). There was a significant inverse association between the frequency of alcohol consumption and the risk

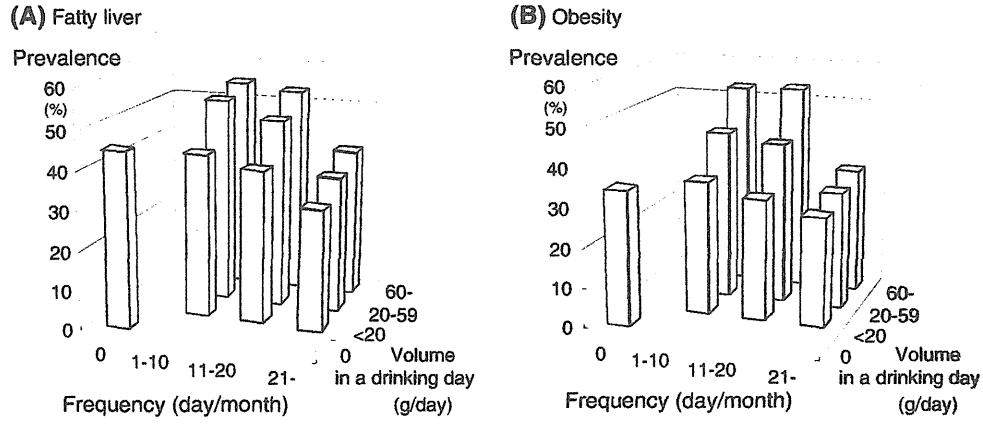

Fig. 1a,b Relationship of drinking patterns (frequency and volume in a drinking day) with the prevalence of fatty liver and obesity

Table 4 Estimated risk of fatty liver and obesity by alcohol drinking patterns among current alcohol drinkers

	Fatty liver		Obesity	
	Number (%)	OR [95% CI][a]	Number (%)	OR [95% CI][b]
Frequency (days/month)				
1–10 (n = 1,953)	921 (47%)	1.0 (referent)	775 (40%)	1.0 (referent)
11–20 (n = 863)	384 (45%)	0.92 [0.75, 1.12]	326 (38%)	1.05 [0.86, 1.27]
21– (n = 5,939)	2,005 (34%)	0.62 [0.53, 0.71]	1,765 (30%)	0.87 [0.76, 1.00]
P value	<0.001[#]	<0.001[##]	<0.001[#]	<0.033[##]
Alcohol volume (g/drinking day)				
1–20 (n = 3,438)	1,206 (35%)	1.0 (referent)	1,042 (30%)	1.0 (referent)
21–59 (n = 4,705)	1,823 (39%)	1.02 [0.90, 1.15]	1,566 (33%)	1.18 [1.05, 1.33]
60– (n = 612)	281 (46%)	0.83 [0.66, 1.04]	258 (42%)	1.41 [1.14, 1.74]
P value	<0.001[#]	0.378[##]	<0.001[#]	<0.001[##]

[#] P values were obtained by χ^2 test

[##] P values for trend were obtained by likelihood ratio test

[a] Odds ratios (ORs) and corresponding 95% confidence intervals [95% CIs] were obtained by logistic regression models using alcohol drinking patterns (frequency/daily alcohol consumption); age; body mass index; height; smoking status; the presence of alanine aminotransferase elevation, hypertension, dyslipidemia, and diabetes mellitus; and serum levels of alanine aminotransferase, γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

[b] ORs and 95% CIs were obtained by logistic regression models using alcohol drinking patterns (frequency/daily alcohol consumption); age; height; smoking status; the presence of fatty liver, alanine aminotransferase elevation, hypertension, dyslipidemia, and diabetes mellitus; and serum levels of alanine aminotransferase, γ-glutamyl transpeptidase, triglycerides, and high-density lipoprotein cholesterol

of FL (P for trend <0.001). On the other hand, alcohol volume was not related to the risk of FL (P for trend = 0.378).

