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Secondary Structure of the Amino-Terminal Region of HCV NS3 and Virological Response to Pegylated Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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The aim of the study was to identify a predictive marker for the virological response in hepatitis C virus 1b (HCV-1b)-infected patients treated with pegylated interferon plus ribavirin therapy. A total of 139 patients with chronic hepatitis C who received therapy for 48 weeks were enrolled. The secondary structure of the 120 residues of the amino-terminal HCV-1b non-structural region 3 (NS3) deduced from the amino acid sequence was classified into two major groups: A and B. The association between HCV NS3 protein polymorphism and virological response was analyzed in patients infected with group A (n = 28) and B (n = 40) isolates who had good adherence to both pegylated interferon and ribavirin administration (>95% of the scheduled dosage) for 48 weeks. A sustained virological response (SVR) representing successful HCV eradication occurred in 33 (49%) in the 68 patients. Of the 28 patients infected with the group A isolate, 18 (64%) were SVR, whereas of the 40 patients infected with the group B isolate only 15 (38%) were SVR. The proportion of virological responses differed significantly between the two groups ($P < 0.05$). These results suggest that polymorphism in the secondary structure of the HCV-1b NS3 amino-terminal region influences the virological response to pegylated interferon plus ribavirin therapy, and that virus grouping based on this polymorphism can contribute to prediction of the outcome of this therapy. ***J. Med. Virol. 82:1364–1370, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis C; interferon; ribavirin; interaction; polymorphism

INTRODUCTION

Hepatitis C virus (HCV) is the major pathogen that causes chronic liver diseases with a risk of progression to cirrhosis and hepatocellular carcinoma. Currently, the standard treatment for chronic hepatitis C is antiviral therapy using pegylated interferon (Peg-IFN) plus ribavirin (RBV), and this approach is most effective for eradication of HCV viremia. However, even with the widely used treatment regimen of 48 weeks, the rate of sustained virological response (SVR), which indicates eradication of viremia, is still approximately 50% for patients infected with the therapy-resistant HCV genotype 1b (HCV-1b) with a high viral load [Manns et al., 2001; Bruno et al., 2004; Hadziyannis et al., 2004]. It would be useful to predict the virological response to this therapy and to identify patients who would obtain beneficial therapeutic effects before treatment, in order to avoid any serious side effect and to eliminate those who would not be helped by the treatment. In the future it will be important to establish a protocol of tailor-made medicine for chronic hepatitis C.

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Both the HCV genotype and pre-treatment viral load are major viral factors that influence the response to IFN-based antiviral therapy, but IFN resistance is also partly due to variation of the amino acid sequence encoded by HCV itself. Enomoto et al. [1996] proposed that variation of 40 amino acids within the NS5A region (aa 2,209–2,248), which is referred to as the IFN sensitivity-determining region (ISDR), is well correlated with IFN responsiveness. ISDR and its adjacent sequence bind and inhibit the enzymatic activity of a double-stranded RNA-activated protein kinase (PKR), which can have an antiviral effect, and therefore the combined region is referred to as the PKR-binding domain (PKR-BD) [Gale et al., 1997, 1998]. A correlation between sequence variation in the PKR-BD and IFN responsiveness has been reported [Nousbaum et al., 2000], and some reports show a correlation between IFN responsiveness and the sequence diversity of variable region 3 (V3) (aa 2,356–2,379) or surrounding regions near the carboxy terminus of NS5A [Murphy et al., 2002; Sarrazin et al., 2002; Puig-Basagoiti et al., 2005]. A high degree of amino acid substitution in the V3 and pre-V3 regions (aa 2,334–2,355) of NS5A, which is referred to as the IFN/RBV resistance-determining region (IRRDR) (aa 2,334–2,379), has been associated with SVR in Peg-IFN/RBV combination therapy for patients infected with HCV-1b [El-Shamy et al., 2007, 2008]. In addition to these findings in non-structural proteins of the virus, amino acid substitution in a structural region of HCV has been reported to be a predictive viral marker for the virological response to PegIFN/RBV therapy. Amino acid polymorphisms in the HCV core region (Arg70 vs. Gln70 and Leu91 vs. Met91) correlate with virological outcome and on-treatment viral kinetics in Peg-IFN/RBV therapy [Akuta et al., 2006, 2007], and a double wild-type HCV core (Arg70 and Leu91) may be a significant predictor of SVR in Peg-IFN/RBV therapy [Akuta et al., 2007].

