TABLE 1. Characteristics of study participants

	Men (n = 1306)	Women (n = 1626)	*P* value[a]
Age (yr)			
40–49	142 (10.9)	188 (11.6)	0.351
50–59	312 (23.9)	426 (26.2)	
60–69	447 (34.2)	546 (33.6)	
≥70	405 (31.0)	466 (28.7)	
Adiponectin (μg/ml)	7.0 (5.1–9.9)	10.4 (7.4–14.9)	<0.001
BMI (kg/m^2)			
<22.0	424 (32.5)	550 (33.8)	0.731
22.0–24.9	485 (37.1)	588 (36.2)	
≥25.0	397 (30.4)	488 (30.0)	
Blood pressure (mm Hg)			
Systolic	136.1 ± 15.7	133.1 ± 16.1	<0.001
Diastolic	81.9 ± 9.9	77.5 ± 9.8	<0.001
Serum lipids (mg/dl)			
Total cholesterol	193.4 ± 31.0	207.3 ± 0.9	<0.001
HDL-C	56.3 ± 14.4	61.6 ± 14.2	<0.001
LDL-C	119.1 ± 28.9	128.9 ± 29.6	<0.001
Triglycerides	95 (69–136)	88 (65–118)	<0.001
Glucose tolerance			
Glucose (mg/dl)	96.9 ± 19.5	92.3 ± 13.3	<0.001
Insulin (μU/ml)	4.2 (3.0–7.0)	5.0 (3.9–8.0)	<0.001
HOMA-IR			
<2.0	1084 (83.0)	1292 (79.5)	0.001
2.0–3.9	184 (14.1)	303 (18.6)	
≥4.0	38 (2.9)	31 (1.9)	
Liver enzymes			
ALT (IU)	21 (17–29)	18 (15–24)	<0.001
γ-GTP (IU)	32 (21–52)	19 (14–26)	<0.001
Alcohol consumption (g/wk)			
None	351 (26.9)	1384 (85.1)	<0.001
<120	366 (28.0)	207 (12.7)	
120–239	285 (21.8)	28 (1.7)	
≥240	304 (23.3)	7 (0.4)	
Smoking habit			
Never	506 (38.7)	1495 (91.9)	<0.001
Current	445 (34.1)	88 (5.4)	
Former	355 (27.2)	43 (2.6)	

χ^2 test, unpaired *t* test, or Mann-Whitney *U* test was used for analyses. Data are n (%) unless otherwise indicated: mean ± SD for blood pressure, total cholesterol, HDL-C, LDL-C, and glucose; median (25th–75th percentile) for adiponectin, triglycerides, insulin, ALT, and γ-GTP.

[a] Men *vs.* women.

the relationship between two continuous or ordered variables. Multiple regression analysis was used with covariance analyses, and log-transformed adiponectin was used as the independent variable. In multivariable analyses, the impact of the effect of 10 g/d alcohol consumption was assessed. The SPSS 15.0 program for Windows (SPSS Inc., Chicago, IL) was used for the statistical analyses. $P < 0.05$ (two sided) was considered statistically significant.

Results

Characteristics of the 2136 subjects are shown in Table 1. There were significant differences in adiponectin levels, lipid levels, glucose, insulin, HOMA-IR, and both systolic and diastolic blood pressure between men and women. Levels of all these variables, except for HDL-C and triglycerides, were significantly higher in women than in men. Only 15% of female subjects were drinkers compared with 73% of men ($P < 0.001$).

The relationship between adiponectin concentrations and potentially confounding factors and alcohol intake are shown in Table 2. Using correlation analysis, we found a small and significant negative correlation for adiponectin concentrations and alcohol consumption in men (r_s) = −0.141; $P < 0.001$) and a weaker negative correlation in women ($r_s = -0.055$; $P = 0.025$). Significant negative correlations with adiponectin concentrations were observed in total cholesterol, LDL-C, triglyceride, BMI, blood glucose, insulin, HOMA-IR, ALT, γ-GTP, systolic and diastolic blood pressure, and smoking habits in both in men and women. A positive correlation was observed in HDL-C levels in both genders.

In the next analysis, we used categorized data on alcohol consumption to investigate the relationship between alcohol intake and serum adiponectin levels. As shown in Fig. 1, adiponectin levels significantly decreased in a dose-

TABLE 2. Relationship between serum adiponectin concentrations and other factors studied

	Men (n = 1306)		Women (n = 1626)	
	Adiponectin levels or correlation coefficient[a]	*P* value	Adiponectin levels or correlation coefficient[a]	*P* value
BMI (kg/m^2)				
<22.0	8.4 (6.2–12.1)	<0.001	12.9 (9.2–17.6)	<0.001
22.0–24.9	6.9 (5.1–9.4)		10.0 (7.3–14.4)	
≥25.0	6.0 (4.4–8.1)		9.0 (6.4–12.7)	
Blood pressure (mm Hg)				
Systolic	−0.009	0.749	−0.029	0.242
Diastolic	−0.100	<0.001	−0.027	0.275
Serum lipids (mg/dl)				
Total cholesterol	−0.113	<0.001	−0.029	0.245
HDL-C	0.329	<0.001	0.355	<0.001
LDL-C	−0.103	<0.001	−0.097	<0.001
Triglyceride	−0.390	<0.001	−0.307	<0.001
Glucose tolerance				
Glucose (mg/dl)	−0.091	0.001	−0.183	<0.001
Insulin (μU/ml)	−0.341	<0.001	−0.441	<0.001
HOMA-IR				
<2.0	7.6 (5.4–10.3)	<0.001	11.4 (8.3–15.9)	<0.001
2.0–3.9	5.3 (3.8–6.7)		7.5 (5.7–10.7)	
≥4.0	4.9 (3.4–7.0)		5.6 (4.3–7.7)	
Liver enzymes				
ALT (IU)	−0.264	<0.001	−0.185	<0.001
γ-GTP (IU)	−0.300	<0.001	−0.223	<0.001
Alcohol consumption (g/wk)	−0.141	<0.001	−0.055	0.025
Smoking habit				
Never	7.5 (5.4–10.4)	<0.001	10.5 (7.5–15.0)	0.002
Current	6.7 (4.7–9.3)		9.1 (5.9–13.9)	
Former	7.2 (5.0–10.0)		9.8 (6.6–14.7)	

ANOVA, Pearson's correlation coefficient, or Spearman's correlation coefficient was used for analyses.

[a] Data are median (25th–75th percentile) of serum adiponectin levels, Pearson's correlation coefficient, or Spearman's correlation coefficient.

dependent manner in men ($P < 0.001$). A similar trend was noted in women ($P = 0.029$), although the relationship was not as clear as that seen in men. In women, a borderline significant decrease of serum adiponectin levels was observed among drinkers who consumed less than 120 g/wk of ethanol compared with abstainers ($P = 0.053$). A decrease in serum adiponectin levels was not noted in those who consumed 120 g/wk or more of ethanol compared with abstainers.

We also examined the established relationship between alcohol consumption and HDL-C levels. Significant positive correlations were demonstrated ($r_s = 0.165$, $P < 0.001$ for men; and $r_s = 0.118$, $P < 0.001$ for women), indicating that these relationships were consistent with previous studies.

Subsequently, we conducted a multiple regression analysis to assess the effect of 10 g/d alcohol intake on adiponectin concentrations, controlling for potential confounding factors. We included age, sex, BMI, systolic blood pressure, LDL-C, HDL-C, triglycerides, glucose, HOMA-IR, ALT, and smoking habits as covariates. Alcohol consumption was independently associated with hypoadiponectinemia: 10 g/d ethanol intake was associated with a 0.028 (95% confidence interval = −0.040 to −0.016; $P < 0.001$) μg/ml decrease of log-transformed adiponectin concentrations (Table 3).

