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RAPID COMMUNICATION

Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: A project of the Kyushu University Liver Disease Study Group

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Abstract

AIM: To determine the efficacy of an interferon alpha and ribavirin combination treatment for Japanese patients infected with hepatitis C virus (HCV) of genotype 2, a multi-center study was retrospectively analyzed.

METHODS: In total, 173 patients with HCV genotype 2 started to receive interferon-alpha subcutaneously thrice a week and 600–800 mg of ribavirin daily for 24 wk.

RESULTS: The overall sustained virological response (SVR), defined as undetectable HCV RNA in serum, 24 wk after the end of treatment, was remarkably high by 84.4%, (146/173) by an intention-to-treat analysis. A significant difference in SVR was found between patients with and without the discontinuation of ribavirin (46.9% *vs* 92.9%), but no difference was found between those with and without a dose reduction of ribavirin. A significant difference in SVR was also found between patients with less than 16 wk and patients with 16 or more weeks of ribavirin treatment (34.8% *vs* 92.0%).

CONCLUSION: The 24-wk interferon and ribavirin treatment is highly effective for Japanese patients with HCV genotype 2. The significant predictor of SVR is continuation of the ribavirin treatment for up to 16 weeks.

Key words: Hepatitis C virus; Interferon; Ribavirin; Genotype 2

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INTRODUCTION

The heterogeneity of the hepatitis C virus (HCV) genome has warranted the classification of the virus into different genotypes, with six major genotypes and more than 50 subtypes of HCV having been described till date[1-3]. The different genotypes may be important to the pathogenesis of the disease[4], response to antiviral therapy[5], and the diagnosis[6], as shown by molecular epidemiological studies and research on vaccine development.

A currently popular treatment regimen for the treat-

ment of chronic HCV infection in the world is pegylated interferon (IFN) alpha in combination with ribavirin. However, there was no data of response to such combination treatment for Japanese patients, because the treatment was just approved by the Japanese Minister of Health, Labour and Welfare in December 2004. Treatment with these drugs has resulted in a high rate of sustained virological response (SVR), over 50%[7,8]; however, the treatment duration is long, 48 wk and it causes various side effects, which are sometimes serious. Such a combination treatment is also expensive; a 24-wk treatment course costs approximately $20 000[9]. The efficacy and economic aspects need to be analyzed. Quite recently, a very short duration treatment for acute hepatitis C was shown to be highly effective[10].

The HCV genotype has been reported to be the most important predictor of IFN treatment response[7-13]. Patients infected with genotypes 2 and 3 have achieved about 65% SVR in a trial of 24-wk IFN alpha in combination with ribavirin, in contrast to patients with genotype 1 who had under 30% SVR[14,15]. Recently, multicenter studies in Europe and North America showed that patients with genotypes 2 and 3 were able to achieve a high SVR in a trial of 14-16 wk of pegylated IFN alpha in combination with ribavirin[16,17]. However, their analysis included very few genotype 2 patients: one included 23 genotype 2 patients and the other had 43 patients.

The distribution of HCV genotypes in Japan includes about 70% genotype 1b, with the remaining 30% genotypes 2a and 2b[18]. The SVRs to treatment of even shorter duration have not yet been reported for Japanese patients. Data are needed to define whether or not the duration of treatment with IFN alpha in combination with ribavirin can be reduced from 24 wk without compromising antiviral efficacy in patients chronically infected with HCV of genotype 2. This investigation has assessed the efficacy of a 24-wk combination treatment of IFN alpha and ribavirin for Japanese patients with HCV genotype 2 infection and focussed on the issue of the relationship between the duration of treatment and the efficacy.

MATERIALS AND METHODS

Patients

A retrospective study was done on Japanese patients treated between December 2000 and March 2004 that included 173 patients, 20 years or older, who satisfied the following criteria: (1) chronically infected with HCV genotype 2a or 2b; and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Criteria for exclusion were: (1) clinical or biochemical evidence of hepatic decompensation; (2) hemoglobin level less than 115 g/L, white blood cell count less than 3×10^9/L, and platelet count less than 50×10^9/L; (3) concomitant liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive); (4) alcohol or drug abuse; (5) suspected hepatocellular carcinoma; (6) severe psychiatric disease; and (7) treatment with antiviral or immunosuppressive agents prior to enrolment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 32 affiliated hospitals in the northern Kyushu area of Japan.

Informed consent was obtained from all the patients before enrollment in this study. The study was approved by the institutional Ethics Committees of the hospitals involved and conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

Study design

All patients were treated with 6-10 MU of IFN alpha-2b (Intron A; Schering-Plough, Osaka, Japan) subcutaneously daily for the first 2 wk, then thrice a week for 22 wk. Ribavirin (Rebetol; Schering-Plough) was administrated orally for 24 wk at a daily dose of 600-800 mg based on the body weight (600 mg for patients weighing less than 60 kg and 800 mg for those weighing 60 kg or more). The above duration and dose were approved by the Japanese Minister of Health, Labour and Welfare. The 48-wk combination treatment and the ribavirin dosage of 1 000-1 200 mg recommended by the international guidelines were not permitted under the rules of the Japanese national health insurance system during the period of this study. The dose of ribavirin was reduced by 200 mg if the hemoglobin level fell to 100 g/L. Patients were considered to have ribavirin-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of ribavirin was required. Both IFN alpha-2b and ribavirin were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, and 2.5×10^9/L, respectively. The treatment was also discontinued if severe malaise developed, the continuation of treatment was judged not to be possible by the attending physician, or the patient desired to discontinue treatment.

Grouping by continuation or discontinuation of treatment

Patients were divided into the following four categories: Group A, patients who well tolerated the 24-wk combination treatment with IFN and ribavirin without a reduction in the dose of either drug; Group B, patients who received the full 24-wk combination treatment but who needed a reduction of the dose of IFN or ribavirin, or both; Group C, patients who discontinued the ribavirin treatment but continued the 24-wk IFN treatment; and Group D, patients who did not complete the 24 wk of treatment, because of adverse effects or who dropped out.

Determination of HCV RNA and HCV genotype and serotype

The serum HCV RNA level was examined with an Amplicor HCV monitor assay (version 2.0) (Roche, Tokyo, Japan), with a lower limit of quantitation of 500 IU (135 copies/mL) and an outer limit of quantitation of 850 000 IU/mL. Samples with HCV RNA over the limit of 850 000 IU/L were not diluted to determine the levels between 850 000-5 000 000 IU/mL. HCV RNA was also examined with the qualitative Amplicor HCV assay (Roche). HCV genotype was determined by type-specific primer from the core region of the HCV genome. The protocol for genotyping was carried out as described earlier[11,12].

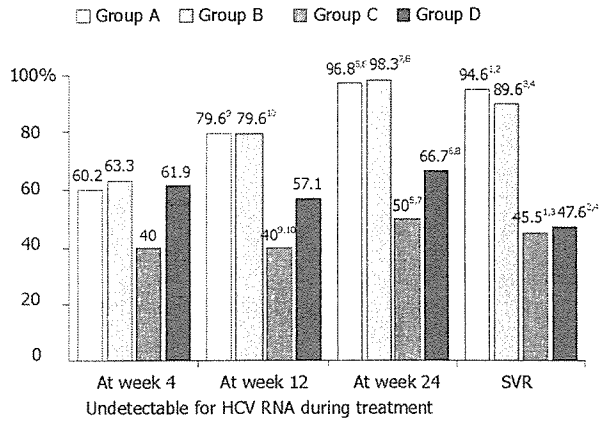

Figure 1 The sustained virological response (SVR) rate and undetectable hepatitis C virus (HCV) RNA rates during the treatment of 173 patients, classified by continuation and discontinuation of interferon and ribavirin combination treatment. Group A patients (n=93) who well tolerated the 24-week treatment with IFN and ribavirin in combination without any reduction in the dose of either drug; Group B patients (n=48) received the 24-week combination treatment, but needed a dose reduction of IFN or ribavirin, or both; Group C patients (n=11) discontinued the ribavirin treatment, but continued the full 24 weeks of IFN treatment; Group D patients (n=21) did not complete the 24 weeks of treatment because of adverse effects (n=17) or dropped out (n=4). [1]P=0.0001; [2]P<0.0001; [3]P=0.0031; [4]P=0.0003; [5]P=0.0001; [6]P=0.0002; [7]P=0.0003; [8]P=0.0002; [9]P=0.124; [10]P=0.0182.

