lifestyle-related diseases in association with metabolic syndrome [14]. Thus, measurement of ALT may identify people in the general population with a risk of these diseases. However, to date, there have been few comprehensive studies of elevated ALT in association with many metabolic factors including an insulin-sensitive adipocytokine in a large population sample.

Recently, the number of people having metabolic syndrome has rapidly increased in many countries. In particular, Asian individuals have been observed to have a high prevalence of visceral fat accumulation [15]. To estimate the spread of metabolic risk for the occurrence of metabolic syndrome–related diseases in the population and to define preventive strategies, investigation of the prevalence of elevated ALT and determination of factors associated with elevated ALT are required in a large population sample. Therefore, we conducted a large-scale cross-sectional study of ALT levels and factors associated with elevated ALT in Japanese adult subjects representative of the general population.

2. Materials and methods

2.1. Subjects

This study was performed as a community-based survey and consisted of a self-administered questionnaire on lifestyle, measurement of physical status, and collection of blood samples from participants. The subjects were the general population aged 40 to 85 years in the town of Takahata, which is located in Yamagata Prefecture, approximately 350 km north of Tokyo. From June 2004 to November 2005, 2401 individuals (1055 men and 1346 women) took part in the research program. Of these people, 236 for whom data were incomplete were excluded from further analysis, leaving 2165 subjects (991 men and 1174 women) aged 40 to 85 years. We examined the prevalence of elevated ALT in a large sample population and determined the factors currently associated with elevated ALT in Japan. The study was approved by the institutional ethics committee, and written informed consent was obtained from all subjects.

2.2. Measurements

The subjects used a self-reported questionnaire to document medical history, current medication, family history, and clinical symptoms. The presence of a smoking habit (current smoker, nonsmoker, or past smoker) and alcohol intake (current drinker, nondrinker, or past drinker) were determined through an interview. Systolic and diastolic blood pressures were determined using a mercury manometer in a sitting position after resting for at least 5 minutes. These measurements were performed twice, and the mean was used for statistical analysis. Body mass index (BMI) was calculated from weight (in kilograms) divided by the height squared (in square meters), and *obesity* was defined as BMI of at least 25 kg/m^2. Blood samples were collected in the morning and shipped to a central laboratory to be assayed. Ordinary biochemical tests for serum levels of ALT, albumin, fasting blood glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, γ-glutamyl transpeptidase (γ-GTP), and cholinesterase were performed. Fasting insulin was measured using a chemiluminescent immunoassay kit (Kyowa Medics, Tokyo, Japan). Insulin resistance was calculated based on the homeostatic metabolic assessment method (HOMA-IR), as follows: HOMA-IR = fasting plasma insulin × fasting plasma glucose/405, where insulin is expressed in microunits per milliliter and glucose in milligrams per deciliter [16]. Insulin resistance was considered to have changed when HOMA-IR was greater than 2, as previously recommended [17]. Adiponectin was measured using an enzyme immunoassay kit (Human Adiponectin ELISA; Otsuka, Tokyo, Japan). Anti–hepatitis C virus (HCV) antibody, hepatitis B surface antigen, and antinuclear antibody were detected with a latex hemagglutination kit (Ortho HCVAb LPIA III; Ortho Clinical Diagnostics, Tokyo, Japan), a chemiluminescent immunoassay kit (Architect HBsAg QT; Abbott, Tokyo, Japan), and an enzyme immunoassay kit (MESACUP ANA Test; MBL, Tokyo, Japan), respectively.

2.3. Metabolic risk factors

According to the National Cholesterol Education Program Adult Treatment Panel III criteria [18] and the Japanese diagnostic criteria for metabolic syndrome published in April 2005 [19], we defined the metabolic risk for the occurrence of metabolic syndrome–related diseases as the presence of 2 or 3 of the following abnormalities: triglycerides of at least 150 mg/dL and/or HDL cholesterol less than 40 mg/dL, systolic blood pressure of at least 130 mm Hg and/or diastolic blood pressure of at least 85 mm Hg, and fasting glucose of at least 110 mg/dL.

2.4. Statistical analysis

Alanine aminotransferase levels were analyzed as the primary data to determine the prevalence of elevated ALT in the subjects. Analysis of the following 17 factors was performed to assess a potential association with elevated ALT levels in 2087 subjects (957 men and 1130 women) who were negative for viral markers for hepatitis B or hepatitis C: age, serum albumin, antinuclear antibody, γ-GTP, cholinesterase, adiponectin, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, BMI, fasting glucose, fasting insulin, HOMA-IR, blood pressure, smoking habit, and drinking habit. The relationship of each factor with elevated ALT was assessed by univariate analysis with a χ^2 test or Fisher exact test for categorical variables, Mann-Whitney test for ordinal data, and unpaired t test for continuous variables. The factors of age and univariate predictors with P less than .10 were included in a multiple

Table 1
ALT levels and seroprevalence of viral hepatitis markers in the study population

	Male (n = 991)		Female (n = 1174)		Total (n = 2165)		*P* value	Test[a]
	n	(%)	n	(%)	n	(%)		
Age group								
40-49	93	(9.4)	128	(10.9)	221	(10.2)	.034	M
50-59	220	(22.2)	294	(25.0)	514	(23.7)		
60-69	338	(34.1)	383	(32.6)	721	(33.3)		
70-79	306	(30.9)	338	(28.8)	644	(29.7)		
>80	34	(3.4)	31	(2.6)	65	(3.0)		
Mean ± SD	64.1 ± 10.2		63.0 ± 10.1		63.5 ± 10.1		.011	T
Seroprevalence of hepatitis B and C								
Both negative	957	(96.6)	1130	(96.3)	2087	(96.4)	.217	F
Positive for HCVAb	12	(1.2)	24	(2.0)	36	(1.7)		
Positive for HBsAg	22	(2.2)	19	(1.6)	41	(1.9)		
Both positive	0	(0.0)	1	(0.1)	1	(0.0)		
ALT (U/L)								
Mean ± SD	24.9 ± 13.8		20.8 ± 11.0		22.7 ± 12.5		<.001	T
Median	21		18		19			
Minimum	6		4		4			
Maximum	122		115		122			

M indicates Mann-Whitney test; F, Fisher exact test; T, *t* test; HCVAb, hepatitis C virus antibody; HBsAg, hepatitis B surface antigen.
[a] Comparison of male with female subjects.

logistic regression model to identify factors associated with elevated ALT levels. We estimated 95% confidence intervals (CIs) with maximum likelihood procedure. A backward-elimination procedure was adopted to remove the most insignificant variable in the regression model at each step until the *P* values for the variables that remained in the working model were all less than .10. The appropriateness of the logistic regression models was confirmed by the Hosmer-Lemeshow test. A 2-tailed *P* value less than .05 was considered statistically significant. Analyses were performed using SAS version 8.2 software (SAS Institute, Cary, NC) or SPSS version 15.0 for Windows (SPSS, Chicago, IL).

3. Results

3.1. ALT levels and seroprevalence of viral hepatitis markers in the study population

The characteristics of the subjects and ALT levels are shown in Table 1. Anti-HCV antibody and hepatitis B

Table 2
Association between the number of metabolic risk factors and ALT levels

No. of risk	Male			Female		
ALT (U/L)	2 or 3 n = 253 Sensitivity	0 or 1 n = 704 Specificity	Accuracy	2 or 3 n = 188 Sensitivity	0 or 1 n = 942 Specificity	Accuracy
≥17	83	28	43	73	42	47
≥18	78	36	47	69	49	53
≥19	72	41	49	63	56	57
≥20	66	47	52	58	62	61
≥21	61	52	54	52	67	64
≥22	56	58	57	48	71	67
≥23	53	62	60	43	75	70
≥24	50	66	62	37	78	72
≥25	46	69	63	34	81	74
≥26	43	72	64	30	84	75
≥27	38	75	65	28	86	76
≥28	36	77	66	25	87	77
≥29	34	79	67	23	88	77
≥30	32	81	68	20	89	78
≥31	29	83	68	19	90	78
≥32	28	85	70	17	92	79
≥33	27	86	70	16	93	80
≥34	26	88	71	15	94	80
≥35	24	88	71	13	94	81
≥36	24	89	72	11	95	81

Table 3
Prevalence of elevated ALT levels in the study population

	ALT ≧30		ALT ≧25				*P* value[a]
	Male (n = 957)		Female (n = 1130)		Total (N = 2087)		
	n	%	n	%	n	%	
Age groups							
40-49	29	31.5	18	14.4	47	21.7	.004
50-59	68	32.2	67	23.4	135	27.2	.032
60-69	72	22.3	94	25.5	166	24.0	.328
70-79	47	15.8	57	17.8	104	16.8	.591
≥80	1	2.9	3	10.0	4	6.3	.333
All ages	217	22.7	239	21.2	456	21.8	.281

[a] Fisher exact test for each age group and age-adjusted Cochran-Mantel-Haenszel χ^2 test for all ages.

surface antigen were positive in 36 (1.7%) and 41 (1.9%) of 2165 subjects, respectively; and 1 subject (1/2165, 0.0005%) was positive for both. The prevalence of anti-HCV antibody and that of hepatitis B surface antigen did not differ between men and women. The mean ALT levels in men and women were (mean ± SD) 24.9 ± 13.8 and 20.8 ± 11.0 U/L, respectively; and ALT was significantly higher in men than in women ($P < .001$).

