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Original Article

Impact of Statin Treatment on the Clinical Fate of Heterozygous Familial Hypercholesterolemia

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Aim: **Familial hypercholesterolemia (FH) patients are at particular risk for premature coronary artery disease (CAD) caused by high levels of low density lipoprotein (LDL). Administration of statins enabled us to reduce LDL-C levels in heterozygous FH patients. To evaluate the impact of statins on the clinical fate of heterozygous FH, a retrospective study was performed.**
Methods: **We analyzed the clinical influence of statins on age at the first clinical onset of CAD in 329 consecutive FH patients referred to the lipid clinic of the National Cardiovascular Center. Among 329 heterozygous FH patients, the onset of CAD was identified in 101.**
Results: **The age at onset of CAD was 58.8 ± 12.5 years in the 25 patients on statins at onset, significantly higher than that in the 76 patients not on statins (47.6 ± 10.5 years) ($p<0.001$). The average age at CAD onset was significantly higher after widespread use of statins (54.2 ± 13.2 years in 48 patients, Group 1) compared to before October 1989 when statins were approved in Japan (46.9 ± 9.6 years in 53 patients; Group 2, $p=0.002$). A significant difference was seen between Groups 1 and 2 in the variables, including sex, prevalence of smoking habit, LDL-C, and the use of statins, aspirin and probucol. After adjusting for these variables, only statin use was independently associated with the difference in age at CAD onset by multivariable analysis.**
Conclusion: **Statins have improved the clinical course of patients with heterozygous FH.**

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Key words; Familial hypercholesterolemia, Statin, Coronary artery disease, LDL cholesterol

Introduction

Familial hypercholesterolemia (FH) is a heritable disease of high prevalence with an autosomal-dominant mode of transmission and is linked to mutations in the low-density lipoprotein (LDL) receptor gene. It is characterized by phenotypes of the elevation of plasma LDL, cutaneous and tendenous xanthomas, arcus corneae, and coronary artery disease (CAD) due to premature atherosclerosis[1]. The earliest clinical sign of heterozygous FH is an elevation of plasma LDL-cholesterol (LDL-C), noted as early as at birth[2]. All other clinical manifestations seem due to an increase of LDL-C in plasma. CAD is the most serious clinical manifestation and determines the prognosis of FH. According to a previous report, Japanese FH heterozygotes generally develop the first CAD event in their 40s or later for men and 50s or later for women[3].

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To reduce plasma LDL-C in FH heterozygotes, bile acid-sequestering resins have been used since the 1970s to upregulate the LDL receptor, but their effect is limited to a 10 to 20% decline because of the concomitant induction of hepatic cholesterol synthesis[4]. Statins, competitive inhibitors of a rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, were introduced onto the market in the late 1980s. Pravastatin, the first approved statin in Japan, became commercially available at the beginning of October 1989 and simvastatin one year later[5]. Synthetic analogues became available in the late 1990s, including several "strong" statins, which lower the level of LDL-C by more than 40%[6]. Many large-scale clinical trials of statins worldwide, including Japan, showed that they reduced the risk of cardiac events or stroke in hypercholesterolemic populations[7-10]. Effective reduction of LDL-C by statins was also shown in FH heterozygotes[11, 12]; however, their clinical benefits in FH patients have not been clearly demonstrated with fixed clinical endpoints. This is partly because of the extremely high risk for CAD in FH patients, thus making controlled clinical trials of sufficient size to yield significant outcomes unethical.

Aim

Substantial numbers of FH patients have been referred to and regularly treated at the lipid clinic of the National Cardiovascular Center (NCVC) since it was founded in 1977. We therefore retrospectively analyzed the clinical records of these patients to assess the impact of the introduction of statins on the clinical prognosis of FH heterozygous patients, using patient age at the development of CAD. This parameter is specific and solid for each patient and the analysis is less influenced or biased by other factors. In addition, Mabuchi and colleagues used the same parameter in their study of Japanese FH reported before statin availability[13].

Methods

Subjects

Of the patients referred to the lipid clinic at NCVC from 1969 to 2007, 329 consecutive patients (139 men, 190 women) were diagnosed as FH heterozygotes using the criteria previously described[14]. Most of the FH patients analyzed in the present paper were referred to our lipid clinic by their general practitioner because of hypercholesterolemia. The medical records of patients were examined according to the analysis protocol approved by our institutional ethics committee (ID#M20-25-2). Of the 329 FH patients, 101 were identified as having CAD, specifically, coronary artery stenosis (less than 75%) on angiography, including 53 patients who had CAD at the first clinic visit. The other 228 patients did not have clinical or angiographic evidence of CAD. For each patient, the age at onset of CAD was determined by the first sign, ascertained by a standardized questionnaire, which included fixed clinical endpoints of CAD, administered by attending physicians at the clinic. The compliance with statins was evaluated from the medical records.

Clinical Risk Factors

Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (kg/m^2). Hypertension was defined as the use of antihypertensive drugs or a blood pressure level higher than 140 mmHg systolic or 90 mmHg diastolic or both at the first clinic visit (the criteria for hypertension of the Japanese Society of Hypertension Guidelines)[15]. Diabetes mellitus was defined according to the 2002 Guideline for the Treatment of Diabetes Mellitus of the Japan Diabetes Society[16]. A family history of CAD was identified by the standardized questionnaire. Smoking was identified from patients' self-reporting. Achilles tendon thickness was measured as previously described[17].

Analysis of Serum Lipids

Fasting plasma lipid concentration was measured before any lipid-lowering treatment. Total cholesterol (TC), triglycerides (TG), and HDL cholesterol (HDL-C) levels were measured enzymatically using an automated system in the clinical laboratory of the NCVC. LDL-C level was calculated by the Friedewald formula when the TG level was less than 400 mg/dL; three patients with 400 mg/dL were omitted from this particular analysis. TG values were expressed as the median, (range), and logarithmically transformed before analysis.

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 (SPSS Inc., Chicago, IL) program. Parametric values are expressed as the mean ± standard deviation (SD). The statistical significance of differences in continuous variables was evaluated by Student's t test for unpaired data or ANOVA. The Pearson's χ^2 test was used to assess differences in the distribution of categorical traits.

Results

Patient Background

The baseline clinical characteristics of the 329

Table 1. Clinical characteristics of heterozygous FH patients with or without coronary artery disease (CAD) at first visit to our center.

	Total subjects	CAD (+)	CAD (−)	*p value*
n	329	101	228	
Age (years)	43.8 ± 16.0	48.9 ± 10.2	41.6 ± 17.6	<0.001
Sex				
Men	139 (42.2%)	66 (65.3%)	73 (32.0%)	<0.001
BMI (kg/m^2)	22.0±3.2	23.0 ± 2.7	22.6 ± 3.3	<0.001
Total cholesterol (mg/dL)	319 ± 70	333 ± 85	313 ± 61	0.039
Triglyceride (mg/dL)	(114) 80–176	(147) 96–193	(109) 76–162	0.263
HDL cholesterol (mg/dL)	50 ± 17	42 ± 14	54 ± 17	<0.001
LDL cholesterol (mg/dL)	241 ± 72	259 ± 84	232 ± 65	<0.001
Hypertension (*n*, %)	54 (16.4%)	33 (32.7%)	21 (9.2%)	<0.001
Diabetes Mellitus (*n*, %)	13 (4%)	8 (7.9%)	5 (2.2%)	0.014
Family history of CAD (*n*, %)	121 (36.8%)	46 (45.5%)	75 (32.9%)	0.028
Smoking habits (*n*, %)	127 (38.6%)	72 (71.3%)	55 (24.1%)	<0.001
Achilles tendon thickness (mm)	13.5 ± 5.4	16.2 ± 5.7	12.1 ± 4.6	<0.001
CAD present at first visit (*n*, %)	53 (16.1)	53 (52.5)	0 (0)	<0.001
Statin treatment at first clinic visit	39 (11.9)	18 (17.8)	21 (9.2)	0.541

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.
BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; CAD, coronary artery disease

heterozygous FH patients analyzed in this study are shown in **Table 1**. Their plasma lipid and lipoprotein profiles are similar to patients in previous reports of Japanese FH[3, 18]. Patients with CAD were older, had higher levels of BMI, TC, and LDL-C, lower HDL-C, and a higher incidence of diabetes mellitus, hypertension, a family history of CAD, and smoking habit, compared to patients without CAD.