Influence of drinking patterns on body composition

The association between alcohol drinking patterns and the prevalence of obesity, which is the most important risk factor for FL, are shown in Fig. 1b and Table 4. The prevalence of FL in non-drinkers was 34.6%. The prevalences of obesity in those who consumed alcohol on 1–10, 11–20, and ≥21 days/month were 34.3, 31.2, and 28.1% (<20 g/day); 43.7, 41.8, and 30.4% (20–59 g/day); and 53.0, 53.7, and 32.6% (≥60 g/day), respectively. As with the prevalence of FL, the prevalence of obesity decreased with increasing frequency of alcohol consumption and increased with the increasing alcohol volume in a drinking day (Table 4).

Logistic regression analysis (Table 4) revealed a significant association between the volume of alcohol in a drinking day and the risk of obesity (P for trend <0.001). In addition, consistent alcohol consumption tended to reduce the likelihood of obesity (OR 0.87, 95% CI 0.76–1.00, P for trend 0.033).

Discussion

This study demonstrated that FL in men was positively associated with factors including the presence of obesity, hypertension, dyslipidemia, and DM, but was negatively associated with age and alcohol consumption. Although our survey was not prospective in nature, these findings confirm that the major risk factors for FL are factors related to adiposity [1–4], not alcohol consumption, findings which agree with recent reports proposing a protective effect of alcohol intake [9–13]. We also confirmed that alcohol consumption tended to be negatively associated with FL in the limited number of subjects who had no history of ALT elevation, hypertension, dyslipidemia, or DM. In addition, our study may provide new evidence to help understand the role of alcohol drinking patterns in the pathogenesis of hepatic steatosis.

While alcohol consumption certainly may be a cause of FL in some cases [7, 8], it potentially plays a protective role against FL regardless of daily alcohol volume. Gunji et al. previously reported that "any drinking" might potentially be protective against FL; light (40–140 g/week) and moderate alcohol (140–280 g/week) consumption decreased the risk of FL, and the prevalence of FL was not increased even by heavy alcohol consumption [12]. Our study is also a report providing evidence of a significant inverse association between FL and alcohol consumption, even in heavy drinkers (≥60 g/day) (Table 3). We consider that alcohol consumption is a double-edged sword in the pathogenesis of hepatic steatosis. The difference in the results of our study and previous studies proposing alcohol consumption as a risk factor for FL may be due to differences in the ethnicity, age, BMI, and lifestyle (drinking style, type of alcohol, dietary habits, etc.) of the subjects in each study.

In the present study, we examined the relationship between drinking patterns (frequency of alcohol consumption and volume of alcohol in a drinking day) and FL. Consistent alcohol consumption (≥21 days/month) reduced the risk of FL independently (Table 4). In addition, consistent alcohol consumption may reduce the likelihood of obesity (Table 4), possibly contributing to a lowered risk of FL. Thus, consistent alcohol consumption may provide a protective effect on FL development in association with or without obesity. Conigrave et al. [22] reported that light to moderate alcohol consumption was inversely associated with an increased risk of DM in men only when consumed frequently (≥5 days/week). Consumption of alcohol on at least 3–4 days per week was associated with a decreased risk of myocardial infarction in men [23]. Consistent alcohol exposure may contribute to the favorable association with FL seen in the present study, as well as contributing to the favorable association with type 2 diabetes and ischemic heart disease reported in the studies cited above [22, 23], suggesting a common mechanism in these metabolic diseases.

We examined the relationship between alcohol volume in a drinking day and FL. Although the prevalence of FL increased with the increase in the daily volume of alcohol consumption (Fig. 1), no significant association between FL prevalence and the daily volume of alcohol consumption could be identified by logistic regression analysis after adjusting for BMI and other factors related to adiposity (Table 4). On the other hand, an increase in the daily volume of alcohol consumption was associated with an increased risk of obesity (Fig. 1; Table 4). We consider that excessive alcohol consumption in a drinking day may cause an alteration of body composition, most likely due to inadequate drinking and eating lifestyles, such as a prolonged duration of eating and increased calorie intake, probably resulting in the increasing prevalence of FL seen in the present study. These factors may influence the conflicting results reported about the relationship between alcohol consumption and the prevalence of FL.