3.2. Determination of normal ALT levels in subjects with a low potential risk for liver injury

Normal ALT levels were determined in subjects with a low potential risk of liver disease. These subjects met the following criteria: normal BMI, normal LDL cholesterol, and normal triglycerides, as described by van der Poorten et al [20]. Subjects with high systolic blood pressure, excessive alcohol consumption, and hepatitis B and C infection were excluded, as defined by Prati et al [21]. For the 120 men and 215 women in the study population who met these criteria, the mean ALT levels were 20.2 ± 7.4 U/L (median, 19) and 17.5 ± 7.7 U/L (median, 16), respectively; and the level was significantly higher in men than in women ($P < .001$).

3.3. Association between the number of metabolic risk factors and ALT levels

The cutoff values of ALT levels for effective screening for metabolic syndrome were determined based on the association between the number of metabolic risk factors found in 2087 subjects who were negative for viral markers for hepatitis B or C and ALT levels, as shown in Table 2. To determine the cutoff required to identify people with a risk of metabolic syndrome, we defined the *upper limit* of ALT as that required to exclude subjects with none or 1 of the 3 metabolic risk factors (as described above) with a specificity of more than 80%. These cutoff levels were determined to be 30 and 25 U/L for men and women, respectively. Using these proposed upper limits, the sensitivities for identifying subjects with 2 or 3 risk factors were 32% and 34% in men and women, respectively.

3.4. Prevalence of elevated ALT levels in the study population without hepatitis B or C

The rates of elevated ALT higher than the upper limits (30 U/L in men and 25 U/L in women) were 217 (22.7%) of 957 men and 239 (21.2%) of 1130 women. The prevalence of elevated ALT in women increased from 14.4% at 40 to 49 years old to 23.4% at 50 to 59 years old and to 25.5% at 60 to 69 years old, whereas those in men did not vary as much with age, with a similar rate of more than 30% at both 40 to 49 and 50 to 59 years old. The rate of elevated ALT was significantly higher in men than in women in the age groups of 40 to 49 ($P < .01$) and 50 to 59 years ($P < .05$) (Table 3).

3.5. ALT levels in subjects classified by the number of metabolic risk factors

The number of subjects with 2 or 3 of the 3 metabolic risk factors were 441 (21.1%) of 2087 total subjects, 253 (26.4%)

Table 4
ALT levels in subjects classified by the number of metabolic risk factors

	Male			Female		
	0 or 1 risk (n = 704)	2 or 3 risk (n = 253)	*P* value[a]	0 or 1 risk (n = 942)	2 or 3 risk (n = 188)	*P* value[a]
ALT (U/L)						
Mean	23.1	29.2	<.001	20.0	24.1	<.001
SD	11.3	17.9		9.9	13.4	
Median	20	24		18	21	
Minimum	6	9		4	8	
Maximum	116	122		111	115	

[a] *t* test (log-transformed value).

Table 5
Factors associated with elevated ALT levels in male subjects (elevated ALT, ≥30)

	Normal ALT n = 740		Elevated ALT n = 217		Univariate test	*P* value	Multivariate test			
							OR[a]	95% CI		*P* value
	n	(%)	n	(%)				Upper	Lower	
Age group										
40-49	63	(8.5)	29	(13.4)	M	<.001				
50-59	143	(19.3)	68	(31.3)						
60-69	251	(33.9)	72	(33.2)						
≥70	283	(38.2)	48	(22.1)						
Albumin (g/dL)										
Low (<3.7)	2	(.3)	0	(.0)	F	1.000				
Middle (3.7-5.5)	738	(99.7)	217	(100.0)						
High (>5.5)	0	(.0)	0	(.0)						
Antinuclear antibody										
Negative	632	(85.4)	183	(84.3)	C	.696				
Positive	108	(14.6)	34	(15.7)						
γ-GTP (U/L)										
Low (<60)	654	(88.4)	118	(54.4)	C	<.001	1.00			
High (≥60)	86	(11.6)	99	(45.6)			5.57	3.80	8.16	<.001
Cholinesterase (U/L)										
Low (<3500)	26	(3.5)	4	(1.8)	M	.165				
Middle (3500-8000)	707	(95.5)	210	(96.8)						
High (>8000)	7	(.9)	3	(1.4)						
Adiponectin (μg/mL)										
Mean ± SD	8.2 ± 4.2		6.1 ± 3.7		T	<.001	0.93	0.88	0.98	.010
Total cholesterol (mg/dL)										
Low (<150)	51	(6.9)	11	(5.1)	M	.005				
Middle (150-219)	568	(76.8)	152	(70.0)						
High (>219)	121	(16.4)	54	(24.9)						
LDL cholesterol (mg/dL)										
Low (<70)	29	(3.9)	8	(3.7)	M	.015	0.79	0.32	1.95	.612
Middle (70-139)	565	(76.4)	148	(68.2)			1.00			
High (>139)	146	(19.7)	61	(28.1)			1.58	1.06	2.35	.024
HDL cholesterol (mg/dL)										
High (≥40)	667	(90.1)	189	(87.1)	C	.200				
Low (<40)	73	(9.9)	28	(12.9)						
Triglyceride (mg/dL)										
Low (≤149)	618	(83.5)	142	(65.4)	C	<.001				
High (≥150)	122	(16.5)	75	(34.6)						
BMI										
Normal (<25)	554	(74.9)	113	(52.1)	C	<.001	1.00			
Obese (≥25)	186	(25.1)	104	(47.9)			1.85	1.28	2.68	.001
Fasting blood glucose (mg/dL)										
Low (<110)	649	(87.7)	176	(81.1)	C	.013				
High (≥110)	91	(12.3)	41	(18.9)						
Insulin (μU/mL)										
Low (<3)	149	(20.1)	16	(7.4)	M	<.001				
Middle (3-18)	584	(78.9)	194	(89.4)						
High (>18)	7	(.9)	7	(3.2)						
HOMA-IR										
0-1.9	630	(85.1)	138	(63.6)	M	<.001	1.00			
2.0-3.9	94	(12.7)	63	(29.0)			1.93	1.25	2.98	.003
≥4	16	(2.2)	16	(7.4)			2.94	1.26	6.86	.013
Blood pressure										
Normal	189	(25.5)	61	(28.1)	C	.449				
Hypertension	551	(74.5)	156	(71.9)						
Smoking habit										
Never	286	(38.6)	87	(40.1)	C	.469				
Current	250	(33.8)	64	(29.5)						
Former	204	(27.6)	66	(30.4)						
Drinking habit										
Never or former	209	(28.2)	54	(24.9)	C	.333				
Current	531	(71.8)	163	(75.1)						

(*continued on next page*)

Table 5 (*continued*)

	Normal ALT n = 740		Elevated ALT n = 217		Univariate test	*P* value	Multivariate test			
							OR[a]	95% CI		*P* value
	n	(%)	n	(%)				Upper	Lower	
Current medication[b]										
No	719	(97.2)	215	(99.1)	F	.132				
Yes	21	(2.8)	2	(0.9)						

C indicates χ^2 test.

[a] Multiple logistic regression analysis. Age group and the variables with *P* less than .1 on univariate analysis were included in the model.

[b] Current medication for hypertension, lipid metabolism abnormality, and diabetes was excluded.