Onset of CAD

In the 101 patients with CAD, age by decade at the first onset of CAD is illustrated in **Fig. 1**. The average age was 45.8±10.6 years in men and 59.0±9.5 years in women, and this is consistent with a previous report of Japanese FH patients[13]. Analysis of CAD onset in relation to the presence (+) or absence (−) of statin treatment showed that in the 66 FH men with CAD, 13 did and 53 did not have statin treatment, and in the 35 FH women with CAD, 12 did and 23 did not have statin treatment. The age distribution at the first onset of CAD in statin (+) or statin (−) patients is shown in **Fig. 2**. The peak was at an older age in statin (+) men and women (Panels A and B, respectively) compared to statin (−). The lipid profile at the time of first onset of CAD in statin (+) and statin (−) patients is shown in **Table 2**. Statin (+) patients were older when CAD was identified and had lower TC and LDL-C levels than statin (−) patients.

To identify the factors that may influence the age

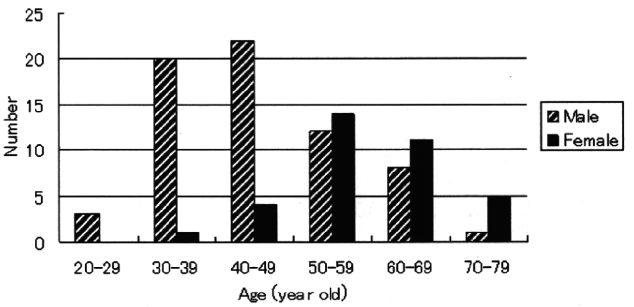

Fig. 1. Distribution of age when CAD was first identified in 101 men and women with heterozygous familial hypercholesterolemia (FH) and coronary artery disease (CAD), for the study period of 1969 to June 2007

at which CAD developed in statin (+) and statin (−) patients, we analyzed covariates (ANCOVA; **Table 3**), which included sex, smoking, BMI, hypertension, diabetes mellitus, family history of CAD, thickness of Achilles tendon, LDL-C levels, and the use of aspirin, probucol, and cholestyramine. We found that statin (+) patients were older when CAD developed, about 10 years older for each variable compared to statin (−) patients, which may be due to the use of statins and the reduction of LDL-C.

To determine the impact of statin treatment on the age at which CAD developed, we analyzed the same data for the pre- and post-statin eras. Pravastatin

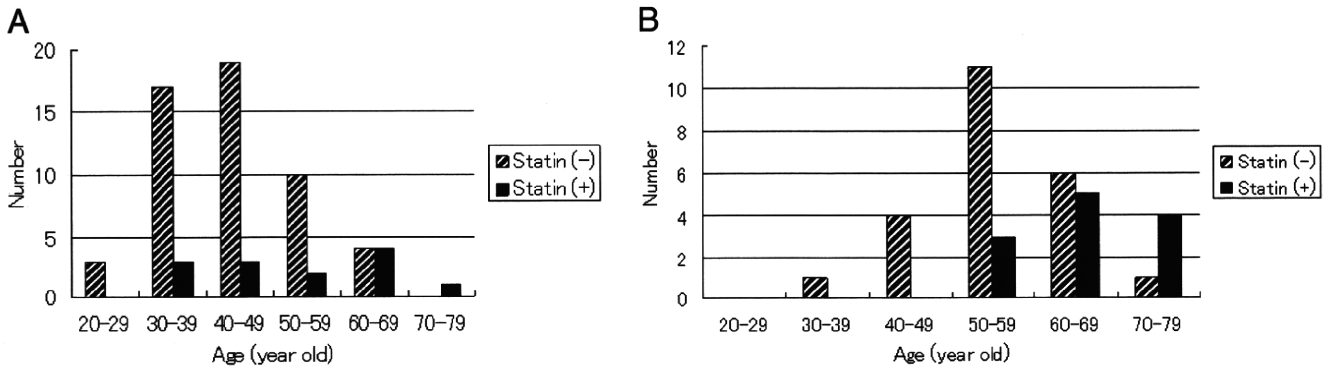

Fig. 2. Distribution of age when CAD was first identified in men (Panel A) and women (Panel B) with CAD taking a statin (+) or not (−)

Table 2. Age, lipid and lipoprotein profiles of FH at the onset of CAD in relation to statin use.

	Statin (+)	Statin (−)	*p value*
n	25	76	
Age of onset of CAD	57.8 ± 12.5	47.6 ± 10.5	<0.001
Lipid and lipoprotein profile at the event			
Total cholesterol (mg/dL)	242 ± 55	315 ± 108	<0.001
Triglycerides (mg/dL)	(127) 93-171	(115) 91-153	0.922
HDL cholesterol (mg/dL)	40 ± 12	38 ± 13	0.569
LDL cholesterol	167 ± 35	250 ± 108	<0.001

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.

Table 3. Onset age of CAD adjusted by each variable.

Variables	Age (95% CI) in Statin (+)	Age (95% CI) in Statin (−)	*p value*
Overall	57.8 (55.3-60.3)	47.6 (46.4-48.8)	<0.001
Smoking habit	58.2 (54.1-62.3)	47.3 (44.8-49.7)	<0.001
Sex	57.2 (53.3-61.0)	48.1 (45.9-50.3)	<0.001
BMI	58.9(54.4-63.3)	47.5 (45.0-50.1)	<0.001
Hypertension	59.4 (54.8-64.4)	47.4 (44.8-49.9)	<0.001
Diabetes mellitus	58.7 (54.3-63.1)	47.7 (45.2-50.3)	<0.001
Family history of CAD	58.8 (54.4-63.2)	47.1 (44.6-49.7)	<0.001
Achilles tendon thickness	58.7 (54.3-63.2)	46.7 (44.0-49.4)	<0.001
LDL cholesterol	58.4 (53.9-63.0)	47.6 (45.0-50.3)	<0.001
Aspirin	57.2 (52.9-61.5)	48.2 (45.7-50.7)	0.001
Probucol	56.0 (51.0-61.0)	48.6 (46.0-51.3)	0.017
Cholestyramine	58.2 (53.0-63.3)	47.9 (45.2-50.6)	0.001

was the first statin approved in Japan. Patients were divided into two groups: Group 1 developed CAD before the end of September 1989 (n=53) and Group 2 developed CAD from October 1989 (to June 2007; n=48). Of the 66 men with CAD, 39 were in Group 1 and 27 in Group 2, and of the 35 women with CAD, 14 were in Group 1 and 21 in Group 2. The men and women whose CAD developed after the beginning of October 1989 were older than those who developed CAD before that date (**Fig. 3A, B**). At the first clinic visit, no clinical differences were seen in these patients in average age, BMI, plasma lipid and lipoprotein profile, Achilles tendon thickness and the incidence of hypertension, diabetes mellitus, and fam-