Discussion

In this population-based cross-sectional study, we found that alcohol intake and serum adiponectin levels were significantly inversely associated in men. A suggested inverse association was demonstrated in women who consumed less than 120 g/wk alcohol. The weak inverse association between alcohol consumption and serum adiponectin concentrations was found even after adjustment for possible confounding factors. These are contradictory observations when compared with several previous epidemiological and experimental reports performed in White and Black populations (4, 16), but they are consistent with experimental studies in animal models (8, 9). Recently, Kawamoto *et al.* (17) reported an inverse relationship between high molecular weight (HMW) adiponectin and alcohol consumption among healthy Japanese men in a cross-sectional study. HMW complex is the most active

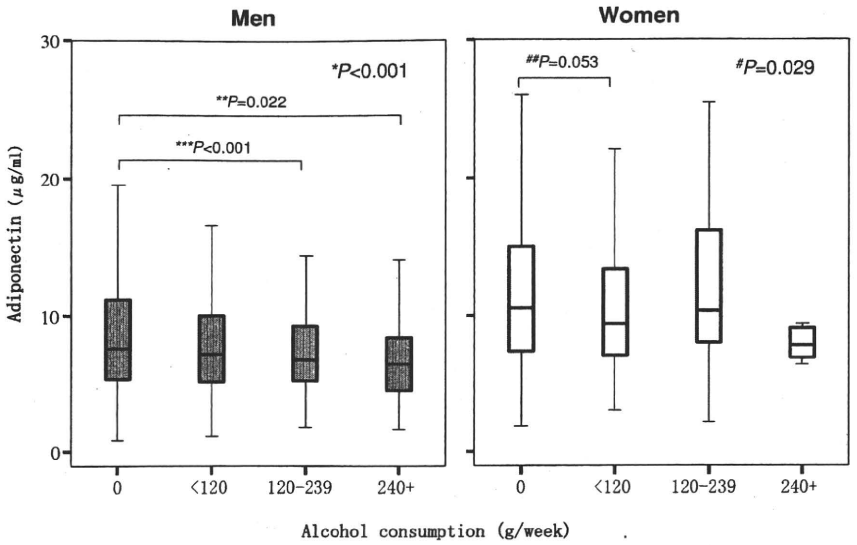

FIG. 1. Box plots illustrating serum plasma adiponectin concentrations for each level of alcohol consumption by gender. *Horizontal lines inside each box* represent medians, and the *top and bottom of the boxes* are the 25th and 75th quartiles, respectively. The *error bars* indicate 95% confidence intervals. *, $P < 0.001$ in men, and #, $P = 0.029$ in women for comparisons by ANOVA; **, $P = 0.022$, and ***, $P < 0.001$ in men, and ##, $P = 0.053$ in women for comparisons with abstainers in each group (Dunnett's test).

form of adiponectin and was closely associated with the type 2 diabetes when compared with total adiponectin (18). Moreover, it was shown that moderate alcohol consumption had different effects on HMW adiponectin, medium molecular weight adiponectin, and low molecular weight adiponectin (19). Further study is necessary to evaluate the effect of HMW on the association between serum adiponectin levels and alcohol consumption in a Japanese population.

Multiple regression analysis demonstrated that serum adiponectin levels were significantly related to sex, age, BMI, HDL-C, triglyceride, HOMA-IR, and ALT. All of the results are in good agreement with previous reports (3, 4, 10, 20, 21). Schulze *et al.* (4) observed an inverse relationship between plasma adiponectin levels and BMI and triglyceride but a positive relationship between plasma adiponectin levels and HDL-C and age in diabetic men. Ferris *et al.* (10) reported that serum adiponectin levels inversely correlated HOMA-IR in White subjects. A sex-based difference in plasma adiponectin levels was supported by previous studies (21, 22) and could be partly explained by differences in body fat distributions (22).

The consistent findings regarding the relationship between serum adiponectin levels and BMI, serum lipids, and insulin resistance and between alcohol consumption and HDL-C levels imply that factors related to ethnic differences, alcohol metabolism, and dietary intake may explain the discrepancies between our results and those of previous studies conducted in humans.

Alcohol is initially oxidized to acetaldehyde, mainly by the alcohol dehydrogenase (ADH) enzyme, and acetaldehyde is subsequently oxidized into acetate by the acetaldehyde dehydrogenase type 2 (ALDH2) enzyme (23). The gene that encodes these two representative alcohol-metabolizing enzymes displays polymorphisms that modulate individual differences in alcohol- and acetaldehyde-oxidizing capacity. Several ethnic differences in distribution of the ADH and ALDH2 genotypes, and in subsequent ethanol metabolism, have been demonstrated. First, the ADH class IV isozyme (σ-ADH), which is present predominantly in the upper gastrointestinal tract but not in the liver and which contributes to gastric ethanol oxidation, is absent or markedly decreased in 80% of Japanese people (24, 25). Second, about 85% of Japanese subjects are carriers of the ADH2*2 allele compared with only 5% or less of European and White American subjects (26). The ADH2*2 encodes an active enzyme and may be expected to generate more acetaldehyde because of this higher activity. Third, the ADH3*1 allele, coding for the rapidly acting ADH3, is more predominant (~95%) in Japanese subjects, whereas it is present in only 40–50% of White subjects (27). Finally, the ALDH2*2 allele, which encodes a catalytically inactive subunit, is present in about 45% of Japanese subjects, although it is extremely rare in White subjects (26). The latter three features indicate a failure to rapidly metabolize acetaldehyde, leading to excessive accumulation of acetaldehyde and higher susceptibility to acetaldehyde among a considerable number of Japanese subjects compared with White subjects. Ethanol and its metabolites, especially acetaldehyde, have been shown to have a toxic influence (23). Acetaldehyde is not only a highly toxic metabolite with extraordinary reactivity but was also shown to induce proinflammatory cytokines, TNF-α, and IL-1β in HepG2 cells (28), whereas TNF-α decreased the levels of adiponectin in human differentiated adipocytes (29). We assume that acetaldehyde and/or acetaldehyde adducts produced through oxidation of ethanol potentially modulate, in part, the association between alcohol intake and serum adiponectin concentrations in the Japanese population. Adjustments for polymorphisms in alcohol-metabolizing genes may explain the differences noted in ethnic groups.

Dietary factors play an important role in the development of type 2 diabetes and ischemic heart disease, because excess caloric intake contributes to the development of obesity, a major risk factor for both diseases. Studies on

TABLE 3. Multivariate-adjusted associations between serum adiponectin concentrations and alcohol consumption in 2932 subjects

Variables	Partial correlation coefficient	SE	Standardized partial correlation coefficient	95% confidence interval		*P* value
				Lower limit	Upper limit	
Sex (men,[a] women)	0.267	0.022	0.244	0.223	0.310	<0.001
Age (yr)	0.106	0.009	0.192	0.089	0.124	<0.001
BMI (<22,[a] 22–24.9, ≥25) (mm Hg)	−0.068	0.012	−0.099	−0.090	−0.045	<0.001
Systolic blood pressure (mm Hg)	0.000	0.001	−0.002	−0.001	0.001	0.902
LDL-C (mg/dl)	−0.001	0.000	−0.029	−0.001	0.000	0.058
HDL-C (mg/dl)	0.008	0.001	0.222	0.007	0.010	<0.001
Triglyceride (mg/dl)	−0.001	0.000	−0.081	−0.001	0.000	<0.001
Glucose (mg/dl)	−0.001	0.001	−0.025	−0.002	0.000	0.144
HOMA-IR (<2.0,[a] 2.1–3.9, ≥4.0)	−0.200	0.021	−0.170	−0.241	−0.158	<0.001
ALT (IU/liter)	−0.002	0.001	−0.060	−0.004	−0.001	<0.001
Smoking status (never,[a] current/former)	−0.031	0.022	−0.027	−0.074	0.011	0.147
Alcohol consumptions (10 g/d)	−0.028	0.006	−0.083	−0.040	−0.016	<0.001

Multiple regression analysis was used in covariance analyses for serum adiponectin concentrations after log transformation as independent variable.

[a] Reference category.

the dietary predictor of plasma adiponectin concentrations in animal models demonstrated that a high-fat diet is related to decreased serum adiponectin levels, just as it related to an increase in insulin resistance (30). Several controversial observations regarding fat intake have been reported when alcohol consumption accompanied this intake. High-fat, ethanol-containing food decreased serum adiponectin concentrations in mice (8) and rats (31). Decreases in serum adiponectin concentrations after ethanol feeding were dependent on the type of fat in the diet. Ethanol-containing diets high in unsaturated fats contributed to ethanol-induced decreases in adiponectin levels, whereas inclusion of saturated fats in the ethanol-feeding protocol prevented decreased adiponectin levels (9). A diet enriched in saturated fatty acids effectively reversed alcohol-induced necrosis, inflammation, and fibrosis despite continued alcohol consumption (32). The precise mechanism through which dietary fatty acids plus ethanol affect adiponectin expression and its secretion has yet to be determined. The protective action of saturated fatty acids is suggested to be partly caused by down-regulation of TNF-α (30, 33), which suppresses an adiponectin expression (29). In the Japanese population, both intake of total fat and that of saturated fats are lower than in the U.S. population (16, 34). The lower intake of saturated fat in the Japanese population may contribute to the different influence of alcohol consumption on adiponectin concentrations between Japanese and White subjects. However, it was not helpful to compare the effect of the intake of saturated fats with that of unsaturated fats in our study, because intake of these two fats was highly correlated ($r_s = 0.87$) among 1457 subjects who had completed the nutritional survey conducted in the same district using a self-administered questionnaire (unpublished data).