Interactions between viral and host proteins in infected cells may influence therapeutic effects and the natural history of infection, since the HCV NS3 region has a significant effect on immunity. The amino-terminal part of this region encodes a serine protease, for which the minimum activity has been mapped to a region between aa 1,059 and 1,204 [Yamada et al., 1998]. The serine protease inactivates Cardif, a caspase recruitment domain (CARD)-containing adaptor protein that interacts with the RNA helicase retinoic acid inducible gene 1 (RIG-1)-dependent antiviral pathway in infected cells [Foy et al., 2003; Meylan et al., 2005; Evans and Seeger, 2006]. This action inhibits phosphorylation and subsequent heterodimerization of interferon regulatory factor-3 (IRF-3), which is essential for activation of IFN signaling through translocation of IRF-3 heterodimers into the nucleus, and eventually blocks IFN-beta production. In addition, inactivation of IRF-3 is postulated to influence the therapeutic effect of IFN-based antiviral therapy, because the IRF-3 heterodimer translocates into the nucleus to bind to the IFN-stimulated response element that produces many antiviral proteins, including 2′,5′-oligoadenylate synthetase and PKR [Nakaya et al., 2001; Grandvaux et al., 2002]. Collectively, these findings suggest that polymorphisms in HCV NS3 structure deduced from sequence variation may influence IFN-related signaling and the antiviral effect of IFN-based anti-HCV therapy.

We have focused on polymorphisms in the secondary structure of the viral polyprotein that interacts with host proteins involved in immunity, with the aim of identification of predictive viral markers for the response to Peg-IFN/RBV therapy. In this study, we examined the potential correlation between polymorphisms in the secondary structure of the HCV NS3 amino-terminal region and virological responses to Peg-IFN/RBV therapy in patients infected with HCV-1b with a high viral load.

PATIENTS AND METHODS

Patients and Treatment Regimen With Peg-IFN Plus Ribavirin

A total of 139 consecutive patients diagnosed with chronic hepatitis C were enrolled in the study from December 2004 to March 2007. These patients included 81 men and 58 women, and were aged from 31 to 75 years old (mean ± SD, 56.8 ± 8.7 years old). All patients were infected with HCV-1b with a high viral load of over 100 KIU/ml, and all received Peg-IFN/RBV therapy. Patients with alcoholic liver injury, autoimmune liver disease, and those who had symptoms of decompensated cirrhosis including ascites were excluded. Briefly, all patients were treated with a combination of Peg-IFN-alpha 2b (Pegintron®; Schering-Plough, Kenilworth, NJ) and RBV (Rebetol®; Schering-Plough) for 48 weeks. Peg-IFN was administered subcutaneously once a week and RBV was given orally twice a day for the total dose. The dosages were determined on the basis of body weight according to the Japanese standard prescription information supplied by the Japanese Ministry of Health, Labour and Welfare, and there was a limit for calculating the optimized dose: patients with body weights of 35–45, 46–60, 61–75, and 76–90 kg were given Peg-IFN at doses of 60, 80, 100, and 120 μg, respectively, and those with body weights of <60, 60–80, and >80 kg were given RBV at doses of 600, 800, and 1,000 mg, respectively. The dose of Peg-IFN or RBV was reduced according to the Japanese standard criteria based on the white blood cell count, neutrophil count, hemoglobin concentration and platelet count [Hiramatsu et al., 2008].

