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Relation of Serum Total Cholesterol and Other Factors to Risk of Cerebral Infarction in Japanese Men With Hypercholesterolemia

— The Kyushu Lipid Intervention Study —

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Background Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT), and few studies have examined the risk factors in the elderly.

Methods and Results Clinical and behavioral risk factors for cerebral infarction were examined in 4,349 Japanese men aged 45–74 years with a serum total cholesterol (TC) concentration of 220 mg/dl or greater who participated in the Kyushu Lipid Intervention Study. A total of 81 men developed definite cerebral infarction in a 5-year follow-up period. The Cox proportional hazards model was used with serum TC at baseline and during the follow-up, serum high-density lipoprotein-cholesterol (HDL-C), HT, diabetes mellitus (DM), and other factors as covariates. Serum TC during the follow-up, not at baseline, was positively associated with cerebral infarction, showing a stronger association in the elderly (≥65 years old) than in the middle-aged (<65 years old). Statin use was related to a moderate decrease in the risk of cerebral infarction when follow-up TC was not considered, but the decrease was almost nullified after adjustment for follow-up TC. A low concentration of serum HDL-C, diabetes mellitus, hypertension, and angina pectoris were each related to an increased risk. No clear association was observed for body mass index, smoking or alcohol use.

Conclusions Lowering cholesterol is important in the prevention of cerebral infarction in men with moderate hypercholesterolemia. A low concentration of HDL-C, DM, and HT are independent predictors of cerebral infarction. (*Circ J* 2005; **69**: 1–6)

Key Words: Cerebral infarction; Diabetes mellitus; High-density lipoprotein-cholesterol; Hypercholesterolemia; Japanese men

Stroke is a leading cause of death and disability in industrialized countries and of the 2 major types of stroke, cerebral infarction predominates, although hemorrhagic stroke remains common in Asian populations[1]. Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT)[2]. Findings regarding the relation between serum total cholesterol (TC) or low-density lipoprotein-cholesterol (LDL-C) and cerebral infarction are inconsistent in observational studies[3–16] whereas cholesterol-lowering trials have shown a decrease in the risk of cerebral infarction among patients assigned to statin treatment[17,18]. The role of serum high-density lipoprotein-cholesterol (HDL-C) is receiving particular interest in the epidemiology of cerebral infarction. Low concentrations of serum HDL-C have been fairly consistently associated with an increased risk of cerebral infarction[4–9,14,19]. Several[5,7,8,12,15,19] but not all[3,6] prospective studies reported that diabetes mellitus (DM) was associated with an increased risk of cerebral infarction. In the study reported here, we examined the relation of serum TC and HDL-C and other factors to the risk of cerebral infarction using data from the Kyushu Lipid Intervention Study (KLIS), a primary prevention trial of coronary heart disease (CHD) events and cerebral infarction in Japanese men with moderately elevated concentrations of serum TC[20–23]. Furthermore, because few studies have investigated the risk factors for cerebral infarction in elderly persons[6,15,19], we examined the association with these factors in middle-aged and elderly men separately.

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Methods

Details of the study design, patient characteristics at baseline, and primary results of the KLIS have been described previously[20–23]. In brief, a total of 5,640 men aged 45–74 years with serum TC concentration of 220 mg/dl or greater were enrolled by 902 physicians in Kyushu District during the period between May 1990 and September 1993.

Table 1 Characteristics of the Study Subjects by Statin Use

	Mean (SD) or proportion		p-value for difference*
	Statin (−)	Statin (+)	
No. of subjects	1,637	2,712	–
Age (years)	58.1 (8.2)	58.0 (7.9)	0.78
Baseline total cholesterol (mg/dl)	244 (17.9)	258 (25.8)	<0.0001
Follow-up total cholesterol (mg/dl)	225 (25.1)	219 (26.7)	<0.0001
Baseline HDL-cholesterol (mg/dl)	50 (12.0)	49 (12.0)	0.05
Body mass index (kg/m^2)	23.9 (2.8)	24.2 (2.7)	0.008
Angina pectoris (%)	8.2	10.5	0.01
Hypertension (%)	42.6	44.5	0.24
Diabetes mellitus (%)	24.9	22.8	0.11
Prior use of hypolipidemics (%)	7.6	14.3	<0.0001
Current smoking (%)	40.0	37.5	0.10
Daily alcohol use (%)†	41.7	39.6	0.16

Values are mean (SD) unless otherwise specified.
*Comparison of means was based on t-test, and the chi-square test was used for proportions.
†Drinking alcohol 5 days per week or more frequently.

All the patients gave consent to participate in the study. Ineligible for the study were a history of myocardial infarction, coronary bypass surgery, coronary angioplasty, cerebral hemorrhage, or cerebral infarction; serum HDL-C concentration of 80 mg/dl or greater; and a life-limiting morbid condition such as severe renal or hepatic disease. Each physician was instructed to randomly allocate patients to either pravastatin treatment or conventional treatment as specified in a sealed envelope, but participating physicians did not necessarily follow that instruction?[0] The patients were followed up until the end of 1997 for the occurrence of coronary events and cerebral infarction. The study was approved by the ethical committee of the principal investigator's affiliated institution.

Subjects

The present analysis included 4,349 of the 5,640 enrolled men; 1,291 were excluded for the following reasons: (1) withdrawal of consent (n=147), (2) no institutional contract (n=616; this category represented the lack of a written agreement between a participating hospital or clinic and a sponsoring pharmaceutical company, which became necessary in the course of the study because of the introduction of a new regulation for clinical trials in Japan), (3) found to be ineligible during follow-up (n=97), and (4) missing data (n=431).