Table 6
Factors associated with elevated ALT levels in female subjects (elevated ALT, ≥25)

	Normal ALT n = 891		Elevated ALT n = 239		Univariate test	*P* value	Multivariate test			
							OR[a]	95% CI		*P* value
	n	%	n	%				Upper	Lower	
Age group										
40-49	107	(12.0)	18	(7.5)	M	.521	1.00			
50-59	219	(24.6)	67	(28.0)			1.51	0.81	2.81	.196
60-69	274	(30.8)	94	(39.3)			1.71	0.94	3.12	.081
≥70	291	(32.7)	60	(25.1)			1.11	0.59	2.08	.756
Albumin (g/dL)										
Low (<3.7)	0	(.0)	0	(.0)	F					
Middle (3.7-5.5)	891	(100.0)	239	(100.0)						
High (>5.5)	0	(.0)	0	(.0)						
Antinuclear antibody										
Negative	697	(78.2)	191	(79.9)	C	.572				
Positive	194	(21.8)	48	(20.1)						
γ-GTP (U/L)										
Low (<60)	875	(98.2)	198	(82.8)	C	<.001	1.00			
High (≥60)	16	(1.8)	41	(17.2)			11.54	6.12	21.75	<.001
Cholinesterase (U/L)										
Low (<3500)	19	(2.1)	2	(.8)	M	.488				
Middle (3500-8000)	848	(95.2)	231	(96.7)						
High (>8000)	24	(2.7)	6	(2.5)						
Adiponectin (μg/mL)										
Mean ± SD	11.5 ± 5.5		9.5 ± 5.5		T	<.001	0.97	0.93	1.00	.047
Total cholesterol (mg/dL)										
Low (<150)	23	(2.6)	0	(.0)	M	<.001				
Middle (150-219)	597	(67.0)	137	(57.3)						
High (>219)	271	(30.4)	102	(42.7)						
LDL cholesterol (mg/dL)										
Low (<70)	11	(1.2)	2	(.8)	M	<.001				
Middle (70-139)	611	(68.6)	136	(56.9)						
High (>139)	269	(30.2)	101	(42.3)						
HDL cholesterol (mg/dL)										
High (≥40)	857	(96.2)	225	(94.1)	C	.165				
Low (<40)	34	(3.8)	14	(5.9)						
Triglyceride (mg/dL)										
Low (≤149)	794	(89.1)	194	(81.2)	C	.001				
High (≥150)	97	(10.9)	45	(18.8)						
BMI										
Normal (<25)	662	(74.3)	118	(81.2)	C	<.001	1.00			
Obese (≥25)	229	(25.7)	121	(18.8)			2.02	1.43	2.84	<.001
Fasting blood glucose (mg/dL)										
Low (<110)	834	(93.6)	199	(83.3)	C	<.001				
High (≥110)	57	(6.4)	40	(16.7)						
Insulin (μU/mL)										
Low (<3)	84	(9.4)	11	(4.6)	M	.003				
Middle (3-18)	801	(89.9)	222	(92.9)						

Table 6 (*continued*)

	Normal ALT n = 891		Elevated ALT n = 239		Univariate test	*P* value	Multivariate test			
							OR[a]	95% CI		*P* value
	n	%	n	%				Upper	Lower	
High (>18)	6	(.7)	6	(2.5)						
HOMA-IR										
0-1.9	731	(82.0)	130	(54.4)	M	<.001	1.00			
2.0-3.9	148	(16.6)	94	(39.3)			2.44	1.68	3.55	<.001
≥4	12	(1.3)	15	(6.3)			4.93	2.14	11.33	<.001
Blood pressure										
Normal	336	(37.7)	67	(28.0)	C	.006				
Hypertension	555	(62.3)	172	(72.0)						
Smoking habit										
Never	821	(92.1)	221	(92.5)	C	.494				
Current	44	(4.9)	14	(5.9)						
Former	26	(2.9)	4	(1.7)						
Drinking habit										
Never or former	760	(85.3)	210	(87.9)	C	.312				
Current	131	(14.7)	29	(12.1)						
Current medication[b]										
No	879	(98.7)	237	(99.2)	F	.746				
Yes	12	(1.3)	2	(0.8)						

[a] Multiple logistic regression analysis. Age group and the variables with *P* less than .1 on univariate analysis were included in the model.
[b] Current medication for hypertension, lipid metabolism abnormality, and diabetes was excluded.

of 957 men, and 188 (16.6%) of 1130 women. The ALT levels in these subjects were 29.2 ± 17.9 U/L in men and 24.1 ± 13.4 U/L in women; and thus, the mean levels were close to the cutoff values determined in this study. These values were significantly higher than those for subjects who had 0 or 1 metabolic risk factor for both men and women ($P < .001$) (Table 4).

3.6. Factors associated with elevated ALT levels

Factors associated with elevated ALT higher than the upper limits were investigated in 2087 subjects who were negative for anti-HCV antibody and serum hepatitis B surface antigen. The results for 957 men and 1130 women are shown in Tables 5 and 6, respectively. In men, 10 factors with a significant association with elevated ALT were identified in univariate analysis: age group, high γ-GTP, low adiponectin, high total cholesterol, high LDL cholesterol, high triglycerides, high BMI, high fasting glucose, high fasting insulin, and high HOMA-IR. In women, 10 factors associated with elevated ALT were identified in univariate analysis: high γ-GTP, low adiponectin, high total cholesterol, high LDL cholesterol, high triglycerides, high BMI, high fasting glucose, high fasting insulin, high HOMA-IR, and hypertension. A current drinking habit was not associated with elevated ALT in either men or women in univariate analysis. Multivariate logistic regression models were constructed for men and women using variables with low *P* values in univariate analysis. This analysis revealed 5 factors in men (high γ-GTP: odds ratio [OR], 5.57; 95% CI, 3.80-8.16; $P < .001$; low adiponectin: OR, 0.93; 95% CI, 0.88-0.98; $P < .02$; high LDL cholesterol: OR, 1.58; 95% CI, 1.06-2.35; $P < .03$; high BMI: OR, 1.85; 95% CI, 1.28-2.68; $P < .01$; and high HOMA-IR [2.0-3.9]: OR, 1.94; 95% CI, 1.26-2.98; $P < .01$; [≥4]: OR, 2.94; 95% CI, 1.26-6.86; $P < .02$) and 4 factors in women (high γ-GTP: OR, 11.54; 95% CI, 6.12-21.75; $P < .001$; low adiponectin: OR, 0.97; 95% CI, 0.93-1.00; $P < .05$; high BMI: OR, 2.02; 95% CI, 1.43-2.84; $P < .001$; and high HOMA-IR [2-3.9]: OR, 2.44; 95% CI, 1.68-3.55; $P < .001$; [≥4]: OR, 4.93; 95% CI, 2.14-11.33; $P < .001$) with a significant association with elevated ALT levels.

4. Discussion

Elevated serum ALT levels in the general population are closely associated with NAFLD, which is a liver phenotype of metabolic syndrome [4-8]. Alanine aminotransferase activities have also been shown to be useful as an indicator of general health [14], and ALT is a predictor of mortality in community residents [13]. Mortality may be due to unrecognized liver diseases, but may also be due to other causes of ALT elevation, such as atherosclerosis, hypertension, and type 2 diabetes mellitus, which are linked to nonliver health risks. This suggests the importance of determining the association of ALT levels with metabolic factors influencing the occurrence of metabolic syndrome–related diseases in a large population sample. Our results clearly indicate that elevated ALT levels unrelated to hepatitis virus infection are closely associated with metabolic syndrome–related features in a study population that is representative of the general Japanese population older than 40 years old. This suggests that measurement of ALT levels is likely to be a useful primary screening test for metabolic syndrome in the population.

In this study, the seroprevalences of hepatitis B and C were 1.7% and 1.9%, respectively, similar to the standard rates in the Japanese population [22]. Because hepatitis B and C infection is associated with elevated ALT levels, subjects positive for hepatitis markers were excluded from further analysis. To date, the upper limits of ALT levels in screening tests for the general population have not been established clearly; and therefore, we reevaluated these limits for effective screening of metabolic syndrome in the Japanese adult population. Previous reports have shown that sex has a significant influence on ALT levels [23,24]; and therefore, we assessed ALT levels separately for men and women. The ALT cutoff levels for effective screening of individuals with metabolic syndrome for men and for women were proposed in this study on the basis of the relationship between ALT levels and the number of the 3 major metabolic risk factors. Upper limits of 30 U/L in men and 25 U/L in women gave a good specificity of more than 80% for exclusion of subjects with none or 1 of the 3 metabolic risk factors: hypertension, lipid metabolism abnormality, and hyperglycemia. Using these cutoff values, we demonstrated that approximately 20% of the male and female subjects older than 40 years had ALT elevation. A current drinking habit was identified in 694 (72.5%) of 957 men and 160 (14.3%) of 1130 women, but a drinking habit itself was not significantly associated with elevated ALT in univariate analyses in this population, although there is no doubt that excess intake of alcohol causes liver injury in each individual. Multivariate analysis clearly showed that metabolic syndrome–related features that reflect obesity and insulin resistance, including high BMI, high LDL cholesterol, high HOMA-IR, and lower adiponectinemia, were associated with elevated ALT in the study population.

Elevated serum γ-GTP also showed a significant association with elevated ALT in both male and female subjects. These results were replicable in subjects without a history of alcohol consumption (data not shown). Previous studies have documented that elevated serum γ-GTP has a risk for metabolic syndrome and type 2 diabetes mellitus in middle-aged Japanese male office workers [25] and may represent an early marker of subclinical inflammation and increased oxidative stress in healthy individuals [26,27]. Our results are consistent with these studies, and we also found that elevated γ-GTP was associated with obesity and insulin resistance in both men and women. Therefore, γ-GTP is a promising marker for metabolic syndrome and particularly for prediction of development of metabolic syndrome–related diseases; and this warrants a further prospective study.

