Relationship between Alcohol Consumption and Serum Adiponectin Levels: The Takahata Study—A Cross-Sectional Study of a Healthy Japanese Population

Yuko Nishise, Takafumi Saito, Naohiko Makino, Kazuo Okumoto, Jun-Itsu Ito, Hisayoshi Watanabe, Koji Saito, Hitoshi Togashi, Chisaki Ikeda, Isao Kubota, Makoto Daimon, Takeo Kato, Akira Fukao, and Sumio Kawata

Departments of Gastroenterology (Y.N., T.S., N.M., K.O., J.-I.I., H.W., K.S., C.I., S.K.); Cardiology, Pulmonology, and Nephrology (I.K.); Neurology, Hematology, Metabolism, Endocrinology, and Diabetology (M.D., T.K.); and Public Health (A.F.), Yamagata University School of Medicine, and Yamagata University Health Administration Center (H.T.), Yamagata 990-9585, Japan

Context: The relationship between alcohol consumption and serum adiponectin levels has not been fully explored in an Asian population.

Objective: Our goal was to determine whether alcohol consumption is associated with a change in adiponectin levels in a healthy Japanese population.

Design: This was a cross-sectional study.

Setting: Subjects were recruited from participants in a health check-up program.

Participants: This study included 2932 subjects (1306 men and 1626 women).

Main Outcome Measures: The effects of total weekly or daily volume of ethanol intake on serum adiponectin levels were evaluated. In addition, the correlation of clinical traits with serum adiponectin levels was examined. A multivariate regression model was used to control for possible confounding factors.

Results: Alcohol consumption was weakly correlated with decreased serum adiponectin levels in men [Spearman's ordered correlation coefficient (r_s) = −0.141; $P < 0.001$]; an even weaker correlation was seen in women (r_s = −0.055; $P = 0.025$). Multivariate analysis demonstrated that alcohol consumption was independently associated with hypoadiponectinemia.

Conclusion: In contrast to reports from the United States and Europe among White and Black subjects, our study demonstrated an inverse association between alcohol intake and serum adiponectin levels in Asian subjects, suggesting ethnic differences in the effects of alcohol consumption on serum adiponectin levels. ***(J Clin Endocrinol Metab* 95: 3828–3835, 2010)**

Adiponectin, predominantly synthesized in adipose tissue, is a major modulator of insulin action and resistance (1). It is also related to lipid metabolism, particularly higher levels of high-density lipoprotein cholesterol (HDL-C) and lower levels of triglycerides (2). Higher adiponectin levels are associated with a lower risk of coronary heart disease (3, 4) and type 2 diabetes (5).

Light to moderate alcohol intake is associated with lower risk for coronary heart disease, potentially by increasing HDL-C levels (6) or enhancing fibrinolysis (7).

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Abbreviations: ADH, Alcohol dehydrogenase; ALDH2, acetaldehyde dehydrogenase type 2; ALT, alanine aminotransferase; BMI, body mass index; FBG, fasting blood glucose; γ-GTP, γ-glutamyltransferase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; HMW, high molecular weight; LDL-C, low-density lipoprotein cholesterol; r_s, Spearman's ordered correlation coefficient.

Several previous studies performed in White and Black populations investigated the association between adiponectin concentrations and the risk of developing cardiovascular disease or type 2 diabetes and showed that alcohol intake was associated with elevated serum adiponectin levels (3). In contrast, recent studies in mice and rats have demonstrated that chronic ethanol feeding decreases circulating adiponectin concentrations (8, 9).

As previously described, there are ethnic differences both in serum adiponectin levels (10) and in the risk of type 2 diabetes and cardiovascular disease between Asian and White individuals that are not explained by conventional risk factors (11). In light of these findings, we hypothesized that alcohol consumption may have a different effect on modulation of adiponectin levels in individuals of Asian descent. This relationship has not been fully elucidated on a large scale because of the limited number of subjects. Given the sample size available to us, we chose to evaluate the relationship between alcohol consumption and serum adiponectin levels among a Japanese general population while adjusting for potential confounding factors.

Subjects and Methods

Study population

This study is a part of the Japanese prospective, population-based study held in an agricultural area located about 350 km north of Tokyo. The design and methods of these studies have been reported elsewhere (12–14). Briefly, the study was designed to evaluate the role of lifestyle, diet, and genetic factors in the subsequent development of many common diseases. The study cohort consists of subjects recruited from participants in the regular health check-up program for residents. Since 2004, the baseline survey and subsequent follow-up surveys have been conducted annually. The survey collects information on lifestyle and anthropometric measurements and collects blood and urine specimens from participants on the morning of the survey. The study protocols were approved by the ethics committee at Yamagata University.

Of 3826 participants in the health check-up program from June 1, 2004, through November 30, 2005, the present study population started with 3166 subjects aged 40 yr or older who agreed to participate (83%). Written informed consent was obtained from all subjects. For this analysis, we restricted subjects to those with available information on drinking status and adiponectin levels (n = 3130). We also excluded those who ate breakfast before blood was drawn or those with missing information regarding biomedical variables, anthropometrical variables, or blood pressure. Thus, data from 2932 subjects (1306 men and 1626 women) who met all eligibility criteria were analyzed.

Data collection and measurements

Height, weight, and blood pressure were measured with the subject in light clothes and without shoes, and the body mass index (BMI) (kilograms per square meter) was calculated. After blood samples were drawn, they were frozen in aliquots at −70 C within 4 h and stored frozen until measurements. Biochemical variables evaluated in this study included levels of total adiponectin, total cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C, triglycerides, fasting blood glucose (FBG), fasting serum insulin, alanine aminotransferase (ALT), and γ-glutamyltransferase (γ-GTP). Plasma glucose, serum lipids, and liver enzymes were assayed by routine automated laboratory methods in a single laboratory (BML Inc., Tokyo, Japan). Serum insulin concentrations were measured using a chemiluminescent immunoassay kit (Kyowa Medics, Tokyo, Japan), with intra- and interassay coefficients of variation of 2.0–3.0 and 0.9–4.7%, respectively. Plasma total adiponectin levels were determined by a human adiponectin ELISA (Otsuka Pharmaceutical Co., Tokyo, Japan). Intra- and inter-assay coefficients of variation were 3.3–3.6 and 3.2–7.3%, respectively. All biochemical measurements were performed using plasma samples collected after an overnight fast. The estimate of insulin resistance was done using the homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated from FBG and fasting insulin levels using the following formula: FBG (milligrams per deciliter) × fasting plasma insulin (microunits per milliliter)/405.