Carbohydrate intake may also be a factor that modulates the relationship between alcohol intake and adiponectin concentrations. In epidemiological studies, high glycemic loads, which were calculated by multiplying the carbohydrate content of each food by its glycemic index, were significantly associated with lower adiponectin concentrations in healthy men (16). For Japanese people, rice is the primary food that contributes to total carbohydrate and energy intake, which is seldom the case in Western populations. Data from the nutritional survey conducted in the same district (unpublished data) have shown that carbohydrate intake accounted for about 59% of total energy intake, and the mean glycemic load was about 206 among subjects aged 40 yr or over. Both parameters were higher than those of White adults (16). Although the effect of the dietary glycemic intake on the relationship between alcohol intake and adiponectin concentrations has not been fully elucidated, the higher intake of carbohydrate in the Japanese population may contribute to the different influence of alcohol consumption on adiponectin concentrations between Japanese and White subjects.

Our study demonstrated an inverse association between alcohol intake with serum adiponectin levels in men, with less clear findings in women. This discrepancy might be explained, in part, by the gender difference in ethanol metabolism. Women differ from men in several factors associated with alcohol metabolism (35), including 1) a lower gastric σ-ADH activity, which mediates the first-pass mechanism of ethanol in women, and 2) a decreased volume of ethanol distribution (body size and distribution space for alcohol, with water space being smaller

in women). However, these properties are not sufficient to explain the gender difference of the effect of alcohol intake on serum adiponectin concentrations. The small number of drinkers among our female subjects (15%) might cause difficulty in evaluating this result. Further study, including increasing the number female drinkers enrolled, is necessary to examine this inference.

There are potential limitations to this study. Because of its cross-sectional nature, this study did not provide a causal inference regarding the association between alcohol intake and serum adiponectin levels. However, information on the drinking habits of subjects was determined before the measurement of adiponectin concentrations; thus, an incorrect finding of an inverse association is unlikely. Data on drinking habits was based on face-to-face interviews, which leads to the possibility of misclassification of exposure (*e.g.* underreporting). However, it is also unlikely that this type of misclassification is directly dependent on adiponectin levels, which could be a nondifferential misclassification. Because our study subjects were recruited from participants in a health screening program, any generalization of these results to the normal population should be made with caution.

In conclusion, alcohol consumption was weakly associated with decreased serum adiponectin concentrations in apparently healthy Japanese subjects. Further investigations in Japanese subjects on alcohol metabolism and nutrition intake are necessary to clarify the factors that modulate this inverse effect, which differs from that seen in White subjects.

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Short Communication

Expression of the RNA-binding protein Musashi1 in adult liver stem-like cells

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Aim: Musashi1 is an RNA-binding protein that regulates the Notch signaling pathway in stem cells. Our previous study revealed that Musashi1 is expressed in early hepatocytes during liver development in the mouse. However, whether this unique protein is expressed with Notch signaling markers in adult liver stem-like cells remains unknown.

Methods: Established hepatic stem-like cells (HSLC), which were derived from adult Sprague–Dawley rats, were used for experiments *in vitro*. HSLC were differentiated into mature cells in terms of producing albumin when co-cultured with epidermal growth factor (EGF). The mRNA expression of *Musashi1*, *Notch* family (*Notch1* and *Notch2*), *Jagged1* and *Hes1* was examined in HSLC before and after cell differentiation using polymerase chain reaction-based techniques. Protein expression of Musashi1 was examined in the HSLC and normal mature hepatocytes by immunofluorescence staining.

Results: The mRNA expression of *Musashi1*, *Notch1*, *Jagged1* and *Hes1* was detected in the original HSLC before culturing with EGF but not in primary cultured mature hepatocytes. The mRNA expression of *Musashi1* and *Hes1* was found to be downregulated in differentiated HSLC that produce albumin. Protein expression of Musashi1 was detectable in the original HSLC but not in both differentiated HSLC and mature hepatocytes.

Conclusion: These findings demonstrate that the RNA-binding protein Musashi1 is expressed with Notch signaling markers in adult liver stem-like cells.

Key words: hepatic stem cell, Musashi1, Notch, liver, RNA-binding protein

INTRODUCTION

IT HAS BEEN demonstrated that liver cell regeneration originates from epithelial cells through two mechanisms. First, mature hepatocytes can proliferate independently by division after the loss of liver cells, as is often observed after a partial hepatectomy.[1] An alternative mechanism, in which liver stem/progenitor cells that subsequently differentiate into hepatocytes, cholangiocytes or other liver components are produced, is involved in reconstruction of the liver after severe liver damage.[2] The signal transduction in liver stem cell differentiation has not been fully investigated.

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Musashi1, a neural RNA-binding protein was first isolated as a mammalian homolog of the *Drosophila* protein, which is required for the asymmetric division of sensory neural precursor cells.[3] It is also known that Musashi1 is a positive regulator of the Notch signaling pathway,[4,5] which is essential for the determination of cell fate,[6] thereby maintaining the self-renewing ability of stem cells. Thus, Musashi1 is closely involved in the regulation of asymmetric cell division of stem-like cells, which generates differentiated cells.

In our previous study, we have shown that Musashi1 is expressed in early hepatocytes during liver development in the mouse.[7] Whether this unique RNA-binding protein has any association with the process of liver stem cell differentiation in adults is of considerable interest. In this study, we investigated the expression of Musashi1 in adult liver stem-like cells that regulates the Notch signaling. Our results suggest a possible association of Musashi1 in liver stem-like cell differentiation.

METHODS

Liver stem cell line and culture

AN ESTABLISHED HEPATIC epithelial stem-like cell (HSLC) line derived from the healthy liver of adult male Sprague–Dawley rats[8] was used for experiments *in vitro*. This cell line has an immature liver cell phenotype with positive expression only for α-fetoprotein and negative for both albumin and cytokeratin (CK)19, and exhibits the potential to differentiate into cells of the hepatocytic lineage and serve as stem-like cells for differentiated hepatocytes.[8] The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C. Spheroidal aggregates of hepatocytes are known to exhibit higher functions than hepatocytes produced by monolayer culture.[9,10] In order to demonstrate the differentiation of HSLC into cells of the hepatocytic lineage *in vitro*, the cells were co-cultured for 24 h with epidermal growth factor (EGF) at a concentration of 10 ng/mL. These cells formed spheroids in culture. The expression of albumin was examined as a marker of cell differentiation in culture cells. The mRNA expression profile for both CK19 and tyrosine aminotransferase (TAT) was also examined in HSLC before and after culturing with EGF. Primary cultured, normal adult hepatocytes were used as control cells.[11]

Western blot analysis of albumin expression in HSLC

Expression of albumin was analyzed in HSLC cultured with or without EGF. The proteins were prepared by treating the cells with cell lysis buffer, followed by centrifugation. A 15-μg sample of proteins was subjected to a 10% sodium dodecylsulfate polyacrylamide ready gel (Bio-Rad Laboratories, Richmond, CA, USA). Resolved proteins were transferred electrophoretically to an Immunobilon-P membrane (Millipore, Bedford, MA, USA) at 4°C and processed for immunodetection. After blocking with 5% nonfat milk for 1 h at room temperature, the membrane was incubated with rabbit antirat albumin antibody (1:200 dilution; Cappel, Aurora, OH, USA) at 37°C for 2.5 h. The membrane was then incubated with alkaline phosphatase-labeled goat antirabbit immunoglobulin (Ig)G antibody (1:1000 dilution; KPL, Gaithersburg, MD, USA) for 1.5 h at room temperature. Detection of the immunoreaction was performed with the BCIP/NBT phosphate substrate system (KPL), according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT–PCR)

The mRNA expression of *CK19*, *TAT*, *Notch* family (*Notch1* and *Notch2*) and its ligand *Jagged1*, and *Hes1* in both HSLC cultured with or without EGF and in primary cultured mature hepatocytes were examined by RT–PCR according to the procedure we previously described.[7] The PCR consisted of 35 cycles at a denaturation temperature of 94°C for 30 s, an annealing temperature of 58°C for 2 min and an extension temperature of 72°C for 1 min using a Perkin-Elmer 9600 thermal cycler platform (Perkin-Elmer, Norwalk, CT, USA). The primers for PCR to detect mRNA expression were: *Musashi1*, 5′-GGC TTCGTCACTTTCATGGACCAGGCG-3′ and 5′-GGGACC TGGTAGGTGTAAC-3′ (PCR product; 542 bp); *Hes1*, 5′-CCACTGCTACCCGTAAAGTC-3′ and 5′-GGCCTGAG GCTCTCAGTTCC-3′ (228 bp); *Notch1*, 5′-GACTATGCC TGCAGCTGTGCC-3′ and 5′-GGCTGCAGGGCACGTA GG-3′ (421 bp); *Notch2*, 5′-ATGTGTGTTACCTACCA CA-3′ and 5′-CCACAGTGGTACAGGTACTT-3′ (371 bp); *Jagged1*, 5′-CATCATAGCCTGTGAGCCTTC-3′ and 5′-ATATCATCCTCTTCCACTTCC-3′ (492 bp); CK19, 5′-TT GCGCGACAAGATTCTTGG-3′ and 5′-CATCTCACTCAG GATCTTGG-3′ (361 bp); and TAT, 5′-TGAACAGCAC TACCACTGTG-3′ and 5′-AGGCATCCTCCGTCTTCT GC-3′ (380 bp). The PCR reaction for β-actin was performed as an internal control (191 bp).[7]

Quantitation of Musashi1 mRNA levels in HSLC before and after differentiation

The total cellular RNA extracted from Hep3B cells positive for *Musashi1* mRNA expression[7] was used as a standard. The methods for RNA isolation and cDNA amplification were performed as previously.[7] To quantitate *Musashi1* mRNA levels in HSLC before and after differentiation, real-time PCR was performed using a LightCycler quick system 350S (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The primers for detection of *Musashi1* mRNA in the real-time PCR were 5′-GGCTTCGTCACTTTCATGGA CCAGGCG-3′ and 5′-GGGACCTGGTAGGTGTAAC-3′. Quantitation test was performed in quadruplicate and the results were expressed as mean ± standard error (SE). Differences at $P < 0.05$ by Mann–Whitney *U*-test were considered significant.