Because high serum ALT levels often reflect hepatic fat accumulation and inflammation, they are well correlated with the prevalence of NAFLD in the population in cases of unexplained ALT elevation. The importance of ALT activity as an indicator of NAFLD has been demonstrated in association with metabolic abnormalities caused by central obesity and insulin resistance [28-30]. Nonalcoholic fatty liver disease is classified into 2 categories: simple fatty liver and nonalcoholic steatohepatitis (NASH), which is intractable and progressive. The population with elevated ALT levels includes those with NASH [7,8,31] as a phenotype of metabolic syndrome in the liver. Fat droplets in liver tissue are often depleted in the advanced stage of NASH, and such cases may be diagnosed as cryptogenic liver cirrhosis or liver cancer [32]. In fact, the prevalence of obesity, hypertriglyceridemia, or type 2 diabetes mellitus is significantly higher in cases of liver cancer that develop from cryptogenic cirrhosis compared with those caused by HCV infection or excess intake of alcohol [33]. Because a cohort study showed prospectively that individuals with NAFLD had a higher mortality due to liver disease–related deaths [34], people in the general population with high ALT levels are of particular concern because those with NASH have a risk for progression to cirrhosis or cancer.

Individuals with minor elevation of serum ALT levels that are close to the upper limits of the reference range are also of concern because elevated ALT itself is closely associated with insulin resistance, even in the absence of NAFLD and obesity [35,36]. Recent studies have shown that elevated ALT could be a prognostic marker for development of metabolic syndrome [11,12]. Because individuals with ALT elevation have a potential risk for development of various metabolic syndrome–related diseases, including type 2 diabetes mellitus [9], cardiovascular disease [10], atherothrombosis [37], and obstructive sleep apnea [38], it may be worthwhile to notify those with minor ALT elevation of the risk of such diseases. In fact, in this study, we found that mean ALT activities in subjects with 2 or 3 metabolic risk factors were not particularly high, tending only to be close to the upper limit. Thus, minor ALT elevation is also an important feature for effective screening of metabolic syndrome. Elevation of ALT beyond the cutoff levels determined in this study was strongly associated with a broad spectrum of metabolic syndrome–related features, including obesity and insulin resistance. A prospective study of the association between elevated ALT levels and the occurrence of metabolic syndrome–related diseases is now in progress in this Takahata cohort, which includes more than 4000 people and is representative of the Japanese adult population.

In conclusion, the results of this study clearly show that elevated ALT levels in the Japanese population older than 40 years are associated with obesity and insulin resistance, which in turn are associated with metabolic syndrome. This suggests that, in addition to detection of liver disease, screening of serum ALT levels may contribute to identifying the potential risk of metabolic syndrome–related diseases in the general population.

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Original Paper

Plasma Level of Granulocyte-Colony Stimulating Factor during Granulocyte and Monocyte Adsorptive Apheresis in Patients with Ulcerative Colitis

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KEY WORDS: Granulocyte-colony stimulating factor; Granulocyte and monocyte adsorptive apheresis; Ulcerative colitis, Cytapheresis

ABBREVIATIONS: Granulocyte-colony stimulating factor (G-CSF); Granulocyte and monocyte adsorptive apheresis (GCAP); Cytapheresis with extra-corporeal circulation (CAP); Tumor necrosis factor-alpha (TNF-α); Interleukin-1 beta (IL-1β); Interleukin-1 receptor antagonist (IL-1ra); White blood cell (WBC); Bone marrow-derived cells (BMCs); Enzyme-Linked ImmunoSorbent Assay (ELISA); Vascular endothelial growth factor (VEGF)

ABSTRACT

Background/Aims: Cytapheresis with extra-corporeal circulation for ulcerative colitis is effective but its mechanisms are still unclear. Granulocyte-colony stimulating factor (G-CSF) strongly mobilizes bone marrow-derived cells and serves as anti-inflammatory factor. We investigated plasma levels of G-CSF during granulocyte and monocyte adsorptive apheresis (GCAP).

Methodology: Nineteen cases of ulcerative colitis were measured plasma concentration of G-CSF during the first session of GCAP therapy.

Results: G-CSF were significantly increased in the column inflow at 30 min compared with the baseline (Wilcoxon test, $p<0.01$), and also increased through the column (Wilcoxon test, $p<0.01$). The ratio of the increase in the column outflow at 60 min was 1.5-fold compared with the baseline. However, we could not show a significant relation between G-CSF level and clinical efficacy.

Conclusions: This is the first report concerning G-CSF during CAP. G-CSF is increased due to GCAP and appears to be a candidate which should be further investigated.

INDRODUCTION

Cytapheresis with extra-corporeal circulation (CAP) has been developed as a standard therapy for ulcerative colitis in Japan. Although the mechanism of its effectiveness is still unclear, it has been proposed that elimination of activated leukocytes,(1) decreased of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) by leukocytes passed through the column,(2,3) increased levels of anti-inflammatory cytokines such interleukin-10 (IL-10) (4) and interleukin-1 receptor antagonist (IL-1ra) (5) in the column, or release of hepatocyte growth factor (HGF) in the column (6) may play a role. Besides of soluble factors, the peripheral white blood cell (WBC) count decreases significantly during CAP due to elimination of leukocytes, but immediately recovers at the end of a session of granulocyte and monocyte adsorptive apheresis (GCAP) (7). WBC shows a transient increase from the baseline after a session of leukocytapheresis (LCAP) (8). This rapid recovery of WBC may be due to recruitment of peripheral blood stem cells as well as immature leukocytes. It has been reported that bone marrow-derived cells (BMCs) may play a role in the repair of mucosal injury (9, 10). These phenomena, release of IL-10 and mobilization of BMCs, may be mediated by granulocyte-colony stimulating factor (G-CSF), which is thought to be a pivotal factor in CAP therapy. However, no previous studies have focused on the role of G-CSF in relation to CAP. In the present study, therefore, we investigated upregulation of G-CSF during the process of GCAP.

METHODOLOGY

Nineteen cases of ulcerative colitis that had been refractory to steroid treatment were enrolled for this study. **Table 1** summarizes the profiles of the patients. Criteria of disease activity are based on the ulcerative colitis severity criteria of the Japanese Ministry of Health, Labor and Welfare. Patients with moderate disease activity underwent one GCAP session per week, and those with severe disease activity underwent two sessions in the first week. Most patients experienced 5 sessions of GCAP. If patients reached the clinical remission, GCAP therapy was discontinued under 5 times. The average number of GCAP sessions per patient was 4.3 times. When administration of steroid hormone can be tapered to the half or below at the tow weeks after the final ses-

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sion, we consider as effective cases.

Blood samples were taken from each patient during the first session of GCAP. Heparinized blood sample was drawn during inflow into the column at 0 and 30 min during circulation, and also during outflow at 30 and 60min. Plasma samples were stored in a freezer at -70°C. Plasma levels of G-CSF were measured using a hG-CSF ELISA kit (IBL-Japan, Takasaki, Japan). For statistical analysis, Wilcoxon signed-rank test was used, and a p-value of <0.01 was considered to indicate significance.

RESULTS

Based on our criteria of clinical efficacy, GCAP therapy was effective in 14 (74%) of the patients, but ineffective in 5 (26%). No serious side effects were observed in any of the patients **(Table 2)**.

Plasma levels of G-CSF in the inflow were increased significantly at 30 min (p<0.01) **(Figure 1)**. The increase was about 30% compared with the baseline **(Figure 2)**. Only one patient showed no increase at 30 min, even though GCAP was effective. At 30 min, a significant increase was also observed in the outflow compared with the inflow (p<0.01). A slight increase was evident in the outflow at 60 min, and finally the G-CSF level in the outflow at 60 min was increased by 50% from the baseline.

There was no significant difference in the increased level of G-CSF (outflow at 60 min – inflow at 0 min) between the GCAP-effective group and the GCAP-ineffective group. Neither the dose of prednisolone nor the WBC count was related to the baseline level of G-CSF or its degree of increase.

Table 1 Profiles of 19 Patients with Ulcerative Colitis Treated with Granulocyte/Monocyte Adsorptive Therapy

Gender	male: 11, female: 8
Age	<30 yr: 5, 31-50 yr: 11, 51 yr<: 3
(Mean)	(42 + 16 yr)
Extension of colitis	total: 14, left: 5
Severity of disease	severe: 2, moderate: 17
No. of sessions (times)	<5: 3, 5: 13, 5<: 3

Total means total colitis type of ulcerative colitis (extending over mid-transverse colon). Left means that extent of inflammation was within the mid-transverse colon. Criteria of disease are based on the ulcerative colitis severity criteria of the Japanese Ministry of Health, Labor and Welfare, and were described at the time of the first session of granulocyte/monocyte adsorptive therapy.