Assessment of alcohol consumption and smoking history

Information on alcohol consumption and smoking habits of each individual was obtained in face-to-face interviews. Alcohol consumption was calculated on the basis of ethanol volume, and each drinker's status was defined according to the total weekly volume of ethanol intake. The amounts of alcoholic beverages, including beer, wine, and whisky, were converted to an equivalent amount of sake (rice wine). One hundred eighty milliliters of sake contains 20 g ethanol; 180 ml sake equals 500 ml beer, 180 ml wine, or 60 ml whisky in alcohol content. Information on smoking habits was categorized as current use, past use, or never. To assess the reliability of the amount of alcohol consumption, we compared the volume of ethanol intake in the present study with the information on similar items in the survey conducted using a self-administered questionnaire during May 16 through May 29, 2005. Among 1457 subjects who completed the lifestyle questionnaire, Spearman's ordered correlation coefficient (r_s) between the two variables was 0.71.

Statistical analysis

Because alcohol habits are gender related (15), the analysis was conducted according to gender. Variables are given as means ± SD for variables with a normal distribution, median (25th–75th percentile) for skewed variables or n (percent) for numerical or categorized variables. The skewed variables (adiponectin, glucose, insulin, and triglyceride levels) were log transformed before statistical analysis.

Alcohol consumption was treated both as a continuous variable and as a categorical variable: abstainer, less than 120 g/wk, 120–239 g/wk, and 240 g/wk or more. BMI (<22.0, 22.0–24.9, and ≥25.0) and HOMA-IR (<2.0, 2.0–3.9, and ≥4.0) were categorized before statistical analysis. One-way ANOVA was used for testing between multiple groups, and Dunnett's test was used for subsequent comparison of abstainers with other groups. An unpaired t test was used to compare continuous data, and the χ^2 test was used for the analysis of proportions between groups. Pearson's correlation coefficient or r_s was calculated to evaluate

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

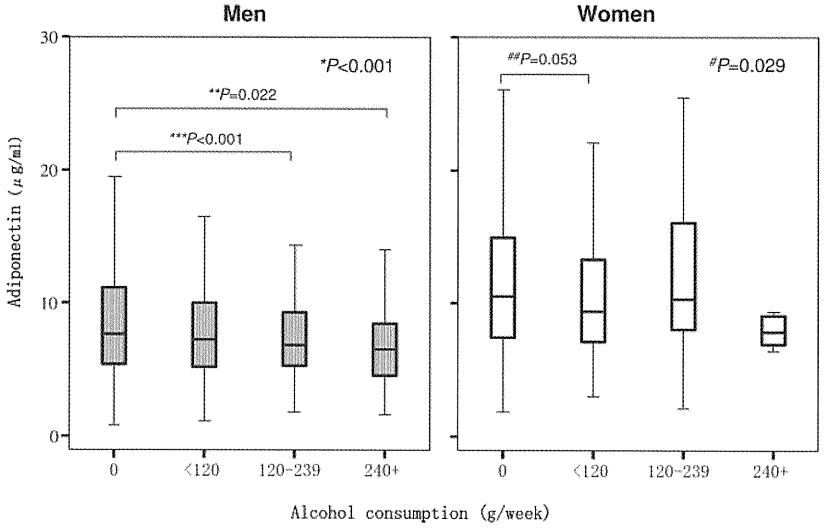

FIG. 1. Box plots illustrating serum plasma adiponectin concentrations for each level of alcohol consumption by gender. *Horizontal lines inside each box* represent medians, and the *top and bottom of the boxes* are the 25th and 75th quartiles, respectively. The *error bars* indicate 95% confidence intervals. *, $P < 0.001$ 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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Address all correspondence and requests for reprints to: Yuko Nishise, M.D., Department of Gastroenterology, Yamagata University School of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan. E-mail: ynishise-gi@umin.ac.jp.

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Therapeutic Apheresis and Dialysis

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

Shoichi Nishise,[1]* Yuji Takeda,[2]* Shoichiro Fujishima,[1] Tomohiko Orii,[1] Takeshi Sato,[1] Yu Sasaki,[1] Yuko Nishise,[1] Hiroaki Takeda,[1] and Sumio Kawata[1]

[1]*Department of Gastroenterology, Yamagata University School of Medicine, Yamagata, and* [2]*Department of Environmental and Preventive Medicine, Hyogo College of Medicine, Nishinomiya, Japan*

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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Address correspondence and reprint requests to Dr Shoichi Nishise, Department of Gastroenterology, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan. Email: nishise-sic@umin.ac.jp
*These authors contributed equally to this work.

release of anti-inflammatory substances and corrects an imbalance between pro- and anti-inflammatory cytokines in active ulcerative colitis.

Complement activation is the key to understanding the biological responses of GMA, such as GM adsorption to cellulose acetate beads and the release of IL-1ra. Complement activation induced by contact between blood and cellulose acetate beads is completely inhibited by nafamostat mesilate, a potent serine protease inhibitor (8). Complement activation generates anaphylatoxins (C5a and C3a) that stimulate the release of IL-1ra with granulocyte adsorption to cellulose acetate beads, and the cascade of complement activation is inhibited by nafamostat mesilate (8). Thus, we speculated that the responses of GMA might be influenced by serine protease inhibitors, and we clarified the effect of other serine protease inhibitors, such as ulinastatin and gabexate mesilate, in the present study. Ulinastatin does not affect complement activation, but gabexate mesilate inhibits complement activation (9).

Ulinastatin, which is extracted and purified from human urine, has an inhibitory effect on serine proteases such as trypsin, chymotrypsin, plasmin, human leukocyte elastase, and hyaluronidase (10). Ulinastatin is also known to suppress the production of pro-inflammatory cytokines. For example, ulinastatin suppresses the TNF-α production of lipopolysaccharide-stimulated monocytes (11) and the over-induction of IL-6 and IL-8 in heart surgery (12,13). In Japan, ulinastatin has been mainly used for the treatment of acute pancreatitis, disseminated intravascular coagulation, and shock (14), and the daily total dose is 150 000–300 000 units. Ulinastatin has also been reported to be effective in steroid-resistant patients with severe ulcerative colitis when steroids are administered concurrently (15,16); however, combination therapy with GMA and ulinastatin has not been attempted in patients with ulcerative colitis.

The effects of ulinastatin on biological responses during treatment with Adacolumn are currently of considerable interest. In the present study, we investigated the effects of ulinastatin on GM adsorption and IL-1ra release to determine the feasibility of combination therapy with GMA and ulinastatin.