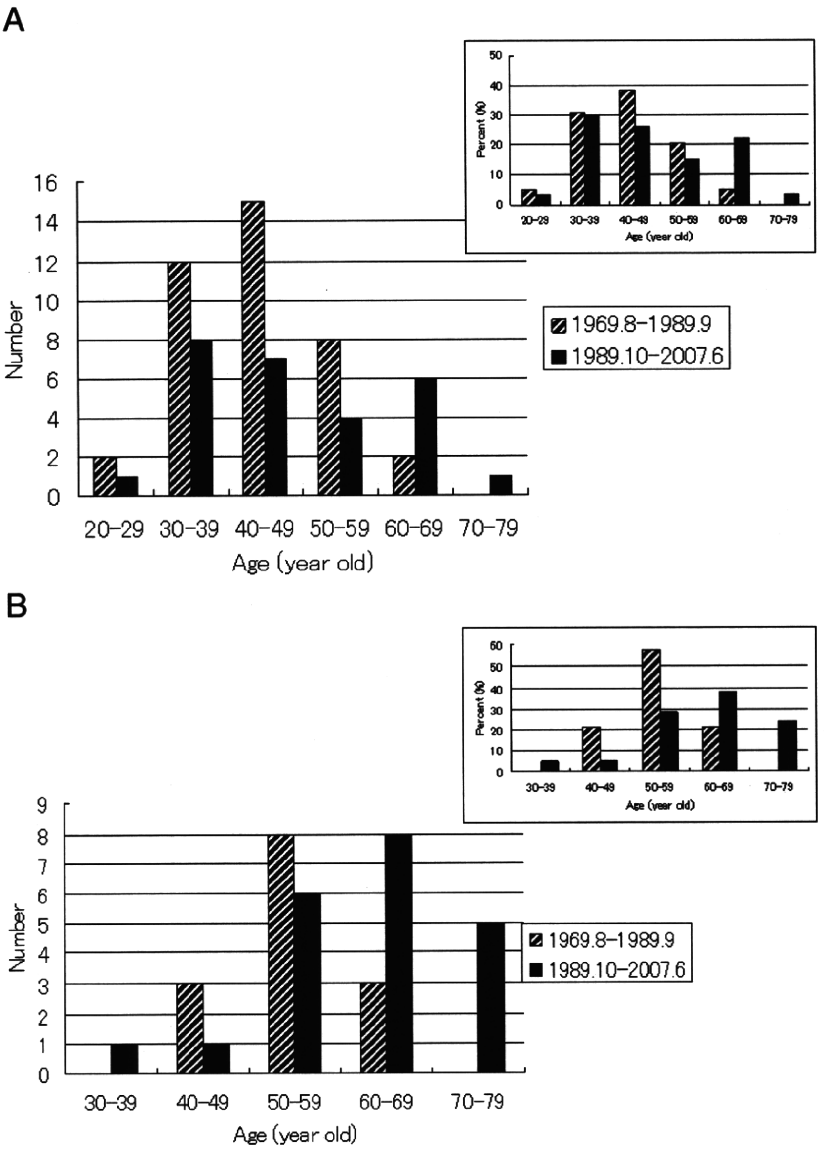

Fig. 3. Distribution of age at CAD onset in men (Panel A) and women (Panel B) who developed CAD before the end of September 1989 from October 1989

Each inset figure shows the percent of distribution, respectively.

ily history of CAD (**Table 4**); however, significantly more of the patients who developed CAD before the end of September 1989 were smokers. Assessment of clinical parameters obtained which CAD was identified shows that patients who developed CAD from October 1989 were older (**Table 5**), reflecting the influence of statins on the onset age of CAD (**Fig. 3A, B**), and that TC and LDL-C levels were lower, reflecting that more of these patients were receiving lipid-lowering treatment than patients who developed CAD before this date.

Analysis of Factors that Affect Age at the First Onset of CAD

Age at the development of CAD in Groups 1 and 2 was analyzed using analysis of covariance (ANCOVA; **Table 6**). Significant differences between groups were seen for sex, prevalence of smoking, LDL-C, and the use of statins, aspirin and probucol. After adjusting for these variables, statin use was inde-

Table 4. Clinical characteristics (at first visit) of FH Patients depending on the onset date of CAD

	Group 1 1969–Sept. 1989	Group 2 Oct. 1989–June 2007	*p value*
n	53	48	
Age	48.4 ± 9.1	49.5 ± 11.4	0.584
Sex			
Male	39 (73%)	27 (56%)	0.068
BMI (kg/m^2)	22.6 ± 2.8	23.5 ± 2.6	0.288
Total cholesterol (mg/dL)	343 ± 84	321 ± 85	0.195
Triglycerides (mg/dL)	(114) 103–193	(148) 82–208	0.785
HDL cholesterol (mg/dL)	40 ± 15	44 ± 13	0.127
LDL cholesterol (mg/dL)	268 ± 80	250 ± 87	0.279
Hypertension (*n*, %)	21 (39.6%)	12 (25.0%)	0.118
Diabetes Mellitus (*n*, %)	2 (4%)	4 (8.3%)	0.535
Family history of CAD (*n*, %)	23 (43.4%)	25 (52.1%)	0.317
Smoking habits (*n*, %)	41 (83.7%)	31 (64.6%)	0.036
Achilles tendon thickness (mm)	16.0 ± 5.3	16.5 ± 6.1	0.710

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.

Table 5. Age, lipid and lipoprotein profiles and medication of FH at the onset of CAD.

	Group 1 1969–Sept. 1989	Group 2 Oct. 1989–June 2007	*p value*
n	53	48	
Age of onset of CAD	46.9 ± 9.6	54.2 ± 13.2	0.002
Lipid and lipoprotein profile at the event			
Total cholesterol (mg/dL)	323 ± 100	267 ± 95	0.011
Triglycerides (mg/dL)	(119) 96–162	(121) 79–152	0.427
HDL cholesterol (mg/dL)	36 ± 13	41 ± 12	0.088
LDL cholesterol	257±100	199 ± 95	0.011
Medication, *n* (%)			
Statin	1 (2.0)	24 (50.0)	< 0.0001
Probucol	6 (11.8)	17 (35.4)	0.005
Cholestyramine	3 (5.7)	11 (22.9)	0.015
Aspirin	1 (2.0)	7 (14.6)	0.021
No medication	44 (83.0)	22 (45.8)	< 0.001

Values are shown as Mean ± SD except for triglyceride. For triglyceride, median (range) is shown.

Table 6. Onset age of CAD adjusted by each variable.

Variables	Age (95% CI) in Group 1	Age (95% CI) in Group 2	*p value*
Overall	46.9 (44.2–50.0)	54.2 (50.3–58.0)	0.002
Smoking habits	46.9 (43.7–50.0)	53.4 (50.2–56.5	0.005
Sex	47.9 (45.2–50.7)	53.1 (50.2–55.9)	0.013
LDL cholesterol	48.2 (44.2–52.3)	54.5 (50.8–58.2)	0.029
Statin	49.1 (45.8–48.3)	51.8 (48.3–55.4)	0.325
Aspirin	47.9 (44.8–51.0)	53.2 (50.0–56.4)	0.021
Probucol	48.1 (45.0–51.2)	53.0 (49.8–56.2)	0.034
Cholestyramine	47.6 (44.4–50.8)	53.6 (50.2–56.9)	0.013

pendently associated with age at the onset of CAD.

Discussion

The mortality rate for CAD is 11 times higher in heterozygous FH patients than in the general population; thus, prevention of CAD is the key therapeutic goal for these patients[14]. Treatment to reduce high levels of LDL-C in FH patients was limited before statins became available, and a clinically meaningful decrease in LDL-C levels was difficult to obtain. Pravastatin was first introduced onto the Japanese market at the beginning of October 1989 and thereafter, LDL-C reductions of 20% to 30%, even in FH heterozygous patients, became possible[19]. Recently, the risk of myocardial infarction in heterozygous FH was reported to be reduced by 76%, similar to the general population of the Netherlands[20]. In the present paper, we assessed the impact of statin use on the clinical prognosis of Japanese FH patients visiting our lipid clinic by retrospectively analyzing their clinical records. The use of statins delayed the first CAD event by about 7 years in FH patients whose first event occurred after the introduction of statins, compared to FH patients whose first event occurred prior to the introduction of statins.

In this study, 101 of 329 (30.6%) consecutive heterozygotes of FH had clinical evidence of CAD. The profile of CAD patients is similar to previous reports, that is, more men than women[3, 21, 22], and higher BMI, higher TC and LDL-C levels, lower HDL-C levels, and a higher incidence of hypertension, diabetes mellitus, family history of CAD, and smoking[3, 13, 23, 24].

The time span of our study allowed us to assess the impact on the development of CAD of the introduction of statins onto the Japanese market at the beginning of October 1989. Comparing clinical parameters at the first clinic visit the patients whose CAD developed before the end of September 1989 with after that date, revealed that only smoking was different, perhaps reflecting the social trend against smoking (**Table 4**). In contrast, interesting differences between these groups were seen in relation to when they developed CAD. Patients who developed CAD prior to the introduction of statins were younger on average (47.6 years old) and had higher levels of TC and LDL-C (6.2 and 4.3 mmol/L, respectively). Two other prominent differences were the improved lipid-lowering drug regimens, including statins, cholestyramine, probucol, and aspirin, and a decline in the number of smokers. Notably, statin use was independently and significantly associated with age at CAD onset in the 101 FH patients on covariate analysis of factors known to affect the age of developing CAD. Besides these factors, many other factors should be considered for the potential influence on the onset age of CAD, such as the widespread recognition of FH and the regimen for the treatment of other risk factors, such as hypertension and diabetes mellitus. Nevertheless, we should conclude from this analysis that the use of statins is a major factor contributing to the improvement of the clinical prognosis of FH patients in Japan.