Table 2 Clinical Efficacy and Increased Level of G-CSF

Effucacy	Cases	Increased level (pg/ml)	Ratio
Effective	14	5.5 +/- 6.1	1.39 +/- 0.46
Ineffective	5	7.1 +/- 2.9	1.86 +/- 0.45

If the patients achieved a state of remission or if the prednisolone dose could be reduced to half two week after the final session, we considered the therapy effective. If the patients did not reach an effective level and required another treatment, we considered the therapy ineffective. Effective rate was 73.7 %. There was no significant difference in the increased level of G-CSF (outflow at 60 min – inflow at 0 min) between the GCAP-effective group and the GCAP-ineffective group. Increased ratio was calculated by outflow at 60 min /inflow at 0 min.

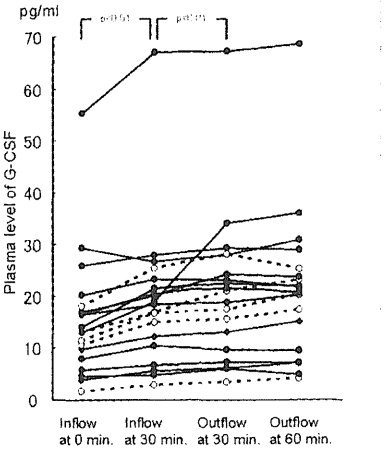

FIGURE 1 Plasma G-CSF levels in each patient. Solid and clear circles indicate effective and ineffective cases, respectively. There was a significant increase of G-CSF at 30 min in the column inflow (Wilcoxon test, p<0.01). There was also a significant increase at 30 min in the outflow compared with the inflow at the same time point (Wilcoxon test, p<0.01). There was no significant difference in the increased level of G-CSF (outflow at 60 min – inflow at 0 min) between the GCAP-effective group and the GCAP-ineffective group.

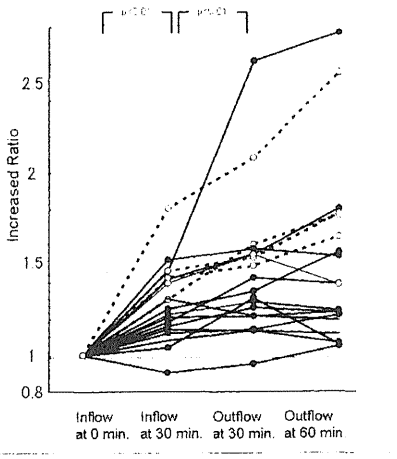

FIGURE 2 Relative plasma G-CSF levels in each patient. Plasma G-CSF at 0 min inflow was designated as the baseline level. Solid and clear circles indicate effective and ineffective cases, respectively. There was a significant increase of G-CSF at 30 min in the column inflow (Wilcoxon test, p<0.01). There was also a significant increase at 30 min in the outflow compared with the inflow at the same time point (Wilcoxon test, p<0.01).

DISCUSSION

A variety of cell types such as monocytes/macrophages, T-lymphocytes, endothelial cells and fibroblasts can produce G-CSF. Plasma level of G-CSF usually increases when there is a demand for granulocyte production in conditions such as acute neutropenia or infection (11) Clinically, G-CSF is used for treatment of patients with leukocytopenia due to chemotherapy, or in the collection of peripheral blood stem cells. In this study, we observed the significant upregulation of G-CSF. WBC counts are decreased during G-CAP. So this upregulation of G-CSF was thought to be based on elimination of activated leukocytes. Interestingly, as the level of G-CSF in the column outflow was significantly higher than that in the inflow at 30 min, it appeared that G-CSF might be produced in the column.

Some recent studies have provided additional

information about the biological role of G-CSF. It has been shown that increased production of G-CSF in the acute phase response is a key physiological component of host defense (12) Administration of G-CSF decreases TNF production by increasing the production of IL-10 and prostaglandin-E2 in lipopolysaccharide-treated model rats (13) Stimulation of monocytes with G-CSF attenuates the release of IL-1β and TNF-α(14) Moreover, G-CSF may exert an anti-inflammatory effect (15) and markedly upregulates IL-10 mRNA levels (16) in a colitis model. Accordingly, an increase of G-CSF during CAP therapy is thought to have a favorable anti-inflammatory effect. In fact, an increased level of IL-10 is observed during GCAP therapy and contributes to clinical efficacy (4).

From the viewpoint of mucosal healing, growth factors such as HGF and vascular endothelial growth factor (VEGF) are thought to be involved. It had been shown that HGF is produced in the column during GCAP (6), and that *in vivo* administration of G-CSF stimulates the production of HGF (17) and VEGF (18).

Recently, stem/progenitor cells have been shown to contribute to repair of mucosal injury.9,10 Furthermore, G-CSF mobilizes BMCs into the peripheral blood (19). Although no previous report has mentioned that CAP therapy mobilizes CD34-positive cells, it seems reasonable to hypothesize that G-CSF might mobilize CD34-positive cells into the peripheral blood.

Currently, three kinds of CAP therapy are available: GCAP, LCAP and centrifugation. Among them, there is no marked difference in therapeutic effect (20). However, all three share a common mechanism in that leukocytes are eliminated (about 3-5 x 109 in one session), and therefore an increased level of G-CSF may be a common key factor. We plan to focus on this possibility, and to test whether addition of G-CSF to CAP therapy may improve clinical efficacy. In fact, in the treatment of Crohn's disease, administration of G-CSF has a considerable clinical effect (21).

In this study, the rate of response to GACP was similar to our previous experience (data not shown) or other reports (20,22). Unfortunately, however, we were unable to examine the relationship between the degree of G-CSF upregulation and clinical effectiveness. The effect of CAP therapy may depend on the individual. In addition to problem of effectiveness, we must discuss about the amount of G-CSF production during GCAP. The increase level of G-CSF may not reach to the pharmacological level. In this point, we need a further investigation.

This is the first report about G-CSF during CAP therapy. The entire mechanism of GCAP therapy cannot be explained in terms of G-CSF alone. GCAP therapy is based on integration of many factors. However, the increase of G-CSF during GCAP possibly creates favorable conditions for ulcerative colitis therapy. G-CSF might therefore be one of the key factors involved in CAP therapy, and warrants further investigation to achieve a better outcome.

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Therapeutic Apheresis and Dialysis

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Biological Effect of Anaphylatoxin C5a on the Generation of Anti-inflammatory Substances in Leukocyte Adsorption

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Abstract: Anaphylatoxins, which are involved in both pro-inflammatory processes and a variety of anti-inflammatory effects, are produced during granulocyte and monocyte adsorptive apheresis. We noticed the anti-inflammatory effects of C5a, the strongest anaphylatoxin, in granulocyte and monocyte adsorptive apheresis. The aim of this study was to investigate the effect of C5a on interleukin-1 receptor antagonist (IL-1ra) and hepatocyte growth factor (HGF) generation in granulocyte and monocyte adsorption. Peripheral blood containing nafamostat mesilate as an endogenous complement activation inhibitor was divided into four groups: (1) no recombinant C5a added, no contact with cellulose acetate (CA) beads (control group); (2) no C5a added, contact with CA beads; (3) C5a added, no contact with CA beads; and (4) C5a added, contact with CA beads. After incubation, IL-1ra and HGF in plasma were measured. IL-1ra was significantly higher in group 3, in which only C5a was added in the absence of CA beads, compared to groups 2 ($P < 0.01$) and 4 ($P < 0.05$). HGF was significantly higher only in group 4, in which C5a was added in the presence of CA beads ($P < 0.05$), but did not increase in the absence of CA beads. C5a can directly induce IL-1ra generation without the granulocyte and monocyte adsorption stimuli to CA beads, but can synergistically induce HGF generation with the adsorption stimuli, indicating C5a has different effects on IL-1ra and HGF generation.
Key Words: Adsorption, Apheresis, Complement C5a, Granulocyte, Hepatocyte growth factor, Interleukin-1 receptor antagonist.

A granulocyte and monocyte (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 (UC) (1,2) and rheumatoid arthritis (3). The device comprises a column filled with 2 mm cellulose acetate (CA) beads that act as carriers for adsorptive leukocyte apheresis (4). Steroid and GMA therapies are similarly effective in relieving the symptoms of UC patients, but the latter causes less severe side-effects (5,6). Therefore, characterization of the biological responses to GMA is useful to elucidate the physiological anti-inflammatory responses in patients with inflammatory diseases.

A decrease in GMs was initially considered to be important to the anti-inflammatory effect, but the cell numbers recover within approximately 24 h after GMA. Although the precise mechanisms of the clinical efficacy of GMA are unclear, GM adsorption possibly triggers various biological responses, such as the release of interleukin-1 receptor antagonist (IL-1ra) and hepatocyte growth factor (HGF) (4,7). These substances are returned to the patients along with the blood and might contribute to the restoration of normal immune function (8–10). We are interested in what type of stimulation is able to generate these anti-inflammatory substances in GMA.