MATERIALS AND METHODS

Reagents

Cellulose acetate beads were prepared by the JIMRO Institute (Takasaki, Japan). Ulinastatin was purchased from Sawai Pharmaceutical Company (Osaka, Japan). Gabexate mesilate and low molecular weight heparin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Propidium iodide, formyl-methionyl-leucyl-phenylalanine (fMLP), SB203580, and PD98059 were obtained from Sigma (St Louis, MO, USA). Mouse anti-human CD62L (Dreg 56, IgG1) was from Becton Dickinson Biosciences (San Diego, CA, USA). All other chemicals were obtained commercially and were of the highest purity available.

Blood samples

After receiving written informed consent from all participants in the study, we collected peripheral blood from six healthy volunteers in plastic syringes (Terumo, Tokyo, Japan).

Blood exposure to cellulose acetate beads

A mixture of heparinized peripheral blood containing 0–10 000 units/mL of ulinastatin or 0–800 μg/mL of gabexate mesilate and cellulose acetate beads at a 1:2 mL/g ratio in 10-mL syringes was rotated at 1 rpm for 1 h at 37°C. Blood samples were removed from the syringes by flash centrifugation at $80 \times g$ for a few seconds. Fractions of granulocytes adsorbed to the cellulose acetate beads were measured using a Coulter Gen-S hematology analyzer (Beckman Coulter, Fullerton, CA, USA), and then plasma was separated by centrifugation at 800 *g* for 5 min at 4°C and stored at –80°C. The ratio (%) of adsorbed granulocytes was calculated as follows: adsorbed granulocytes (%) = 100 × (number of granulocytes incubated without beads – number of granulocytes incubated with beads)/number of granulocytes incubated without beads. Cytotoxicity was examined by a Trypan blue exclusion assay and propidium iodide assay.

Measurement of IL-1ra

The level of IL-1ra was measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The optical density of test samples at 450 nm was determined using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA, USA). The ratio (%) of increased IL-1ra was calculated as follows: increased IL-1ra (%) = 100 × (concentration of IL-1ra after incubation – concentration of IL-1ra before incubation)/concentration of IL-1ra before incubation.

Statistical analysis

Statistical analyses were conducted as described in the figure legends, and $P < 0.05$ was considered significant. Data are presented as mean ± standard error unless otherwise noted.

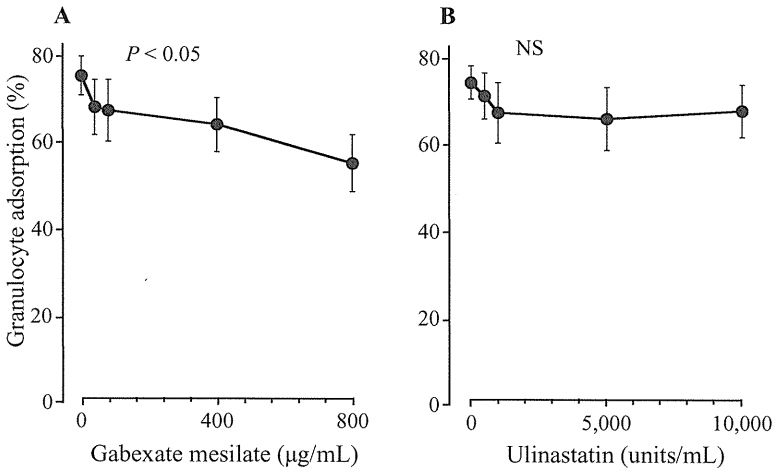

FIG. 1. Effect of ulinastatin on granulocyte adsorption to cellulose acetate beads. Peripheral blood from healthy volunteers was mixed with serial dilutions of either (A) gabexate mesilate or (B) ulinastatin. Test samples were then incubated with cellulose acetate beads for 1 h, and fractions of adsorbed granulocytes were measured with a hemocytometer. Ratios (%) of granulocyte adsorption were calculated as described in the Materials and Methods section. Results are presented as mean ± standard error from six blood donors. NS, not significant, by Friedman test.

RESULTS

Granulocyte adsorption to cellulose acetate beads is unaffected by ulinastatin

We first verified the effect of two serine protease inhibitors on granulocyte adsorption to cellulose acetate beads. Peripheral blood containing various concentrations of ulinastatin or gabexate mesilate was incubated with cellulose acetate beads. Granulocyte adsorption was significantly decreased with increasing gabexate mesilate concentrations (Fig. 1A). While the adsorption was slightly decreased at 1000 units/mL of ulinastatin, the decrease was not significant and reached a plateau at 10 000 units/mL (Fig. 1B). Neither ulinastatin nor gabexate mesilate affected leukocyte viability (data not shown).

Effect of ulinastatin on IL-1ra release in GM adsorption

Next, we examined the effect of ulinastatin and gabexate mesilate on the release of IL-1ra in GM adsorption to cellulose acetate beads. The increased ratio of IL-1ra significantly decreased with increasing gabexate mesilate concentrations (Fig. 2A). The ratio of increased IL-1ra in the presence of 500 and 1000 units/mL ulinastatin was lower than in the absence of ulinastatin, although this difference was not statistically significant; however, the IL-1ra ratio was significantly augmented by the addition of 10 000 units/mL ulinastatin (Fig. 2B). These results indicate that a high concentration of ulinastatin augments the release of IL-1ra in GM adsorption to cellulose acetate beads, although a low concentration of ulinastatin conversely suppresses IL-1ra release.

Extracellular signal-regulated kinase 1/2 inhibitor on granulocyte adsorption and IL-1ra release

To identify signaling pathways involved in the suppression and augmentation of IL-1ra release, p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK) 1/2 were

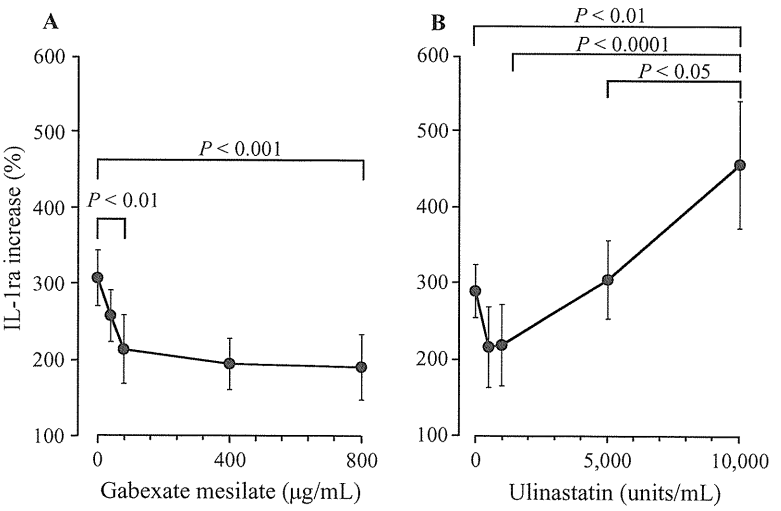

FIG. 2. Effect of ulinastatin on interleukin-1 receptor antagonist (IL-1ra) release in granulocyte and monocyte adsorption. Peripheral blood from healthy volunteers was mixed with serial dilutions of either (A) gabexate mesilate or (B) ulinastatin and incubated with cellulose acetate beads for 1 h. After incubation, plasma was separated by centrifugation for measurement of IL-1ra. Increase ratios (%) of IL-1ra were calculated as described in the Materials and Methods section. Results are presented as mean ± standard error from six blood donors. The *P*-values are based on repeated-measures one-way ANOVA (post-hoc test with Bonferroni).