Recent investigations have elucidated some of the mechanisms by which alcohol alters liver metabolism. Two critical nuclear transcription factors, sterol regulatory element binding protein (SREBP) [24] and peroxisome proliferator activated receptor alpha (PPARα) [25], are altered with alcohol consumption. You et al. [26] reported a role

for AMP activated protein kinase activity in the action of ethanol on the liver. In addition, disturbances in the cytokine network, including alterations in the tumor necrosis factor-α (TNF-α) [27] level, were shown to be involved in ethanol-induced steatosis. These pivotal factors, however, appear to be common in the pathogenesis of both NAFLD [28–30] and AFLD. Therefore, the inverse association between FL and alcohol consumption cannot be explained by these alterations alone.

It has been reported that moderate alcohol intake enhances insulin sensitivity [19, 20], contributing to a lower risk of DM. It was shown that moderate alcohol consumption was associated with a lower prevalence of both nonalcoholic steatohepatitis (NASH) and DM [11]. Sierksma et al. [21] hypothesized that the increase in adiponectin after chronic moderate alcohol consumption would cause an increase in insulin sensitivity in relatively insulin-resistant men. In addition, alcohol consumption also alters apolipoprotein profiles. Elevations in HDL cholesterol levels confer a lower risk of chronic heart disease [16]. Recently, studies examining the pathogenesis of NASH have demonstrated an association of hepatic apolipoprotein synthesis/secretion with the development of steatosis [31, 32]. Our study provides evidence that the risk of FL is decreased across the categories of alcohol consumption, despite an increase in serum triglyceride levels. Alcohol dehydrogenase-mediated ethanol metabolism generates a redox shift in the liver, which stimulates the synthesis of fatty acids. Subsequent removal of these fatty acids may be of benefit to prevent the development of FL.

Although the level of AST is considered to be higher than that of ALT in the majority of alcoholic liver diseases, the AST level was similar to that of ALT in moderate and heavy drinkers in our study (Table 2). In addition, our study demonstrated that liver injury assessed by AST and γ-GTP was positively associated with alcohol consumption regardless of the presence or absence of FL (Table 2). It was reported that the distribution of ratios of AST to ALT (AST/ALT) <1 and >1 was not different between healthy non-drinkers and moderate drinkers of normal weight or with obesity [33]. The ALT level was also similar to the AST level in moderate drinkers of normal weight, and it was observed that the effect of moderate alcohol consumption on liver-derived enzymes, including AST, ALT, and γ-GTP, increased with increasing BMI [33]. These results indicate that the relationships between alcohol consumption, BMI, and different serum liver-derived enzymes in drinkers should be considered. We should also pay attention to the finding that the absence of FL assessed by ultrasonography does not necessary rule out liver injury in drinkers.