Cellulose acetate was originally used in membrane-based hemodialysis devices (11–13) and it is known that CA membranes activate the complement system by contact with blood (11). Similarly, complement activation and generation of complement activation fragments containing anaphylatoxins

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(such as C3a and C5a) are observed in the GMA column using CA beads (7,14,15). Anaphylatoxins C3a and C5a are known causes of pro-inflammatory processes (16–20); however, they are also involved in a variety of anti-inflammatory effects, including augmentation of anti-inflammatory cytokines (15,21,22).

GMA is a safe therapy for patients, without anaphylatoxin-induced side-effects. Thus, understanding the anti-inflammatory effects of anaphylatoxins in GMA is important for understanding the mechanisms of apheresis therapy. Based on this background, in this study we revealed the effects of complement C5a on the generation of anti-inflammatory substances (IL-1ra and HGF) in GMA.

MATERIALS AND METHODS

Reagents

Cellulose acetate beads were prepared by JIMRO Institute (Takasaki, Japan) and nafamostat mesilate was prepared by Torii Pharmaceutical (Tokyo, Japan). Recombinant human complement C5a (rhC5a) was purchased from Sigma (St Louis, MO, 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 four healthy volunteers into plastic syringes (Terumo, Tokyo, Japan).

Exposure of blood to CA beads

A mixture of peripheral blood containing serial dilutions (1–100 μmol/L) of nafamostat mesilate, as anticoagulant and complement activation inhibitor, and CA 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 CA beads were measured using a COULTER Gen-S hematology analyzer (Beckman Coulter, Fullerton, CA, USA), and then plasma separated by centrifugation at $800 \times g$ for 5 min at 4°C was 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 trypan blue exclusion assay.

Addition of rhC5a to complement activation inhibited blood

Peripheral blood containing 100 μmol/L nafamostat mesilate, which can almost completely inhibit both endogenous complement activation raised by contact between blood and CA beads and GM adsorption to CA beads (15), was divided into four groups: (1) no rhC5a added, no contact with CA beads (control group); (2) no rhC5a added, contact with CA beads; (3) 100 ng/mL rhC5a added, no contact with CA beads; and (4) 100 ng/mL rhC5a added, contact with CA beads. These were rotated at 1 rpm for 1 h at 37°C. The plasma was then separated and stored as mentioned above.

Measurement of anaphylatoxin C5a

Complement C5a was measured using a cytometric bead array anaphylatoxin kit (BD Biosciences, San Jose, CA, USA) with a flow cytometer (FACSCalibur; BD Biosciences) according to the manufacturer's instructions. The ratio (%) of increased C5a was calculated as follows: increased C5a (%) = 100 × (concentration of C5a after incubation − concentration of C5a before incubation)/concentration of C5a before incubation.

Measurement of IL-1ra and HGF

IL-1ra and HGF were measured using enzyme-linked immunosorbent assays (ELISAs) (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 or HGF was calculated as follows: increased IL-1ra or HGF (%) = 100 × (concentration of IL-1ra or HGF after incubation − concentration of IL-1ra or HGF before incubation)/concentration of IL-1ra or HGF before incubation.

Statistical analysis

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

RESULTS

Positive correlation between C5a increase and IL-1ra and HGF generation

We first verified the association between complement C5a increase and anti-inflammatory substance generation in the syringe filled with CA beads. Peripheral blood containing various concentrations (1–100 μmol/L) of nafamostat mesilate, serving not only as an anticoagulant but also as a complement activation inhibitor, was incubated with CA beads for 1 h to make various concentrations of complement

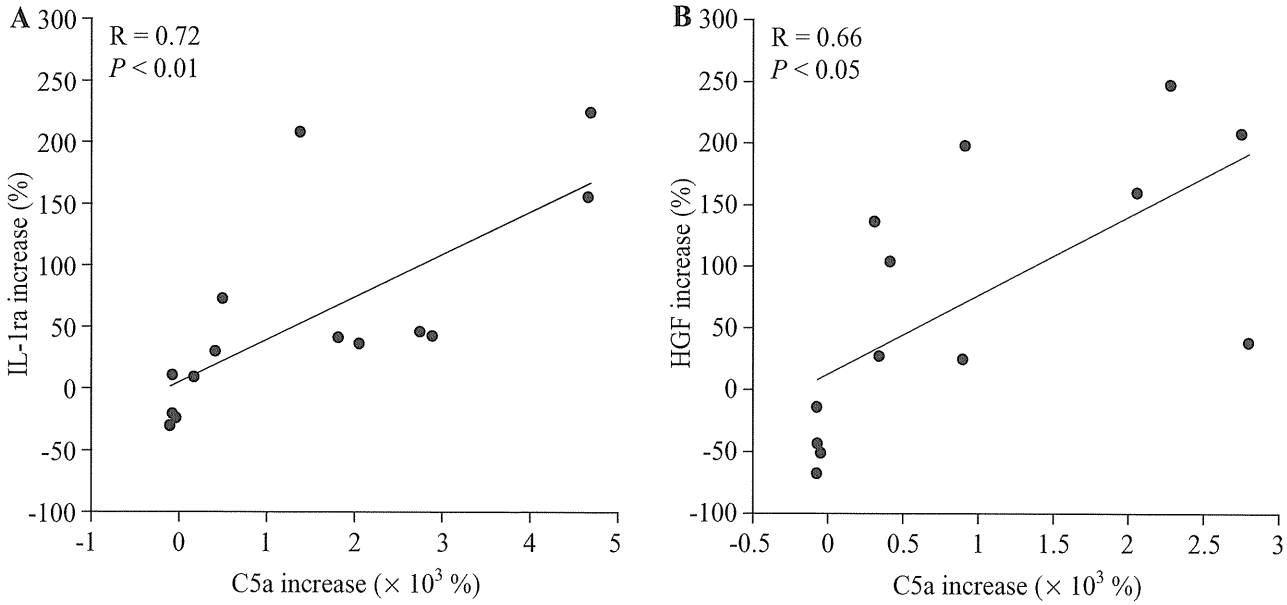

FIG. 1. Correlation of the increases in (A) interleukin-1 receptor antagonist (IL-1ra), and (B) hepatocyte growth factor (HGF) with C5a increase. Peripheral blood from healthy volunteers was mixed with serial dilutions of nafamostat mesilate. Test samples were then incubated with cellulose acetate beads for 1 h and the plasma was separated by centrifugation for the measurement of C5a, IL-1ra, and HGF after incubation. The increase ratios (%) of C5a, IL-1ra, and HGF were calculated as described in the Materials and Methods section. The *P* values are based on Spearman's rank correlation.

activation fragment C5a in blood. In the experiments described here, the number of granulocytes in the peripheral blood was 3236 ± 676.3 cells/μL. The C5a increase ratio was positively correlated with both the IL-1ra (Fig. 1A) and HGF (Fig. 1B) increase ratios. These results suggest that C5a generation is related to the generation of IL-1ra and HGF.

Effect of C5a on the generation of IL-1ra and HGF in GM adsorption

The C5a concentration in plasma reflects the degree of complement activation raised by contact between blood and CA beads, and complement activation induces GM adsorption to CA beads according to the degree of activation (15). Furthermore, the release of IL-1ra and HGF is related to the GMs absorbed to CA beads (7). In other words, the three phenomena (C5a generation, GM adsorption, and release of IL-1ra and HGF) are closely related. In this study, Figure 2A and B show that both IL-1ra and HGF increase ratios were positively correlated with the ratio of adsorbed granulocytes.

Previous reports were not enough to explain the relationship between C5a and the generation of IL-1ra and HGF, because C5a generation occurred simultaneously when GM adsorbed to CA beads in previous studies. Thus, to clarify the effect of C5a on the generation of IL-1ra and HGF, we prepared the complement inactive condition of blood using 100 μmol/L nafamostat mesilate and added rhC5a in the presence or absence of CA beads. We prepared four groups: (1) no rhC5a added, no contact with CA beads; (2) no rhC5a added, contact with CA beads; (3) rhC5a added, no contact with CA beads; and (4) rhC5a added, contact with CA beads. The concentration of plasma C5a before adding rhC5a was 6.9 ± 1.0 ng/mL, which is the baseline of C5a. After incubation, each aliquot of plasma was separated by centrifugation, and the concentrations of IL-1ra and HGF were measured. The amount of IL-1ra in the four groups was: (1) 215.9 ± 15.9 pg/mL; (2) 332.1 ± 89.0 pg/mL; (3) 1379.2 ± 188.4 pg/mL; and (4) 937.0 ± 156.3 pg/mL. The highest amount of IL-1ra was in group 3 (rhC5a added, no contact with CA beads) (Fig. 3A). The amount of HGF in the four groups was: (1) 773.2 ± 24.5 pg/mL; (2) 704.5 ± 93.3 pg/mL; (3) 840.9 ± 67.0 pg/mL; and (4) 1156.3 ± 124.8 pg/mL. The highest amount of HGF was in group 4 (rhC5a added, contact with CA beads) and the amount in group 3 increased slightly, but the level was not significant compared to the control group (Fig. 3B). These results show that C5a increases anti-inflammatory substances in GM adsorption to CA beads and has different effects on IL-1ra and HGF generation. We conclude that C5a can induce IL-1ra release without adsorption, while HGF release requires adsorption to CA beads.