Immunofluorescence staining for Musashi1 in HSLC

Expression of Musashi1 was analyzed by indirect immunofluorescence staining in HSLC cultured with or

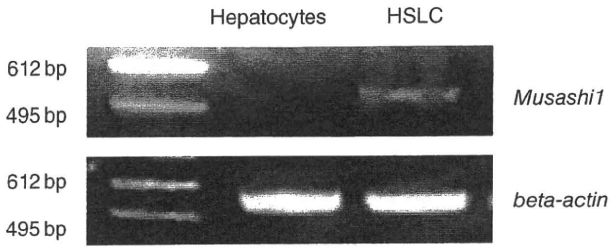

Figure 1 Reverse transcription polymerase chain reaction (RT–PCR) analysis of *Musashi1* mRNA expression in hepatic stem-like cells (HSLC) and primary cultured mature hepatocytes. The mRNA expression of *Musashi1* was detected in HSLC but not in hepatocytes. The predicted size of the PCR-amplified *Musashi1* product was 542 bp.

without EGF. A polyclonal rabbit anti-Musashi peptide antibody (Chemicon International, Temecula, CA, USA) that recognizes human and rodent Musashi1 was used as a primary antibody. Fluorescein isothiocyanate-labeled $F(ab')_2$ fragments of goat antirabbit IgG (Dako-Cytomation, Kyoto, Japan) were used as a secondary antibody. The cells were examined with the aid of a fluorescence microscope.

RESULTS

Expression of Musashi1 mRNA and Musashi1 protein in HSLC

THE MRNA EXPRESSION of *Musashi1* was detected in HSLC but not in primary cultured mature hepatocytes by RT–PCR (Fig. 1). The RT–PCR product of the *Musashi1* mRNA amplified using specific primers predicted a band of 542 bp. Protein expression of Musashi1 was detected in HSLC by immunofluorescence staining (Fig. 2), but it was not detected in primary cultured hepatocytes.

Downregulated expression of Musashi1 mRNA and Musashi1 protein in differentiated HSLC producing albumin

Hepatic stem-like cells were cultured with 10 ng/mL EGF for 24 h and harvested when they formed spheroids. Albumin expression in these cells was examined as a marker of cell differentiation from an immature to a mature state. Albumin expression was not detected in HSLC before culturing with EGF, but was detectable by western blot analysis after culturing with EGF. Quantitative analysis revealed that the level of *Musashi1* mRNA in the differentiated HSLC was significantly lower than that of original HSLC ($1.06 \pm 1.34 \times 10^{10}$ copies/mL vs $4.33 \pm 2.68 \times 10^{13}$ copies/mL, mean ± SE, $P < 0.05$) (Fig. 3). Expression of Musashi1 protein was not detected in differentiated HSLC producing albumin by immunofluorescence staining.

Changes in mRNA expression of the Notch signaling markers in HSLC differentiation

Because the expression of *Musashi1* mRNA was found to be downregulated in the cell differentiation process, the expression of the *Notch* family mRNA was investigated in HSLC before and after culturing with EGF by RT–PCR analysis. *Notch1* mRNA expression was detected in HSLC before and after culturing with EGF, but its expression was not detected in primary cultured mature hepatocytes. *Notch2* mRNA expression was found in HSLC before and after culturing with EGF as well as in primary cultured mature hepatocytes. The notch ligand *Jagged1* mRNA expression was detected in HSLC before and after culturing with EGF, but its expression was not detected in primary cultured mature hepatocytes. *Hes1* mRNA expression was detected in the original HSLC, but not in those producing albumin after culturing with EGF, and nor was it detected in primary cultured mature hepatocytes. The biliary cell marker, CK19 mRNA expression was not detected in any cells examined. The hepatocyte marker, TAT mRNA expression was not detected in the original HSLC, but its expression was detectable in those

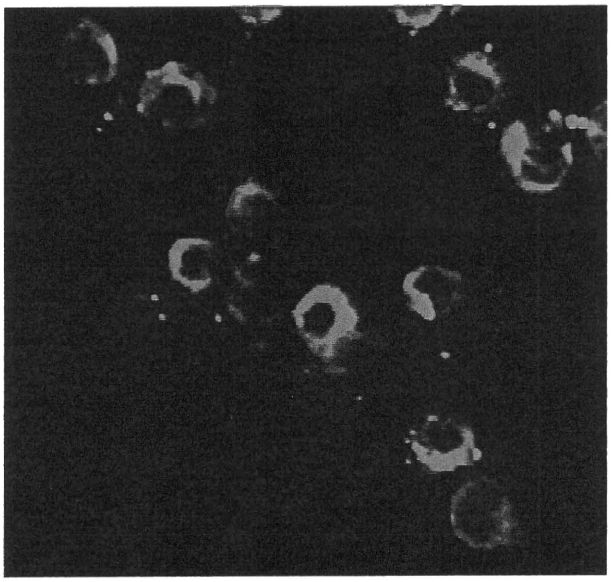

Figure 2 Immunofluorescence staining for Musashi1 protein in the cytoplasm of hepatic stem-like cells (original magnification ×400).

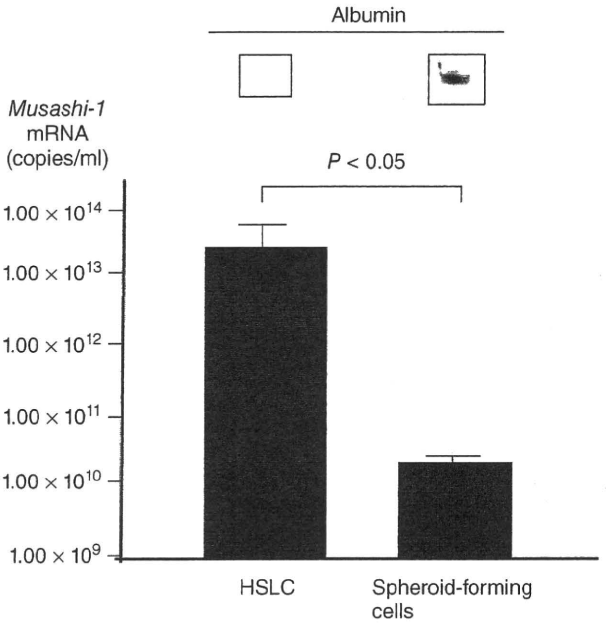

Figure 3 Western blot analysis of albumin expression in hepatic stem-like cells (HSLC). Albumin expression was not detected in the original HSLC, but was detected in spheroid-forming cells after culturing with epidermal growth factor (EGF). Real-time polymerase chain reaction analysis of *Musashi1* mRNA expression in HSLC before and after culturing with EGF. The level of *Musashi1* mRNA in the spheroid-forming differentiated cells was significantly lower than that of original HSLC. Quantitation test was performed in quadruplicate.

producing albumin after culturing with EGF as well as in primary cultured mature hepatocytes (Fig. 4).

DISCUSSION

ALTHOUGH A CLOSE association has been shown to exist between Musashi1 and Notch signaling in neural stem cell differentiation,[4] the involvement of such a mechanism in the differentiation of stem cells in digestive organs has not been fully elucidated. Recently, it was shown that Musashi1 is expressed in putative intestinal stem cells [12] and can be used as a marker of stem cells and early-lineage progenitor cells in murine intestinal tissue.