Virological Tests and Response to Peg-IFN Plus Ribavirin

Virological responses were evaluated at 12 weeks after the start of treatment with an early depletion of viremia referred to as an early virological response (EVR), at the end of treatment with depletion of viremia referred to as an end of treatment virological response (ETR), and at 24 weeks after completion of treatment,

with a clinical outcome of a sustained virological response (SVR) representing successful HCV eradication. All patients were negative for hepatitis B surface antigen. Quantification of serum HCV RNA was performed using an RT-PCR-based commercial kit (Amplicor HCV monitor test, ver. 2.0, Roche Diagnostics, Tokyo, Japan). This Amplicor HCV RNA assay has a lower limit of detection of 50 IU/ml. SVR was determined by monitering negativity for HCV RNA monthly for 6 months. The real-time PCR assay kit (COBAS TaqMan HCV Auto, Roche Diagnostics) for more precise quantitation of HCV viremia has recently become available and pre-treatment viral titers were re-evaluated using preserved serum samples. This real-time PCR assay has a lower limit of detection of 15 IU/ml. The study protocol was approved by the Ethics Committee of Yamagata University Hospital. Informed consent was obtained from all patients.

PCR Amplification of the Amino-Terminal Region of NS3

RNA was extracted from 50 μl of serum using an RNeasy Mini kit (Qiagen, Tokyo, Japan). To amplify the region of the HCV genome encoding the amino-terminal region of NS3 (1,027–1,206), a one-step PCR was performed in a tube using the Superscript One-Step RT-PCR kit with Platinum Taq (Gibco-BRL, Tokyo, Japan) and an outer set of primers: NS3-F1 (sense primer; 5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3,295–3,316) and NS3-AS2 (antisense primer; 5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4,040–4,019), as reported previously [Ogata et al., 2002a, 2003]. PCR was initially performed at 45°C for 30 min at RT and then at 94°C for 2 min, followed by the first-round PCR for forty 3-min cycles at 94°, 55°, and 72°C for 1 min each. The second-round PCR was performed with *Pfu* DNA polymerase (Promega, Tokyo, Japan) and an inner set of primers: NS3-F3 (sense primer; 5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3,390–3,407) and NS3-AS1 (antisense primer; 5′-GCC ACT TGG AAT GTT TGC GGT A-3′; nucleotides 4,006–3,985). The second-round PCR was performed for 35 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C. This method allowed amplification of the corresponding portion of the HCV genome from HCV-1b RNA-positive samples. The amplified fragments were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced (without being subcloned) in both directions using a dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 sequencer (Applied Biosystems, Tokyo, Japan).

Classification of the Secondary Structure of the HCV-1b NS3 Amino-Terminal Region

The secondary structure of the amino-terminal region of HCV NS3 was predicted by computer-assisted Robson analysis [Garnier et al., 1978] with Genetyx-Mac software (ver.10.1; Software Development Co., Tokyo, Japan). Previously, the full-length secondary structure of the HCV-1b NS3 region was analyzed, and this showed that the secondary structure deduced from the carboxy-terminal 60 residues was well conserved in terms of linear structure, without any turn structure [Ogata et al., 2002a]. We have shown that the secondary structure of the 120 residues in the amino-terminal region of HCV-1b NS3 can be classified into two major groups: A and B (Fig. 1) [Ogata et al., 2002a, 2003]. Briefly, the criteria for this classification are as follows: in group A isolates, the carboxy-terminal 20 residues (aa 1,125–1,146) are oriented leftward relative to a domain composed of the remaining amino-terminal region; whereas in group B isolates, the same 20 residues are oriented rightward relative to the rest of the amino-terminal domain.

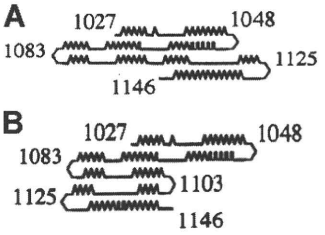

Fig. 1. Secondary structure of the 120 amino-terminal residues of HCV-1b nonstructural 3 (NS3) region classified into two major groups: A and B. The looped, zigzag, straight, and bent lines represent α-helix, β-sheet, coil, and turn structures, respectively. The numbers indicate amino acid positions. **A**: Group A, (**B**) Group B.