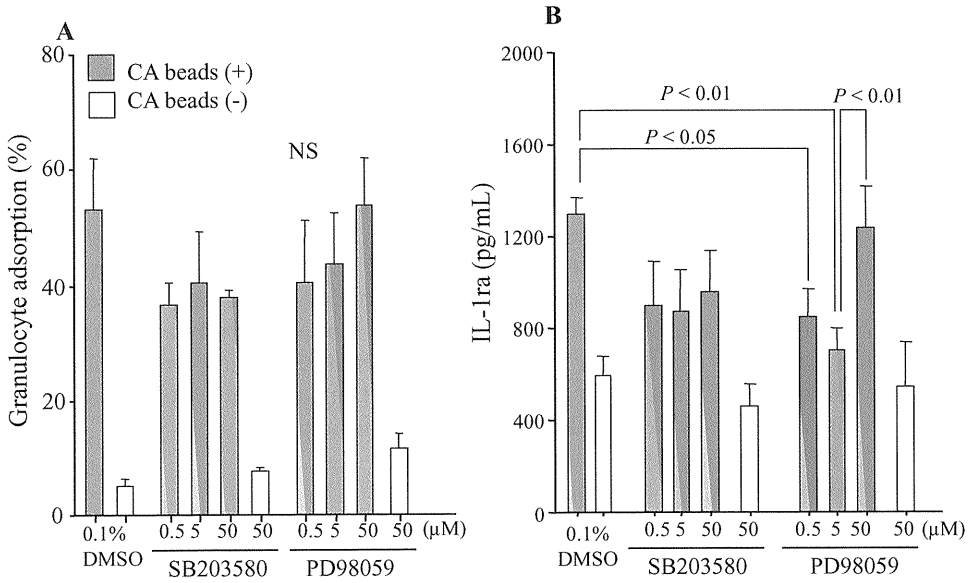

FIG. 3. Effects of signal transduction inhibitors on (A) the adsorption to cellulose acetate (CA) beads and (B) IL-1ra release. Blood cells were pre-incubated with various concentrations of SB203580 or PD98059 for 15 min at room temperature and then cultured with (■) or without (□) cellulose acetate 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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Differential immunophenotypic analysis of dendritic cell tumours

Tomohiko Orii,[1,2] Hiroaki Takeda,[2] Sumio Kawata,[2] Kunihiko Maeda,[1] Mitsunori Yamakawa[1]

[1]Department of Pathological Diagnostics, Faculty of Medicine, Yamagata University, Yamagata, Japan
[2]Second Department of Internal Medicine, Yamagata University School of Medicine, Yamagata, Japan

Correspondence to
Professor Mitsunori Yamakawa, Department of Pathological Diagnostics, Faculty of Medicine, Yamagata University, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan;
myamakaw@med.id.yamagata-u.ac.jp

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ABSTRACT

Aims The phenotypic and biological characteristics of dendritic cell (DC) tumours have not been fully elucidated. The aim of this study was to compare the immunophenotypic characteristics of DC-related markers and cell-cycle-associated markers among DC tumours and finally to utilise them for differential diagnosis of DC tumours.

Methods Tissue sections from 28 patients with DC tumours were immunohistochemically examined using DC-related and cell-cycle-associated markers.

Results The Langerhans cell histiocytosis (LCH) and Langerhans cell sarcoma (LCS) samples were positive for S-100 protein, CD1a, Langerin, fascin, DEC-205 and DC-SIGN. Interdigitating dendritic cell sarcoma (IDCS) was positive for S-100 protein and fascin and negative for Langerin. In addition, two IDCS samples were positive for CD1a, DEC-205 and DC-SIGN. The labelling indices of Ki-67, cyclin A, cyclin B1 and acetylated histone H3 on the LCS and IDCS specimens were significantly higher than those on the LCH specimens. The expression of p53 was also significantly higher in the LCS specimens than in the LCH specimens. The numbers of infiltrating $CD123^+$ and $FOXP3^+$ cells were also significantly higher in the LCS samples than in the LCH and IDCS samples. Follicular dendritic cell sarcoma was distinguished from other DC tumours by the lack of DC-SIGN, Langerin and DCE-205.

Conclusions These results suggest that Langerin can be used to distinguish LCS from IDCS, and DC-SIGN and DEC-205 can be used to identify DC tumour cells. The frequency of cell-cycle-associated markers can be used for the differential diagnosis of malignant and benign DC tumours.

INTRODUCTION

Langerhans cell histiocytosis (LCH) is caused by the abnormal accumulation and/or proliferation of pathological LCs which are S-100 $protein^+$, $CD1a^+$ and Birbeck $granule^+$.[1] It displays a heterogeneous clinical feature that ranges from the involvement of a single organ system (SS) (primarily skin or bone) to the involvement of multiple organ systems (MS) complicated by organ dysfunction.[1 2] Only about 20 cases of Langerhans cell sarcoma (LCS) have been previously reported in English literature,[1 3 4] which can be considered as a higher-grade variant of LCH, and it can present de novo or progress from antecedent LCH. Interdigitating dendritic cell sarcoma (IDCS) is also a very rare disease, and fewer than 60 cases have been reported.[1 5–8] IDCS is a neoplastic proliferation with phenotypes similar to those of IDCs. The immunophenotype of LCS and IDCS has not been classified because of their rarity. Furthermore, it is not easy to morphologically distinguish LCS or IDCS tumours from lesions of the MS subtype of LCH. Follicular dendritic cell sarcoma (FDCS) is categorised as a neoplastic proliferation of spindle to ovoid cells showing morphological and immunophenotypic features of FDCs.[1]

The expression of the tumour suppressor gene p53 occurs at low levels in normal cells due to a very short half life; however, p53 is often abnormal in malignant tumours. There have been only a few reports addressing the levels of p53 expression in dendritic cell (DC) tumours.[9–11] $CD4^+CD25^+$ regulatory T cells (Tregs) express forkhead box protein 3 (FOXP3).[12 13] Recent studies have reported that high numbers of Tregs are present in cancer tissue and suppress the anti-tumour immune response; however, there has been no reported correlation between Tregs and DC tumours.[14]

The objective of this study was to evaluate the immunophenotype of DC-related markers and the expression of cell-cycle markers, acetylated histone H3 and p53 in the four types of DC tumours in order to determine their distinguishing characteristics. We also examined the degree of the infiltration of non-neoplastic plasmacytoid DC (pDC) and Treg in DC tumours to clarify the difference of the resistance of DC tumours to antitumour immune response mediated via the impact of pDCs and Tregs especially between benign (LCH) and malignant (LCS) LC tumours. The present study evaluated the identity of markers that would enable the differentiation of benign DC tumours from malignant DC tumours.