More recently, "strong" statins have become available, making it possible to reduce LDL-C levels to much lower levels compared to conventional statins in FH patients[25-27]. The possible impact of these stronger statins on delaying the development of CAD in FH patients will be of interest.

One diagnostic criterion for heterozygous FH in the existing guidelines is a family history of premature CAD[28-30]. However, our results suggest that this criterion may need to be reconsidered because of the proven ability of statin treatment to delay the development of CAD to an age similar to that in persons who do not have heterozygous FH.

We showed in this retrospective analysis that the development of CAD was delayed by about 7 years in FH patients whose CAD developed after the introduction of statins in Japan compared to those whose CAD developed before the current statin era.

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In vivo siRNA delivery with dendritic poly(L-lysine) for the treatment of hypercholesterolemia†‡

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Intravenous delivery of apolipoprotein B-specific siRNA with a sixth-generation of dendritic poly(L-lysine) (KG6) resulted in siRNA-mediated knockdown of ApoB in healthy C57BL/6 mice without hepatotoxicity, and with a significant reduction of serum low-density lipoprotein cholesterol in apolipoprotein E-deficient mice.

siRNA-mediated specific gene silencing has generated great interest in its use as a research tool and as a therapeutic agent for a wide spectrum of disorders that include cancer, infectious disease, and metabolic conditions. Effective *in vivo* siRNA delivery is essential for siRNA-based applications and a variety of nonviral and viral systems are being developed.[1]

Apolipoprotein B (ApoB) is an essential protein for the formation and secretion of very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) in the metabolism of dietary and endogenous cholesterol. ApoB is expressed in hepatocytes. Elevated ApoB and LDL levels are correlated with increased risk of coronary artery disease (CAD), and ApoB and LDL-cholesterol (LDLc) levels are suggested to be controlled in patients with a high risk of CAD. Statins, a class of drugs that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are currently widely used to reduce this risk. Although statins are effective cholesterol-lowering drugs, two-thirds of statin-treated patients still experience adverse coronary events. In addition, LDLc levels of homozygous familial hypercholesterolemia can't be controlled by oral administration, and extracorporeal treatment is needed for these patients. Therefore, new alternative strategies are needed.[2] ApoB is too large a protein to collect the 3D structural data; accordingly, it is not a suitable target for conventional small-molecule drug development. Therefore, as an alternative strategy, ApoB silencing by RNAi is quite promising.

Once *in vivo*, intravenous delivery of cholesterol-conjugated siRNA against ApoB (si–ApoB) and subsequent reduction of serum LDLc had been achieved,[3,4] *in vivo* ApoB knockdown experiments were reported, using various siRNA delivery systems. Lipid-based systems, known as stable nucleic acid–lipid particles (SNALP) and interfering nanoparticles (iNOP), have increased nuclease stability and decreased the injection dose of siRNA to clinically relevant levels.[5,6] A polymer-based system called Dynamic PolyConjugates, which is functionalized with a *N*-acetyl-galactosamine ligand for hepatocyte targeting and linked to siRNA with a disulfide bond for reductive release, offers efficient and nontoxic delivery of siRNA.[7] Recently, a new class of lipid-like delivery agents, termed lipidoids, was synthesized and evaluated for safe and effective *in vivo* siRNA delivery.[8] The efficiency to deliver siRNA to hepatocytes was evaluated based on the changes of ApoB mRNA levels after injection of si–ApoB formulated in various lipidoid nanoparticles. Thus si–ApoB is not only a new therapeutic agent for hypercholesterolemia, but also a research tool available for characterization of newly developed hepatocyte-targeting carriers in terms of transfection efficiency.

We previously reported that a sixth generation of dendritic poly(L-lysine) (KG6, Fig. 1),[9] consists of amino acid, and has advantages in having a monodispersed structure that can be synthesized using easy protocols. It has a high plasmid DNA transfection ability with low cytotoxicity *in vitro*.[9–12] The efficiency was comparable to commercially available transfection reagents such as Lipofectin, JetPEI, and Superfect. In particular, during plasmid DNA transfection, the addition of chloroquine, which enhances endosome escape to the cytosol, was not required, and serum could be added to the medium during transfection. It can be concluded that KG6 is a simple and highly efficient transfection reagent like the commercially available transfection reagents that work *in vitro*. KG6 can be also used as a siRNA carrier into cells. After adding the siRNA complex of KG6 to cultivated cells, efficient uptake of the siRNA into the cells was observed with low cytotoxicity. The efficiency was higher and the cytotoxicity was lower than those of Lipofectamine 2000, a commercially available transfection reagent.[13] We recently evaluated the performance of this promising molecule, KG6, as a gene carrier *in vivo*.[14] We investigated the biodistribution of plasmid DNA delivered with KG6 in mice after intravenous administration. Southern blotting analysis revealed that more than 20% of the plasmid

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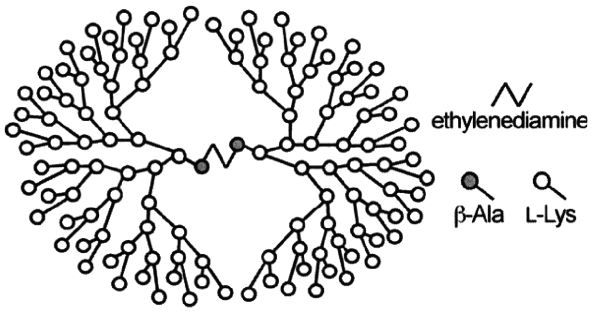

Fig. 1 The structure of the sixth-generation dendritic poly(L-lysine) (KG6).

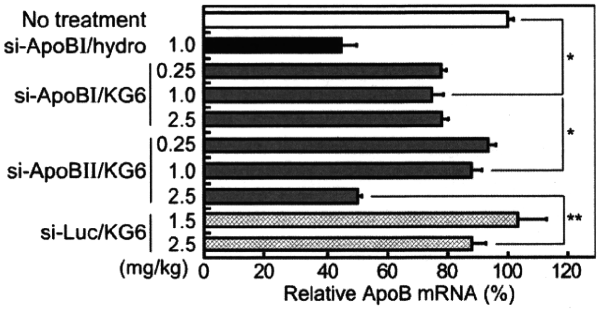

Fig. 2 Reduction of ApoB mRNA levels in the liver treated with the siRNA complexes of KG6. RT-qPCR of liver ApoB levels relative to GAPDH was performed 24 h after injection of si–ApoBI, si–ApoBII or si–Luc with KG6 as a carrier (siRNA/KG6), or by hydrodynamic-based administration as a positive control (siRNA/hydro). Data were normalized to untreated mice. Significances of differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$). Data are means ± S.E. ($n > 3$).

DNA had accumulated in the liver and 10% of the DNA complexes with KG6 at a cation/anion (C/A) ratio of 8.0 remained circulating in the blood for 30 min after intravenous injection. Almost all of the DNA circulating in the blood would also be trapped in the liver and then gradually degraded. Thus the hepatotropic accumulation of intact plasmid DNA is one of the noteworthy characteristics of KG6. Based on these characteristics, efficient siRNA delivery to hepatocytes should be possible using KG6.

We started with siRNA complex of KG6 at C/A ratio of 8.0 formed in 5% dextrose, and the particle size of the complex was determined by dynamic light scattering. The size was 168 ± 9.9 nm (Table S1, ESI‡). We had previously confirmed that the size of the pDNA complex of KG6 at the C/A ratio of 8.0 was 238 ± 5.2 nm.[9] In addition, we determined the electrophoretic mobilities of the siRNA complexes of KG6 at different C/A ratios using 20% native polyacrylamide gel electrophoresis in TBE buffer at pH 8.0. No migration of the siRNA band occurred at a C/A ratio of 1.0 or above (Fig. S1‡). For the KG6 pDNA complex, electrophoresis retardation occurred at the same C/A ratio.[9] Therefore, the characteristics of the siRNA complex of KG6 were expected to be similar to those of the pDNA complex, and hepatotropic accumulation of the siRNA complex after intravenous injection was anticipated.