There are several limitations in our study. Firstly, we did not enroll women subjects, although 7097 women subjects were investigated, because most of the women did not drink, or they drank 20 g/day at most; the number of subjects who drank more than 20 g/day was only 153 and the number of subjects with FL was 33. Thus, the sample size was insufficient to elucidate the association between alcohol drinking patterns and FL risk in women by the methods used in the present study. Secondly, subjects with other liver diseases, including autoimmune hepatitis and primary biliary cirrhosis, were not excluded. In addition, the association between alcohol consumption and FL prevalence was estimated by multivariate analysis after adjusting for age; BMI; the presence of hypertension, dyslipidemia, or DM; and smoking status. However, it is possible that additional factors for which we did not adjust may have influenced the results. One such factor is adult-onset type II citrullinemia (CTLN2) [34], an inherited metabolic disease caused by a deficiency of mitochondrial aspartate/glutamate carrier protein. Because of an impairment of cytosolic NADH oxidation, CTLN2 patients show both steatohepatitis and alcohol intolerance [35]. Thus, there may have been some differences in metabolic background, such as the capacity for alcohol oxidation or NAD^+/NADH metabolism, across the categories of alcohol consumption that could have affected the prevalence of FL. Thirdly, drinking habits are influenced by a polymorphism in the aldehyde dehydrogenase 2 (*ALDH2*) gene, and this polymorphism may affect our analysis. However, our study did not examine this polymorphism, and the alcohol-flushing response, which can be used to roughly estimate the presence of this polymorphism, was not investigated in the questionnaire. Furthermore, although treatment with an angiotensin II type 1 receptor blocker or peroxisome proliferator-activated receptor agonists is known to alleviate FL, we did not obtain information from the subjects about their use of these medications. Fourthly, the diagnosis of FL was made using abdominal ultrasonography, which defines the presence or absence of fatty steatosis. Diagnosis by ultrasonography may overlook a subset of advanced AFLD or NAFLD, so called "burnt-out steatohepatitis", in which fatty steatosis is reduced. Lastly, although direct interviews were carried out by trained medical staff, self-reported information on alcohol consumption may lead to under- or over-reporting. The influence of the type of alcoholic beverage was also not taken into account in this study. Further studies will be needed to clarify these issues.

In conclusion, this study demonstrates that the major risk factors for FL in Japanese men are factors related to adiposity, not alcohol consumption, and that consistent consumption of alcohol may play a protective role against FL. These results suggest that lifestyle modifications aimed at fighting central obesity and metabolic abnormalities should be the most important recommendations for the management of FL. In addition, it seems unlikely that the

risk of FL can be reduced by the discontinuation and/or reduction of alcohol consumption alone. Further studies are required to better understand FL pathogenesis and management.

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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