Analysis of Amino Acid Substitutions in the Core Region

To amplify a region of the HCV genome encoding the core region including positions 70 and 91, reverse transcription and the first-round PCR were performed in a tube by the Superscript One-Step RT-PCR kit with Platinum *Taq* (Gibco-BRL) and an outer set of primers, followed by second-round PCR with an inner set of primers in accordance with procedures reported previously [Ogata et al., 2002b]. The sequences of the amplified fragments were determined by direct sequencing.

Statistical Analysis

Data were analyzed by a χ^2 test for independence with a two-by-two contingency table and a Student *t*-test. A *P*-value <0.05 was considered significant.

RESULTS

Virological Response *and Adherence* to the Peg-IFN Plus Ribavirin Regimen

Rates of virological responses in patients treated with PegIFN/RBV combination therapy for 48 weeks are shown in Figure 2. Of the 139 patients enrolled in the study, SVR, non-SVR and cessation of therapy occurred in 58 (42%), 62 (45%), and 19 (14%), respectively. Serious

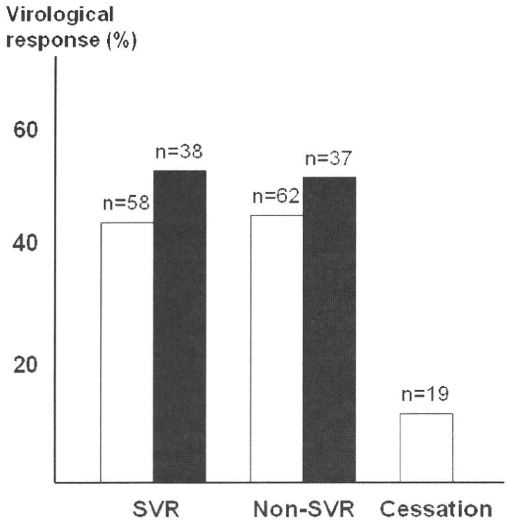

Fig. 2. Virological response in patients treated with peginterferon plus ribavirin for 48 weeks. The results are shown for all 139 subjects (open bars) and for 75 cases with good adherence of >80% of the scheduled dosages (closed bars). SVR, sustained virological response.

adverse events that necessitated discontinuation of this therapy were depression in one patient, thyroid function disorder in 2, general itching in 2, infection in 2, anorexia in 2, occurrence of hepatocellular carcinoma in 2, and a decreased neutrophil count in 2. Six patients also terminated this therapy at their own request. Of the 139 patients, 75 (54%) received >80% of the scheduled dosage of Peg-IFN and RBV designated before treatment, and of these 75 cases SVR and non-SVR occurred in 38 (51%) and 37 (49%), respectively.

Prevalence of Types of Secondary Structure of the Amino-Terminal Region of HCV NS3

The prevalence of the types of secondary structure of HCV NS3 in the 139 subjects is shown in Table I. Among these subjects, 43 (31%), 70 (50%), and 26 (19%) were classified into groups A, B, and others, including 3 of mixed type (A plus B) and 23 of non-A, non-B type. Of the 75 cases with good adherence to administration of >80% of the scheduled dosage, 28 (37%), 40 (53%) and 7 (9%) were classified into groups A, B, and others. The amino acid data of group A and B in the cases with good adherence to administration are available in the DDBJ/EMBL/GenBank databases with the accession numbers AB548070–AB548137. Our analysis revealed no specific correlations between amino acid sequences and the secondary structure deduced by the Robson method, as we have reported previously [Ogata et al., 2003].