Histological examination

Liver biopsy was done for 117 patients infected with genotype 2 within the 6 months before the start of the treatment. For each specimen, a stage of fibrosis and a grade of activity were established according to the following criteria. Fibrosis was staged on a scale of 0-4: F0=no fibrosis, F1=portal fibrosis without septa, F2=few septa, F3=numerous septa without cirrhosis, F4=cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0=no histological activity, A1=mild activity, A2=moderate activity, A3=severe activity. Liver biopsy was not available from 56 patients who declined to have a biopsy.

Efficacy of treatment

The SVR was defined as undetectable HCV RNA by the qualitative Amplicor HCV assay (Roche) and a normal ALT level (under 40 IU/L) at 6 months after the end or stoppage of the treatment. Patients not achieving a SVR were considered as non-SVR. Patients who had undetectable HCV RNA within 4 wk of the start of treatment were considered to have had an early virological response (EVR).

Statistical analysis

The analysis of SVR was done on an intention-to-treatment basis, including dropouts, who were counted as non-sustained virological responders, and patients who stopped treatment. The χ^2 test or Fisher's exact test was used to examine the association between baseline characteristics and SVR. The Mann-Whitney U test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. Independent factors associated with SVR were studied using forward stepwise logistic regression analysis of the variables. Forward stepwise logistic regression analysis was done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. A P-value of less than 0.05 was considered significant. All P-values were two tailed.

RESULTS

Patient characteristics, dose reduction and discontinuation of treatment regimen

The distribution of Groups A, B, C, and D patients was 93 (53.8%), 48 (27.7%), 11 (6.4%), and 21 (12.1%), respectively. Completing the 24-week ribavirin treatment were 141 patients in Groups A and B. Thirty-two patients of Groups C and D discontinued the ribavirin treatment.

The pretreatment characteristics of these four groups of patients are summarized in Table 1. The median age was significantly younger in Group A (51 years) than in Groups B (56 years) and C (59 years). Significantly more men were in Group A (71.0%) than in Group B (33.3%). The median creatinine clearance was significantly higher in Group A (110 mL/min) than Groups B (92 mL/min) and C (85 mL/min). The median hemoglobin level was significantly higher in Group A (150 g/L) than Groups B (136 g/L), C (134 g/L), and D (134 g/L). The median platelet count was significantly higher in Group A (168 $\times 10^9$/L) than in Group C (127$\times 10^9$/L). No notable differences between the groups were found in body weight, ribavirin dose, HCV RNA level, genotype, or histology.

Virological response

SVR was achieved by 146 (84.4%) of 173 patients. The SVR did not differ between patients with genotypes 2a and 2b (83.1% *vs* 84.6%). The SVRs were 82.4% (14 of 17) (under 100 kIU/mL), 84.2% (16 of 19) (100-199 kIU/mL), 85.7% (24 of 28) (200-299 kIU/mL), 83.3% (15 of 18) (300-399 kIU/mL), 100% (12 of 12) (400-499 kIU/mL), 76.9% (10 of 13) (500-599 kIU/mL), 77.8% (7 of 9) (600-699 kIU/mL), 90.9% (10 of 11) (700-799 kIU/mL), and 82.6% (38 of 46) (800 and over kIU/mL). The SVRs were 76.9-100%. The SVRs of the HCV genotype 2 patients with any level of viremia level did not significantly differ.

Figure 1 shows the SVR and undetectable HCV viremia rate during the treatment of 173 patients, classified by continuation and discontinuation of combination treatment. The SVRs were significantly higher in Groups A (94.6%) and B (89.6%) than in Groups C (45.5%) and D (47.6%). A significant difference of SVR was found between patients with and without discontinuation of ribavirin (46.9%, 15 of 32 of Groups C and D patients *vs* 92.9%, 131 of 141 of Groups A and B patients, P<0.0001). During the treatment period, except for at week 4, the rates of undetectable HCV RNA were also significantly higher in Groups A and B than in Groups C and D.

Figure 2 shows the relationship between SVR and the ribavirin treatment period in all the patients. A significant difference was found between patients with less than 16 wk of treatment period and patients with longer periods

Table 1A Baseline characteristics

Characteristic	Complete Ribavirin treatment (n = 141)		Discontinued Ribavirin treatment (n = 32)		All patients (n = 173)
	Group A (n = 93)	Group B (n = 48)	Group C (n = 11)	Group D (n = 21)	
Median age (yr)	51[1,2]	56[1]	59[2]	50	53
(range)	(20-73)	(25-70)	(53-73)	(29-73)	(20-73)
Male (%)	66 (71.0)[3]	16 (33.3)[3]	5(50.0)	13 (61.9)	100 (57.8)
Body weight					
60 kg or more (%)	60 (64.5)	23 (47.9)	6 (54.5)	12 (57.1)	101 (58.3)
Ribavirin dose by weight					
12 mg/kg or more (%)	24 (25.8)	20 (41.7)	4 (36.4)	9 (42.8)	57 (32.9)
Creatinine clearance (mL/min)	110[4,5]	92[4]	85[5]	101	102
(range)	(53-261)	(46-167)	(60-111)	(41-203)	(41-261)
HCV RNA level					
500 kIU/mL or more (%)	44 (47.3)	22 (45.8)	4 (36.4)	9 (42.8)	79 (45.7)
Genotype 2a (%)	67 (72.0)	28 (58.3)	6 (54.5)	13 (61.9)	114 (65.9)

[1]P=0.0401; [2]P=0.0044; [3]P<0.0001; [4]P=0.0002; [5]P=0.0248

Table 1B Baseline characteristics (continued)

Characteristic	Complete Ribavirin treatment (n = 141)		Discontinued Ribavirin treatment (n = 32)		All patients (n = 173)
	Group A (n = 93)	Group B (n = 48)	Group C (n = 11)	Group D (n = 21)	
Histology					
Stage of fibrosis					
F0 - F1 (%)	27 (43.5)	17 (50.0)	4 (50.0)	9 (42.9)	56 (47.9)
F2 - F3 (%)	35 (56.5)	15 (44.1)	4 (50.0)	6 (28.6)	59 (50.4)
F4 (%)	0 -	2 (5.9)	0 -	0 -	2 (1.7)
Not determined	31	14	3	6	54
Grade of activity					
A0 - A1 (%)	27 (43.5)	17 (50.0)	4 (50.0)	9 (42.9)	43 (47.9)
A2 (%)	35 (56.5)	15 (44.1)	4 (50.0)	6 (28.6)	58 (50.4)
A3 (%)	0 -	2 (5.9)	0 -	0 -	16 (1.7)
Not determined	31	14	3	6	54
Median hemoglobin (g/L)	150[6,7,8]	136[6]	134[7]	134[8]	144
(range)	(117-171)	(116-163)	(121-152)	(12.1-153)	(116-171)
Median platelet count ($\times 10^9$/L)	168[9]	167	127[9]	157	162
(range)	(79-385)	(58-363)	(55-181)	(57-240)	(55-385)

[6]P=0.0003; [7]P=0.0063; [8]P=0.0225; [9]P=0.0120

(34.8%, 8 of 23 *vs* 92.0%, 138 of 150, P<0.0001), showing that 16 wk of ribavirin treatment significantly contributed to a SVR. Of the 173 studied patients, 104 (60.1%) had an EVR, defined as undetectable HCV RNA within 4 wk of the start of treatment. The SVR was 94 (90.4%) of these 104 patients with EVR, which was significantly higher than the non-EVR patients (52 of 69, 75.4%) (P=0.0142). No significant differences were found between patients with and without undetectable HCV RNA at 8 or 12 wk of the start of treatment. Moreover, we analyzed the relationship between SVR and the length of ribavirin treatment in the 104 patients with EVR. A significant difference was found between patients with less than 16 wk of ribavirin treatment and those with a longer treatment period (46.2%, 6 of 13 *vs* 96.7%, 88 of 91, P<0.0001). These findings showed that 16 wk of ribavirin treatment significantly contributed to a SVR, even in patients with EVR.