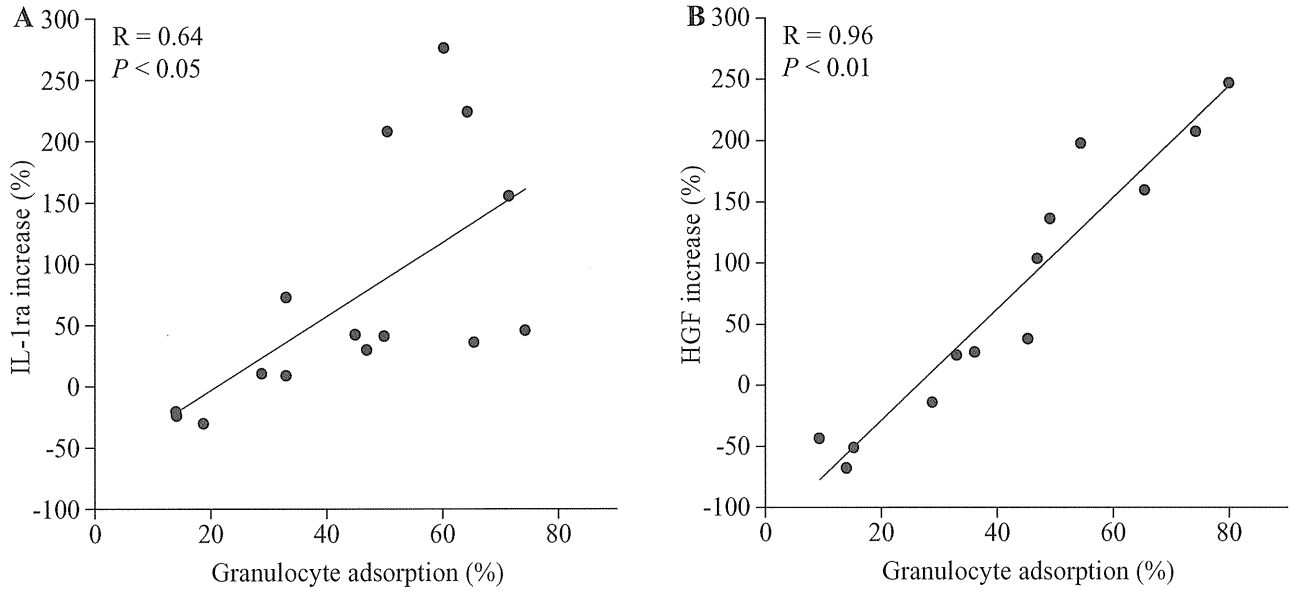

FIG. 2. Correlation of the increases in (A) interleukin-1 receptor antagonist (IL-1ra), and (B) hepatocyte growth factor (HGF) with granulocyte adsorption to cellulose acetate (CA) beads. Peripheral blood from healthy volunteers was mixed with serial dilutions of nafamostat mesilate and incubated with CA beads for 1 h. The fractions of granulocytes adsorbed to the CA beads were measured after incubation and then plasma was separated by centrifugation for measurement of IL-1ra and HGF. The ratio (%) of granulocyte adsorption and increase ratios (%) of IL-1ra and HGF were calculated as described in the Materials and Methods section. The *P* values are based on Spearman's rank correlation.

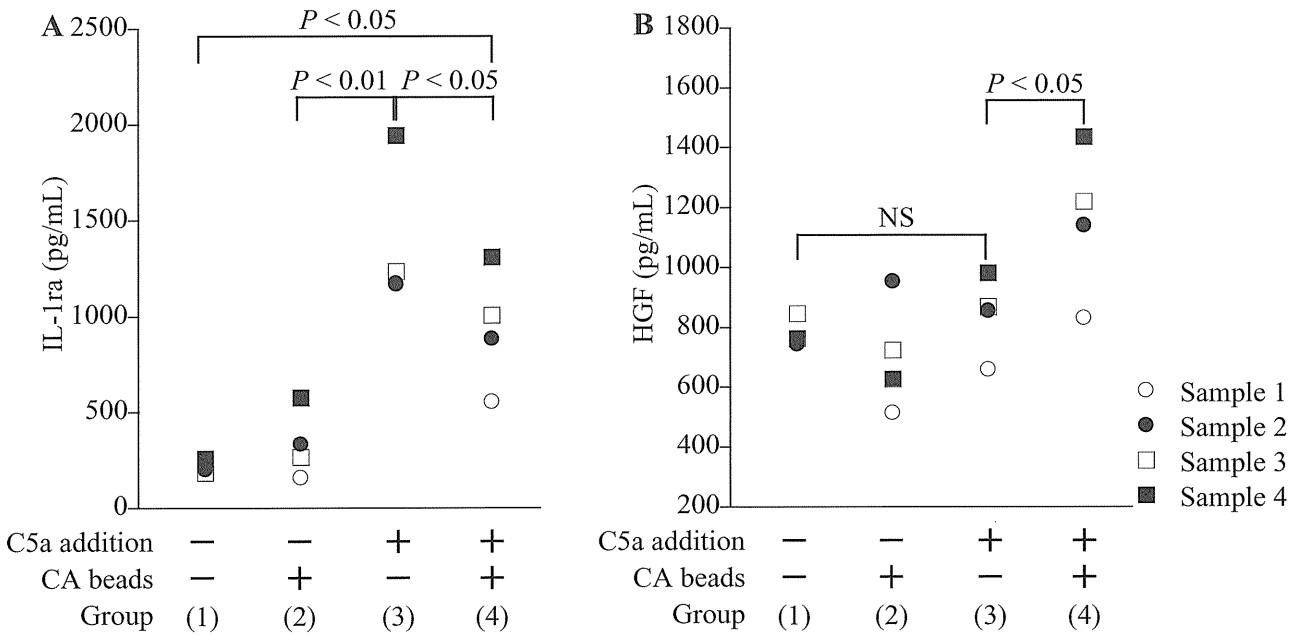

FIG. 3. Effect of recombinant human complement C5a (rhC5a) and cellulose acetate (CA) beads on (A) interleukin-1 receptor antagonist (IL-1ra), and (B) hepatocyte growth factor (HGF) generation. Peripheral blood containing 100 µmol/L nafamostat mesilate was divided into four groups: (1) no rhC5a added, no contact with CA beads (control group); (2) no rhC5a added, contact with CA beads; (3) 100 ng/mL rhC5a added, no contact with CA beads; and (4) 100 ng/mL rhC5a added, contact with CA beads. These were incubated for 1 h. After incubation, plasma was separated by centrifugation for measurement of IL-1ra and HGF. The *P* values are based on paired *t*-test.

DISCUSSION

CA beads are used for GMA therapy, and not only induce GM adsorption, but also generate C5a and the release of IL-1ra and HGF. These phenomena are interrelated; however, the precise role of C5a stimulation is still unclear. In this study we indicated that C5a can induce IL-1ra release without GM adsorption, and that C5a and GM adsorption to CA beads affect HGF release synergistically.

Activation and subsequent cleavage of C5 by C5 convertase produces the complement activation fragment C5a, an anaphylatoxin. Anaphylatoxins are involved in a variety of pro-inflammatory processes, and C5a has the strongest known biological effects (16). For example, because C5a induces the production of mediators that cause both vasodilatation and an increase in vascular permeability, it is partly responsible for the pathogenesis of sepsis, including hypovolemia due to both arterial and venous dilatation and leakage of plasma into the extravascular space (17–19). On the other hand, it is known that anaphylatoxins also have anti-inflammatory effects in some situations. C5a is essential during the early priming stages of hepatocyte regeneration in mice (21), and recombinant C5a induces the production of IL-1ra in peripheral mononuclear cells (22). In GM adsorption it has been reported that IL-1ra release is augmented by adding C5a (15). Additionally, an increase in HGF release by adding C5a was also revealed in this study. It has been reported that patients with UC, who responded to GM apheresis treatment, show a significant increase in IL-1ra in the Adacolumn outflow (9), and that HGF administration accelerates colonic mucosal repair in rats with dextran sulfate sodium-induced colitis (10). These reports suggest that IL-1ra and HGF might contribute to healing in UC patients. Thus, C5a produced during GMA is believed to provide clinical efficacy in patients with UC through an increase in anti-inflammatory substances.