Laboratory and Clinical Data

Serum concentrations of TC, HDL-C, triglycerides (TG), and other clinical and biochemical variables were determined at baseline and subsequently in the follow-up. Laboratory measurements were done at different laboratories, but each physician was requested to use the same laboratory throughout the study period. Average serum TC concentrations during the follow-up were determined on the basis of periodical follow-up measurements at 3 months, 6 months, and every year thereafter; the number of measurements ranged from 1 to 10 with a median of 6. Serum LDL-C was not used in the present study because the Friedewald method[24] was not applicable for 7% of the men who even at baseline had serum TG concentrations of 400 mg/dl or greater (n=282) or who had missing data (n=5). HDL-C was not measured in 46 men during the follow-up.

Hypertension was defined as present if a patient had systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or if was under medication for HT. Subjects were defined as having DM if they had either fasting plasma glucose ≥140 mg/dl or hemoglobin A1c ≥6.5% or if they were under medication for DM. The presence of angina pectoris (AP) and prior use of hypolipidemic drugs were based on the report of the study physicians. Statin use was defined if any statin drugs were prescribed during the follow-up period. Height and body weight were recorded, and body mass index (kg/m^2) was calculated as an index of obesity. Current habits of smoking and alcohol drinking were ascertained, together with the number of cigarettes smoked per day and frequency of alcohol drinking per week.

Endpoints

Cerebral infarction was the secondary endpoint and coronary events were the primary endpoint. Definite cerebral infarction was diagnosed when typical symptoms and signs were accompanied with diagnostic findings on brain imaging or cerebral angiography. Diagnosis based only on clinical signs and symptoms was regarded as a suspected case?[0] These endpoints were determined by the Endpoint and Adverse Effect Committee on the basis of periodical reports from the study physicians and, if necessary, by supplementary inquiry. From January to May 1998, an ad hoc survey was carried out to ascertain the occurrence of coronary events and cerebral infarction up to the end of 1997. Vital status was unknown for 36 men, and cerebral infarction and coronary events were not ascertained for 97 men. A total of 81 definite cases and 10 suspected cases of cerebral infarction were identified in an average observation period of 5.05 years. One definite case and 2 suspected cases of cerebral infarction occurred subsequent to a coronary event. None developed coronary events after cerebral infarction. Only definite cases of cerebral infarction were used in the present study, and the follow-up period continued until the event of a definite cerebral infarction regardless of coronary events.

Statistical Analysis

The Cox proportional hazards model was used to examine the relation of clinical and behavioral factors to the risk of cerebral infarction. The principal model included indicator variables for age (5-year class), baseline serum TC (<240, 240–259, ≥260 mg/dl), follow-up TC (<220, 220–239, ≥240 mg/dl), serum HDL-C (<40, 40–59, ≥ 60 mg/dl), BMI (<22.5, 22.5–24.9, ≥25.0 kg/m^2), AP, HT, DM, prior

use of lipid-lowering drugs, current smoking (0, 1–19, ≥20 cigarettes per day), and alcohol use (0, 1–4, ≥5 days per week). The association with statin use was examined using the model with and without follow-up TC. Adjusted relative risk and 95% confidence intervals (CI) were obtained from a regression coefficient and standard error for the corresponding indicator variable. Statistical significance of the interaction was assessed by the likelihood ratio test. Statistical significance was declared when the 95%CI did not include unity or when the two-sided p-value was less than 0.05. Statistical computations were done with the SAS software version 8.2 (SAS Institute, Inc, Carry, NC, USA).

Results

The mean age of the study subjects was 58.0 years, and the mean concentrations of baseline and follow-up serum TC were 253 mg/dl and 221 mg/dl, respectively. The characteristics of the study subjects are summarized by statin use in Table 1. Serum TC concentrations were higher at baseline and lower during the follow-up among men taking statins than in those not taking the medication. The differences were highly significant. The number of cerebral infarctions was 47 (1.7%) in men taking statins and 34 (2.1%) in those who were not.

Although baseline TC was not associated with the risk of cerebral infarction, the follow-up TC concentration was strongly, positively associated with cerebral infarction (Table 2). A nearly 4-fold increase in the risk was observed for men with a follow-up serum TC ≥240 mg/dl compared with those with a concentration <220 mg/dl. High concentrations of serum HDL-C (≥60 mg/dl) was associated with a lower risk of cerebral infarction, although the decrease was not statistically significant (p=0.08). Diabetes mellitus was associated with a statistically significant increase in the risk of cerebral infarction. Men with AP or HT also showed a moderate increase in the risk, and the increased risk associated with HT was statistically significant. No clear association was observed for BMI. A statistically nonsignificant decrease in the risk was observed for each of the intermediate categories of smoking and alcohol use.

Statin use was associated with a statistically non-significant, moderate decrease in the risk of cerebral infarction without adjustment for follow-up TC; adjusted relative risk for statin use was 0.73 (95%CI 0.46–1.16) when follow-up TC was replaced with statin use in the model used for Table 2. When both statin use and follow-up TC were included in the model, the adjusted relative risk for statin use was 0.91 (95%CI 0.57–1.47), and adjusted relative risks for the follow-up TC concentrations of <220, 220–239, and ≥240 mg/dl were 1.00 (referent), 1.69 (95%CI 0.97–2.97), and 3.76 (95%CI 2.15–6.56), respectively. Further, the association between follow-up TC and cerebral infarction was examined by statin use. In that analysis, follow-up TC was used as continuous variable to avoid unstable estimation because of the smaller number of cases. Adjusted relative risks for an increase of 10 mg/dl in TC were 1.31 (95%CI 1.14–1.51) in non-statin users and 1.11 (95%CI 0.99–1.25) in statin users. The increased risk associated with follow-up TC seemed to be attenuated in the latter group, but the interaction was not statistically significant (p=0.16).