Factors contributing to SVR

To assess the independent role of the IFN and ribavirin combination treatment on SVR, an adjustment by forward stepwise logistic regression analysis for all other independent risk factors identified was done. The continuation of ribavirin treatment (P<0.0001) was significantly associated with SVR in analysis of all the patients. A higher SVR (odds ratio = 13.15) was found for patients who continued to receive ribavirin treatment than for those who discontinued it. Other factors such as sex, age, HCV genotype, pretreatment-HCV RNA level, histological findings, pretreatment platelet count and creatinine clearance, history

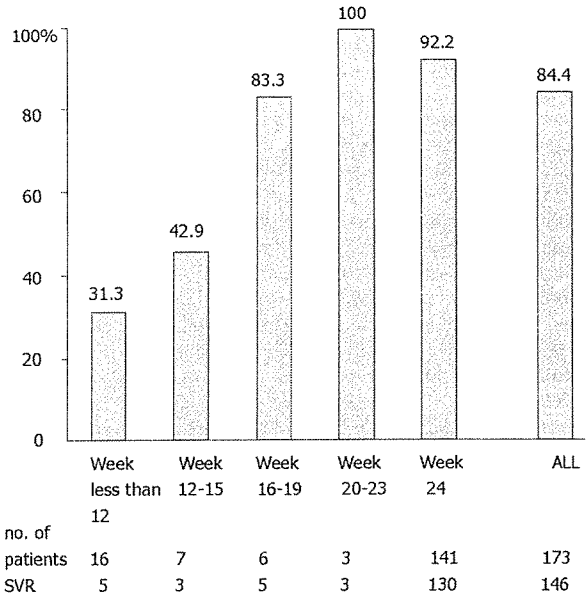

Figure 2 Relationship between the sustained virological response rates and the length of ribavirin treatment period of the 173 studied patients.

of prior IFN, and dose reduction of IFN or ribavirin were not significantly, independently associated with a SVR.

DISCUSSION

The large number of Japanese HCV genotype 2 patients enrolled in this study was sufficient to provide for meaningful statistical analysis, even though it was retrospective. This study demonstrated that a 24-wk IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR (84.4%) in genotype 2 patients, as expected. Importantly, we also showed that dose reductions of ribavirin were not associated with a poor outcome in these patients, only ribavirin discontinuation, and that the addition of ribavirin for up to 16 wk contributed to the high SVR.

In December 2004, pegylated IFN plus ribavirin combination treatment received the official approval in Japan. The combination treatment was not yet approved for clinical use for patients with chronic HCV viremia by the Japanese Ministry of Health, Labour and Welfare at the time of the present study. So far, our most effective and available treatment is the 6-month IFN-alpha plus ribavirin combination.

Remarkably high SVRs were observed for our patients with genotype 2 who took the IFN and ribavirin combination treatment. IFN monotherapy does not result in a satisfactory outcome for patients with chronic hepatitis C, particularly those with genotype 1, which is known to be IFN-resistant, whereas genotype 2 is IFN-sensitive[11-13]. The addition of ribavirin, a synthetic purine nucleoside analog, to IFN enhances the virological response[8,9,13-17]. Our research group, KULDS, also analyzed the data of patients with genotype 1 who were treated with this 24-wk combination treatment: SVR was achieved by 21% of 528 patients with genotype 1 by intention-to-treat analysis (data not published). Differences between genotype 1 and 2 patients still existed following the ribavirin combination treatment. Moreover, a striking finding in our study was that there were no differences among the patients with genotype 2 of any HCV RNA level (76.9-100%). The precise mechanism is unclear, although it possibly originates in different nucleotide sequence of their genome. Further study is needed to clarify the reasons for the differences in antiviral effect, by the use of novel and new tools for the quantification of the HCV replication system[19,20].

How long the ribavirin needs to be administrated to achieve the best efficacy with IFN alpha-treated patients of genotype 2 is unclear. In the present study, SVR after 16 or more weeks of treatment ranged from 83.3% to 100% and was not dependent on the dose reduction of ribavirin treatment but on the discontinuation of IFN or ribavirin treatment. A pilot study from Norway showed that patients with genotype 2 and an EVR obtained a high SVR after 14 weeks of pegylated IFN and ribavirin combination treatment[21]. The Zeuzem group also demonstrated a very high SVR in a 24-week pegylated IFN and ribavirin treatment for genotype 2 patients, and 16-week treatment duration was observed to be a significant independent predictor[17]. In view of the adverse effects, high cost of ribavirin, and the above mentioned findings along with our results, a 16-week ribavirin addition to IFN treatment would seem to produce a high rate of SVR for patients with genotype 2, especially for those with EVR, defined as undetectable HCV RNA within 4 wk of the start of the treatment.

The Davis group attempted to confirm that an EVR in patients with chronic hepatitis C undergoing initial treatment with a combination therapy of pegylated IFN alpha and ribavirin was predictive of SVR[22]. Retrospective analysis of data from other trials[23] has also suggested that patients who do not attain EVR have a nominal chance of SVR with additional weeks of treatment. While the primary goal, or "holy grail", of treatment of chronic hepatitis C is SVR, it must be acknowledged there are other secondary goals that compel physicians to continue treatment without EVR. In fact, patients who do not achieve EVR or SVR may have histological benefit[24], leading to a decreased risk of hepatocellular carcinoma[19]. Thus, it remains to be determined whether or not early discontinuation of treatment would reduce economic costs if a long-term perspective is taken.

Several adverse reactions are associated with ribavirin. One of the most significant reactions is hemolytic problems, especially anemia[15]. Most of our patients who had to have a dose reduction or who discontinued ribavirin were observed to have anemia. It is important to reduce the dose of ribavirin at as early a stage as possible to allow the safe continuation of the combination treatment. The Nomura group pointed out that careful administration is necessary in patients over 60 years, in female patients, and in patients receiving a ribavirin dose by body weight of 12 mg/kg or more[21]. Our forward stepwise logistic regression analysis showed that the continuation of ribavirin treatment was significantly associated with SVR. This combination treatment, which could depend on hemolytic adverse reaction, has a high efficacy, if physicians are able to continue the ribavirin treatment for as short a period as

16 wk, even when taking into account of the dose reductions necessary for patients with a dangerous decrease of hemoglobin caused by ribavirin, as often seen in genotype 2 patients with a low hemoglobin level at pretreatment.

In conclusion, the 24-week IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR in Japanese HCV patients with genotype 2 from the retrospective study of ours. The most significant predictor was continuation of the ribavirin treatment for up to 16 wk. These findings are not pertinent to the other different genotypes.

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Association between fast-migrating low-density lipoprotein subfraction as characterized by capillary isotachophoresis and intima-media thickness of carotid artery

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Abstract

Background: A mildly modified LDL subfraction that is characterized by an increased negative charge exists in plasma. This electronegative LDL separated by ion-exchange chromatography has been shown to be inflammatory and its proportion is increased in patients with hyperlipidemia and diabetes mellitus. The present study examined the association between the level of fast (f)-migrating LDL subfraction characterized by capillary isotachophoresis (cITP) and carotid-artery intima-media thickness (CA-IMT).

Methods and results: This study included 469 subjects who underwent a physical examination. CA-IMT was determined by high-resolution B-model ultrasonoraphy. Levels of charge-based LDL subfractions were measured by cITP on a Beckman P/ACE MDQ system. An increased serum LDL-C level and cITP fLDL level were associated with increased CA-IMT after adjusting for age. The extent of the associations between cITP fLDL and CA-IMT and between LDL-C and CA-IMT were similar as assessed by a receiver-operating characteristic curve analysis. LDL-C, triglyceride, and remnant-like particle cholesterol levels were independently correlated with cITP fLDL, and the LDL-C level had the strongest correlation with cITP fLDL. The association between the cITP fLDL level and CA-IMT was significant in the high LDL-C stratum but not in the low stratum, indicating that it is modified by the LDL-C level. The high-LDL-C-high-fLDL group had the highest relative risk for a high CA-IMT among the groups with each combination of LDL-C and cITP fLDL level.

Conclusion: The cITP fLDL level was associated with CA-IMT and its combination with the LDL-C level is a stronger indicator for a high CA-IMT.

Keywords: Low density lipoprotein (LDL) cholesterol; Intima-media thickness; Carotid atherosclerosis; Capillary isotachophoresis; Fast-migrating LDL subfraction

1. Introduction

Serum level of low-density lipoprotein cholesterol (LDL-C) is an established risk factor of coronary artery disease (CAD), and a reduction in LDL-C levels has been shown to be associated with reduced death rates caused by CAD. LDL is composed of heterogeneous particles that differ in size, composition, and electric charge. Qualitatively modified forms of LDL have been shown to exist in human plasma, including small dense LDL, oxidative modified LDL, glycated LDL, and diasylated LDL [1–5], and they are

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all atherogenic. These forms of modified LDL all have an increased negative charge. The electronegative subfraction of LDL [LDL(−)] has been separated from plasma by anion-exchange chromatography techniques [6,7]. LDL(−) in plasma can also be generated by other processes including enrichment with nonesterified fatty acids or enzymatic modification by phospholipase A2 or cholesteryl esterase/trypsin [7].