TABLE I. Prevalence of the HCV NS3 Secondary Structure Type

	Group A (%)	Group B (%)	Others (%)
Enrolled cases (n = 139)	43 (31)	70 (50)	26 (19)
Adherent cases (n = 75)	28 (37)	40 (53)	7 (9)

Characteristics of Adherent Patients Based on Different HCV NS3 Structure Types

The virological responses to Peg-IFN/RBV combination therapy for patients infected with group A and B isolates were assessed in the 68 subjects with good adherence to the scheduled dosage of Peg-IFN and RBV. The characteristics of patients infected with group A and B isolates are shown in Table II. Age, gender, pre-treatment level of serum HCV RNA and ALT, and frequency of fibrosis stage did not differ significantly between the two groups. Peg-IFN/RBV combination therapy was completed in all the patients, and the total administered dosages of Peg-IFN and RBV was >95% of the scheduled dosage in both groups.

Relationship Between Virological Responses and Polymorphisms in the HCV NS3 Amino-Terminal Region

In the 68 patients who received >95% of the scheduled doses of Peg-IFN and RBV for 48 weeks, SVR and non-SVR occurred in 33 (49%) and 35 (51%), respectively. The EVR, ETR, and SVR rates in patients infected with group A and B isolates are shown in Table III. There was a significant difference in the rates of EVR between subjects infected with group A and B isolates: EVR was achieved in 19 of 28 (68%) patients with group A infection, compared to 17 of 40 (43%) with group B infection ($P < 0.05$). The final outcome also differed significantly between subjects infected with group A and B isolates: SVR was achieved in 18 of 28 (64%) patients with group A infection, compared to 15 of 40 (38%) with group B infection ($P < 0.05$).

Polymorphisms in Core Amino Acids 70/91 and in the HCV NS3 Secondary Structure

The wild-type core sequence (Arg70, Leu91) has been associated with SVR in Peg-IFN/RBV combination therapy, while the non-double wild-type containing one or two substitutions at positions 70 and/or 91 was associated with non-SVR [Akuta et al., 2007]. Therefore, we examined substitutions at positions 70 and 91 in the HCV core region in pre-treatment serum samples of 44 cases that were available for testing. The double wild-type 70/91 sequence was found in 22 of the 44 cases (50%), of which 12 were SVR and 10 were non-SVR. Combination analysis of polymorphisms of the HCV core 70/91 positions and the NS3 amino-terminal region showed that 10 (83%) of the 12 SVR cases and only 3 (30%) of the 10 non-SVR cases with the double wild-type core had a group A polymorphism in HCV NS3 (Table IV). Thus, combination analysis of the core and NS3 regions may improve prediction of the outcome of Peg-IFN/RBV therapy.

TABLE II. Characteristics of Adherent Patients Infected With HCV Group A and B Isolates

	Group A (n = 28)	Group B (n = 40)	*P*
Age (years)	55.5 ± 9.5	55.5 ± 8.9	NS[a]
Sex (men/women)	18/10	21/19	NS[b]
Pre-treatment HCV RNA (KIU/ml)	1,635 ± 930	2,087 ± 1,422	NS[a]
Alanine aminotransferase level (U/L)	80 ± 62	71 ± 47	NS[a]
Stage of liver fibrosis			
F1 or F2/F3 or F4	19/9	28/12	NS[b]
Drug adherence dosage (%)			
Pegylated interferon	97.7 ± 5.2	95.2 ± 7.3	NS[a]
Ribavirin	96.8 ± 6.4	95.3 ± 7.7	NS[a]

NS, not significant.
[a]*t*-test.
[b]χ^2 test.

Re-Evaluation of Pre-Treatment HCV Viremia Status Using Real-Time PCR

Since the viral titer before treatment is a major predictive marker of the outcome of Peg-IFN/RBV therapy, we re-evaluated the pre-treatment viral titers more precisely using preserved serum samples taken within 1 month before treatment, using a real-time PCR assay. The pre-treatment viral titers did not differ significantly between sera with group A and B isolates (5.98 ± 0.94 vs. 6.25 ± 0.62 logIU/ml) (Table V). The secondary structure polymorphisms of HCV NS3 were independent of the pre-treatment viral titers.