We next determined the ability of KG6 to deliver siRNA to hepatocytes and to silence ApoB expression *in vivo*. The si–ApoBI complex, si–ApoBII complex, and luciferase-specific siRNA (si–Luc) complex as a control, were delivered to C57BL/6 mice by intravenous injection. Livers from injected mice were harvested 24 h after the injection and assayed for ApoB mRNA levels by reverse transcriptase quantitative PCR (RT-qPCR). The ApoB mRNA levels were relative to the level of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. As shown in Fig. 2, the ApoB mRNA levels in mice treated with the si–ApoBI complex were reduced by approximately 22% compared with those in the no-treatment group, at all of the doses evaluated. si–ApoBII complex showed significant reduction of the mRNA. Especially, a 50% reduction was observed at 2.5 mg kg^{-1} dose, and it was a significantly stronger effect compared with the case of si–Luc (2.5 mg kg^{-1}), which showed a slight reduction.

The reduced levels of ApoB mRNA mediated by si–ApoBI were lower than those previously reported using the SNALP and iNOP systems (approximately 77% and 50% reduction, respectively).[5,6] In the case of si–ApoBII, approximately 87% reduction was observed by using Dynamic PolyConjugates.[7] The difference in efficiencies between our KG6 system and their systems may be due to the chemical structure of the siRNA. In their systems, the siRNAs contained a 2′-*O*-methyl (2′-OMe), 2′-fluoro modification (2′-F) or a phosphorothioate (PS) linkage.[5–7] In general, chemical modification of siRNA not only improves stability but also reduces off-target effects.[15] Furthermore, appropriate modifications do not reduce the siRNA activity and the 2′-OMe modification, in particular, is calculated to maintain siRNA activity by retaining the canonical right-handed A-form helical geometry required for RNAi. Here, we adopted to deliver unmodified siRNAs using KG6. Therefore, it is considered that our less active and stable si–ApoB would result in the lower efficiency of ApoB knockdown than that achieved using the lipid- and polymer-based systems mentioned above, and si–Luc, as a negative control, might nonspecifically interfere in its expression in a dose-dependent manner.[16,17]

The other reason is that the ability of KG6 to deliver siRNA to hepatocytes might be insufficient. In fact, ApoB mRNA levels reduced by 55% in mice administered with only 1.0 mg kg^{-1} of chemically unmodified si–ApoBI by a hydrodynamic injection, which is a positive control procedure to deliver an siRNA gene or oligonucleotide to hepatocytes.[18] The insufficient delivery with KG6 could be attributed to the size of the complex. It has been suggested that complexes of > 100–200 nm would be restricted by the fenestrations in liver blood vessels and thus unable to access the hepatocytes.[19] As the particle size of siRNA complex of KG6 is larger than 100 nm (Table S1, ESI‡), some of the complexes would be unable to cross the fenestrations and become trapped in the Kupffer cells.

The potential toxicity of the KG6 siRNA complex was assessed by measuring the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are two enzymes located in liver cells and which leak into the circulation when liver cells are injured. Slight elevations of AST and ALT activities were detected in mice treated with 2.5 mg kg^{-1} of the KG6 siRNA complex compared with untreated mice at 24 h after injection. The levels of AST and

ALT (IU L^{-1}) were as follows: 19.4 ± 1.2 and 6.1 ± 0.5 respectively, in untreated mice, and 22.7 ± 1.1 and 10.7 ± 0.4, respectively, in mice injected with the complex. Because the increased levels were not significant, the intravenous delivery of siRNA with KG6 appears to be well tolerated.

Finally, improvement of hypercholesterolemia was observed after treating apolipoprotein E (ApoE)-deficient ($ApoE^{-/-}$) mice with the KG6 siRNA complex. ApoE is a ligand for cell-surface lipoprotein receptors such as the LDL-receptor (LDLr) and LDLr-related proteins (LRP). Homozygous deletion of the *ApoE* gene in mice results in a pronounced increase in the plasma levels of LDL and VLDL, which is attributable to the failure of LDLr- and LRP-mediated clearance of these lipoproteins.[20] $ApoE^{-/-}$ mice are one of the most widely used hypercholesterolemic mouse models. In ApoB knockdown experiments, it was reported that intraperitoneal administration of a mouse-specific ApoB antisense oligonucleotide, ASO ISIS 147764, to $ApoE^{-/-}$ mice reduced the plasma levels of LDLc by 40%.[21] Here, we focused on si–ApoBI, which matches with not only mouse ApoB mRNA but also human's, while si–ApoBII has a mismatch with human ApoB mRNA.[3,7] We intravenously injected $ApoE^{-/-}$ mice (weighing 30–40 g) with 50 μg of the KG6 si–ApoBI complex. A single dose of the si–ApoBI complex resulted in a decrease in VLDLc and LDLc levels for up to 96 h, whereas no decrease in their levels was observed with the 5% dextrose only or the si–Luc complex (Fig. 3). Change of total serum cholesterol was similar to that of VLDLc and LDLc (data not shown). We also determined the ApoB mRNA levels 96 h after injection and found that the ApoB mRNA levels remained reduced only in the si–ApoBI-treated group (reduction in ApoB mRNA levels: 24.6 ± 2.6% *vs.* 7.6 ± 9.4%, respectively). These results indicate that systemic siRNA delivery with KG6 could provide a clinically useful; approach to reducing cholesterol levels in patients with hypercholesterolemia.

In summary, our results show that KG6 is a promising carrier to deliver siRNA to silence endogenous genes in clinically acceptable doses and improve hypercholesterolemia. In prior reports in which si–ApoB was delivered *in vivo*, a reduction in serum total cholesterol levels in healthy C57BL/6 mice was observed.[3–7] However, mice lack the cholesterol ester transferase protein (CETP) and carry the majority of their plasma cholesterol as HDL. Because plasma lipoprotein compositions in $ApoE^{-/-}$ mice resemble those in patients with hypercholesterolemia more closely than in C57BL/6 mice, our ApoB knockdown experiment with siRNA in $ApoE^{-/-}$ mice is valuable for assessing RNAi therapeutics to target hypercholesterolemia. For clinical use of KG6, however, further investigations of the KG6 complex in terms of its physical properties and physiological effects are needed. Recently, it was suggested that an electrostatically formed complex of siRNA differs from pDNA in terms of encapsulation efficiency, stability and other physical properties.[22] As we have investigated the biodistribution of plasmid DNA delivered with KG6, an assessment of the difference between plasmid DNA and siRNA should be performed.

Our results also indicate that KG6 is applicable for intravenous delivery of other types of therapeutic oligonucleotides such as antisense and decoy oligonucleotides to the liver.

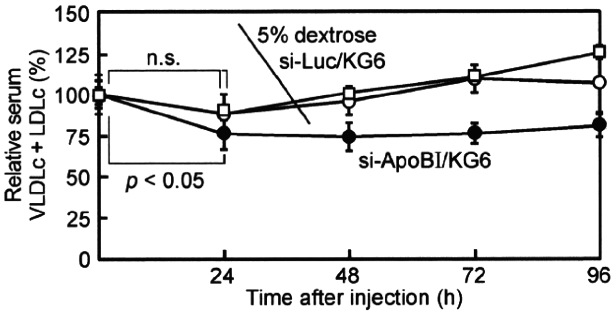

Fig. 3 Therapeutic effect of the KG6 si–ApoBI complex in a mouse model of hypercholesterolemia. $ApoE^{-/-}$ mice (30–40 g) were injected intravenously with 50 μg of the si–ApoBI complex of KG6 (closed circles), si–Luc complex (open circles) or 5% dextrose only (open squares) ($n > 3$, respectively). Serum was obtained from 6-hour-fasted mice every 24 h after administration for determination of LDLc and VLDLc levels. Values represent the rate of change in each cholesterol level. Data are shown as means ± S.E. Significances of differences from each group before treatment are indicated. n.s. indicates no significance.