Although the origins of LDL(−) are complex and not fully understood, LDL(−) has been shown to have proinflammatory activity on endothelial cells [8] and its proportion is increased in patients with hypertriglyceridemia [9], familial hypercholesterolemia (FH) [9], and diabetes mellitus (DM) [10], patients on hemodialysis [11], and patients with anigographically documented CAD [12].

However, there is still little information available on whether or not the LDL(−) subfraction level is a marker for atherosclerosis and whether or not its association with atherosclerosis is independent of the LDL-C level, partly because of the lack of a routine analytical technique for this modified LDL subfraction.

The current ion-exchange chromatography method for measuring the LDL(−) subfraction gives the proportion of LDL(−) protein content in total LDL separated by ultracentrifugation [6]. Therefore, it is disadvantageous for routine analysis in that it is time-consuming and requires a relatively large amount of samples, and the absolute level of LDL(−) in plasma cannot be determined.

Capillary isotachophoresis (cITP) is another technique that separates and quantifies LDL subfractions according to electric charge. It was originally developed by the research group of Schmitz [13,14]. Fast-migrating LDL (fLDL) carries more negative charge than slow-migrating LDL (sLDL) [13,14]. Since lipoproteins are pre-stained with a fluorescent lipophilic dye, LDL subfractions can be measured directly in plasma and with high sensitivity (only several microliters of sample are necessary). Separation and on-line detection can both be performed within just a few minutes. Therefore, analytical cITP technique may be useful for the routine analysis of lipoprotein profiles. We previously showed that the absolute levels of lipoprotein subfractions can be determined as a peak area relative to an internal marker and the levels of cITP fLDL and sLDL were proportional to the protein content of LDL [15–17].

Measurement of the thickness of the intima and media of carotid arteries by high-resolution B-mode carotid ultrasonography has been used as a non-invasive method for detecting early carotid atherosclerosis [18,19]. Carotid-artery intima-media thickness (CA-IMT) is associated with the prevalence of cardiovascular disease and with cardiovascular risk factors [20].

We investigated the hypothesis that the cITP fLDL subfraction level is associated with CA-IMT. We also hypothesized that the cITP fLDL level contributes to the ability of LDL-C to predict the risk of CA-IMT after controlling for conventional cardiovascular risk factors.

2. Methods

2.1. Subjects

This study included 469 male subjects (aged between 21 and 88 years) who participated in a health examination. This study was approved by the Ethics Committees of Kyushu University Hospital, and samples were collected only after the participants had given their informed consent.

The prevalence of hypertension, diabetes mellitus, and smoker in the study subjects was 39.7% ($n=186$), 13.9% ($n=65$), and 32.6% ($n=153$), respectively. Twelve subjects (2.6%) had a history of stroke, and 12 (2.6%) had a history of coronary heart disease. Hypertension was defined as systolic blood pressure $\geq$140 mmHg, diastolic pressure $\geq$90 mmHg, or treatment with antihypertensive medications. Diabetes mellitus was defined as a self-reported history of diabetes, a fasting plasma glucose concentration $\geq$126 mg/dl, or the use of anti-diabetic drugs. Smokers were defined as those who had smoked past or who were present smokers. Subjects who refused ultrasound examination or who had a fasting blood glucose concentration $\geq$400 mg/dl or triglyceride (TG) level $\geq$400 mg/dl were excluded from the study.

Blood was drawn between 9 and 12 a.m. after an overnight fast and stored at −80 °C until analysis. Storage of samples at −80 °C for up to 5 months does not apparently affect measurements for cITP LDL subfractions [17].

2.2. Ultrasonographic measurement

Common carotid-artery lesions were assessed by high-resolution B-mode ultrasonography with a 7.5 MHz mechanical sector transducer on an Aloka SSD-2000 (Aloka Co. Ltd., Tokyo, Japan), as described previously [21,22]. All assessment of carotid arteries was performed by three specially trained technicians who were unaware of the clinical history or risk factor profile. IMT was measured at points 20, 25, 30 mm proximal to the flow divider on the far wall of the right and left common carotid arteries at the end of the diastolic phase. Using this information, mean CA-IMT was determined for each individual.

2.3. Measurement of serum lipids and lipoproteins

Serum levels of total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C) were measured by enzymatic methods. Serum LDL-C levels were calculated indirectly using the Friedewald formula.

Remnant-like particle cholesterol (RLP-C) levels were measured by an RLP-Cholesterol Immunoseparation Assay using a commercially available kit (JIMRO-II, Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan) [23,24]. Briefly, the RLP immunoseparation gel was washed before use three times with RLP buffer by low-speed centrifugation and suspended by repeatedly inverting the container. After 150 μl of the suspended gel was aliquoted into Hitachi

microsample cups, 5 μl serum samples were added and the mixture was stirred using a steel bead for 2 h at room temperature on an RLP Mixer J-100 (Otsuka Electrics Co., Ltd, Tokyo, Japan). After the gel had settled for 15 min, the cholesterol level in the supernatant was measured with cholesterol reagents included in the assay kit using an auto-analyzer (Hitachi 7600-020S).

2.4. Quantification of lipoprotein subfractions by cITP

Capillary isotachophoresis of serum lipoproteins was performed on a Beckman P/ACE MDQ system (Beckman-Coulter Inc., Tokyo, Japan) according to the method of Bottcher et al. [13] with some modifications, as previously described [15–17,25,26]. Briefly, 6 μl of serum was diluted with 14 μl of leading buffer consisting of 10 mM HCl and 18 mM ammediol (2-amino-2-methyl-1,3-propanediol) (pH 8.8), prestained with 10 μl 0.1 mg/ml NBD C6-ceramide (Molecular Probe Inc., OR, USA) for 5 min at room temperature, and mixed with 50 μl of the mixture containing leading buffer with 0.35% hydroxypropylmethylcellulose (HPMC), spacers, and 5-carboxy-fluorescein as an internal marker. The spacers were *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), D-glucuronic acid, 1-octanesulfonic acid sodium salt, 3-(*N*-tris[hydroxymethyl]methylamino)-2-hydroxypropanesulfonic acid (TAPSO), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), L-serine, L-glutamine, L-methionine, and glycine. The terminating buffer contained 24 mM β-alanine and 13 mM ammediol, and was adjusted to pH 10.5 with saturated barium hydroxide solution. A dimethylpolysiloxane-modified fused silica capillary (AT^{TM}-1) was purchased from Alltech Japan Inc. (Tokyo, Japan). The sample was injected at 20 psi for 18 s into a 30-cm long capillary (i.d. 180 μm), and separation was performed at a constant 30 μA for 1 min and 10 kV for 7 min. The separated zones were monitored with argon-laser-induced fluorescence detection (excitation, 488 nm; emission, 520 nm). Each peak was identified and the peak area in relative fluorescence units was analyzed using 32 Karat Software version 5.0 (Beckman-Coulter Inc., Tokyo, Japan). Levels of cITP lipoprotein subfractions were expressed as the peak area relative to the internal marker.

2.5. Statistical analysis

All of the statistical analysis was performed using the SAS (Statistical Analysis System) Software Package (Version 9.1, SAS Institute, CA, USA) at the Fukuoka University. The distribution of variables were examined by the Shapiro–Wilk test [27]. The 33.3th and 66.7th percentiles were used to produce tertiles of CA-IMT. Linear trends of risk factors across tertiles of CA-IMT after adjusting for age were examined by an analysis of covariance (ANCOVA) using a general linear model. Correlation between variables was examined by Spearman correlation. Log-transformed values of TG and RLP-C were used in the data analysis. Low and high LDL-C strata were defined as $<$ and $\geq$ the median value of LDL-C (118 mg/dl), respectively, and low and high CA-IMT were defined as $<$ and $\geq$ the median value of CA-IMT (0.77 mm). The strength of the associations between the cITP fLDL and LDL-C levels was compared using a receiver operating characteristic (ROC) curve analysis. An ROC-curve (plot of sensitivity versus 1-specificity) analysis is a powerful tool for assessing the ability of a continuous variable to discriminate between two groups of subjects, and does not depend on the cutoff value selected. The area under the ROC curve represents the probability for a randomly chosen low CA-IMT subject to exhibit a value lower than the level observed among randomly chosen high CA-IMT subjects. A value of 0.5 means that the distributions of the values in the two groups are similar; conversely, a value of 1.0 means that the distributions of the values in the two groups do not overlap. We determined the area under the ROC curve by the trapezoidal rule and evaluated its significance by the Wald chi-square test, as described previously [28]. Stepwise multiple regression analysis was used to examine the independent variables that are related to cITP fLDL. The significance of the association between the combination of LDL-C and cITP fLDL and CA-IMT after controlling for age and other related variables was examined by a multivariate logistic regression analysis using dummy variables. The odds ratio and 95% confidence interval (CI) were given for each combination of LDL-C and cITP fLDL. All p values are two-tailed. The significance level was considered to be 5% unless indicated otherwise.