MATERIALS AND METHODS

Patients and specimens

Tissue samples were obtained from 26 patients who were diagnosed as having LCH, LCS, IDCS or FDCS in our hospital between 1980 and 2008. Twenty-two patients were male, and four were female, and the mean age was 28 years (6–71). Seventeen patients were diagnosed as having LCH (14 males and three females with a mean age of 11 years (6–35)), including 13 cases of SS and four cases of MS LCH. Four patients had LCS (four males with a mean age of 57 years (40–64)), and five patients were diagnosed as having IDCS based on lymph node (four males and one female with a mean age of 59 years (52–71)). Two patients were also treated with FDCS (two males with a mean age of 63 years (60 and 66)). Resected specimens were fixed in 10% formalin. These specimens were embedded in paraffin to use for Haematoxylin & Eosin staining and

Table 1 Primary antibodies used in this study

Antibody (clone)	Ig subclass	Source
S-100 protein	Rabbit, heterologous	Nichirei, Tokyo, Japan
CD1a (O10)	Mouse IgG1κ	Immunotech, Marseille, France
Langerin (CD207; 12D6)	Mouse IgG2b	YLEM, Rome, Italy
Fascin (55K-2)	Mouse IgG1κ	DAKO, Glostrup, Denmark
CD83 (1H4b)	Mouse IgG1κ	Novocastra, Newcastle upon Tyne, UK
DC-SIGN (CD209)	Rabbit IgG	Santa Cruz, Delaware Avenue, California
DEC-205 (CD205; 11A10)	Mouse IgG1	Novocastra
CD123 (S-12)	Mouse IgG1	Santa Cruz
FOXP3 (263A/E7)	Mouse IgG1	Abcam, Cambridge, UK
Ki-67 (MIB-1)	Mouse IgG1	Immunotech
Cyclin A (6E6)	Mouse IgG1κ	Novocastra
Cyclin B1 (7A9)	Mouse IgG1κ	Novocastra
p53 (DO-7)	Mouse IgG2b	Novocastra
Acetylated histone H3	Rabbit, heterologous	Upstate, Lake Placid, New York
CD163 (10D6)	Mouse IgG1	Novocastra

immunohistochemical study. All the patients provided informed consent, and the study was approved by the Yamagata University School of Medicine Ethics Committee.

Immunohistochemistry

Four-micrometre-thick sections were prepared and mounted on glass slides. The primary antibodies used in this study are listed in table 1. The labelled streptavidin-biotin-peroxidase method (Ultratech HRP Streptavidin-biotin Universal Detection System, Immunotech, Marseille, France) or the alkaline phosphatase method (streptavidin/AP; DAKO, Glostrup, Denmark) was used. A positive reaction was detected as a brown colour with 3,3′-diaminobenzidine (Dojin Chemicals, Kumamoto, Japan) or as a red colour with new fuchsin (DAKO). In the case of the former, the sections were counterstained with haematoxylin. The double immunostaining was done to evaluate whether S-100 protein$^+$ DC tumour cells simultaneously express DC-SIGN. The alkaline phosphatase method was applied for DC-SIGN (new fuchsin, red) in the first-step staining. After sections were subjected to heat-induced antigen retrieval for 20 min, the labelled streptavidin-biotin-peroxidase method was carried out for S-100 protein (TrueBlue, blue; Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) in the second-step staining.

For the positive controls, we similarly immunostained tissue sections of lymph nodes obtained from three patients with reactive lymphadenitis. In addition, tissue sections were incubated with non-immune mouse or rabbit immunoglobulin (DAKO), respectively, instead of the primary antibody as negative control.