In addition, precise functionalization of the surface primary amino groups can be achieved by modification with hydrophilic polymer such as a polyethylene glycol for stable circulation in blood flow,[23] glycation and peptide ligand modification for site-specific delivery.[7,24] Thus KG6 is expected to be a base molecule for efficient and targeted nucleotide delivery *in vivo*.

Experimental

Chemicals and instruments

Organic solvents used in all synthetic procedures and RNA extraction, ethylenediamine and transaminase CII-test Wako were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N*-Boc-protected β-alanine and lysine were purchased from Novabiochem, Merck Ltd. (Tokyo, Japan). The coupling reagents, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazol (HOBt) were purchased from Watanabe Chemical (Hiroshima, Japan). Trifluoroacetic acid (TFA) was purchased from Kanto Chemical (Tokyo, Japan). siRNAs were purchased from Gene Design Inc. (Osaka, Japan). RNA iso Plus, SYBR PrimeScript RT-PCR Kit II (Perfect Real Time), and primers for RT-qPCR were purchased from Takara Bio Inc. (Shiga, Japan). The particle sizes of the KG6 siRNA complexes were measured with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom). RT-qPCR was performed with a LightCycler ST300 (Roche Diagnostics, Basel, Switzerland). Plasma lipoproteins were analyzed by high-performance liquid chromatography (HPLC) using molecular sieve columns, Skylight Biotech, Inc. (Akita, Japan).

siRNAs

The siRNAs had the following sequences: si–ApoBI, sense 5′-GUCAUCACACUGAAUACCAAU-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGACAC-3′;[3–5] si–ApoBII, sense 5′-GAAUGUGGGUGGCAACUUUAG-3′, antisense 5′-AAAGUUGCCACCCACAUUCAG-3′;[7] si–Luc (GL-3 luciferase

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reporter gene), sense 5′-CUUACGCUGAGUACUUCGAUU-3′, antisense 5′-UCGAAGUACUCAGCGUAAGUU-3′.[7]

Synthesis of dendritic poly(L-lysine)

Dendritic poly(L-lysine) was synthesized as previously described.[7] In brief, for the initial core synthesis, *N*-Boc-protected β-alanines were coupled with ethylenediamine in DMF by the HBTU-HOBt method, and deprotection was then performed by TFA treatment. For the synthesis of the first and higher generations, the coupling reaction between the amino group-free previous generation of dendrimers and *N*-Boc-protected lysines was performed in DMF by the HBTU–HOBt method, and Boc-groups were then removed by TFA. We synthesized dendrimers up to the sixth generation (KG6) by repetition of these coupling and deprotection procedures. The molecular weights of these synthesized dendrimers were measured by MALDI-TOF-MS (data not shown).

Preparation of the siRNA complex

For administration of appropriate amounts of siRNA (if we consider the amount as X μg), X μl of 1.0 μg μl^{-1} siRNA was added to 60 μl of 25% dextrose. After the addition of (240 − 2X) μl of water, X μl of 0.2 mM KG6·128TFA salt solution was added to the mixture and incubated for 10 min at room temperature to form KG6 siRNA complexes at a C/A ratio of 8.0. The C/A ratio means a molar ratio of cationic amino groups (KG6)/anionic phosphate groups (siRNA). All aqueous solutions were prepared with DEPC-treated water.

Animals

Male C57BL/6N mice (7-weeks old, 19–21 g) were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). Male $ApoE^{-/-}$ mice on C57BL/6J background mice (8-weeks old) were obtained from Jackson Laboratories (Bar Harbor, Me., USA). All animal experiments were carried out in accordance with Guidelines for the Animal care and Use committee, Kyushu University and the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Mice were housed in a room at 24 ± 2 °C with a 12 h light–dark cycle before being used in the experiments. Food and water were available *ad libitum*.

In vivo siRNA administration procedure

For intravenous administration, 300 μl of KG6 siRNA complexes at a C/A ratio of 8.0 in 5% dextrose was injected *via* the tail vein over 5 s. For hydrodynamics-based administration, which is an established method for *in vivo* gene or oligonucleotide transfer to mouse liver,[16] mice were injected *via* the tail vein with a volume equivalent to 10% of the body weight within 5 s.

Liver harvest, RNA isolation and RT-qPCR assay

Twenty-four hours after injection, mice were sacrificed and perfused *via* the portal vein before the liver was harvested. Total RNA was isolated from the liver immediately after harvest using RNA iso Plus and reverse transcribed using the PrimeScript RT Reagent Kit according to the manufacturer's protocol. Relative quantification of the target gene mRNA compared with the housekeeping gene GAPDH mRNA was determined by quantitative PCR assay using SYBR Premix Taq II and gene-specific primers on a LightCycler ST300 according to the manufacturer's protocol.

Hepatotoxicity analysis

Three hundred μl of KG6 siRNA complexes at a C/A ratio of 8.0 in 5% dextrose was administered *via* the tail vein to 7-week-old male C57BL/6N mice. Blood was collected 24 h after administration. Serum was prepared by incubation of collected blood for 30 min at room temperature and centrifugation at 2000 × *g* for 20 min at 4 °C. The serum AST and ALT activities were measured using a transaminase CII-test Wako.

Determination of plasma lipoprotein compositions

Three hundred μl of the complexes containing 50 μg of siRNA at a C/A ratio of 8.0 in 5% dextrose was administered *via* the tail vein to $ApoE^{-/-}$ mice (31, 34, 40 or 52 weeks old, 30–40 g). Mice were starved for 6 h and blood was collected from a tail vein every 24 h after administration. Serum HDL or LDL/VLDL cholesterol levels were measured in each blood sample, analyzed by HPLC.[25]

Statistical analysis

Treatment effects were analyzed by one-way ANOVA. For statistically significant F-values, means were compared by Fisher's multiple range tests. All data described in context also follow this analytic method and are shown as means ± S.E.

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Opinion

Proposed Guidelines for Hypertriglyceridemia in Japan with Non-HDL Cholesterol as the Second Target

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The Japan Atherosclerosis Society (JAS) guidelines for the prevention of atherosclerotic diseases, proposing management for LDL cholesterol as the primary target, have successfully contributed to the prevention of cardiovascular events; however, recently, the impact of hypertriglyceridemia as an additional cardiovascular risk has become understood, especially in light of the rise in obesity, metabolic syndrome, and diabetes in the Japanese population. Rather than waiting to obtain conclusive domestic data confirming that hypertriglyceridemia is a cardiovascular risk factor and that its management is efficacious, we propose guidelines for hypertriglyceridemia using non-HDL cholesterol as a second target.

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Key words; Hyperlipidemia, Dyslipidemia, Triglycerides, HDL cholesterol, LDL cholesterol

Introduction

Many prospective epidemiological studies have indicated a positive relationship between serum triglyceride (TG) levels and the incidence of coronary heart disease (CHD)[1, 2]. TG-rich lipoproteins such as remnant lipoproteins and small dense LDL particles are increased in hypertriglyceridemia and have been established to be atherogenic by numerous clinical and experimental studies[3-6]; however, classification of the plasma TG level as an independent risk factor for atherosclerosis has been controversial. This is partly because plasma TG levels are inversely intercorrelated by other well-established risk factors, such as low HDL cholesterol. To date, large scale trials for intervention targeting plasma TGs with TG reducing agents such as fibrates have not reached definitive conclusions about their effectiveness on primary endpoints, although fibrates have some impact on both primary and secondary prevention in small scale studies[7-9].