When separate analyses were done for men aged less than 65 years and those aged 65 years or older (Table 3), the increased risk of cerebral infarction associated with elevated concentrations of serum TC during the follow-up was slightly more evident in the elderly men than in the middle-aged men. A decreased risk associated with high concentrations of HDL-C was more apparent in the middle-aged men, but neither of the decreases in risk for the 2 age groups was statistically significant. Diabetes mellitus and AP were each associated with a statistically significant increase in the risk of cerebral infarction in the elderly only. Hypertension was statistically non-significantly associated with an increased risk in both middle-aged and elderly men. As regards alcohol use, a statistically significant decrease in the risk was observed for the category of 1–4 days per week in elderly men only.

Table 2 Adjusted Relative Risks of Cerebral Infarction According to Selected Risk Factors

Variable	*No. of men*	*No. of cases*	*Adjusted RR (95%CI)**
Baseline TC (mg/dl)			
<240	*1,463*	*21*	*1.00 (referent)*
240–259	*1,538*	*38*	*1.42 (0.83–2.46)*
≥260	*1,348*	*22*	*0.78 (0.42–1.46)*
Follow-up TC (mg/dl)			
<220	*2,097*	*29*	*1.00 (referent)*
220–239	*1,338*	*23*	*1.72 (0.99–3.00)*
≥240	*914*	*29*	*3.86 (2.23–6.62)*
HDL-cholesterol (mg/dl)			
<40	*970*	*22*	*1.00 (referent)*
40–59	*2,485*	*49*	*0.94 (0.56–1.57)*
≥60	*894*	*10*	*0.50 (0.23–1.09)*
Body mass index (kg/m²)			
<22.5	*1,197*	*22*	*1.00 (referent)*
22.5–24.9	*1,609*	*31*	*1.15 (0.66–2.01)*
≥25.0	*1,543*	*28*	*1.16 (0.65–2.07)*
Angina pectoris			
None	*3,930*	*66*	*1.00 (referent)*
(+)	*419*	*15*	*1.74 (0.98–3.08)*
Diabetes mellitus			
None	*3,325*	*54*	*1.00 (referent)*
(+)	*1,024*	*27*	*1.81 (1.13–2.89)*
Hypertension			
None	*2,445*	*30*	*1.00 (referent)*
(+)	*1,904*	*51*	*1.65 (1.04–2.63)*
Cigarettes per day			
0	*2,679*	*60*	*1.00 (referent)*
1–19	*474*	*6*	*0.54 (0.23–1.26)*
≥20	*1,196*	*15*	*0.82 (0.46–1.46)*
Alcohol use (days/week)			
0	*1,683*	*44*	*1.00 (referent)*
1–4	*910*	*10*	*0.50 (0.25–1.01)*
≥5	*1,756*	*27*	*0.85 (0.52–1.41)*

RR, relative risk; CI, confidence interval; TC, total cholesterol.
**Based on the Cox proportional hazards model controlling for age (5-year class), prior use of cholesterol-lowering drugs, and listed variables.*

Discussion

Methodological problems need to be clarified before interpreting the present findings. Because the patients were non-randomly allocated to pravastatin treatment, cardiovascular risk factors were generally more prevalent among statin users, as shown in Table 1. Although statistical adjustment was done for these factors, residual confounding effects possibly remained. Statin use was strongly associated with lower concentrations of follow-up TC, and it may be difficult to conclude which is more importantly related to the risk of cerebral infarction on statistical grounds. All the patients were treated for hypercholesterolemia regard-

Table 3 Adjusted Relative Risk of Cerebral Infarction According to Selected Risk Factors in Men Aged Less Than 65 Years and Older Men

Variable	<65 years (n=3,115)		≥65 years (n=1,070)	
	No. of cases	Adjusted RR (95%CI)*	No. of cases	Adjusted RR (95% CI)*
Baseline TC (mg/dl)				
<240	7	1.00 (referent)	14	1.00 (referent)
240–259	16	1.91 (0.78–4.70)	22	1.15 (0.57–2.31)
≥260	15	1.65 (0.64–4.26)	7	0.38 (0.15–0.98)
Follow-up TC (mg/dl)				
<220	13	1.00 (referent)	16	1.00 (referent)
220–239	12	1.40 (0.63–3.12)	11	1.91 (0.87–4.19)
≥240	13	2.45 (1.07–5.60)	16	5.26 (2.53–10.95)
HDL-cholesterol (mg/dl)				
<40	12	1.00 (referent)	10	1.00 (referent)
40–59	23	0.70 (0.35–1.43)	26	1.33 (0.63–2.82)
≥60	3	0.33 (0.09–1.18)	7	0.70 (0.25–1.94)
Body mass index (kg/m²)				
<22.5	7	1.00 (referent)	15	1.00 (referent)
22.5–24.9	14	1.26 (0.51–3.15)	17	1.20 (0.59–2.46)
≥25.0	11	1.45 (0.59–3.56)	11	1.01 (0.45–2.27)
Angina pectoris				
None	34	1.00 (referent)	32	1.00 (referent)
(+)	4	0.99 (0.35–2.82)	11	2.70 (1.32–5.53)
Diabetes mellitus				
None	26	1.00 (referent)	28	1.00 (referent)
(+)	12	1.39 (0.70–2.77)	15	2.42 (1.25–4.68)
Hypertension				
None	16	1.00 (referent)	14	1.00 (referent)
(+)	22	1.50 (0.77–2.90)	29	1.89 (0.98–3.67)
Cigarettes per day				
0	27	1.00 (referent)	33	1.00 (referent)
1–19	2	0.45 (0.11–1.92)	4	0.59 (0.21–1.72)
≥20	9	0.83 (0.38–1.81)	6	0.83 (0.33–2.04)
Alcohol use (days/week)				
0	17	1.00 (referent)	27	1.00 (referent)
1–4	7	0.79 (0.32–1.92)	3	0.28 (0.08–0.94)
≥5	14	0.83 (0.40–1.73)	13	0.94 (0.47–1.90)

RR, relative risk; CI, confidence interval; TC, total cholesterol.
**Based on the Cox proportional hazards model controlling for age (5-year class), prior use of cholesterol-lowering drugs, and listed variables.*

less of statin use, and cardiovascular risk factors ascertained at baseline may have changed in varying degrees. The relation of clinical risk factors at baseline to the risk of cerebral infarction may have been attenuated because comorbid conditions such as HT and DM were probably well treated during the follow-up[25] Finally, results from the subgroup analysis should be interpreted cautiously. In the analysis by statin use or by age group, the estimated relative risks were more subject to random fluctuation because of the smaller number of cases.