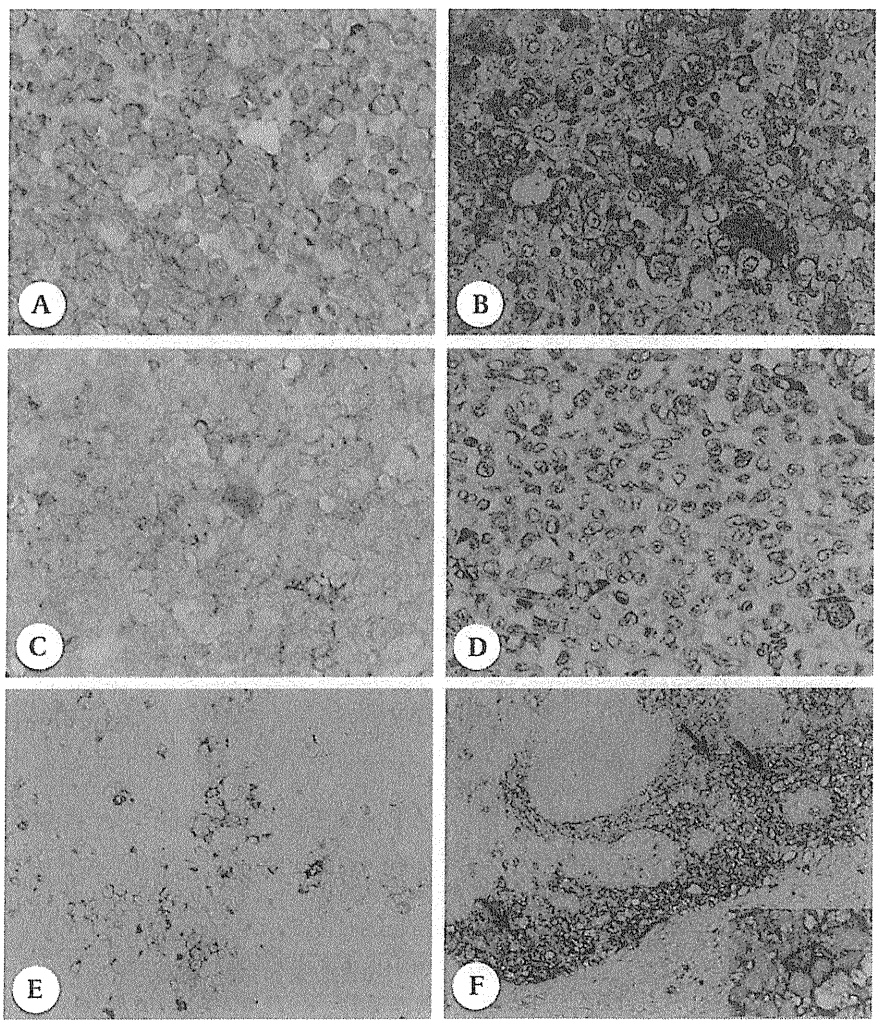

Figure 1 Immunophenotype of dendritic cell tumours. (A) Almost all Langerhans cell sarcoma cells express CD1a. (B) A majority of tumour cells in the case of interdigitating dendritic cell sarcoma express CD1a. (C) Almost all Langerhans cell sarcoma cells express DEC-205. (D) A majority of tumour cells in this case of Langerhans cell sarcoma also express DC-SIGN. (E) A part of follicular dendritic cell sarcoma cells express fascin. (F) Double immunostaining for DC-SIGN (red) and S-100 protein (blue) in Langerhans cell sarcoma showing a small number of double positive tumour cells (arrows). (Inset) High-power view of a double positive cell. (A–D) Counterstained with haematoxylin, original magnification ×400; (E–F) not counterstained, original magnification ×300 (inset ×800).

The tissue sections were immersed in 10 mM citrate buffer, pH 6.0 (Mitsubishi Kagaku Iatron, Tokyo, Japan), or in 50 mM Tris-HCl buffer, pH 9.0, containing 1 mM ethylenediaminetetraacetic acid for the CD123 immunostain. The sections were subjected to autoclave treatment for 20 min at 120°C to determine the antigenicity.

Counting of immunostained cells

Photographs of 10 high-power fields at ×400 magnification under a microscope with CCD camera were taken and stored as Photoshop files (Photoshop 5.0 Limited Edition, Adobe, San Jose, California). The ratios of tumour cells positive for S-100 protein, CD1a, Langerin, fascin, CD83, DC-SIGN, DEC-205, Ki-67, cyclin A, cyclin B1, acetylated histone H3 (H3Ac) and p53 per total tumour cells (%) were counted by two observers independently and expressed as the mean±SD. The number of cells positive for CD123 and FOXP3 was counted in 10 high-power fields, and the mean number±SD of positive cells per a microscopic field (×400) was calculated.

Statistical analysis

Statistical analysis was carried out using StatView 4.5 software package (Abacus Concepts). The Mann–Whitney U test was used for immunohistochemical data. P values <0.05 were considered to be significant.

RESULTS

Immunophenotype of DC-related markers in DC tumour cells

The majority of tumour cells in the 26 cases of LCH, LCS and IDCS tumours expressed S-100 protein and fascin. However, none of the tumour cells expressed CD83. In most cases of LCH and LCS, at least 75% of the tumour cells expressed CD1a (figure 1A), Langerin and DEC-205 (figure 1C). Most of the cases of LCH and LCS expressed DC-SIGN in variable frequencies (figure 1D). The double immunostaining demonstrated the presence of DC-SIGN$^+$ S-100 protein$^+$ tumour cells (figure 1F). Langerin was not expressed in IDCS cells, and the other DC markers, CD1a, DEC-205 and DC-SIGN, were positive in only two cases (figure 1B). CD163$^+$ cells were scattered throughout the DC tumours in various degrees. Both cases of FDCS were often positive for S-100 protein and fascin but negative for CD1a, DC-SIGN, Langerin and DEC-205.

The frequency of tumour cells positive for DC markers (S-100 protein, CD1a, Langerin, fascin and DEC-205) was compared statistically among LCH, LCS and IDCS (table 2). Although there was a significant difference in the frequency of Langerin$^+$ tumour cells among the IDCS and LCH or LCS cells ($p<0.05$), there was no significant difference detected in the frequencies of any other DC markers.

Table 2 Frequency of S-100 protein, CD1a, Langerin, fascin, DC-SIGN, and DEC-205 in Langerhans cell histiocytosis (LCH), Langerhans cell sarcoma (LCS), interdigitating cell sarcoma (IDCS) and follicular dendritic cell sarcoma (FDCS)

		The frequency of immunopositive tumor cells (%)(mean ± standard deviation)		
Histology	Number of cases	S-100 protein	CD1a	Langerin
LCH (total)	17	87.44 ± 11.57	89.02 ± 13.13	78.00 ± 19.27
LCH (SS)	13	86.31 ± 12.86	92.05 ± 9.07	80.28 ± 17.00
LCH (MS)	4	91.13 ± 5.29	79.18 ± 20.51	70.59 ± 27.98
LCS	4	95.78 ± 2.24	96.43 ± 2.14	80.96 ± 13.83
IDCS	5	79.97 ± 18.02	43.99 ± 51.41	0.00 ± 0.00
FDCS	2	15.12 ± 1.28	0	0

Histology	Number of cases	Fascin	DC-SIGN	DEC-205
LCH (total)	17	82.97 ± 16.92	33.80 ± 24.13	87.85 ± 19.81
LCH (SS)	13	79.98 ± 18.00	29.36 ± 22.73	91.01 ± 17.29
LCH (MS)	4	92.68 ± 8.18	48.22 ± 26.01	77.59 ± 26.72
LCS	4	93.93 ± 3.96	35.38 ± 22.80	98.27 ± 1.70
IDCS	5	93.74 ± 5.37	34.45 ± 46.25	49.40 ± 55.87
FDCS	2	29.03 ± 5.97	0	0

SS; single system, MS; multisystem, [a]: $p < 0.01$ and [b]; $p < 0.05$ by Mann-Whitney U-test
FDCS was excluded from statistical analysis because of a lower number of cases.

The expression of cell-cycle markers, acetylated histone H3 (H3Ac) and p53 in DC tumour cells

The frequency of tumour cells positive for Ki-67, cyclin A, and cyclin B1 on LCS and IDCS was significantly higher than that on the LCH tumour cells ($p<0.01$)(table 3; figure 2A–C). Furthermore, there was a significant difference in the frequencies of Ki-67 and cyclin A expression between the LCS or IDCS specimens and the samples from the LCH subtypes (SS and MS). There was a significant difference in the frequency of cyclin B1 expression between the LCH MS samples and the IDCS samples. There were no significant differences in the frequencies of Ki-67, cyclin A and cyclin B1 between the SM and MM subtypes of LCH and between the LCS and IDCS tumour cells.