The precise estimation of plasma TGs as a cardiovascular risk is confounded by other risk factors, such as obesity, diabetes, hypertension and smoking. In addition, a cluster of metabolic risk factors, such as visceral obesity and insulin resistance with hypertriglyceridemia, referred to as metabolic syndrome, indicates that plasma TG concentrations are tightly linked to other strong risk factors for CHD. Thus, patients with elevated TGs are at increased risk for CHD, although greater risk cannot be independently explained by TGs. Meanwhile, recent meta-analyses suggested that plasma TGs could be an independent factor for CHD[1, 2]. Supportively, many experimental studies indicated that triglyceride-rich lipoproteins as well as LDL are atherogenic. Taken together, these data suggest that hypertriglyceridemia should be regarded as a semi-independent risk factor and should be included as a clinical target for the prevention of CHD. Considering the increasing prevalence of obesity, metabolic syndrome, and diabetes in this country, guidelines specialized for patients with hypertriglyceridemia need to be immediately established. In this study, we propose new guidelines for Japanese patients with hypertriglyceridemia

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Table 1. Plasma lipid profile of severe and mild type IIb hyperlipidemic patients sub-grouped by non-HDL cholesterol level

Male	severe type IIb non-HDLc >190 mg/dL	mild type IIb non-HDLc <190 mg/dL	*p*
n	51	54	
Total Cholesterol	270±41.8	234±40.3	0.001
Triglycerides	347±286	236±110	0.031
HDL Cholesterol	42.4±8.0	54.9±15.2	0.000
LDL Cholesterol	159±51.6	135±38.1	0.029
non-HDL Cholesterol	228±41.6	182±39.1	0.000
Female	**severe type IIb non-HDLc >180 mg/dL**	**mild type IIb non-HDLc <180 mg/dL**	***p***
n	52	48	
Total Cholesterol	265±29.6	231±20.2	0.000
Triglycerides	242±120	218±56	0.1
HDL Cholesterol	47.3±14.1	63.2±19.5	0.000
LDL Cholesterol	175±40.4	125±17.9	0.000
non-HDL Cholesterol	224±30.2	168±14.9	0.000

Subjects were patients who visited the outpatient clinic of the Endocrinology and Metabolism Unit of Tsukuba University Hospital on a regular basis (monthly or bimonthly) as described in Materials and Methods. Data are the means±SD (mg/dL).

using non-HDL as a secondary target after the goal for LDL cholesterol as the primary target is achieved.

Materials and Methods

A total of 1,124 patients in Tsukuba University hospital in 2006 were consecutively included in the study (**Table 1**). Patients with severe illness were excluded. Plasma total cholesterol (TC), LDL-C, TG, HDL-C, glucose and HbA1c in either the fasted or fed state were determined enzymatically with the Hitachi 7070. Plasma HDL-C concentration was measured by a direct method using polyethylene-glycoso-pretreated enzymes. We calculated LDL-C concentration with Friedewald's formula (TC-TG/5-HDL-C) when TG was less than 400 mg/dL. Plasma non-HDL-C concentration was calculated as TC−HDL-C. One hundred and five male and 100 female patients were diagnosed with Type IIb hyperlipidemia (TC>220 mg/dL and TG>150 mg/dL). They were subcategorized into two groups according to their non-HDL cholesterol level (**Table 1**).

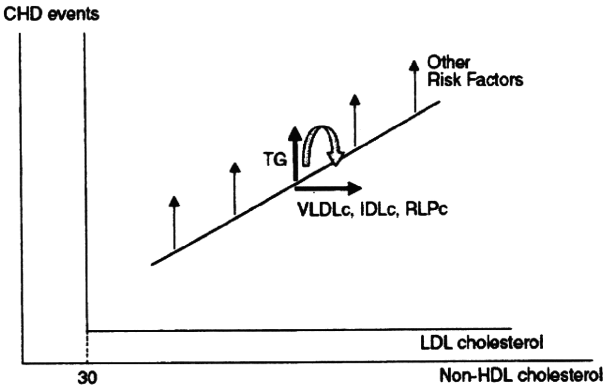

Fig. 1. Rationale for usage of non-HDL cholesterol: impact of TG and other risk factors on correlation between LDL-cholesterol CHD event.

nonHDL cholesterol = Total cholesterol − HDL cholesterol = VLDL cholesterol + IDL cholesterol (remnant lipoprotein cholesterol) + LDL cholesterol (Friedewald formula).
VLDL cholesterol + IDL cholesterol (RLP cholesterol) = TG/5
The risk of hypertriglyceridemia is approximated to VLDL, IDL, and RLP cholesterol estimated as TG/5, and incorporated into non-HDLC. The difference between non-HDL cholesterol and LDL cholesterol on X-axis was set up at 30 mg/dL based upon the data from Fig. 2.

Results and Discussion

Advantage of Non-HDL Cholesterol as a Marker for Hypertriglyceridemia

LDL cholesterol has been established as the most potent predictor of CHD and is currently the primary target for treatment and prevention. Other risk factors, including TG, diabetes, obesity, and metabolic syndrome, do not directly elevate plasma LDL cholesterol, but could enhance the risk of LDL cholesterol by shifting up the curve, as depicted in **Fig. 1**. To evaluate and manage the risk of hypertriglyceridemia, the TG level must be interpolated into the risk of plasma cholesterol. In patients with high TGs, most VLDL cholesterol resides in the smaller (remnant) VLDL fraction. Cholesterol of remnant lipoproteins (VLDL and IDL), which is concomitantly increased by elevation of plasma TG is an appropriate surrogate marker of hypertriglyceridemia. TG-rich remnant lipoproteins have been established as atherogenic lipoproteins[4, 5]. Thus, RLPc, a commercially available laboratory test for remnant lipoprotein cholesterol, could be a suitable marker for the atherogenicity of hypertriglyceridemia; however, this test is expensive and is not practical for use as a routine parameter. In contrast, non-HDL cholesterol, defined as total cholesterol − HDL cholesterol, is easily calculated, and represents the sum-

mation of VLDL/IDL (remnant) cholesterol and LDL cholesterol. It reflects the risks for all apoB-containing lipoproteins and could be an excellent marker for atherogenic lipoproteins. Plasma TG itself is not an appropriate marker for CHD risk due to its internal and dietary variability. In contrast, non-HDL cholesterol is not affected by dietary states and has much less daily variability than TG.

Predictive Power of Non-HDL Cholesterol

Non-HDL cholesterol reflects the risks of both hypertrigyceridemia and LDL-cholesterol[10, 11]. Several studies have indicated that non-HDL cholesterol is better than LDL cholesterol in its predictive power of cardiovascular diseases, indicating that VLDL cholesterol could contribute to CVD[12]. Non-HDL cholesterol is also a useful marker in a variety of subpopulations: men, the elderly, and patients with high-risk diseases such as diabetes and end-stage renal disease[13-16]. Our current clinical data from patients with type IIb hyperlipidemia also support the usefulness of non-HDL cholesterol (**Table 1**). In our outpatient clinic, 70% of patients had diabetes and roughly 10% were type IIb hyperlipidemia (cholesterol > 220 mg/dL and TG > 150 mg/dL). These type IIb hyperlipidemic patients were equally divided into two sub-groups: severe (non-HDL cholesterol levels ≥ 190 mg/dL for male patients and 180 mg/dL for female patients) and mild < 190 mg/dL for male patients and 180 mg/dL for female patients. When the severe and mild IIb groups were compared, total, LDL, HDL cholesterol, and TG levels were significantly different among these two groups for both genders, except for serum triglyceride in females (**Table 1**). These data indicate that non-HDL cholesterol is an excellent marker representing all the components of dyslipidemia. The usefulness of non-HDL cholesterol rather than low-density lipoprotein cholesterol as a tool for lipoprotein cholesterol screening and assessment of risk and therapy has been already recognized in the USA[17, 18]. Another candidate marker for both remnant and LDL cholesterol is plasma apoB level[19]. ApoB is a direct marker for the particle number of apoB-containing lipoproteins and reflects risks of both remnants and LDL. Non-HDL cholesterol is highly correlated with apoB, and should replace this specialized and expensive laboratory test despite some reports indicating that apoB is better than non-HDL cholesterol for the predictive power of CHD[13, 20].

However, according to the Friedewald formula, the TG risk in non-HDL cholesterol represents only one fifth of TG levels as remnant cholesterol, and thus, the contribution of the risk is relatively weak compared to that of LDL cholesterol. Our previous data indicated that the correlation of non-HDL cholesterol to LDL cholesterol was much stronger than that to the TG level (**Fig. 2**)[21]. It should be noted that non-HDL cholesterol is not a specific marker for hypertriglyceridemia. Rather, non-HDL cholesterol should be regarded as a general single marker for both hypercholesterolemia and/or hypertriglyceridemia.