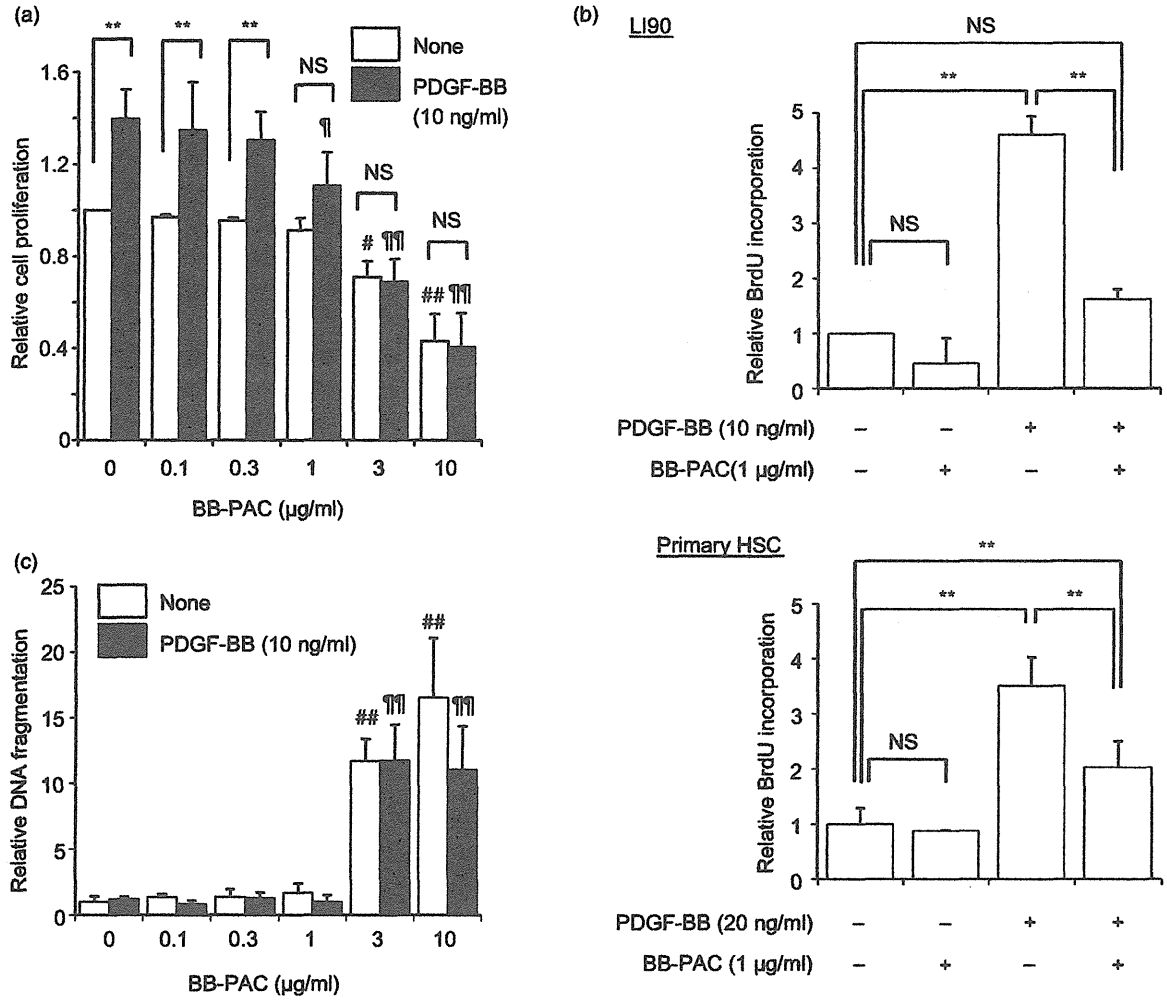

Figure 1 Suppressive effects of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-induced proliferation and DNA synthesis in LI90 hepatic stellate cells. (a) LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed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