In this study, we have demonstrated that Musashi1 is expressed in putative rat liver stem-like cells at the mRNA and protein level. Interestingly, the mRNA expression of *Hes1* was downregulated along with *Musashi1* mRNA expression in the differentiated cells that produced albumin. Notch proteins were initially identified in *Drosophila* and *Caenorhabditis elegans*, but have subsequently been identified in vertebrate species.[13] It has been reported that there is an association between expression of the *Notch* family, and bile duct formation,[14] liver cell regeneration after partial hepatectomy[15] and neovascularization in the human diseased liver,[16] although no such association has been demon-

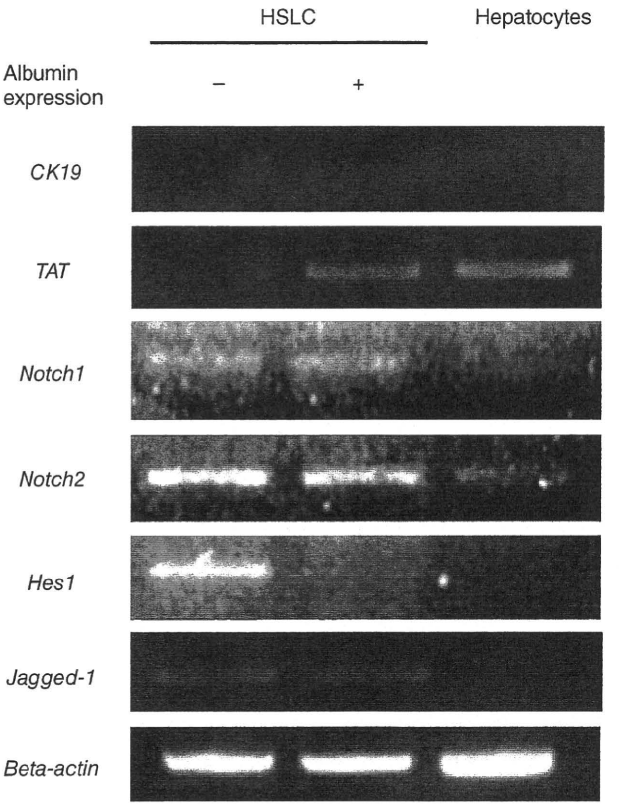

Figure 4 The mRNA expression for cytokeratin (CK)19, tyrosine aminotransferase (TAT) and *Notch* family in hepatic stem-like cells (HSLC), as revealed by reverse transcription polymerase chain reaction (RT-PCR) analysis. CK19 mRNA expression was not detected in all cells. TAT mRNA expression was not detected in the original HSLC, but it was detectable in differentiated cells producing albumin as well as in mature hepatocytes. The mRNA expression for *Notch1* and *Jagged1* was detected in HSLC, but it was not detected in mature hepatocytes. *Notch2* mRNA expression was detected in all cells. The mRNA expression of *Hes1* was detected in HSLC, but was undetectable in differentiated cells producing albumin as well as in mature hepatocytes. The predicted size of the PCR-amplified product was 361 bp for CK19, 380 bp for TAT, 421 bp for *Notch1*, 371 bp for *Notch2*, 492 bp for *Jagged-1* and 228 bp for *Hes1*.

strated in liver stem cells. Both Notch-1 and its ligand Jagged-1 have been detected in the hepatic progenitor cells, referred to as oval cells, in the liver of the 2-acetylaminofluorene 70% hepatectomy models.[17] In this study, *Notch1* and *Jagged1* mRNA expressions were detectable in HSLC , but not in mature rat hepatocytes. The absence of Notch1 expression in mature hepatocytes has also been demonstrated in humans.[17] *Hes1* mRNA expression is activated by a nuclear translocation of the Notch intracellular domain.[11] In the present study, we could show that *Hes1* mRNA expression was also detectable in HSLC at a location downstream of this signaling. The mRNA expression of *Notch1–Hes1* signaling was upregulated in Musashi1-positive HSLC and was undetectable in the differentiated cells producing albumin. To confirm the association of Musashi1 with an activation of the Notch signaling, it would be important to see if changes in Musashi1 expression level by the gene knockdown influence of liver stem-like cell differentiation. In addition, expression of Musashi1 in the liver tissue remains unknown. Further studies are needed to elucidate these issues.

The roles of Musashi1 in the development of liver morphology and function remain unknown. A report on the *Musashi1* gene disruption model revealed that homozygous newborn mice are not prone to immediate death, but frequently develop obstructive hydrocephalus with aberrant proliferation of ependymal cells.[5] As Musashi2, another member of the RNA-binding protein family,[18] is co-expressed in this model, gene compensation of *Musashi2* in the *Musashi1* disruption model might contribute to organ development, and hence improve the chances of survival. Analyses of alteration of liver-specific mRNA expression as well as liver morphology in such a model would provide information that could extend our understanding of the role of Musashi1 in the development of liver morphology and function.

In conclusion, the results of this study suggest that Musashi1 is expressed with Notch signaling markers in liver stem-like cells as well as in neural stem cells in adults. The role of Musashi1 in liver regeneration warrants further investigation.

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Release of Interleukin-1 Receptor Antagonist by Combining a Leukocyte Adsorption Carrier With Ulinastatin

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Abstract: Both granulocyte/monocyte adsorptive apheresis (GMA) and ulinastatin, a serine protease inhibitor, are reported to be effective in patients with ulcerative colitis; however, combination therapy with GMA and ulinastatin has not been attempted. Investigating the effect of ulinastatin on GMA is required for combination therapy since the inhibition of serine protease suppresses the reaction of GMA. To clarify the effects of ulinastatin on GMA, we investigated whether granulocyte adsorption to cellulose acetate beads (carriers for GMA) and interleukin-1 receptor antagonist (IL-1ra) release were inhibited by ulinastatin. Peripheral blood containing ulinastatin, a different serine protease inhibitor (gabexate mesilate), or signal-transduction inhibitors was incubated with cellulose acetate beads in vitro, and the ratios of adsorbed granulocytes and IL-1ra release were measured. Granulocyte adsorption and IL-1ra release were significantly suppressed with increasing gabexate mesilate concentrations; however, the adsorption was not significantly inhibited by ulinastatin. Furthermore, IL-1ra release was augmented by the addition of a high dose of ulinastatin or PD98059 as compared to a low dose. The activation levels of extracellular signal-regulated protein kinase may regulate IL-1ra release induced by the carrier, because both ulinastatin and PD98059 inhibit extracellular signal-regulated protein kinase. High concentrations of ulinastatin increased IL-1ra release without inhibiting granulocyte adsorption to cellulose acetate beads. This result warrants clinical trials of a combination of ulinastatin and GMA for the treatment of ulcerative colitis. **Key Words:** Adsorption, Granulocytes, Interleukin-1 receptor antagonist, Ulcerative colitis, Ulinastatin.

Cytokines, which are categorized into pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and IL-8, or anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra) and IL-10, play a key role in the modulation of the intestinal mucosal immune system (1). A major source of cytokines is activated granulocytes and monocytes (GM) (2). In patients with active ulcerative colitis, it has been reported that leukocytes produce significantly more TNF-α and IL-1β compared with normal controls (3), and that the mucosal levels of TNF-α, IL-1β, IL-6, and IL-8 are significantly higher, and the IL-1ra/IL-1β ratio significantly lower, compared with the control group (4). Such imbalance between pro- and anti-inflammatory cytokines is considered to be an important feature of active ulcerative colitis and a target of therapy, although the etiology and pathogenesis remain unclear.

A GM adsorptive apheresis (GMA) device (Adacolumn; Jimro Institute, Takasaki, Japan) can deplete excess and activated GMs from the peripheral blood of patients with ulcerative colitis (5). The device comprises a column filled with 2-mm cellulose acetate beads that act as carriers for adsorptive leukocyte apheresis (6). After GMA therapy, the blood level of pro-inflammatory cytokines decreases (3), anti-inflammatory cytokines including IL-1ra increase, but IL-1β is not detectable in outflow of the GMA column (6–8); also, the mucosal level of pro-inflammatory cytokines decreases, and the IL-1ra/IL-1β ratio increases (4). Although the precise mechanisms of the clinical efficacy of GMA are unclear, GM adsorption likely triggers various biological responses, such as the

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*These authors contributed equally to this work.