DISCUSSION

Antiviral therapy with Peg-IFN/RBV for 48 weeks fails to eradicate HCV in about half of patients infected with a high titer of HCV genotype 1b, and the severe adverse events and high costs associated with this therapy require outcome prediction to allow targeted treatment for chronic hepatitis C. The pre-treatment viral titer, viral factors that influence the virological response to IFN-based anti-HCV therapy have been widely investigated. Viral kinetics showing prompt seronegativity after the start of treatment is a critical factor for achieving SVR, and thus the possible correlation between an early virological response and genetic sequence variation of the HCV has been studied. In particular, amino acid substitutions in the HCV core region at positions 70 and 91 or multiple mutations detected in the IRRDR of the HCV NS5A region are useful markers for predicting EVR and subsequent SVR.

TABLE III. Virological Responses in Subjects With Different Polymorphisms in the Secondary Structure of HCV NS3

	EVR*	ETR**	SVR*
Group A (n = 28)	19 (68%)	23 (82%)	18 (64%)
Group B (n = 40)	17 (43%)	25 (63%)	15 (38%)

EVR: early virological response at 12 weeks after the start of treatment.
ETR: virological response at the end of treatment.
SVR: sustained virological response 24 weeks after completion of treatment.
*$P < 0.05$.
**$P = 0.08$; χ^2 test.

To date, the influence of several single amino acid substitutions and accumulation of these changes in the viral genome on the effect of IFN-based anti-HCV therapy has been examined. Since interactions between host and viral proteins in infected cells may influence the therapeutic effect of an antiviral agent, we focused on the association of structural polymorphism of a viral protein with the effect of Peg-IFN/RBV combination therapy in this study. Our results suggest that polymorphism analysis of secondary structure deduced from sequence variations in the HCV NS3 amino-terminal region can be used to predict viral responses to this therapy.

Amino acid sequences of the HCV NS3 amino-terminal region, which encodes a serine protease, vary greatly among HCV isolates. Interactions between HCV NS3 and host proteins may influence both oncogenesis and immunity, and thus elucidation of the biological significance of these interactions could result in a new prognostic marker for HCC or a predictive marker for anti-HCV therapy. First, HCV NS3 interacts with the p53 tumor suppressor to suppress p53-dependent apoptosis or p21 transcriptional activity [Ishido and Hotta, 1998; Kwun et al., 2001; Deng et al., 2006]. Transfection of a plasmid expressing the amino-terminal portion of HCV NS3 induces cell transformation in vitro, and transplanted cells proliferate with sarcoma-like features in vivo [Sakamuro et al., 1995]. These findings suggest that NS3 may be involved in the oncogenic pathway in HCV infection. We have shown that the secondary structure of the 120-residue amino-terminal region of NS3 (1,027–1,146) is classifiable into two major groups: A and B. This region encodes a serine protease and also includes p53-binding sites. Our

TABLE IV. Treatment Outcome of Cases With a Double Wild-Type Core Region and Different HCV NS3 Structural Polymorphism

	Group A (%)	Group B (%)	*P*
SVR (n = 12)	10 (83)	2 (17)	0.02[a]
Non-SVR (n = 10)	3 (30)	7 (70)	

SVR, sustained virological response.
[a]χ^2 test.

TABLE V. Pre-Treatment HCV RNA Levels Measured by Real-Time PCR for Subjects With Different HCV NS3 Structural Polymorphism

	Group A	Group B	*P*
SVR (n = 33)	5.78 ± 1.05	6.I3 ± 0.71	NS[a]
Non-SVR (n = 35)	6.33 ± 0.59	6.32 ± 0.55	NS[a]
Total (n = 68)	5.98 ± 0.94	6.25 ± 0.62	NS[a]

SVR, sustained virological response. NS, not significant.
[a]*t* test.

previous cross-sectional studies revealed that the prevalence of group B infection is significantly higher in HCC cases than in non-HCC cases [Ogata et al., 2003], and that the group B infection is an independent risk factor for development of HCC in patients with chronic HCV infection [Nishise et al., 2007]. Second, NS3 interacts with host proteins associated with IFN signaling and thus influences cellular immunity. Since the serine protease encoded by the amino-terminal region of NS3 inhibits the IFN-signaling pathway, polymorphism of this region is likely to influence the effect of Peg-IFN/RBV combination therapy.