The present study demonstrated an evident, positive association between the serum TC concentration in the follow-up period, but not at baseline, and cerebral infarction. Statin use was associated with a moderate, statistically nonsignificant decrease in the risk of cerebral infarction when follow-up TC was not taken into consideration. The magnitude of the decrease in the risk associated with statin use was the same as reported for pravastatin use previously in the KLIS[21] However, the decreased risk associated with statin use was almost nullified after adjustment for follow-up TC. The positive association with follow-up TC remained after adjustment for statin use. These findings indicate that lowering cholesterol itself is important in the prevention of cerebral infarction among men with moderate hypercholesterolemia.

It remains a matter of controversy whether the reduced risk of stroke or ischemic stroke associated with use of statins can be ascribed to the cholesterol-lowering effect of statins or to other properties[26,27] Statins are known to ameliorate endothelial dysfunction, stabilize atherosclerotic plaques, and modify inflammatory responses and thrombus formation[28] The observation that statin treatment confers a reduced risk of ischemic stroke among hypertensive patients with average or below-average cholesterol concentrations suggests a role of the nonlipid-lowering effects of statins[29] On the other hand, a meta-analysis of randomized controlled trials indicated that the beneficial effect on stroke incidence was seen only when the final cholesterol concentration was <232 mg/dl (6.0 mmol/L), suggesting the importance of lowering cholesterol[30] Another meta-analysis of 7 prospective observational studies showed a statistically significant decrease of 15% in the risk of thromboembolic stroke for a 1.0 mmol/L decrease in LDL-C[31] In this regard, the interaction between statin use and follow-up TC is of particular interest. In the present study, the positive relation between follow-up TC and cerebral infarction seemed weaker among statin users. The findings may be interpreted as suggestive of a protective effect of statins other than their cholesterol-lowering effect, but it is also possible that uncontrolled risk factors other than elevated concentrations of serum TC may be major determinants of the risk of cerebral infarction among statin users because they are generally at a higher risk of cardiovascular diseases on entry. A larger study is needed to clarify the relation between

achieved concentrations of TC or LDL-C and cerebral infarction among patients under treatment with different classes of cholesterol-lowering drugs.

The positive association between serum TC in the follow-up and cerebral infarction seemed to be stronger in the elderly men than in the middle-aged men in the present study. Not many studies have addressed the relation between cholesterol and cerebral infarction in the elderly. In a prospective study of those aged 60 years or older[6] serum TC was unrelated to the risk of cerebral infarction. In a clinical trial of the elderly in Europe[32] statin use did not result in a decrease in the incidence of stroke, not specifically of cerebral infarction, but there was a substantial decrease in coronary events. On the other hand, in Japanese men in Hawaii[15] a statistically significant increase in the risk of ischemic stroke was observed for the highest vs lowest quartile of serum TC in men aged 60–74 years, but not in those aged 51–59 years. The seemingly differential association with serum TC according to age group in the present study could be a random fluctuation as discussed earlier, but the findings are consistent with the progression of atherosclerosis; that is, atherosclerosis in the cerebral arteries occurs later in life than coronary atherosclerosis[15]

Although HDL-C has been emphasized as a predictor of coronary heart disease in Western countries[33] and in Japan[34] it is only very recently that low concentrations of serum HDL-C were found to be associated with an increased risk of cerebral infarction[4–9,14,19] Our findings on HDL-C add to evidence for a protective association between HDL-C and cerebral infarction. The non-HDL-C concentration may be more useful than that of TC in predicting the risk of cardiovascular diseases[35] When the non-HDL-C concentrations at base line and during the follow-up, categorized at each quartile, were included in the model used for Table 2 instead of the baseline and follow-up TC concentrations and HDL-C at baseline, the relation between non-HDL-C concentration during the follow-up and the risk of cerebral infarction was not as strong as observed for follow-up TC; adjusted relative risk for the highest (≥188 mg/dl) vs lowest (<153 mg/dl) quartile was 3.16 (95%CI 1.62–6.16). The role of serum TG in the development of atherosclerotic diseases is also of recent interest because of its relevance to metabolic syndrome[33,35] In the present study, however, the baseline concentrations of serum TG were unrelated to the risk of cerebral infarction; when serum TG were additionally included in the model used for Table 2, adjusted relative risks for triglycerides <150, 150–199, and ≥200 mg/dl were 1.00 (referent), 1.17 (95%CI 0.68–2.04), and 0.95 (95%CI 0.56–1.63), respectively.

Diabetes mellitus, HT and AP were found to be related to an increased risk of cerebral infarction, especially among the elderly men. Further confirmation is needed regarding possible differential relations according to age group. The present findings regarding smoking and alcohol use were difficult to interpret because past smokers and past drinkers were not distinguished from lifelong nonsmokers and nondrinkers. Occasional alcohol use was related to a substantial decrease in the risk of cerebral infarction among elderly men. In general, no material association between alcohol use and cerebral infarction has been found in prospective studies[3,7,10–12] Exceptionally, alcohol use was associated with a decreased risk of atherosclerotic stroke in a study of elderly persons[19] Further studies are warranted in view of much evidence that moderate alcohol consumption confers protection against atherosclerosis[36,37]

In summary, in men undergoing treatment for moderately elevated concentrations of serum TC, higher concentrations during the treatment, but not at baseline, were strongly related to an increased risk of cerebral infarction and the decreased risk associated with statin use was almost nullified when follow-up TC was taken into account. Lowering cholesterol itself, rather than the choice of cholesterol-lowering regimen, is important in the prevention of cerebral infarction in patients with hypercholesterolemia.