3. Results

Table 1 shows the mean levels of conventional risk factors of CAD, serum levels of lipids and lipoproteins, and RLP-C levels according to tertiles of CA-IMT. Increased age was associated with increased CA-IMT (tertile III versus tertile II versus tertile I: 64.4 ± 0.9 year versus 59.7 ± 0.9 year versus 48.9 ± 1.0 year, $p < 0.05$, by an analysis of variance). The prevalence of DM and serum levels of TC and LDL-C were positively and significantly associated with CA-IMT after adjusting for age, as assessed by an analysis of covariance (Table 1). Body mass index (BMI), prevalence of HT and smoking, and serum levels of TG, HDL-C, and RLP-C were not significantly associated with CA-IMT after adjusting for age (Table 1).

Fig. 1 shows the typical cITP lipoprotein profiles of subjects with low (0.54 mm) and high CA-IMT (1.17 mm). As shown, capillary isotachophoresis clearly separated lipoproteins into eight fractions within 8 min. Peaks 6 and 7 are the two LDL subfractions with fast and slow electrophoretic mobility. Subject with high CA-IMT had apparently higher levels of both fLDL and sLDL than that with low CA-IMT (Fig. 1).

Table 2 shows that age-adjusted mean levels of intermediate-migrating HDL decreased and cITP fLDL and sLDL increased across tertiles of CA-IMT. This result indi-

Table 1
Age-adjusted mean levels of risk factors according to tertiles of carotid-artery intimal-media thickness (CA-IMT)

	Tertiles of CA-IMT			p^a
	Low (<0.67 mm)	Middle (0.67–0.83 mm)	High (≥0.83 mm)	
No. of subjects	142	159	168	
Age (year)	48.9 ± 1.0	59.7 ± 0.9	64.4 ± 0.9	<0.05
Body mass index (kg/m^2)	22.6 ± 0.3	23.9 ± 0.2	23.5 ± 0.2	n.s.
Hypertension (%)	23	40	53	n.s.
Diabetes mellituss (%)	5	12	22	<0.05
Smoking (%)	37	31	30	n.s.
TC (mg/dl)	191 ± 3	199 ± 2	204 ± 3	<0.05
log(TG)	4.7 ± 0.0	4.8 ± 0.0	4.8 ± 0.0	n.s.
HDL-C (mg/dl)	53 ± 1	54 ± 1	52 ± 1	n.s.
LDL-C (mg/dl)	113 ± 3	118 ± 2	123 ± 2	<0.05
log(RLP-C)	2.7 ± 0.0	2.8 ± 0.0	2.9 ± 0.0	n.s.

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RLP-C, remnant-like particle cholesterol. The units of TG and RLP-C were mg/dl.

[a] Assessed by an analysis of covariance or logistic regression analysis after adjusting for age. Continuous variables were adjusted for age by means of linear regression, and categorial variables were adjusted for age by means of logistic regression.

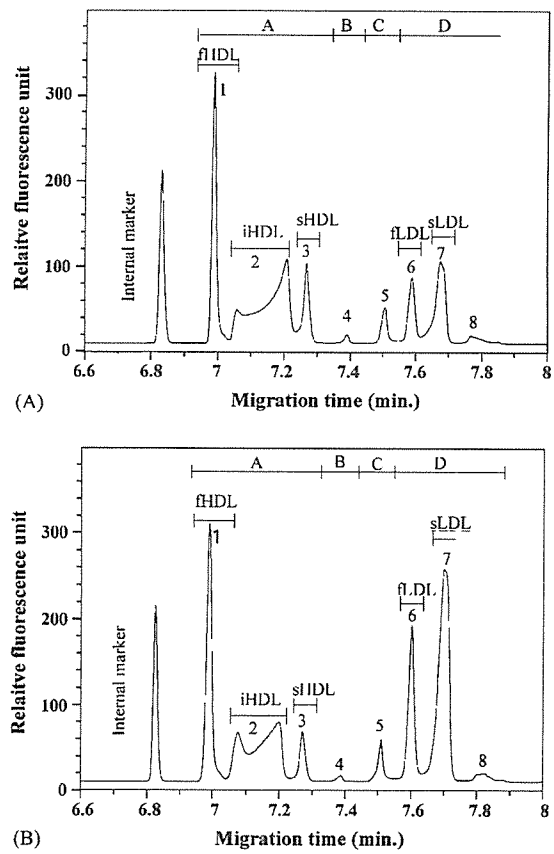

Fig. 1. Lipoprotein profiles as determined by capillary isotachophoresis in serum from subjects with low (A) and high (B) carotid-artery intima-media thickness (CA-IMT: 0.54 and 1.17 mm, respectively). The various lipoprotein subfractions are depicted as follows [13,14]: A, HDL; B, chylomicron/remnants; C, VLDL/IDL; D, LDL. fHDL, fast-migrating HDL; iHDL, intermediate-migrating HDL; sHDL, slow-migrating HDL; fLDL, fast-migrating LDL; sLDL, slow-migrating LDL.

cates that both cITP fLDL and sLDL were positively associated with CA-IMT independent of age. The strength of the associations between cITP fLDL and CA-IMT (two levels) and between LDL-C and CA-IMT were compared by an ROC curve analysis. Fig. 2 shows the plot of sensitivity (true positive) versus 1-specificity (false positive) for the LDL-C level and cITP fLDL level. The area under the ROC curve was similar for cITP fLDL and LDL-C (0.578 and 0.582, respectively).

The cITP fLDL levels were negatively correlated with HDL-C levels ($r=-0.135$, $p<0.01$) and significantly ($p<0.01$) and positively correlated with age, BMI, and serum levels of TC, TG, LDL-C, and RLP-C ($r=0.156$, 0.168, 0.524, 0.208, 0.545, and 0.147, respectively). Stepwise multiple regression analysis selected LDL-C, TG, and RLP-C as independent variables that were related to cITP fLDL (Table 3). The LDL-C level had the strongest correlation with cITP fLDL (Table 3). Fig. 3 shows the correlation between cITP fLDL and LDL-C levels in subjects with low, middle, and high CA-IMT. As shown, cITP fLDL levels were significantly correlated with LDL-C levels in all the three groups of subjects. As also shown in Fig. 3, the regression lines of cITP fLDL versus LDL-C levels in subjects with middle and high CA-IMT (dotted lines) were shifted towards higher cITP fLDL levels as compared with that in subjects with low CA-IMT (solid line). This result indicates that cITP fLDL levels were higher in subjects with middle and high CA-IMT than in subjects with low CA-IMT after controlling for LDL-C levels.

Therefore, LDL-C levels were stratified into low and high strata and the association between cITP LDL and CA-IMT was examined according to LDL-C strata to test its relation to LDL-C levels. As shown in Table 4, the association between cITP fLDL and CA-IMT was significant in the high LDL stratum [odds ratio (95% CI): 2.2 (1.2–3.8)] but not in the low LDL stratum after adjusting for age by a multiple logistic regression analysis. This result indicates that

Table 2
Age-adjusted mean levels of lipoprotein subfractions as measured by capillary isotachophoresis (cITP) according to tertiles of carotid-artery intimal-media thickness (CA-IMT)

	Tertiles of CA-IMT			p^a
	Low (<0.67 mm)	Middle (0.67–0.83 mm)	High (≥0.83 mm)	
cITP fHDL	1.46 ± 0.04	1.47 ± 0.04	1.40 ± 0.04	n.s.
cITP iHDL	2.22 ± 0.04	2.14 ± 0.03	2.10 ± 0.03	<0.05
cITP sHDL	0.41 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	n.s.
cITP VLDL/IDL	0.91 ± 0.04	1.04 ± 0.04	1.01 ± 0.04	n.s.
cITP fLDL	1.09 ± 0.03	1.16 ± 0.02	1.20 ± 0.03	<0.05
cITP sLDL	1.29 ± 0.05	1.36 ± 0.04	1.46 ± 0.04	<0.05

Levels of cITP lipoprotein subfractions are expressed as peak area relative to the internal marker. fHDL, iHDL, and sHDL, fast-intermediate, and slow-migrating high-density lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; fLDL and sLDL, fast- and slow-migrating low-density lipoprotein.
[a] Assessed by an analysis of covariance. Variables were adjusted for age by means of linear regression.