Several factors associated with the virological response to this therapy are well known, with adherence to both IFN and RBV strongly influencing outcome [Pearlman, 2004; Arase et al., 2005; Yamada et al., 2008]. In this study, we analyzed 75 cases in which >80% of the scheduled dosage of both drugs was administered. Of these cases, 28 (37%) and 40 (53%) were infected with group A and B isolates, respectively, which were similar rates to those for the 139 cases in the overall study. Age, gender, viral load before treatment, ALT level, proportion of fibrosis stage and adherence to Peg-IFN and RBV did not differ between the group A and B cases. However, the frequencies of SVR and EVR were significantly higher in group A, and those for non-EVR and non-SVR were significantly higher in group B. The results suggest that infection with the group B isolate, which correlates with a higher rate of HCC, is resistant to Peg-IFN/RBV therapy. The pre-treatment viremia status in the 68 cases with group A or B isolates showed no significant differences between the two groups of patients. Therefore, these results suggest that the secondary structure of the HCV NS3 amino-terminal region may be useful for prediction of the outcome of Peg-IFN/RBV combination therapy. In this initial study setting, the relationship of these polymorphisms to the frequency of rapid viral response at 4 weeks after the start of treatment was not evaluated. It will be important to assess this relationship in a future study.

The polymorphism in HCV core region (Arg70/Leu91) is a useful predictive marker for virological responses in Peg-IFN/RBV therapy [Akuta et al., 2007]. Interestingly, a combined analysis of polymorphisms of the core region (which encodes a structural protein) and HCV NS3 (a nonstructural protein) improved the prediction rate. Therefore, analysis of NS3 polymorphism in combination with the core structural polymorphism appears to improve prediction of the outcome of Peg-IFN/RBV therapy. A larger, multi-center prospective study would be necessary to validate the present results. In conclusion, the results of this study suggest that secondary structure polymorphism in the amino-terminal region of HCV NS3 is a useful predictive marker of the effect of Peg-IFN/RBV combination therapy for chronic hepatitis C. Although the present findings are clinically important, and will be helpful for predicting the outcome of Peg-IFN/RBV therapy, further in vitro studies will be needed to elucidate the molecular mechanism underlying the association of HCV NS3polymorphisms with clinical outcome.

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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

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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