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Appendix 1

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Positive family history for coronary heart disease and 'midband lipoproteins' are potential risk factors of carotid atherosclerosis in familial hypercholesterolemia

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Abstract

Patients with heterozygous familial hypercholesterolemia (FH) were examined with B-mode ultrasound in order to determine intima-media thickness (IMT) in the common carotid artery, and to uncover potential risk factors responsible for the development of IMT. Ninety seven FH subjects and 132 non FH type IIa hyperlipidemic subjects were involved in the present study. Age was found to correlate positively with IMT in both FH and non FH groups. FH individuals showed a higher IMT, along with elevated low density lipoprotein (LDL) cholesterol levels, compared with age-matched non FH individuals. To clarify potential factors contributing to the formation and development of carotid atherosclerosis, we divided the FH subjects into two subgroups, namely FH with high IMT group (HIG), and those with low IMT group (LIG). We investigated those two subgroups on the presence of angiographically documented coronary heart disease (CHD), of family history of CHD and of 'midband lipoproteins' by polyacrylamide gel electrophoresis (PAGE) analysis, by matching for age and LDL-cholesterol (LDL-C) level. Fifty percent of FH men in HIG was found to have CHD, whereas only 14% of those in LIG had CHD ($P < 0.05$). Thirty-three percent of FH women in HIG was found to have CHD, whereas only 12% of those in LIG had CHD ($P < 0.05$). Fifty percent of FH men in HIG was found to have 'midband lipoproteins', whereas only 7% of those in LIG had 'midband lipoproteins' ($P < 0.01$). Seventy-three percent of FH women in HIG had 'midband lipoproteins', whereas only 21% of those in LIG had 'midband lipoproteins' ($P < 0.0005$). Fifty-five percent of FH men in HIG was had positive family history for CHD, whereas only 14% of those in LIG had positive family history for CHD ($P < 0.05$). Sixty-three percent of FH women in HIG was found to have positive family history for CHD, whereas only 29% of those in LIG had positive family history for CHD ($P < 0.05$). Based on these findings, we propose that, besides age and elevated levels of LDL-C, positive family history for CHD and 'midband lipoproteins' are important determinants for the development of carotid atherosclerosis in FH individuals in Japanese population.

Keywords: Familal hypercholesterlemia (FM); Intima-media thickness (IMT); Low density lipoprotein (LDL); Midband lipoproteins

1. Introduction

Familial hypercholesterolemia (FH) is a common inherited disease characterized by elevated low density lipoprotein (LDL) cholesterol levels in the plasma and tendon xanthomas. The disease is well known to be associated with an increased risk for premature coronary heart disease (CHD) [1]. It is believed that CHD may progress for many years before coronary symptoms occur, but little is known about the clinically silent phase of disease development.

Recently, B-mode ultrasound is increasingly used in epidemiological and clinical research to non-invasively study the atherosclerotic process in the carotid artery. It is assumed that the atherosclerotic changes in the

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carotid artery mirror general atherosclerosis, and ultrasound measurements of the thickness of the intima-media complex in the carotid artery are partly used as surrogate variables for coronary atherosclerosis [2,3]. An increased thickness of the intima-media complex in the common carotid artery has previously been reported in FH individuals, compared with a sex- and age-matched control group [4]. However, the degree of the development of atherosclerosis seems to differ widely by individuals with FH, indicating that other factors than plasma LDL-cholesterol (LDL-C) level and age would contribute greatly to the development of CHD in FH.

Several studies have suggested that 'midband lipoprotein' in the plasma detected by PAGE was closely associated with the development of coronary atherosclerosis [5–8]. We previously reported that in FH subjects with CHD, which was coronary angiographically assessed, the prevalence of positive for 'midband lipoprotein' was significantly higher, compared to those without CHD, when matched for TC, TG, HDL-C and Lp(a) [5].

In this study we compared intima-media thickness (IMT) in the common carotid artery in FH subjects with that in age-matched non FH type IIa hyperlipidemic subjects, by broken down gender and then investigated other factors than age and plasma LDL-C, which might be responsible for the development of carotid atherosclerosis, by measuring IMT in the common carotid artery with B-mode ultrasound in FH subjects in Japan.

2. Methods

2.1. Study groups

Thirty-four men and 63 women with heterozygous FH from Chiba University Hospital, Chiba, Japan, were involved in this study.

Subjects were diagnosed as heterozygous FH [9] following the criteria; (1) type II hyperlipidemia >6.7 mmol/l cholesterol with tendon xanthomas, (2) type II hyperlipidemia >6.7 mmol/l cholesterol and the presence of the subjects with type II hyperlipidemia >6.7 mmol/l cholesterol with tendon xanthomas in the proband's first- or second-degree relatives, or (3) type II hyperlipidemia >6.7 mmol/l cholesterol and the LDL-receptor activity of the proband's fibroblasts lower than that of a normal control. Although these criteria are less stringent than those in the Western countries [10], they are believed to be adequate for diagnosing heterozygous FH in the Japanese population because of the lower plasma cholesterol levels due to the traditional Japanese low-fat diet [11]. The average age of the FH patients was 50 ± 15 years old for men and 57 ± 12 years old for women. The average levels of plasma total and LDL-C were 353 ± 47 mg/dl and 283 ± 47 mg/dl for men, and 377 ± 54 mg/dl and 304 ± 57 mg/dl for women, respectively. Fifty percent of the 97 subjects in the present study were found to have CHD by coronary angiography, whereas in our previous preliminary data, frequencies of existence of CHD in 795 FH individual in Chiba region were 23% for men with average plasma TC levels of 330 ± 62 mg/dl and 13% for women with average plasma TC levels of 325 ± 67 mg/dl. Hyperlipidemic subjects other than FH were defined as 'control' in the present study. However, type I or V hyperlipidemic subjects were excluded from this study. Subjects with uncontrolled diabetes mellitus with HbAlc greater than 7.0%, those with endocrinological disorders or kidney disease were excluded from this study.