Table 3
Stepwise multivariable regression analysis of the independent variables related to fast-migrating low-density lipoprotein (fLDL) as determined by capillary isotachophoresis (cITP)

Step	Variable entered	Partial correlation coefficient	F	p
1	LDL-C	0.573	190.1	<0.001
5	log(TG)	0.306	67.3	<0.001
4	log(RLP-C)	0.204	25.6	<0.001

Levels of cITP fLDL are expressed as peak area relative to the internal mark.

the association between cITP fLDL and CA-IMT was modified by the LDL-C level. Fig. 4 shows a three-dimensional plot of the age-adjusted relative risk for a high CA-IMT for each combination of cITP fLDL and LDL-C levels. The high-LDL-C-high-fLDL group had the highest risk for a high CA-IMT among the four groups: the low-LDL-C-low-fLDL group, the low-LDL-C-high-fLDL group, the high-LDL-C-low-fLDL group, and the high-LDL-C-high-fLDL group. Similar results were obtained after additionally adjusting for HT, DM, and smoking (data not shown). These results indicate that the combination of cITP fLDL and LDL-C level was a stronger indicator for a high CA-IMT than either cITP fLDL or LDL-C alone.

4. Discussion

With advances in techniques in lipoprotein analysis, a new LDL subfraction in plasma that is characterized by a greater negative charge than native LDL has attracted considerable attention. The electronegative LDL subfraction separated by ion-exchange chromatography has been shown to contain mildly modified LDL that could be produced from multiple origins [7] and is associated with a pathogenic state that is related to atherosclerosis [9–11]. Therefore, this negatively charged LDL subfraction could be a novel marker for atherosclerosis. However, there is still little evidence to support this point because of the lack of routine analytical techniques for this LDL subfraction. Ion-exchange chromatography is excellent for the separation of LDL(−) and for preparative use [6]. However, since it requires the separation of LDL by ultracentrifugation, the absolute level of LDL(−) in plasma cannot be measured with this technique and routine analysis is also difficult.

Analytical capillary isotachophoresis is a new technique for routine analysis of LDL subfractions according to their electric charges, which was established by the research group of Schmit et al. [13,14]. Several microliters of serum or plasma can be directly analyzed and separation and detection of cITP fast- and slow-migrating LDL can be performed within minutes. However, little attention has been paid to this technique [15–17,25,26], and therefore the clinical significance of the cITP fLDL subfraction is still unclear. We have previously shown that cITP can be used to quantify charge-based LDL subfractions [17] and express the absolute levels of cITP lipoprotein subfractions as the peak area relative to an internal marker [15–17,25,26].

We are the first to report that cITP fLDL and sLDL levels are associated with carotid-artery IMT. This finding is not unexpected because serum levels of LDL-C are associated with CA-IMT and levels of cITP LDL subfractions were correlated with LDL-C levels (Table 3, Fig. 3). We also found using an ROC curve analysis that the ability of cITP fLDL to predict for a high CA-IMT was similar to that of LDL-C (Fig. 2).

Table 4
Multiple logistic regression analysis of the association between fast-migrating LDL determined by cITP and carotid-artery intima-media thickness after adjusting for age in low and high LDL-C strata

	Regression coefficient ± S.E.	Odds ratio (95% confidence interval)	Wald chi-square	p
Low LDL-C	0.12 ± 0.37	1.1 (0.54–2.3)	0.10	n.s.
High LDL-C	0.78 ± 0.29	2.2 (1.2–3.8)	7.36	<0.01

The median value of LDL-C (118 mg/dl) was used to produce low and high LDL-C strata.

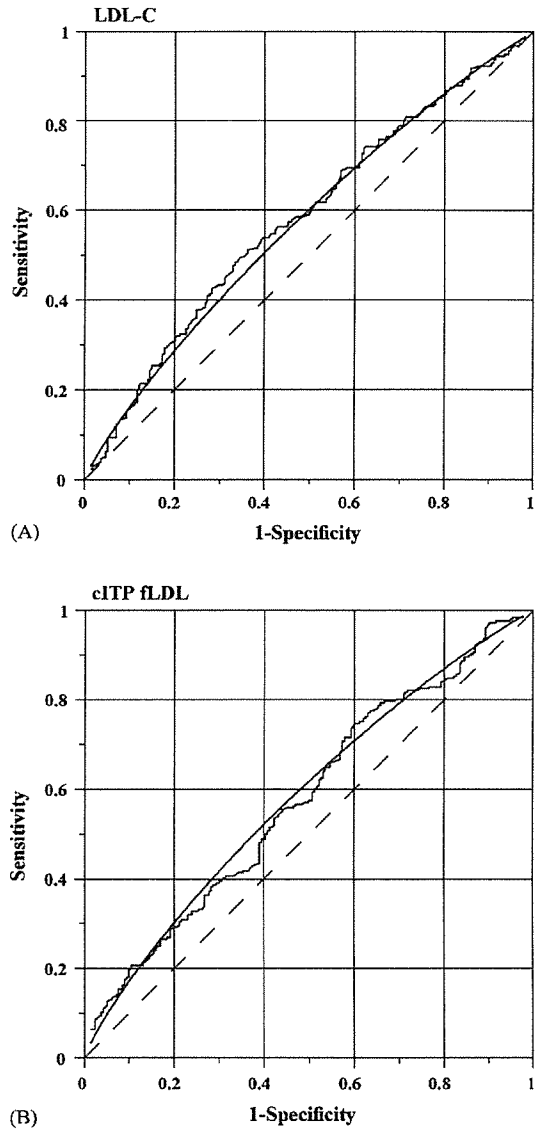

Fig. 2. ROC curves of the true-positive rate (sensitivity) vs. the false-positive rate (1-specificity) for LDL-C (A) and cITP fLDL (B). The smooth curves are model-fitted curves by the method of Swets [31].

Our finding that the cITP fLDL level was significantly related to the serum TG level (Table 3) agrees with that of Sanchez-Quesada et al., who reported that patients with hypertriglyceridemia had an increased proportion of LDL(−) [9]. Therefore, a high TG level could contribute to the increased electronegativity of LDL. We also observed a significant correlation between cITP fLDL and RLP-C levels (Table 3). The RLP-C level has been shown to be associated with CA-IMT independent of LDL-C and TG levels in a group of 50-year-old Caucasian men [29]. In our study subjects who had a wide range of ages, we observed no statistically significant association between the RLP-C level and CA-IMT after adjusting for age. The mechanism by which

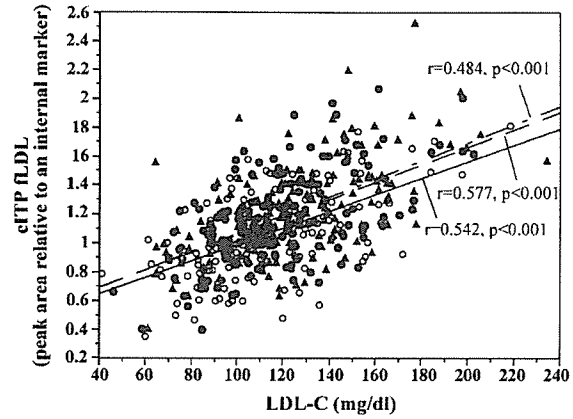

Fig. 3. Correlation between levels of fast-migrating LDL as determined by capillary isotachophoresis (cITP fLDL) and LDL-C levels in subjects with low (○), middle (●) and high (▲) carotid-artery intima-media thickness (CA-IMT).

RLP-C is related to cITP fLDL and whether or not it contributes to the association between cITP fLDL and atherosclerosis need further investigation.