In this study, both IL-1ra and HGF generation were augmented by adding rhC5a, but different experimental groups had the highest amount of each. The IL-1ra amount in group 3 (rhC5a added, no contact with CA beads) was the highest. Since IL-1ra increased by adding only rhC5a in the absence of CA beads, we believe that in GMA, the C5a produced by contact between blood and CA beads itself can induce IL-1ra generation from leukocytes without the stimulation of GM adsorption to CA beads. Strangely, the IL-1ra amount in group 4 (rhC5a added, contact with CA beads) was significantly lower than in group 3, although higher than in group 1 (no rhC5a added, no contact with CA beads). The reason why the presence of CA beads caused suppression of IL-1ra is unclear, though it might be that rhC5a or IL-1ra adsorbed to the CA beads. Further study of the effects of CA beads with respect to IL-1ra generation is necessary.

In contrast, the amount of HGF in group 4 was the highest. Since HGF did not significantly increase in the absence of CA beads, even in the presence of rhC5a, we believe that C5a, which itself induces little HGF generation from leukocytes, acts synergistically with CA beads to generate HGF. HGF is released by degranulation neutrophils (23), and adhesion-dependent degranulation of neutrophils requires Src family kinase (24). On the other hand, a C5a signal via the C5a receptor, known as a protein Gi-coupled seven membrane-spanning receptor, regulates Ras and mitogen-activated protein (MAP) kinase (25). The C5a signal cannot induce activation of the Src family. Thus, the stimulation caused by GM adsorption to CA beads may induce outside-in signals synergistically with C5a receptor signals and then induce Src family kinase phosphorylation.

CONCLUSION

The present study found that the generation of IL-1ra and HGF in GM adsorption was positively correlated with the degree of generation of anaphylatoxin C5a. C5a induced IL-1ra generation by itself and increased HGF generation synergistically with CA beads. Our results indicate that anaphylatoxin C5a plays important, anti-inflammatory roles in GMA therapy. This provides important insight into the various biological responses induced by GM adsorption and into understanding physiological anti-inflammatory responses.

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The relationship between the expression of the glucocorticoid receptor in biopsied colonic mucosa and the glucocorticoid responsiveness of ulcerative colitis patients

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KEYWORDS
Ulcerative colitis;
Glucocorticoid receptor;
Glucocorticoid responsiveness;
Regulatory T cell;
Foxp3

Abstract The objective of this study was to clarify the relationship between the frequency of infiltrating cells expressing the glucocorticoid receptors (GR) α and β in biopsied colonic mucosa and the glucocorticoid (GC) responsiveness of ulcerative colitis (UC) patients. Active UC patients ($n=38$) were divided into GC-sensitive and GC-resistant groups. GRβ$^+$ cells were significantly higher in the GC-resistant group than in the GC-sensitive and control groups. GRα mRNA was expressed in all UC patients, while GRβ mRNA was expressed in only 1 patient in the GC-sensitive group ($n=6$) and 7 patients in the GC-resistant group ($n=8$). Double-positive cells for GRβ and CD4 or CD19 were frequently observed. The Foxp3$^+$ cell count was significantly higher in the GC-sensitive group than in the GC-resistant group, but double Foxp3$^+$GRβ$^+$ cells were not observed. These results indicated that the sensitivity of GC therapy could probably be predicted by immunostaining biopsy specimens for GRβ and Foxp3.

Introduction

Glucocorticoid (GC) therapy is recognized as an effective therapy for inflammatory bowel disease, autoimmune disease, and allergic disorders; furthermore, GCs are widely used as the primary drug of choice for the active stage of ulcerative colitis (UC) [1]. However, the mechanism of action of GCs has not yet been completely elucidated, and the reasons why they have different effects on individual patients in different clinical settings also remain a mystery.

The expression of glucocorticoid receptors (GRs) in target cells is an essential component of the pharmacological action of GCs: GCs bind to GRs and result in the effect. The GR has 2 isoforms, GRα and GRβ, which are produced by alternate splicing of mRNA from the same gene [2]. GRα binds to GCs, translocates from the cytoplasm to the nucleus, and forms homodimers [3]. Thereafter, it binds to the GC response elements present in the promoter region of the target gene

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and regulates the transcriptional activity of the various genes involved in inflammation, producing an anti-inflammatory response [4,5]. In contrast, GRβ is considered to be a possible inhibitor of GRα. This may be explained by the fact that GRβ cannot bind to GCs [6,7] or that it forms heterodimers with GRα and thus cannot bind with the GC response elements of the target gene [8,9].

In the peripheral blood mononuclear cells of UC patients, GRβ mRNA has been reported to be highly expressed in groups with a poor response to GC therapy and minimally expressed in groups with a good response to GC therapy [10]. In addition, there have been similar reports for bronchial asthma and rheumatoid arthritis [11,12]. In contrast, GRβ has been reported to be unable to control the action of GRα [13], while the expression of GRβ does not cause a poor GC response in UC [14,15]. Clearly, the role of GRβ remains controversial. Previous reports have examined the expression of GRα and GRβ in peripheral blood mononuclear cells and cultured cells like HeLaS3 and COS7, but no reports have extensively examined the expression of GRα and GRβ in the inflammatory cells of colonic mucosal tissue, a locus of inflammation.

$CD4^+$ T cells play a crucial role in eliciting an immune reaction to foreign antigens. Regulatory T cells (Tregs) account for 5–10% of the fraction of $CD4^+$ T cells and express CD25. Tregs are involved in maintaining immunological self-tolerance by suppressing autoreactive lymphocytes [16]. The transcription factor Foxp3 is specifically expressed in Tregs, and acts as the master control gene for the development of Tregs [17]. Tregs are known to exist at various sites, including the intestinal mucosa [18,19]. Patients with active UC have decreased numbers of peripheral Tregs and increased numbers of lamina propria Tregs, suggesting a correlation between increased Treg numbers and decreased severity, and supporting the hypothesis that Tregs traffic to sites of inflammation in an attempt to restore immune homeostasis [20,21]. Therefore, if Tregs express GRβ, they would be closely associated with GC resistance/refractoriness. However, to our knowledge, there have been no reports on the correlation between $Foxp3^+$ Treg localization in the lamina propria, the GC responsiveness of patients with active UC, or GRβ expression on these Tregs. The current study was designed to assess these issues.

The aim of this study was to clarify the relationship between the frequency and type of infiltrating cells expressing GRα, GRβ, and Foxp3 in biopsied colonic mucosa, and the GC responsiveness of UC patients.

Materials and methods

Patients and materials

The subjects were patients with initial onset or relapse of UC. Specimens were taken via colonoscopic biopsy from 38 active UC patients with a clinical activity index (CAI) [22] of 5 or higher. Patients with a CAI of 4 or lower were omitted from the present study, because they were clinically in remission and therefore did not require GC therapy. All patients had previously received a 5-aminosalicylic acid preparation but no immunomodulators like azathioprine, methotrexate, or Remicade. A GC (20 mg or more) was administered to all patients, and a biopsy was performed within a week of the start of treatment. The patients were divided into 2 groups, GC-sensitive and GC-resistant in accordance with a previous report [10], and GC was administered to active UC patients with a CAI of 5 or higher. Subjects with a CAI of 4 or lower and to whom 20 mg or less of GC was administered within 4 weeks were defined as sensitive, while those with a CAI of 5 or higher after 4 weeks or those who required surgery were defined as resistant. The GC-sensitive group consisted of 18 patients with a mean age of 41.5±13.7 years (19–67 years), and a mean CAI of 7.1±1.9 (5–13) before GC administration (Table 1). The GC-resistant group consisted of 20 patients with a mean age of 38.3±13.8 years (15–57 years), and a mean CAI of 10.1±3.4 (5–15) before GC administration. Specimens that were taken via a rectal mucosal biopsy from 10 patients who underwent a colonoscopic polypectomy and who had a mean age of 47.3±8.7 years (39–64 years) were used as control. Informed consent was obtained from all patients. The tissue specimens were fixed in 10% buffered formalin for 12 h at room temperature, and were then embedded in paraffin for immunohistochemistry of GRα, GRβ, and Foxp3; immunodouble staining for GRβ and CD4, CD8, CD20, or CD68; and fluorescence immunodouble staining for GRβ and Foxp3. Some parts of the tissue specimens were fixed in 4% paraformaldehyde (PFA) for 6 h at 4 °C, immersed in sucrose gradient buffers, and frozen in a Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) for immunodouble staining of GRβ and CD19. The frozen specimens were kept at −80 °C until cryostat sectioning. Some parts of the tissue specimens were stored at 4 °C for reverse transcription-polymerase chain reaction (RT-PCR) of GRα and GRβ mRNAs.

Table 1 Cases used in this study.

Clinical data	Disease/control		
	Ulcerative colitis		Control (n=10)
	GC-sensitive (n=18)	GC-resistant (n=20)	
Male/female	12/6	15/5	6/4
Age (years)	41.5±13.7	38.3±13.8	47.3±8.7
Clinical activity index	7.1±1.9	10.1±3.4	–
Disease duration (years)	7.5±7.1	6.1±4.5	–

GC; glucocorticoid. Data of age, clinical activity index, and disease duration are represented as mean±SD.