2.2. Family history

Family history data were obtained from the chart of the parents with FH. The presence of CHD was based on hospital or physician reports of one or more of the following events; sudden cardiac death, typical exercise-induced angina pectoris, myocardial infarction, coronary artery bypass surgery, percutaneous transluminal coronary angiography.

2.3. Blood pressure

Resting blood pressure was measured phonographically in the right arm after about 30 min of supine rest at the time of the ultrasound examination.

2.4. Smoking

Information on smoking habits was obtained by a self-administered questionnaire.

2.5. Biochemical analysis

Blood samples for measuring cholesterol and triglyceride (TG) levels in the plasma and lipoproteins fractions were drawn after a fasting period of 10–12 h. Cholesterol and triglycerides levels were determined by using fully enzymatic techniques. High density lipoprotein (HDL) cholesterol was determined after precipitation of apo B-containing lipoproteins with manganese chloride and heparin. LDL cholesterol was calculated as described by Friedewald et al. [12]. 'Midband lipoproteins' were detected by polyacrylamide gel electrophoresis (PAGE) previously reported [13].

2.6. Measurement of intima-media thickness of the common carotid artery

Examination of IMT was carried out with an ultrasound scanner (SSD-1200CV, ALOKA) equipped with a linear 7.5-MHz transducer. IMT was defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface of the far wall. The measurement of IMT in the common carotid artery was made along a 10-mm long section just proximal to the carotid bulb [14]. And average IMT (avgIMT) was calculated from right and left IMT of common carotid arteries.

2.7. Measurement of plaque size

This analysis included plaques in the near as well as far walls of the common carotid artery, the carotid bulb, and the internal carotid artery. A plaque was defined as a distinct area with an IMT more than 50% thicker than neighboring sites [15].

2.8. Statistical analysis

Stat View-J 4.11 software was used for all statistical analysis. Data were expressed as means ± S.D. The significance of differences between the groups was determined using the χ^2-test. Data were compared with the control using the Dunnett test. A difference with a P-value < 0.05 was considered to be statistically significant.

3. Results

Background, lipid and lipoprotein profiles of the heterozygous FH and non FH type IIa hyperlipidemic subjects (= control) involved in the study of Fig. 1 are shown in Table 1. Average age and body mass index (BMI) of FH men did not significantly differ from that of control men, while avgIMT as well as LDL-C level was significantly higher in FH men than those in control men (Table 1(a)). Similar associations to men were observed in women subjects (Table 1(b)).

To determine how age affects IMT in FH and non FH for both genders, we investigated the correlation between age and avgIMT in those subjects Fig. 1. In both FH and control men, age showed a positive correlation with avgIMT (Fig. 1(a)). In both FH and control women, age correlated positively with avgIMT and the values of avgIMT increased rapidly with age, especially over 50 years old, which did not apply to men (Fig. 1(a)–(c)). In both genders, the age-dependent

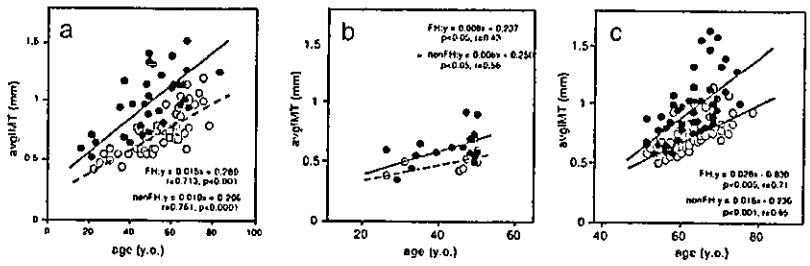

Fig. 1. Correlation between age and average Intima-Media Thickness (avg IMT) in subjects with familial hypercholesterolemia and those with type IIa hyperlipidemia. Scatter plot of relation between age and average wall thickness in the carotid artery in subjects with familial hypercholesterolemia (●) and non FH type IIa hyperlipidemia (○), for men (a), for women aged under/over 50 years old (b, c).

Table 1
Background and lipid profile of subjects (values are given as mean ± S.D.)

	Men			Women		
	FH ($n = 34$)	Control (IIa) ($n = 50$)	t-test	FH ($n = 63$)	Control (IIa) ($n = 82$)	t-test
Age (years)	50 ± 15	53 ± 15	ns	56 ± 10	59 ± 8	ns
BMI (kg/m^2)	23.1 ± 2.2	24.3 ± 3.2	ns	21.7 ± 2.9	22.3 ± 2.8	ns
TC (mg/dl)	353 ± 47	264 ± 30	$P < 0.0001$	377 ± 54	259 ± 33	$P < 0.0001$
LDL-C (mg/dl)	283 ± 47	192 ± 25	$P < 0.0001$	304 ± 57	108 ± 37	$P < 0.0001$
HDL-C (mg/dl)	44 ± 8	47 ± 16	ns	49 ± 15	55 ± 11	ns
TG (mg/dl)	147 ± 105	120 ± 22	ns	125 ± 65	138 ± 69	ns
Lp (a) (mg/dl)	25 ± 24	40 ± 38	ns	53 ± 36	41 ± 33	ns
Smoke	13/34	17/50	ns (χ^2-test)	0/63	2/82	ns (χ^2-test)
Hypertension	5/34	9/50	ns (χ^2-test)	6/63	15/82	ns (χ^2-test)
Average IMT (mm)	1.13 ± 0.27	0.74 ± 0.19	$P < 0.0001$	0.93 ± 0.31	0.71 ± 0.16	$P < 0.005$

Table 2
Background and lipid profile in FH subjects with high intima-media thickness and with normal thickness