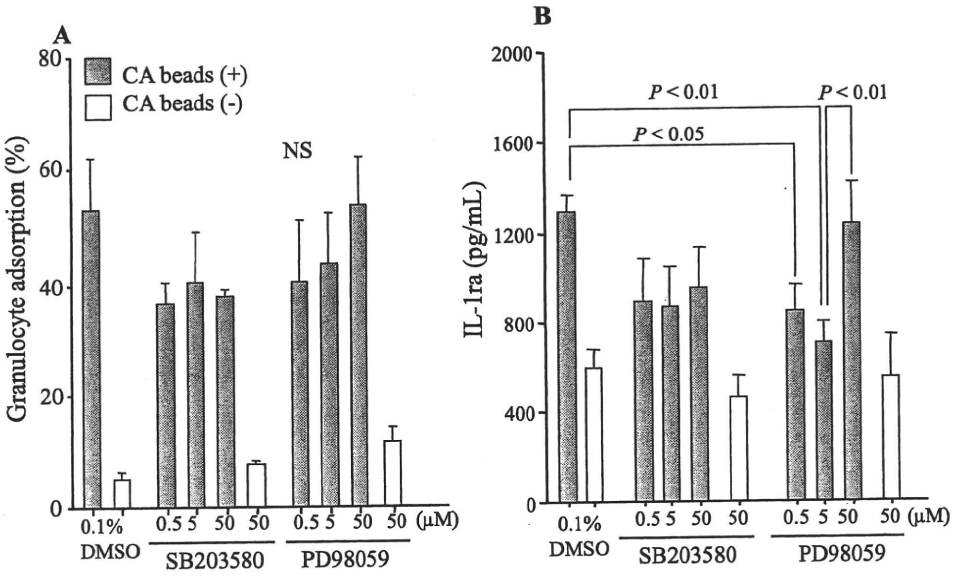

FIG. 3. Effects of signal transduction inhibitors on (A) the adsorption to cellulose acetate (CA) beads and (B) IL-1ra release. Blood cells were pre-incubated with various concentrations of SB203580 or PD98059 for 15 min at room temperature and then cultured with (■) or without (□) cellulose acetate beads for 1 h. For the control treatment, 0.1% dimethyl sulfoxide (DMSO) was used. Data are presented as mean ± standard error from three different blood donors. NS, not significant, by repeated-measures one-way ANOVA (post-hoc test with Bonferroni) in cellulose acetate beads (+).

inhibited using SB203580 and PD98059, respectively. These inhibitors did not show any cytotoxic effects on leukocyte viability after examination (flow cytometric assessment of viable cells using propidium iodide staining showed more than 95% viable cells), and the number of cells did not decrease after incubation. The down-regulation of L-selectin (CD62L) induced by fMLP (1 μmol/L) stimulation was clearly suppressed by treatment with SB203580 and PD98059 (data not shown). These inhibitors slightly, but not significantly, inhibited granulocyte adsorption, as with ulinastatin (Fig. 3A). SB203589 did not significantly inhibit IL-1ra release (Fig. 3A); however, IL-1ra release was significantly inhibited by the addition of a low dose of PD98059 (0.5 and 5 μmol/L), and a high dose of PD98059 (50 μmol/L) augmented IL-1ra release as compared to a low dose (Fig. 3B).

DISCUSSION

Ulinastatin has two functions: one is as a serine protease inhibitor, and the other is as a suppressor of pro-inflammatory cytokine production. In the present study we found that ulinastatin did not inhibit GM adsorption to cellulose acetate beads, and that 10 000 units/mL of ulinastatin augmented the release of IL-1ra induced by GM adsorption. Ulinastatin and cellulose acetate beads are separately used for patients with ulcerative colitis, and IL-1ra release is correlated with clinical efficacy (15,17); thus, the present study demonstrates the feasibility of combination therapy with ulinastatin and GMA.

Complement activation fragments produced by contact between cellulose acetate beads and blood play important roles in biological responses to GMA therapy. Our previous studies have demonstrated that IL-1ra release requires both granulocyte adhesion to cellulose acetate beads and C5a (anaphylatoxin) stimulation (8,18,19). The release of IL-1ra by cellulose acetate beads is similar to that of elastase, a serine protease. Granulocytes stimulated in suspension show little degranulation, but C5a induces the release of elastase from adherent cells in minutes (20). It is well known that granulocyte adhesion and C5a stimulation cause activation of ERK1/2 and p38 MAPK signaling pathways in granulocytes (21,22); thus, ERK1/2 and p38 MAPK signaling pathways should be involved in the release of IL-1ra.

Another serine protease inhibitor, gabexate mesilate, did not augment IL-1ra release (Fig. 2A), suggesting that IL-1ra release is unrelated to serine protease inhibition. Interestingly, gabexate mesilate also inhibits cytokine production, but this effect is mediated by the suppression of nuclear factor-κB (NF-κB) activation (11). On the other hand, ulinastatin inhibits phosphorylation of ERK1/2 and decreases expression of early growth response

factor-1 induced by lipopolysaccharide without affecting the activation of NF-κB and activator protein-1 (23). Since C5a/C5a receptor signaling requires the phosphorylation of ERK1/2 (21), it is reasonable to postulate that a low dose of ulinastatin and an ERK1/2-specific inhibitor (PD98059) inhibit the release of IL-1ra.

That high doses of these reagents augmented the release of IL-1ra is difficult to explain. Interestingly, PD98059 blocks neutrophil chemotaxis, but does not alter superoxide anion production and paradoxically enhances degranulation responses to stimuli (24). Indeed, the effect of PD98059 on IL-1ra release was similar to that of a previous report on degranulation (Fig. 3). The blocking of ERK signaling by ulinastatin may augment alternative signaling by a functional diversion of ERK signaling pathways, such as the activation and maintenance of cytosolic phospholipase A_2 activity (22).

Gabexate mesilate has an inhibitory effect on complement activation (9). We measured the production of C5a in blood samples from two volunteers after incubation with cellulose acetate beads using flow cytometry and found that the level of C5a in blood containing 800 μg/mL of gabexate mesilate was lower than in blood containing 10 000 units/mL of ulinastatin (Nishise, unpublished results). Since complement activation is required for granulocyte adsorption to cellulose acetate beads and the release of IL-1ra (8), the difference between ulinastatin and gabexate mesilate on GM adsorption should be attributed to the inhibitory effect on complement activation.

In patients with ulcerative colitis, ulinastatin has been used with steroids and is thought to be an effective therapy. It has been reported that rectal mucosal blood flow is decreased in active ulcerative colitis patients as compared with healthy controls, but it is improved after the intravenous injection of ulinastatin and prednisolone (15), and that intravenous injection of 200 000 units of ulinastatin weekly for three months in patients taking corticosteroids is effective in 64% of patients with moderate to severe ulcerative colitis who are resistant to steroid therapy (16). Combination therapy with ulinastatin and GMA has not been administered in patients with ulcerative colitis, and the biological effects of ulinastatin for GM adsorption to cellulose acetate beads are still unknown. Our study found that a high concentration of ulinastatin augmented the release of IL-1ra in GM adsorption to cellulose acetate beads. It has been reported that patients with ulcerative colitis who responded to GMA treatment show a significant increase in IL-1ra in the Adacolumn outflow (17), and that the mucosal tissue ratio of IL-1ra/IL-1β is significantly increased in ulcerative colitis patients with clinical remission after Adacolumn therapy (4). Therefore, ulinastatin administered during GMA may provide clinical efficacy in patients with ulcerative colitis through an increase in IL-1ra. There are some practical issues that remain to be resolved before ulinastatin can be applied with GMA in a clinical setting. First of all, it must be verified that a high dose of ulinastatin does not have adverse affects on the human body, because a larger dose of ulinastatin than that used in previous studies might be needed for ulcerative colitis patients during Adacolumn therapy.

CONCLUSION

The present study found that a high concentration of ulinastatin increased the release of IL-1ra without inhibiting granulocyte adsorption to cellulose acetate beads. Our results should help to clarify the anti-inflammatory effects of ulinastatin and potentially lead to new combination therapies for patients with ulcerative colitis.

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BASIC—ALIMENTARY TRACT

Up-regulation of Activation-Induced Cytidine Deaminase Causes Genetic Aberrations at the *CDKN2b-CDKN2a* in Gastric Cancer

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BACKGROUND & AIMS: The DNA/RNA editing enzyme activation-induced cytidine deaminase (AID) is mutagenic and has been implicated in human tumorigenesis. *Helicobacter pylori* infection of gastric epithelial cells leads to aberrant expression of AID and somatic gene mutations. We investigated whether AID induces genetic aberrations at specific chromosomal loci that encode tumor-related proteins in gastric epithelial cells. **METHODS:** Human gastric epithelial cell lines that express activated AID and gastric cells from AID transgenic mice were examined for DNA copy number changes and nucleotide alterations. Copy number aberrations in stomach cells of *H pylori*–infected mice and gastric tissues (normal and tumor) from *H pylori*–positive patients were also analyzed. **RESULTS:** In human gastric cells, aberrant AID activity induced copy number changes at various chromosomal loci. In AID-expressing cells and gastric mucosa of AID transgenic mice, point mutations and reductions in copy number were observed frequently in the tumor suppressor genes *CDKN2A* and *CDKN2B*. Oral infection of wild-type mice with *H pylori* reduced the copy number of the *Cdkn2b-Cdkn2a* locus, whereas no such changes were observed in the gastric mucosa of *H pylori*–infected AID-deficient mice. In human samples, the relative copy numbers of *CDKN2A* and *CDKN2B* were reduced in a subset of gastric cancer tissues compared with the surrounding noncancerous region. **CONCLUSIONS: *H pylori* infection leads to aberrant expression of AID and might be a mechanism of the accumulation of submicroscopic deletions and somatic mutations in gastric epithelial cells. AID-mediated genotoxic effects appear to occur frequently at the *CDKN2b-CDKN2a* locus and contribute to malignant transformation of the gastric mucosa.**

Keywords: Gastric Cancer; Activation-Induced Cytidine Deaminase; Genetic Alterations; Tumor-Related Genes.