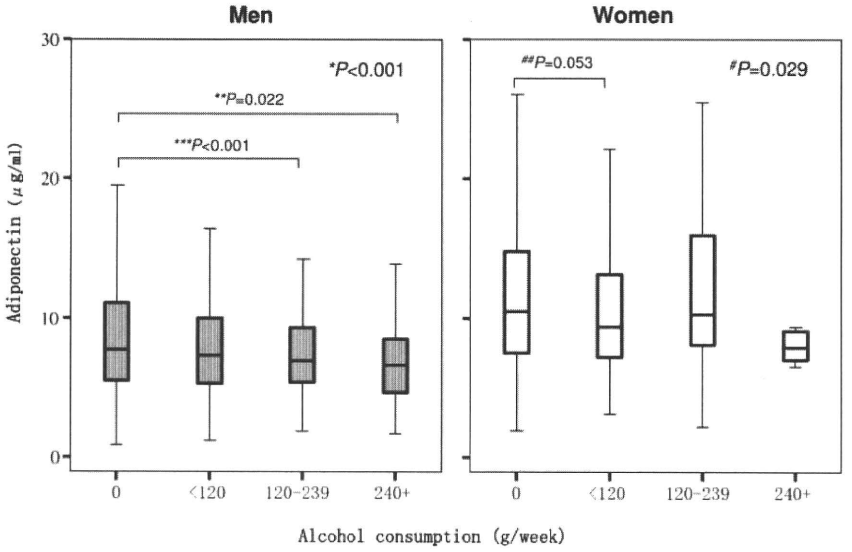

FIG. 1. Box plots illustrating serum plasma adiponectin concentrations for each level of alcohol consumption by gender. *Horizontal lines inside each box* represent medians, and the *top and bottom of the boxes* are the 25th and 75th quartiles, respectively. The *error bars* indicate 95% confidence intervals. *, $P < 0.001$ 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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Therapeutic Apheresis and Dialysis

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

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

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

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

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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.

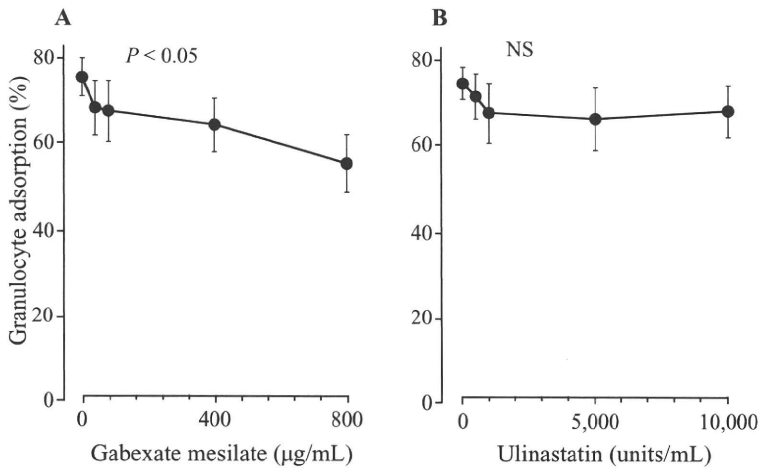

FIG. 1. Effect of ulinastatin on granulocyte adsorption to cellulose acetate beads. Peripheral blood from healthy volunteers was mixed with serial dilutions of either (A) gabexate mesilate or (B) ulinastatin. Test samples were then incubated with cellulose acetate beads for 1 h, and fractions of adsorbed 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

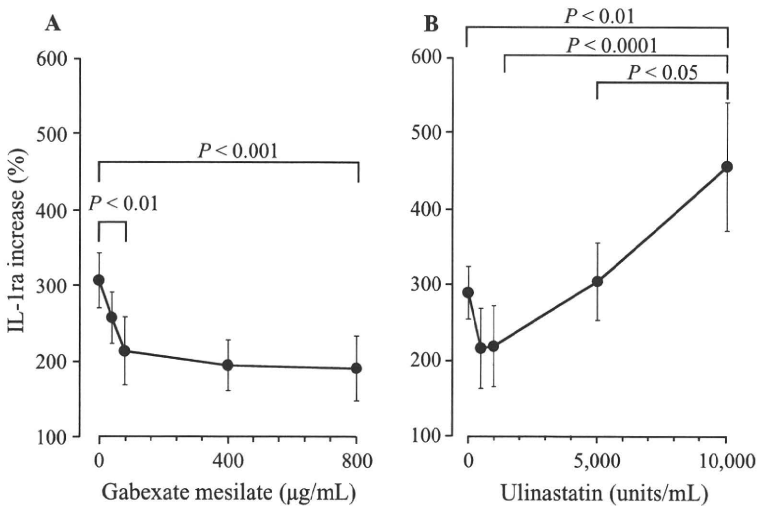

FIG. 2. Effect of ulinastatin on interleukin-1 receptor antagonist (IL-1ra) release in granulocyte and monocyte adsorption. Peripheral blood from healthy volunteers was mixed with serial dilutions of either (A) gabexate mesilate or (B) ulinastatin and incubated with cellulose acetate beads for 1 h. After 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).