	Men			Women (postmenoposal)		
	HIG ($n = 20$)	NIG ($n = 14$)	t-test	HIG ($n = 30$)	NIG ($n = 24$)	t-test
Age (years)	52 ± 12	50 ± 18	ns	61 ± 9	58 ± 8	ns
BMI (kg/m^2)	23.5 ± 2.4	22.6 ± 1.6	ns	22.3 ± 2.4	21.5 ± 2.6	ns
TC (mg/dl)	343 ± 31	367 ± 54	ns	385 ± 55	386 ± 55	ns
LDL-C (mg/dl)	270 ± 36	297 ± 54	ns	312 ± 60	311 ± 59	ns
HDL-C (mg/dl)	41 ± 8	49 ± 6	$P < 0.005$	45 ± 16	48 ± 16	ns
TG (mg/dl)	159 ± 98	106 ± 47	$P < 0.05$	141 ± 72	134 ± 60	ns
Lp (a) (mg/dl)	31 ± 31	16 ± 6	ns	59 ± 37	40 ± 22	ns
ApoEphenotype						
3/3	$n = 14$	$n = 9$	ns (χ^2-test)	$n = 22$	$n = 18$	ns (χ^2-test)
3/4	$n = 6$	$n = 4$	ns (χ^2-test)	$n = 8$	$n = 5$	ns (χ^2-test)
4/4	$n = 0$	$n = 1$	ns (χ^2-test)	$n = 0$	$n = 1$	ns (χ^2-test)
Smoke	8/21	5/14	ns (χ^2-test)	0/30	0/24	ns (χ^2-test)
Hypertension	3/20	2/14	ns (χ^2-test)	4/30	2/24	ns (χ^2-test)
Average IMT (mm)	1.17 ± 0.15	0.80 ± 0.12	$P < 0.001$	1.10 ± 0.25	0.74 ± 0.07	$P < 0.001$

Values are given as mean ± S.D. HIG, high intima-media thickness group; NIG, normal intima-media thickness group.

progressions of IMT were obviously increased in FH compared to those in control subjects, suggesting the importance of higher LDL-C levels and genetic backgrounds in FH for the progression of IMT in any ages. Thus, age is a more important determinant of IMT in the common carotid artery for FH individuals, compared to control subjects.

In order to know the other potential risk factors of the progression of IMT in FH in addition to LDL-C and age, we then divided FH subjects to two subgroups, namely high IMT group (HIG) and low IMT group (LIG), based on the degree of the thickness of intima-media complex in the carotid arteries Table 2. For women, individuals with FH were limited to postmenoposal state at this time. These two subgroups were matched for age and LDL-C level to assess additional factors responsible for the development of carotid arteries thickness. The avg IMTs in HIG and in LIG were 1.17 ± 0.15 and 0.70 ± 0.12 mm for FH men, respectively, 1.10 ± 0.25 and 0.61 ± 0.07 mm for postmenoposal FH women, respectively. Table 3 shows the frequency of having angiographically documented CHD in FH patients in HIG or LIG for both genders. Fifty percent of FH men in HIG was found to have CHD, whereas only 14% of those in LIG had CHD ($P < 0.05$). Thirty-three percent of FH women in HIG was found to have CHD, whereas only 12% of those in LIG had CHD ($P < 0.05$). These results explained that the atherosclerotic changes in the carotid artery mirror general atherosclerosis, especially for coronary atherosclerosis. Frequencies of existence of plaque lesions in carotid artery in HIG were significantly higher than those in LIG for FH men (85 vs 43%, $P < 0.01$), whereas those were almost same for FH women (53 vs. 50%, ns). The mean plaque score in individuals who had plaque lesions in carotid artery was significantly bigger in HIG for FH men (7.2 ± 3.5 vs. 3.1 ± 1.8 mm, $P < 0.05$), and at the score, that was also significantly bigger in HIG for women (5.8 ± 4.2 vs. 2.8 ± 2.3 mm, $P < 0.05$). Table 4 shows potential risk factors for the development of IMT in the common carotid artery using the analyses of two above groups. Fifty percent of FH men in HIG was found to have 'midband lipoproteins' in the plasma detected by PAGE, whereas only 7% of those in LIG had 'midband lipoproteins' ($P < 0.01$). Seventy-three percent of FH women in HIG was found to have 'midband lipoproteins', whereas only 21% of those in LIG had 'midband lipoproteins' ($P < 0.0005$). We also analyzed the frequency of positive family history for CHD for both genders. Fifty-five

Table 3
Comparison for frequency of coronary heart disease, frequency of plaque formation and average plaque score in carotid artery

	Men			Women (postmenoposal)		
	HIG ($n = 30$)	LIG ($n = 14$)	t-test	HIG ($n = 30$)	LIG ($n = 24$)	t-test
CHD	10 (50%)	2 (14%)	$P < 0.05$ (χ^2-test)	10 (33%)	3 (13%)	$P < 0.05$ (χ^2-test)
Plaque formation	17 (85%)	6 (43%)	$P < 0.01$ (χ^2-test)	16 (53%)	12 (50%)	ns (χ^2-test)
Plaque score (mm)	7.2 ± 3.5 ($n = 17$)	3.1 ± 1.8 ($n = 6$)	$P < 0.05$	5.8 ± 4.2 ($n = 16$)	2.8 ± 2.3 ($n = 12$)	$P < 0.05$
Average IMT (mm)	1.17 ± 0.15	0.80 ± 0.12	$P < 0.001$	1.10 ± 0.25	0.74 ± 0.07	$P < 0.001$

Values are given as mean ± S.D. HIG, high intima-media thickness group; LIG, low intima-media thickness group.

percent of FH men in HIG was found to have positive family history for CHD, whereas only 14% of those in LIG had positive family history for CHD ($P < 0.05$). Sixty-three percent of FH women in HIG was found to have positive family history for CHD, whereas only 29% of those in LIG had positive family history for CHD ($P < 0.05$).