Increasing evidence indicates that cancer arises from a stepwise accumulation of genetic changes and that the incipient cancer cells acquire mutant alleles of tumor suppressor genes and/or proto-oncogenes.[1] Genetic alterations observed in cancers include point mutations, chromosomal number alterations, chromosomal translocations, and gene deletions or amplifications.[2] A number of human cancers have deletions and/or point mutations at specific gene loci, causing the putative inactivation of tumor suppressor genes. Thus, elucidation of the molecular mechanisms underlying the genetic alterations that occur at the loci encoding tumor suppressor proteins is important to gain a better understanding of tumorigenesis.

A novel mechanism of genetic alterations (ie, DNA/RNA editing by members of cytidine deaminases) was recently reported.[3–5] Among the 11 human cytidine deaminases identified, activation-induced cytidine deaminase (AID) is the only molecule that exerts genetic effects on human DNA sequences under physiologic conditions. AID is an essential enzyme for somatic hypermutation, class switch recombination (CSR), and gene conversion, all of which are crucial steps to achieve the diversification of the *immunoglobulin* (*Ig*) genes in activated B lymphocytes.[3] In sharp contrast to the physiologic role of AID in the editing of the *Ig* genes, we recently demonstrated a pathologic role of AID linking the accumulation of nucleotide alterations in tumor-related genes and human cancer development.[6–8] Indeed, aberrant expression of AID is induced in response to proinflammatory cytokine stimulation in gastric epithelial cells, colon epithelial cells, biliary ductal cells, and hepatocytes and leads to the accumulation of somatic mutations in various tumor-related genes in vitro.[9–13] Moreover, we showed that infection with *Helicobacter pylori*, a class 1 carcinogen for gastric cancer, induces aberrant AID expression in gastric epithelial cells, resulting in the accumulation of *TP53* tumor suppressor gene mutations.[13] Consistent with

Abbreviations used in this paper: **AID, activation-induced cytidine deaminase; BCL6, B-cell CLL/lymphoma 6; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; CGH, comparative genomic hybridization; CSR, class switch recombination; DSB, double-strand break; PCR, polymerase chain reaction; Tg, transgenic; WT, wild-type.**

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these in vitro findings, a mouse model with continuous and ubiquitous expression of AID develops cancers in several epithelial organs, including stomach, liver, and lung, via the accumulation of somatic mutations.[14–16] These findings elucidated a novel molecular mechanism linking inflammation, genetic mutations, and cancer development.

Because AID can trigger a CSR of the *Ig* gene, it is reasonable to assume that AID can also mediate chromosomal aberrations by triggering double-strand DNA breaks (DSBs) in lymphoid cells, in addition to somatic point mutations. Indeed, recent studies have shown that AID is required for the generation or accumulation of chromosomal translocations during lymphoma development.[17] For example, translocations between *c-myc* and the IgH locus (*Igh*) are induced in primary B cells within hours of AID expression, whereas *c-myc-Igh* translocations are absent in AID-deficient mice.[18–23] These findings prompted us to speculate that aberrant expression of AID in epithelial cells might cause not only somatic point mutations but also chromosomal alterations, both of which would play critical roles in the activation and/or inactivation of tumor-related genes. In this study, therefore, we investigated whether the genotoxic activity of AID could underlie the emergence of genetic aberrations at specific chromosomal loci encoding tumor-related proteins in human gastric epithelial cells and thus contribute to the development of gastric cancers.

Materials and Methods

Mice

The generation of transgenic (Tg) mice with constitutive and ubiquitous AID expression and AID-deficient mice was described previously.[15,24] Wild-type (WT) C57BL/6J mice were purchased from Japan SLC, Inc (Shizuoka, Japan). For infection, mice were challenged with 1.5×10^7 colony-forming units of *Cag*PAI-positive (TN2GF4) *H pylori* as described previously.[25–27] All experiments involving mice conformed to the relevant regulatory standards and were reviewed and approved by the Kyoto University School of Medicine Institutional Animal Care and Use Committee.

Cell Culture and Transfection

AGS human gastric epithelial cells and stable transfection of AID-expressing vector into AGS cells was described previously.[13]

Genomic Polymerase Chain Reaction, Reverse-Transcription Polymerase Chain Reaction, and Quantitative Real-Time Genomic and Reverse-Transcription Polymerase Chain Reaction

The oligonucleotide primers used are shown in Supplementary Table 1. Quantification of gene expression or gene copy numbers was performed by quantitative real-time reverse-transcription polymerase chain reaction (PCR) or genomic PCR using a Light Cycler 480 and Fast Start Universal Probe or SYBR Master (Roche, Mannheim, Germany).[9] To assess the quantity of isolated DNA, target DNAs were normalized to the DNA levels of the housekeeping reference gene human *ACTB* or mouse *Actb*. For simplicity, the ratios are represented as relative values of target gene/human *ACTB* or mouse *Actb*.

Comparative Genomic Hybridization Microarray Analysis

Genomic analyses were performed on Human 44K Agilent arrays by comparative genomic hybridization (CGH; Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. DNA extracted from AID-overexpressing AGS cells and reference DNA were both labeled by random priming with Cy3-deoxycytidine triphosphate and Cy5-deoxycytidine triphosphate for dye-swap experimental design. Arrays were scanned on an Agilent microarray scanner. Data were extracted and flagged with the Feature Extraction software. Agilent CGH Analytics software was used to identify regions of copy number alteration.

Fluorescence In Situ Hybridization Analysis

DNA probes specific to *CDKN2b-CDKN2a* locus and *ELAVL2* gene were amplified using the primers shown in Supplementary Table 1. These probes were labeled by nick-translation with either SpectrumGreen- or SpectrumOrange-labeled deoxyuridine triphosphate (Abbott Molecular Inc, Des Plaines, IL) and hybridized to chromosomes of AGS cells expressing AID for 21 days. Images were taken with the fluorescence microscope MD5000B (Leica, Wetzlar, Germany).

DNA Polymorphism Analysis

The DNA polymorphism analyses of restriction fragment length polymorphisms were performed to detect loss of heterozygosity. PCR was performed using the primer sets shown in Supplementary Table 1, and PCR products were digested with *Dde* I (for human *CDKN2A*) or *Psp* 1406 I (for human *CDKN2B*).

Southern Blot Analysis

Southern blot analysis of the PCR products was performed using AlkPhos Direct Labelling Reagents (GE Healthcare, Buckinghamshire, England), with DNA probes labeled using alkaline phosphatase, according to the manufacturer's protocol. The primer sets used are shown in Supplementary Table 1.

Subcloning and Sequencing of Tumor-Related Genes

The oligonucleotide primers used are shown in Supplementary Table 1. Amplification of the target sequences was performed using high-fidelity Phusion Taq polymerase (Finnzymes, Espoo, Finland), and the products were subcloned into a pcDNA3 vector (Invitrogen, Carlsbad, CA). The resulting plasmids were subjected to sequence analysis.[11]

Patients

The study group comprised 28 patients who had undergone potentially curative resection of primary gastric cancer at Kyoto University Hospital from 2006 to 2007. Written informed consent for the use of the resected tissues was obtained from all patients in accordance with the Declaration of Helsinki, and the Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved the study.

Statistics

Statistical significance ($P < .05$) was evaluated using the χ^2 test for sequence and fluorescence in situ hybridization analyses and the Mann–Whitney U test for quantitative real-time PCR analysis.

Results

AID Expression Induces Chromosomal Aberrations in Gastric Epithelial Cells

To view the overall landscape of the genetic alterations caused by AID activation in human gastric epithelial cells, we used a system that allows for conditional AID activation by constructing a stable transfectant of AID fused with the hormone-binding domain of the human estrogen receptor in the human gastric epithelial cell line AGS (Supplementary Figure 1). We then conditionally activated AID in the cells by introducing an estrogen analogue, 4-hydroxytamoxifen,[13,28] followed by CGH analyses performed on DNA samples extracted from the cells with or without AID activation. Copy number changes emerged in a number of submicroscopic areas in almost all chromosomes of the cells with AID activation compared with the control cells (Supplementary Figure 2). Most of the changes observed in the AID-expressing cells were submicroscopic deletions represented by copy number losses of various chromosomal loci, whereas large-scale deletions or changes in chromosomal number, such as monosomy, were not apparent in the cells with AID expression. In contrast, a submicroscopic copy number gain was observed in a few chromosomal loci on 3p, 10q, and 19p in AID-expressing cells (Supplementary Figure 2). Analysis of the time course changes in the copy numbers revealed that the number of submicroscopic chromosomal deletions increased depending on the duration of AID activation in gastric cells (Figure 1*A* and Supplementary Table 2). These findings suggested that AID expression caused the copy number changes, mainly by inducing submicroscopic chromosomal deletions, in gastric epithelial cells.