The frequency of H3Ac nuclear expression in LCS and IDCS tumour cells was significantly higher than that in the total LCH or the SS subtype of LCH tumour cells ($p<0.05$; figure 2D). There was also a significant difference in the frequency of nuclear p53 expression between the LCS tumour cells and of the total LCH or SS type of LCH tumour cells ($p<0.05$; figure 2E); however, there was no significant difference in the p53 frequency between the IDCS and LCH specimens.

Infiltration of CD123$^+$ and FOXP3$^+$ cells in DC tumours

The number of infiltrating CD123$^+$ cells (figure 2F) and FOXP3$^+$ cells (figure 2G) was significantly higher in the LCS specimens than in the LCH or the IDCS specimens (table 4).

DISCUSSION

The present study did not detect any remarkable immunophenotypic differences between LCH and LCS cells. The cells in both types of tumours expressed S-100 protein, CD1a, Langerin, fascin and DEC-205. Neither of the tumour types expressed CD83. The immunoreactivity of S-100 protein, CD1a, Langerin, fascin and CD83 on LCH cells was consistent with the observations in previous reports.[1 9] The immunoreactivity of DC-SIGN and DEC-205 in DC tumours has not been fully examined, but this study is the first to demonstrate the usefulness of these markers to pick up LCH, LCS and IDCS cells on routine formalin-fixed and paraffin-embedded tissue sections, although these markers are not available to make a differential diagnosis among LCH, LCS and IDCS. Generally, IDCS cells lack CD1a,[1 9] but some reports are controversial.[15 16] Many recent reports defined LCS as CD1a$^+$ and Langerin$^+$ in the Birbeck granules, which is identical to LCs. Some reports also define IDCS as Langerin$^-$ independent of CD1a immunoreactivity.[9] The present study showed similar results to these earlier reports.

The Ki-67 labelling index in DC tumours has been evaluated in some previous reports, but a wide variation among cases has been reported.[17] This study demonstrated that the frequency of expression of Ki-67 (non-cycle-specific), cyclin A (S-G2 phase) and cyclin B1 (G2-M phase) was significantly higher in LCS cells and IDCS cells in comparison with the frequency in LCH cells, indicating that LCS may have a higher malignant potential than

Table 3 Frequency of Ki-67, cyclin A, cyclin B1, acetylated histone H3, and p53 in Langerhans cell histiocytosis (LCH), Langerhans cell sarcoma (LCS), interdigitating cell sarcoma (IDCS) and follicular dendritic cell sarcoma (FDCS)

		The ratio of immunopositive tumor cells per total tumor cells (%)(mean ± standard deviation)					
Histology	Number of cases	Ki-67		Cyclin A			
LCH (total)	17	30.85 ± 11.23	a	a	7.10 ± 3.71	a	a
LCH (SS)	13	28.11 ± 10.52	b	a	6.01 ± 2.81	a	a
LCH (MS)	4	31.88 ± 9.02	b	b	10.64 ± 4.49	b	b
LCS	4	53.77 ± 10.21			22.59 ± 2.18		
IDCS	5	62.15 ± 19.82			26.29 ± 8.41		
FDCS	2	25.20 ± 2.08			11.60 ± 0.80		

Histology	Number of cases	Cyclin B1			Acetylated histone H3			p53	
LCH (total)	17	1.81 ± 1.68	a	a	83.16 ± 8.99	b	b	43.75 ± 22.25	b
LCH (SS)	13	1.25 ± 0.99	a	a	82.47 ± 8.42	b	b	39.35 ± 20.59	a
LCH (MS)	4	3.63 ± 2.31	b		85.42 ± 11.78			58.06 ± 24.22	
LCS	4	11.29 ± 3.11			94.07 ± 2.28			72.90 ± 7.22	
IDCS	5	7.15 ± 2.67			93.76 ± 3.84			62.24 ± 24.60	
FDCS	2	1.13 ± 0.08			79.52 ± 2.48			24.35 ± 3.87	

SS; single system, MS; multisystem, [a]: $p < 0.01$ and [b]; $p < 0.05$ by Mann-Whitney U-test
FDCS was excluded from statistical analysis because of a lower number of cases.

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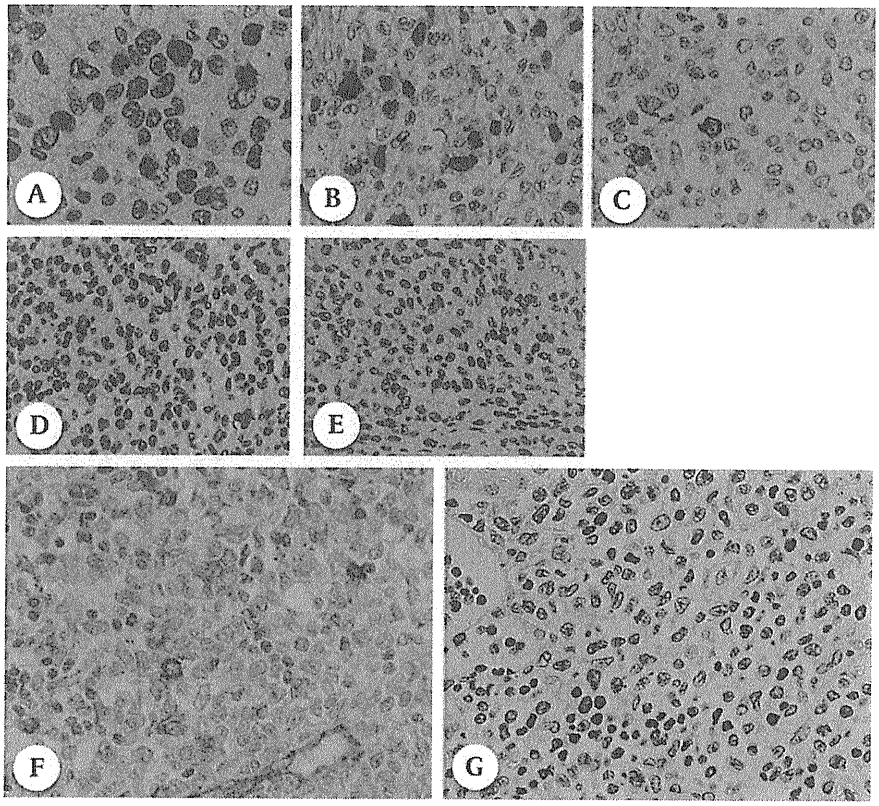

Figure 2 Immunostaining of cell cycle markers, acetylated histone H3, p53, CD123 and FOXP3 in Langerhans cell sarcoma. (A) The nuclei of a part of tumour cells are labelled with Ki-67. (B) Some tumour cells are labelled with cyclin A. (C) Occasional tumour cells express cyclin B1. (D) Almost all tumour cells are labelled with acetylated histone H3. (E) A majority of tumour cells are labelled with p53. (F) Some $CD123^+$ cells are scattering in the tumour. (G) Many $FOXP3^+$ cells are infiltrating in the tumour. Counterstained with haematoxylin, original magnification: ×400

LCH. The superiority of cyclin A rather than Ki-67 and cyclin D1 as an indicator of poor prognosis has previously been demonstrated.[18] A lower p value was found in the case of cyclin A ($p<0.0001$) in comparison with the p values for other cell-cycle markers used in this study. This observation strongly suggested that cyclin A may be a potent indicator for distinguishing LCH from LCS.