Despite a strong correlation between cITP fLDL and LDL-C levels, we found that the association between the cITP fLDL level and CA-IMT was modified by LDL-C levels (Table 4) and the combination of cITP fLDL and LDL-C levels is a better indicator for a high CA-IMT (Fig. 4). Therefore, increased cITP fLDL could be a potentially useful marker for a high CA-IMT when the LDL-C level is high. Although the result of this cross-sectional study cannot be used to determine whether or not cITP fLDL subfraction is a causal factor for a high CA-IMT, our finding suggests that mildly modified

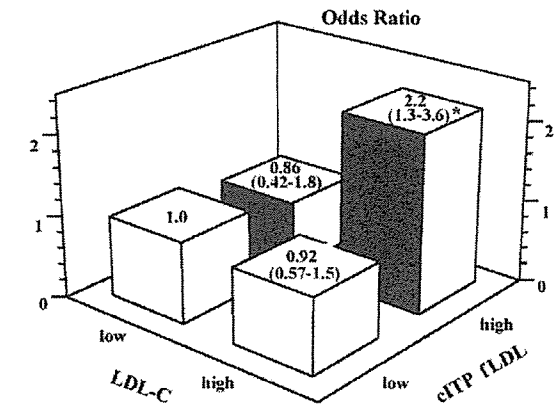

Fig. 4. Age-adjusted odds ratios [95% confidence interval (CI)] for a high carotid-artery intima-media thickness (CA-IMT) in each combination of LDL-C level and cITP fLDL level (low-LDL-C-low-fLDL, low-LDL-C - high fLDL, high-LDL-C-low-fLDL, and high-LDL-C-high-fLDL groups). Two levels of CA-IMT were produced using the median value (given a value of 0 if CA-IMT < 0.77 mm and 1 if CA-IMT ≥ 0.77 mm). The median value of LDL-C (118 mg/dl) and the 66.7th percentile value of cITP fLDL (1.37) were used to make dummy variables for each group. $^{*}p < 0.01$, as assessed by a multiple logistic regression analysis.

LDL in human blood could be important in the pathogenesis of atherosclerosis, especially under a high LDL-C level.

Our finding that cITP fLDL was associated with CA-IMT supports the notion that the electronegative subfraction of LDL is associated with risk factors of CAD, as reported by other authors [8–11], and the prevalence of angiographically documented CAD [12]. However, in the present study, the absolute levels of cITP fLDL were examined in its relation to CA-IMT, while other authors reported an association between the proportion of LDL(−) in total LDL and risk factors for CAD [8–11] or the prevalence of CAD [12]. The absolute plasma level of LDL(−) cannot be determined by anion-exchange chromatography because LDL has to be separated from plasma by ultracentrifugation or other technique before it is used for the separation of LDL(−). Therefore, the proportion of LDL(−) reported in previous studies is not equivalent to the level of cITP fLDL in the present study. However, the more negative-charged LDL subfractions separated by the two different techniques are closely related. We have previously shown that cITP fLDL represents an electronegative fraction of LDL because the cITP sLDL subfraction was converted to the fLDL subfraction when LDL was subjected to in vitro oxidation by $CuSO_4$ [17]. Bittolo-Bon et al., who separated plasma LDL into four subfractions using a different buffer system in capillary isotachophoresis, also reported that the ratio of fast-migrating (LDL1 and LDL2) and slow-migrating (LDL3 and LDL4) LDL subfractions determined by cITP was strongly and positively correlated with the proportion of LDL(−) determined by anion-exchange chromatography [30]. We observed no significant associations between the proportion of cITP fLDL in total cITP calculated from the absolute levels of cITP fLDL and sLDL and the ratio of cITP fLDL to sLDL and CA-IMT (data not shown). Therefore, our findings indicate that the absolute level of cITP fLDL but not the proportion of cITP fLDL in total LDL is important as a marker for a high CA-IMT.

In conclusion, fast LDL as characterized by analytical cITP was associated with carotid-artery intima-media thickness and could be a potentially useful marker for early atherosclerosis in combination with the LDL-C level. Further investigations are needed to clarify whether or not this conclusion can be applied to coronary atheosclerosis.

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Both Hepatitis C Virus and *Chlamydia Pneumoniae* Infection are Related to the Progression of Carotid Atherosclerosis in Patients Undergoing Lipids Lowering Therapy

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of the Max-IMT (mm) after 24 months. Multiple linear regression analysis was done to detect factors that influenced the rate of change of Max-IMT from among HAV seropositivity, HBV seropositivity, HCV seropositivity, age, sex, smoking history, diabetes, and hypertension. Variables were selected for entry into the model by the backward stepwise method. A probability value of less than 0.05 was considered to indicate statistical significance in all analyses. Data were analyzed on an intention-to-treat basis.

RESULTS

Baseline characteristics and comparability

The mean age of the patients was 65.7 years and 24% were men. Average systolic blood pressure and diastolic blood pressure were 131 and 76 mm Hg, respectively. Of the 165 patients, 46% were recent or former smokers, 38% had a history of hypertension, and 17% had diabetes mellitus. Baseline serum total cholesterol and LDL cholesterol levels were 251 mg/dL and 164 mg/dL, respectively. The HDL cholesterol level was 57 mg/dL, while the serum triglyceride level was 150 mg/dL. The Max-IMT was 1.59 mm. There were no statistically significant differences between the two groups for any of these baseline characteristics. Anti-HAV was detected in 67.5%, HBsAg was positive in 2.4%, and anti-HCV was positive in 15.2% of the patients. HCV-RNA was detected in all of the anti-HCV positive patients.

Percent reduction of total cholesterol after 24 months stratified by HAV, HBV, HCV, and *C. pneumoniae* status

There was a significant reduction of the serum total cholesterol level by 21.7% between baseline (247.2 ± 24.6 mg/dl) and 24 months of therapy (192.5 ± 25.9 mg/dl) in the patients without HAV infection and a decrease of 20.8% in the infected patients (both $p<0.001$, Student's t-test). Significant reduction of the serum total cholesterol level by 21.3% was also seen between baseline (248.9 ± 24.3 mg/dl) and 24 months of therapy (195.9 ± 30.3 mg/dl) in the patients without HBV infection and there was a decrease of 10.8% in the infected patients ($p<0.05$ and $p<0.001$, respectively; Student's t-test). Moreover, a significant reduction of serum total cholesterol by 21.1% occurred between baseline (246.8 ± 23.7 mg/dl) and 24 months (194.1 ± 25.6 mg/dl) in the patients without HCV infection, as well as a decrease of 21.1% in the infected patients (both $p<0.001$, Student's t-test). There were no significant differences between probucol and pravastatin therapy with respect to the reduction of total cholesterol in patients with or without HAV, HBV, and HCV infection after 24 months of treatment (Table 1).

Percent reduction of LDL cholesterol after 24 months stratified by HAV, HBV, HCV, and *C. pneumoniae* status

Significant reduction of the serum LDL cholesterol level by 26.9% was found between baseline (154.0 ± 30.7 mg/dl) and 24 months of therapy (110.0 ± 27.1 mg/dl) in the patients without HAV infection, while the decrease was 27.6% in the infected patients (both $p<0.001$, Student's t-test). Significant reduction of the LDL cholesterol level by 27.4% was also seen between baseline (157.2 ± 27.7 mg/dl) and 24 months (113.6 ± 31.4 mg/dl) in the patients without HBV infection and there was a decline of 27.3% in the infected patients ($p < 0.05$ and $p < 0.001$, respectively;

Table 1 Changes of total cholesterol after 24 months of treatment stratified by HAV, HBV, and HCV status

	No.	Total cholesterol (mg/dl, mean ± SD)		P value (Student's t-test)	Percent change (mg/dl, mean ± SD)	P value (paired t-test)
		before	after 24 months			
anti-HAV						
+	113	248.4+23.3	196.5+30.4	<0.0001	20.8+10.8	NS
−	52	247.2+24.6	192.5+25.9	<0.0001	21.7+10.6	
HBs Ag						
+	4	244.0+12.7	217.8+18.0	<0.05	10.8+4.6	NS
−	161	248.0+87.5	194.7+29.1	<0.0001	21.3+10.7	
anti-HCV						
+	25	242.8+18.8	191.3+20.9	<0.0001	20.8+9.8	NS
−	140	248.9+24.3	195.9+30.3	<0.0001	21.1+10.9	
ant-*C. pneumoniae*-IgA and/or IgG #						
+	115	247+22	195+29	<0.0001	21	NS
−	50	249+27	197+30	<0.0001	21	
Treatment						
Probucol	82	249.4+23.6	196.5+32.8	<0.0001	21.1+12.0	NS
Pravastatin	83	246.8+23.7	194.1+25.6	<0.0001	21.1+9.5	

Data quoted from Sawayama et al. Atherosclerosis 2003; 171 (2): 281-285.