AID Induces Reductions of the CDKN2A *and* CDKN2B *Copy Numbers in Gastric Epithelial Cells*

Repeated CGH analyses on AGS cells showed that deletions at 2 specific loci, 9p21 and 3q27, commonly occurred after 1-week and 3-week AID activation, although there were many deleted regions observed. Notably, these chromosomal regions harbored the tumor suppressor genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) at 9p21 and B-cell CLL/lymphoma 6 (*BCL6*) at 3q27 (Figure 1*A*). It has been well recognized that *CDKN2A* and *CDKN2B* play crucial roles as tumor suppressor genes in the development of various human tumors.[29] Therefore, we further examined whether AID expression caused the deletion of the *CDKN2b-CDKN2a* locus at 9p21 using fluorescence in situ hybridization analyses with the probes specific for *CDKN2b-CDKN2a* locus and control *ELAVL2* gene. We found that significantly more deletions of *CDKA2b-CDKN2a* locus were present in human gastric cells with AID activation than those in the control cells (27.6% and 6.9%, respectively; $P < .001$; Figure 1*B* and Table 1). In contrast, there was no significant difference in the frequency of deleted signals for *ELAVL2* gene between AID-expressing cells and cells without AID activation (11.9% and 11.2%, respectively; Table 1). These results suggest that AID preferentially induces submicroscopic deletions of *CDKN2A* and *CDKN2B* genes in gastric epithelial cells.

Next, we analyzed the gastric mucosa of AID Tg mice, which develop various tumors, including gastric cancer, in association with the accumulation of somatic mutations.[16] We examined the relative copy number ratio of *Cdkn2a* and *Cdkn2b* at chromosome 4, and *Bcl6* at chromosome 16, in noncancerous gastric mucosa as well as in the gastric cancer tissue of AID Tg mice (Figure 2*A*). As a reference, we selected several genes, such as *Acot7* at chromosome 4 and *Actb* at chromosome 5, that were located at stable chromosomal sites in the AID-expressing cells in vitro. The relative copy number ratios of *Cdkn2a* and *Cdkn2b* were significantly lower in gastric epithelial cells of AID Tg mice compared with those of the WT mouse ($P < .05$; Figure 2*B* and *C*). Moreover, the gastric cancer tissues had substantially reduced amounts of *Cdkn2a* and *Cdkn2b* compared with the noncancerous gastric mucosa in the AID Tg mice (Figure 2*B* and *C*). All of the AID Tg mice also had significantly reduced copy number levels of *Bcl6* in the gastric mucosa compared with the WT mouse ($P < .01$; Figure 2*D*). In contrast, there was little difference in the copy numbers of the *Acot7* gene between the gastric mucosa of the WT and AID Tg mice (Figure 2*E*). Southern blotting analyses revealed that signals derived from the *Cdkn2a* gene in the gastric mucosa of the AID Tg mice were substantially reduced compared with that in the WT mouse (Figure 2*F*). These findings together suggested that constitutive expression of AID in normal gastric epithelial cells resulted in submicroscopic *Cdkn2a* and *Cdkn2b* gene defects at high frequency in vivo.

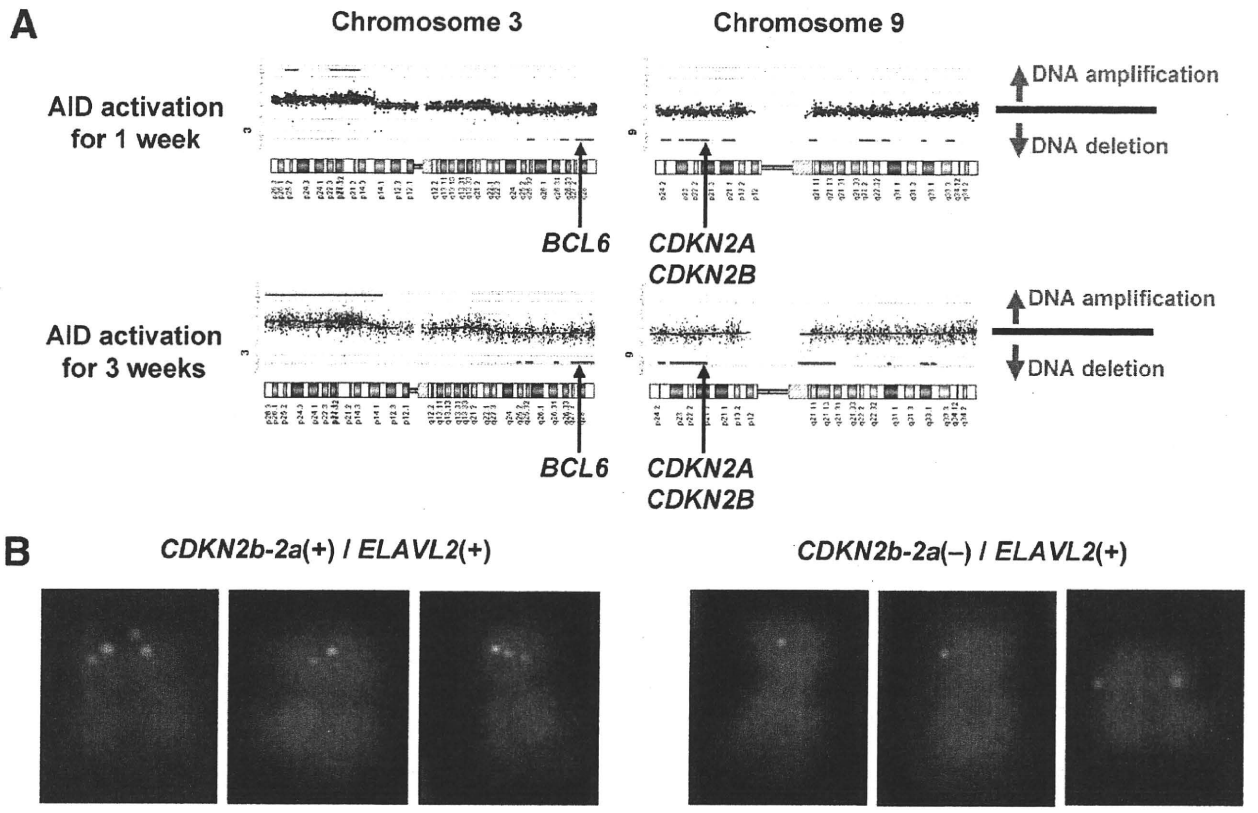

Figure 1. Copy number analyses of *CDKN2A* and *CDKN2B* genes in AGS cells with or without AID activation. (*A*) Copy number profiles on chromosomes 3 and 9 of AGS cells with AID activation for 1 or 3 weeks, using CGH analyses, are shown. The *BCL6* gene is located on chromosome 3, and the *CDKN2A* and *CDKN2B* genes are located on chromosome 9. *Red* and *green dots* represent copy number amplification and reduction, respectively. (*B*) Dual-color fluorescence in situ hybridization analyses for AID-expressing AGS cells. Representative images for the *CDKN2b-CDKN2a* locus (*green signals*) and *ELAVL2* gene (*red signals*) in cells with AID activation for 3 weeks. Chromosomes with both *CDKN2b-CDKN2a* locus and *ELAVL2* gene had green and red signals (*CDKN2b-2a*(+)/*ELAVL2*(+); *left panels*). Chromosomes without *CDKN2b-CDKN2a* signals had only red signals (*CDKN2b-2a*(−)/*ELAVL2*(+); *right panels*).

AID Expression in Gastric Epithelial Cells Caused Somatic Mutations in the CDKN2A *and* CDKN2B *Genes*

To further determine if the *CDKN2A* and *CDKN2B* genes are preferential targets of AID-mediated genotoxic effects, we examined whether somatic mutations are induced by AID activation in *CDKN2A* and *CDKN2B* genes of gastric cells in vitro and in vivo. In vitro, control AGS cells without AID activation contained only a single or 2 nucleotide alterations of the *CDKN2A* or *CDKN2B* gene sequences (Table 2). In contrast, gastric cells with AID activation had significantly higher frequencies of nucleotide alterations in the *CDKN2A* and *CDKN2B* genes than those in control cells ($P < .01$; Table 2). Of the 19 mutations in *CDKN2A* of AID-activated AGS cells, one was a nonsynonymous mutation and an-

Table 1. Frequency of Chromosomes With Deleted Signals Identified in AID-Expressing AGS Cells Using *CDKN2b-CDKN2a/ELAVL2* Dual-Color Fluorescence In Situ Hybridization Analyses

	AID(+)		Control	
Deleted genes	Frequency of the chromosomes without signals	Chromosomes without signals/total chromosomes	Frequency of the chromosomes without signals	Chromosomes without signals/total chromosomes)
CDKN2b-CDKN2a	27.6%	(97/352)[a]	6.9%	(24/347)
ELAVL2	11.9%	(42/352)	11.2%	(39/347)

NOTE. Data represent the frequency of chromosomes with the deletions of targeted genes, and values in parentheses indicate the number of chromosomes with deleted genes per number of total chromosomes examined.
[a]$P < .001$ vs control.