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,

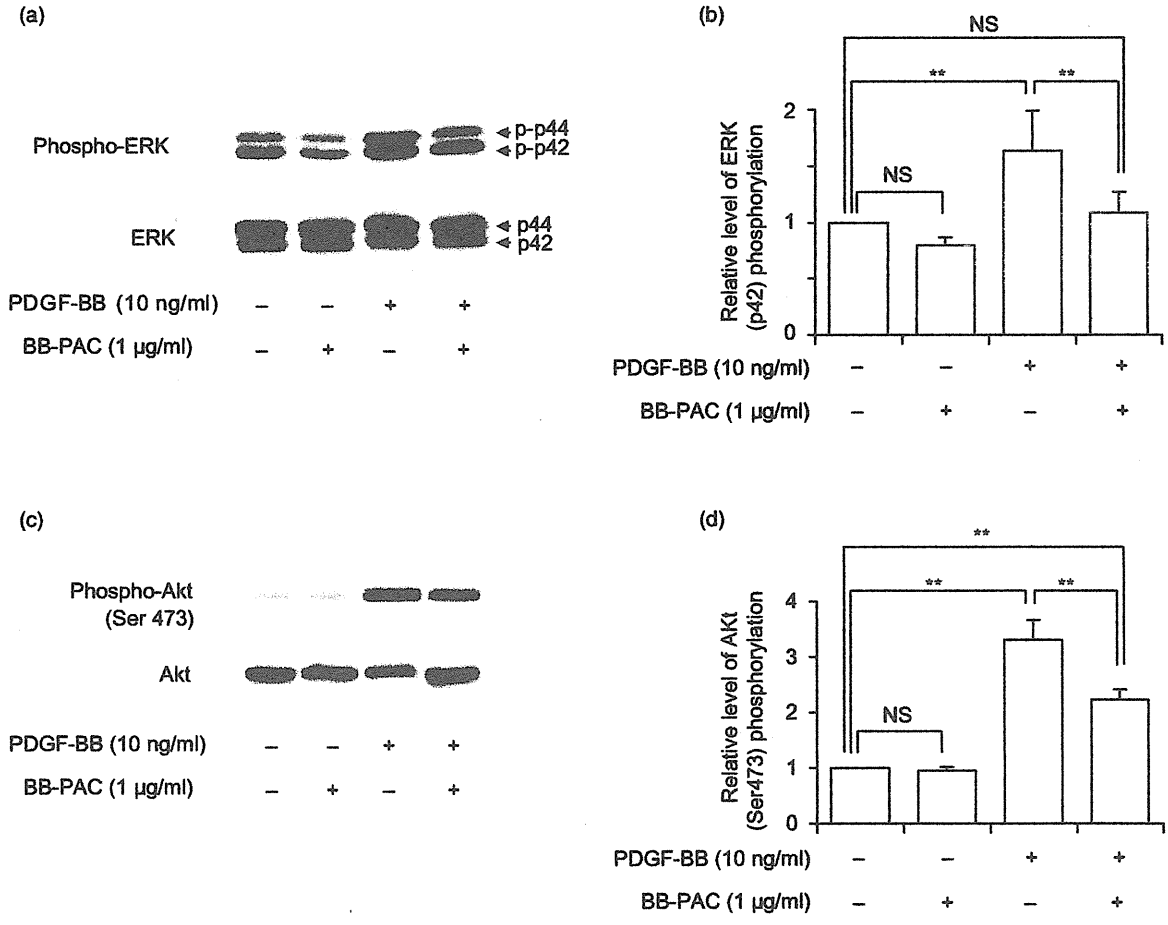

Figure 2 Effects of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-mediated phosphorylation of extracellular signal-regulated kinase (ERK) and Akt. LI90 cells were pre-incubated for 1 h with 1 μg/mL BB-PAC, followed by a 15-min incubation with or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

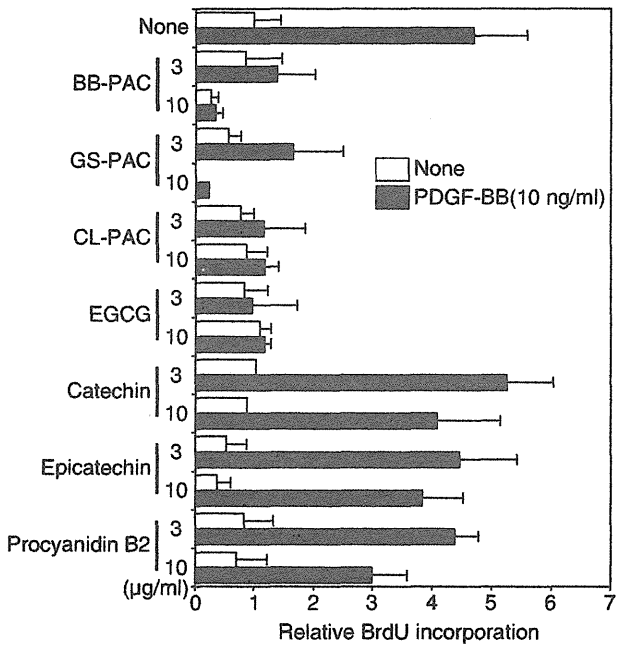

Figure 3 Comparison of the inhibitory effects of proanthocyanidin extracted from blueberry leaves (BB-PAC), PAC from grape seeds (GS-PAC) and *Croton lechleri* (CL-PAC) and monomeric and dimeric PAC. LI90 cells were pre-incubated for 1 h with 3 or 10 μg/mL BB-PAC, GS-PAC, CL-PAC, epigallocatechin-3-gallate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

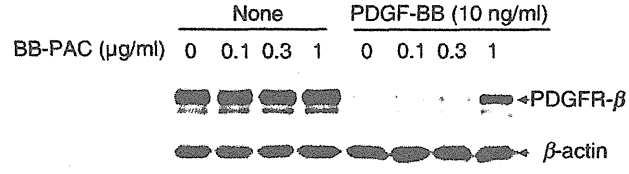

Figure 4 Effect of proanthocyanidin extracted from blueberry leaves (BB-PAC) on platelet-derived growth factor (PDGF)-BB-induced PDGF receptor-β (PDGFR-β) degradation. LI90 cells were pre-incubated for 1 h with the indicated concentrations of BB-PAC, followed by a 24-h incubation with or without 10 ng/mL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