Table 2 Changes of LDL cholesterol after 24 months of treatment stratified by HAV, HBV, and HCV status

	No.	Total cholesterol (mg/dl, mean ± SD)		P value (Student's t-test)	Percent change (mg/dl, mean ± SD)	P value (paired t-test)
		before	after 24 months			
anti-HAV						
+	113	158.3+26.1	114.8+33.4	<0.0001	27.6+19.2	NS
−	52	154.0+30.7	111.0+27.1	<0.0001	26.9+15.9	
HBs Ag						
+	4	146.2+25.7	112.4+39.5	<0.0001	24.1+21.6	NS
−	161	157.2+27.7	113.6+31.4	<0.0001	27.4+18.2	
anti-HCV						
+	25	154.9+25.2	118.0+23.9	<0.0001	22.6+16.2	NS
−	140	157.3+28.1	112.8+32.6	<0.0001	28.2+18.4	
ant-*C. pneumoniae*-IgA and/or IgG #						
+	115	157+27	112+32	<0.0001	28	NS
−	50	159+29	117+30		26	
Treatment						
Probucol	82	162.7+24.8	125.6+31.7	<0.0001	22.3+18.6	<0.0001
Pravastatin	83	151.9+29.0	103.2+27.5	<0.0001	31.8+16.7	

Data quoted from Sawayama et al. Atherosclerosis 2003; 171 (2): 281-285.

Student's t-test). Moreover, a significant reduction of LDL cholesterol by 28.2% occurred between baseline (157.3 ± 28.1 mg/dl) and 24 months (154.9 ± 25.2 mg/dl) in the patients without HCV infection, while the decrease was 22.6% in the infected patients (both $p<0.001$, Student's t-test). A significantly greater reduction of LDL cholesterol levels was achieved by pravastatin than probucol in patients with HAV, HBV, HCV, and *C. pneumoniae* infection after 24 months of treatment (Table 2).

Percent reduction of Max-IMT after 24 months stratified by HAV, HBV, HCV, and *C. pneumoniae* status

A significant decrease of Max-IMT by 9.7% was found between baseline (1.28 ± 0.57 mm) and 24 months of therapy (1.10 ± 0.45 mm) in the patients without HAV

Table 3 Changes of Max-IMT after 24 months of treatment stratified by HAV, HBV, and HCV status

	No.	Total cholesterol (mg/dl, mean ± SD)		P value (Student's t-test)	Percent change (mg/dl, mean ± SD)	P value (paired t-test)
		before	after 24 months			
anti-HAV						
+	113	1.54+0.88	1.36+0.78	<0.0001	9.1+21.5	NS
−	52	1.28+0.57	1.10+0.45	<0.0001	9.7+23.7	
HBs Ag						
+	4	1.28+0.15	1.13+0.15	<0.0577	11.7+7.9	NS
−	161	1.46+0.81	1.28+0.71	<0.0001	9.2+22.4	
anti-HCV						
+	25	1.51+0.91	1.44+0.83	NS	0.3+22.5	<0.05
−	140	1.45+0.78	1.25+0.67	<0.0001	10.9+121.7	
ant-*C. pneumoniae*-IgA and/or IgG #						
+	115	1.27+0.62*	1.19+0.64*	NS	6	<0.01
−	50	1.33+0.60*	1.08+0.50*	<0.01	19	
Treatment						
Probucol	82	1.59+0.90	1.35+0.67	<0.0001	9.6+24.0	NS
Pravastatin	83	1.35+0.70	1.21+0.73	<0.0001	9.0+20.5	

Data quoted from Sawayama et al. Atherosclerosis 2003; 171 (2): 281-285.
* IMT is the mean IMT value.

infection and there was a decrease of 9.1% in the infected patients (both $p<0.001$, Student's t-test). A reduction of Max-IMT by 9.2% occurred between baseline (1.46 ± 0.81 mm) and 24 months (1.28 ± 0.71 mm) in the patients without HBV infection while there was a decrease of 11.7% in the infected patients ($p<0.001$ and $p=0.057$, respectively; Student's t-test). In contrast, a reduction of Max-IMT by 10.9% was seen between baseline (1.45 ± 0.81 mm) and 24 months (1.25 ± 0.67 mm) in the patients without HCV infection versus a decrease of only 0.3% in the infected patients ($p<0.001$ and $p=0.4104$, respectively; Student's t-test). Although the reduction of IMT was 9.2% versus 11.7% in the patients with and without HBV infection, respectively, showing no significant difference, there was a significant difference of the change of Max-IMT between the patients with and without HCV infection (0.3% versus 10.9%, respectively). There were no significant differences between probucol and pravastatin therapy with regard to the reduction of Max-IMT after 24 months in relation to HAV, HBV, HCV, and *C. pneumoniae* infection status (Table 3).

Multiple linear regression analysis

Backward stepwise multiple linear regression analysis revealed that not only *C. pneumoniae* infection, but also HCV infection showed a strong independent association with a smaller reduction of IMT ($p=0.0276$). There was no significant association between the change of IMT and any of the other variables investigated (Table 4).

Serious adverse events

Among the 25 patients with HCV infection, two had a major cardiovascular event (2 deaths from coronary heart disease) compared with 0 of the 140 uninfected patients. Of the four patients in this study who died, 3 had HCV infection. There was a lower incidence of death in the patients without HCV infection than in the infected patients, but the difference was not significant. There were no significant differences of adverse events between probucol and pravastatin therapy.

Table 4 Multiple linear regression analysis of factors influencing Max-IMT

Fixed	Difference	95% CI		P value
anti-HAV	−1.5412	−9.1996	6.1172	0.6897
HBs Ag	−3.0118	−25.3981	19.3745	0.7917
anti-HCV	9.0000	−0.6367	18.6367	0.0692
C. pneumonia-IgA	−0.6227	−10.7632	9.5178	0.9205
C. pneumonia-IgG	1.0917	−8.5300	10.7134	0.8234
C. pneumonia-IgA and/or IgG	7.5701	−6.0267	21.1669	0.2771
Age >75 years	5.3447	−3.1304	13.8198	0.2180
Male sex	−4.7624	−13.6453	4.1205	0.2959
Smoking	−4.1964	−11.9445	3.5517	0.2895
Hypertension	5.2711	−2.0015	12.5437	0.1573
Diabetes	6.2849	−3.4671	16.0369	0.2078
Backward stepwise	Difference	95% CI		P value
anti-HCV	9.5040	0.2886	18.7194	0.0448
C. pneumonia-IgA and/or IgG	8.5635	1.3738	15.7532	0.0208

DISCUSSION

The present study adds new information to the growing pool of data regarding lipid-lowering therapy for carotid atherosclerosis in patients with HCV infection. A significant reduction of Max-IMT was found in HCV-negative patients, while no significant reduction was seen in the HCV-positive patients, even though both groups of patients showed significant improvement of total cholesterol and LDL cholesterol. These results suggest that HCV infection influences the carotid artery IMT, as does *C. pneumoniae* infection. In contrast, there was no association between HAV or HBV infection and the changes of Max-IMT.

Our results are in concordance with those of another Japanese study[11] performed on company employees undergoing regular health checks that found a relationship between HCV positivity and carotid atherosclerotic plaque or IMT. We previously reported that the prevalence of HCV infection was 3.3-19.7% in northern Kyushu Island, including the area surveyed in the present study[17)~19)]. In general, the clinical course of chronic HCV infection is characterized by a series of exacerbations and remissions, but it may eventually progress to hepatic decompensation and the development of cirrhosis. Usually, the onset of cirrhosis appears to be associated with a decreased risk of atherosclerosis, which may be explained by the decreased production of clotting factors and the reduction of certain conventional risk factors such as total cholesterol and lipoprotein (a)[20]. Therefore, the positive correlation between HCV infection and carotid atherosclerosis observed in the present study was rather unexpected. Because serum total cholesterol and LDL cholesterol levels were not significantly different between our HCV-positive and HCV-negative subjects, it seems that liver dysfunction was not severe in the majority of the HCV-positive patients. Kiechl et al.[21] found no significant association between chronic hepatitis and carotid plaque, although whether their subjects had HBV or HCV infection was not specified. These different results may have been obtained because they analyzed patients with chronic active hepatitis, whereas most of the subjects in our study did not have been active.

Several previous studies have suggested that certain microorganisms may contribute