The inhibition of oncogene p53 expression during DNA damage stops cell proliferation and induces apoptotic cell death. The detection of mutant p53 or wild type p53 expression in IDCS and LCH tumour cells is controversial.[19 20] In this study, a significant difference in the frequency of p53 expression was found only between the LCH and LCS specimens; however, in combination with the cell-cycle-marker analysis, p53 may be an effective prognostic indicator in DC tumours.

Recent papers have demonstrated that the acetylation and deacetylation of histones regulate the cell cycle and the activation and suppression of transcription.[20 21] The present analysis demonstrated a higher frequency of H3Ac expression in LCS and IDCS cells in comparison with LCH cells. This result indicated

Table 4 Frequency of infiltrating $CD123^+$ and $FOXP3^+$ cells in Langerhans cell histiocytosis (LCH), Langerhans cell sarcoma (LCS), interdigitating cell sarcoma (IDCS) and follicular dendritic cell sarcoma (FDCS)

		Number of infiltrating cells/High-power field (mean ± standard deviation)			
Histology	Number of cases	CD123		FOXP3	
LCH (total)	17	0.18 ± 0.27	a	5.44 ± 3.25	a
LCH (SS)	13	0.15 ± 0.29	a	5.15 ± 3.55	a
LCH (MS)	4	0.28 ± 0.21	b	6.38 ± 2.11	b
LCS	4	6.30 ± 4.85	b	23.85 ± 8.05	b
IDCS	5	0.05 ± 0.10		4.13 ± 4.92	
FDCS	2	0.16 ± 0.03		2.86 ± 0.32	

SS; single system, MS; multisystem, [a]: $p < 0.01$ and [b]; $p < 0.05$ by Mann-Whitney U-test
FDCS was excluded from statistical analysis because of a lower number of cases.

the presence of active transcription and accelerated cell proliferation in LCS and IDCS.

The infiltration of both CD123$^+$ pDCs and FOXP3$^+$ Tregs was frequently detected in the LCS tumour samples in this study. The antitumour immune response in some patients with cancer is suppressed by Tregs.[22] A relationship between CD123$^+$ cells and FOXP3$^+$ cells has been demonstrated.[23] Tregs express chemokine receptors such as CCR4 and CCR6,[24] and LCH cells secrete CCL22 (a ligand for CCR4) and CCL20 (a ligand for CCR6).[25 26] LCS tumour cells may become resistant to antitumour immunity by producing these chemokines and inducing Treg infiltration, whereas LCH tumour cells occasionally experience spontaneous regression during the clinical course of the disease. The issue of the impact of infiltrating Tregs on tumour progression is a complex one, as the efficacy of effector T cell suppression by Tregs can be affected by a plethora of cellular and molecular players populating the tumour microenvironment. Therefore, data on the activation status of Tregs, on the ratio between Tregs and T-effectors as well as on the composition of the inflammatory cytokine microenvironment could provide useful elements to support the hypothesis of a differently effective antitumour response in LCH and LCS.

In conclusion, the present study confirmed that Langerin is a useful marker to distinguish LCS from IDCS, and DC-SIGN and DEC-205 can also be used to pick up DC tumours. In addition, we demonstrated the increased frequency of cell-cycle markers, H3Ac, and p53 in LCS and IDCS cells. It should be noted that these frequencies could be used to develop a differential diagnosis of malignant or benign DC tumours (figure 3).

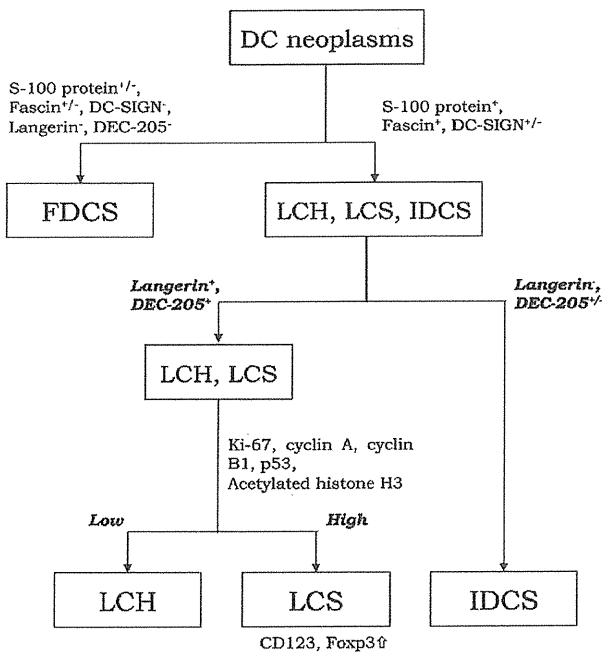

Figure 3 Diagnostic flow chart of dendritic cell neoplasms. Follicular dendritic cell sarcoma is negative for DC-SIGN, Langerin and DEC-205. Langerhans cell tumours and interdigitating cell sarcoma (IDCS) are recognised by S-100 protein$^+$, fascin$^+$ and DC-SIGN$^{+/-}$. Langerhans cell histiocytosis (LCH) and Langerhans cell sarcoma (LCS) are distinguished from IDCS by the immunostaining of Langerin and DEC-205. Expression of cell-cycle markers, acetylated histone H3 and p53 is low in LCH, whereas it is high in LCS.

Take-home messages

- It is not easy to morphologically distinguish LCS or IDCS tumours from lesions of the multiple organ systems subtype of LCH.
- The immunostain of DC-SIGN and DEC-205 on routine formalin-fixed and paraffin-embedded tissue sections is useful to pick up LCH, LCS and IDCS cells.
- Cyclin A may be a potent indicator for distinguishing LCH from LCS.

This study also demonstrated that the frequent infiltration of pDCs and Tregs in LCS tumours facilitates a resistance to the antitumour immune response.

Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by the Yamagata University School of Medicine Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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