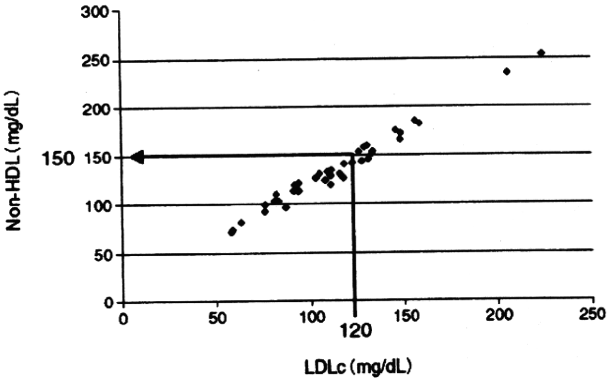

Fig. 2. Distribution of non-HDL cholesterol vs. calculated LDL cholesterol in normolipidemic patients.

Non-HDL cholesterol and LDL cholesterol calculated from Friedewald formula were highly correlated. Subjects were from the outpatient clinic of Tsukuba University Hospital[21].

Proposed Guidelines for Hypertriglyceridemia

Based upon these considerations, we propose guidelines for hypertriglyceridemia in Japanese patients using non-HDL cholesterol as a secondary target, as shown in **Table 2**. This is an extended version of the 2007 edition of the Japan Atherosclerosis Society (JAS) guidelines for the prevention of atherosclerotic diseases in which LDL cholesterol is the primary marker and target. It is essentially similar to the AHA-ATPIII guidelines for hyperTG in USA[22]. ATPIII recommends using non-HDL cholesterol as a secondary target when plasma TG is greater than 200 mg/dL because VLDL cholesterol is not significantly accumulated if TG is less than 200 mg/dL[23]. We do not have enough clinical data for Japanese on the relationship between TG and VLDL cholesterol to provide the appropriate TG level where the use of a non-HDL marker should be considered. Currently, we recommend using non-HDL for patients with hypertriglyceridemia (TG > than 150 mg/dL). Even for patients with hypertriglyceridemia, the primary target is still LDL cholesterol. In the 2007 JAS guidelines, goals of LDL for the secondary prevention group and the primary prevention group with category I, II, and III are 100, 120, 140, and 160 mg/

Table 2. Proposed Japanese Guidelines for Hypertriglyceridemia

Treatment	Categories		Goal for plasma lipids (mg/dL)		
		Coronary Risk Factors other than LCL-C	Primary LDL-C	Secondary nonHDL-C	HDL-C
Primary Prevention Improving lifestyle as the first line, followed by medication	I (Low Risk Group)	0	<160	<190	≥40
	II (Intermediate)	1~2	<140	<170	
	III (High)	≥3	<120	<150	
Secondary Prevention Improving lifestyle & medication	Past History of CHD		<100	<130	

Goals for control depend upon categories of LDL cholesterol and non-HDL cholesterol. The primary target in hypertriglyceridemia is LDL-cholesterol. If the goal for LDL-cholesterol in the Japanese Guidelines for Atherosclerosis 2007 is already achieved, nonHDL-C is the secondary target. For the patients with TG > 500 mg/dL, potential genetic disorders and the prevention of acute pancreatitis should be considered. Coronary risk factors other than LDL-cholesterol include low HDL cholesterol, aging, diabetes, hypertenstion, smoking, past history of CHD, and obesity (visceral obesity).

dL, respectively. Goals for non-HDL cholesterol in each group are those for LDL cholesterol plus 30 mg/dL. This is based upon our outpatient clinic data that non-HDL cholesterol was 30 mg/dL higher than LDL cholesterol (**Fig. 2**)[21]. ATPIII also recommends using LDL cholesterol goal + 30 mg/dL[24]. This also corresponds to the calculated VLDL cholesterol of the cut-off point of normal TGs (150/5 mg/dL). This goal is arbitrarily set and could be modified in the future, especially when the relative atherogenicity of remnants and LDL cholesterol are more precisely determined. In the case of TGs of greater than 500 mg/dL, the risk of pancreatitis should be carefully considered as a potential acute complication.

Treatment of Hypertriglyceridemia Based upon Non-HDL Cholesterol Level

Treatment of patients with hypertriglyceridemia for primary prevention should be initiated with lifestyle modifications, especially reducing weight and increasing physical activity. Lifestyles exacerbating hypertrigylyceridemia, such as overweight, obesity, physical inactivity, cigarette smoking, excess alcohol intake, and very high carbohydrate diets, need to be improved. Other disorders and drugs that cause secondary hypertriglyceridemia, including diabetes, chronic renal filure, nephrotic syndrome, and steroid therapy, should also be treated first. In the event that lifestyle modification for at least three months is not effective to achieve the goal of non-HDL cholesterol, medication should be considered. Currently, due to lack of evidence to fully justify the use of fibrates for high TGs prior to statins, it is recommended to use a statin as the first line choice for high non-HDL cholesterol. If statin therapy is already used to control LDL cholesterol, management of non-HDL should be targeted by increasing the dose of the statin or switching to a stronger form. This is based upon the notion that remnant lipoproteins, as well as LDL, are taken up through LDL receptors that are up-regulated by statins. In the case of type III hyperlipidemia, or if high non-HDL cholesterol is much more prominent than LDL cholesterol because of hypertriglyceridemia, fibrates could be considered as they specifically reduce plasma TGs and are effective against type III hyperlipidemia. However, LDL cholesterol should be carefully monitored since fibrates occasionally raise LDL cholesterol following a decrease in TGs (VLDL cholesterol). In case the goal for LDL cholesterol is not attainable, the addition of cholestimide and/or ezetimibe to statin could be considered, whereas EPA could be considered for hypertriglyceridemia. A positive result from a recent large scale Japanese study using both EPA and pravastatin to estimate the prevention of atherosclerotic events, justifies superimposing EPA on statin therapy, although the contribution of the plasma TG-lowering effect of EPA to the prevention of cardiovascular events is not yet determined[25]. The complexity of the choice of medication for high non-HDL cholesterol is currently inevitable because no agents specifically decrease non-HDL cholesterol. Drug information strongly warns against the use of both statins and fibrates because of increasing the risk of the life-threatening side effect of rhabdomyolysis. Joint use is justified only when the benefit exceeds the risk, which requires expertise in this field; however, considering the very few reports of rhabdomyolysis as a severe side effect in recent post-market studies in Japan, carefully prescribing both agents for high-risk patients such as those with type IIb hyperlipidemia could be re-considered. Joint use might be restricted in the elderly or renal compromised patients. In addition, monitoring mus-

cle symptoms and plasma creatine phosphokinase is necessary in patients prescribed either statins or fibrates.

Conclusions and Future Prospect of the Guidelines

Non-HDL cholesterol containing both LDL cholesterol and remnant cholesterol, is an excellent predictor of atherosclerotic risk, and should be a treatment target. Non-HDL cholesterol is simple, convenient, and free from dietary variations. These advantages are crucial for nation-wide use of the guidelines and health check activity. This simple measurement could also make it possible to re-evaluate previous clinical studies using this parameter to offer a good chance of estimating the usefulness and importance of this marker in a large meta-analytical scale.

In the current study, we propose that LDL cholesterol is the primary target and non-HDL cholesterol should be the secondary target for elevated TG. Considering that non-HDL and LDL cholesterol are partially redundant, non-HDL could replace LDL as the primary target and as a general marker for both elevated cholesterol and TG. As **Table 1** shows, non-HDL cholesterol could be used as a general and convenient lipid marker for type IIb hyperlipidemia.

This proposal still faces the recent problem of selecting lipid markers for the initial assessment for dyslipidemia. The recent GL focus has been on LDL cholesterol rather than TC, while LDL cholesterol has a problem the lower reliability for direct measurement. In addition, a considerable portion of hypertriglyceridemia is not applicable to this equation. For subjects with hypertriglyceridemia, application of this new GL eventually requires all TC, TG, HDL, and LDL cholesterol measurements to assess both LDL and non-HDL cholesterol. Currently, however, the Japanese medical system covers only three out of four lipid measurements as healthcare services provided by health insurance. Further Japanese clinical studies and careful evaluation of the data, as well as technical improvements of reliable LDL cholesterol measurements, are required to determine the most efficient protocol to select lipid measurements as the initial assessment of dyslipidemia to prevent CVD in Japan. Furthermore, guidelines for HDL cholesterol should also be established, although the relative importance and positioning of non-HDL and HDL is yet to be determined.

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