Moreover, in the opposite direction, we divided those subjects, FH men and postmenoposal FH women, into two subgroups, one had subjects with 'midband lipoprotein' detected by PAGE and the other had those without (Table 5). In subjects with 'midband lipoprotein' HDL-C levels were significantly lower than those in subjects without for both FH men and postmenoposal FH women. And in subjects with 'midband lipoprotein' avg IMTs were significantly higher than those in subjects without for both FH men and postmenoposal FH women, despite no differences of age and LDL-C levels for those subgroups (Fig. 2).

4. Discussion

The main findings of the present study in FH subjects were as follows: (1) age is positively associated with the development of IMT in the common carotid arteries in for both genders; (2) IMT progresses more rapidly with age in FH compared to that in non FH. (3) 'Midband lipoprotein' and positive family history of CHD are important determinants for the development of IMT in the carotid artery in FH individuals for both genders, in addition to the level of LDL-C and age.

Wendelhag et al. [16] have reported that in FH men and women combined, age has been closely associated

Table 4
Other risk factors for intima-media thickness of carotid artery in FH men or postmenoposal women

	Men			Women (postmenoposal)		
	HIG ($n = 20$)	LIG ($n = 14$)	χ^2-test	HIG ($n = 30$)	LIG ($n = 24$)	χ^2-test
Midband formation	10 (50%)	1 (7%)	$P < 0.01$	22 (73%)	5 (21%)	$P < 0.0005$
Family history of CHD	11 (55%)	2 (14%)	$P < 0.05$	19 (63%)	7 (29%)	$P < 0.05$

HIG, high intima-media thickness group; LIG, low intima-media thickness group.

Table 5
Intima-media thickness in FH subjects with 'midband lipoproteins' and in those without (values are given as mean ± S.D.)

	Men			Women		
	Midband (+) ($n = 10$)	Midband (−) ($n = 17$)	t-test	Midband (+) ($n = 23$)	Midband (−) ($n = 21$)	t-test
Age (years)	53 ± 13	51 ± 15	ns	62 ± 9	60 ± 7	ns
BMI (kg/m^2)	23.6 ± 2.9	22.9 ± 1.8	ns	22.6 ± 3.2	21.5 ± 2.2	ns
TC (mg/dl)	337 ± 23	365 ± 56	ns	389 ± 55	367 ± 46	ns
LDL-C (mg/dl)	265 ± 25	294 ± 54	ns	316 ± 58	287 ± 42	ns
HDL-C (mg/dl)	39 ± 6	48 ± 7	$P < 0.001$	44 ± 15	56 ± 14	$P < 0.01$
TG (mg/dl)	165 ± 108	115 ± 54	ns ($P = 0.05$)	145 ± 89	122 ± 46	ns
Lp (a) (mg/dl)	36 ± 34	16 ± 6	ns	59 ± 22	38 ± 39	ns

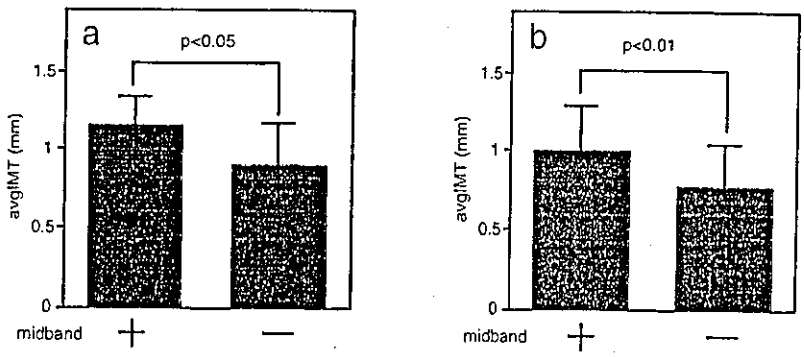

Fig. 2. Comparison of average IMTs in the subjects with or without 'midband lipoproteins' in FH. (a) men, (b) women.

with IMT in the common carotid artery. In the present study, we conducted all investigations in men and women separately, and this association was observed both in FH men and women separately. Several groups of authors have suggested that heterozygous FH individuals had already higher degree of IMT or higher frequency of presence of atheromatous lesions in the carotid arteries than age-matched control individuals had [17,18]. Previous substantial evidence shows that in FH female gender is a strong protective factor [19,20]. In the present study, the progressions of IMT were not much different between subjects with FH and control women under 50 years old, which were almost premenoposal state, compared to men and women over 50 years old.

It has been suggested that 'midband lipoprotein' in the plasma detected by PAGE was closely associated with the development of coronary atherosclerosis [5–8]. Indeed, we previously reported that in FH subjects with CHD, which was coronary angiographically assessed, the prevalence of positive for 'midband lipoprotein' was significantly higher compared to those without CHD, when matched for TC, TG, HDL-C and Lp(a) [5]. Yanagi et al. [6] have reported that in FH subjects abnormal glucose metabolism accelerate the development of CHD due to an increase in 'midband lipoprotein' as well as LDL-C. Moreover Kawano et al. [7] have demonstrated in vitro that a fraction of 'midband lipoprotein' promoted the formation of foam cells from J774 macrophages much more drastically than native LDL. In the present study we revealed that 'midband lipoprotein' was an important risk factor for IMT in the carotid artery in FH individuals.

Other possible factors potentially contributing to the development of the carotid artery thickness are impaired glucose tolerance, high blood pressure and smoking. In this study, the distribution of such factors did not significantly differ in the HIG and NIG in the present study, probably suggesting the potential risk is lower than those of factors determined in this study. However, further detailed analysis is needed to determine the potential of determinants for the progression of carotid athersclerosis in FH.

On the basis of this work, we propose that having family history of CHD and 'midband lipoproteins' in the plasma, besides age, sex, and plasma LDL-C levels, are important determinants of the carotid arteries thickness. Several papers [2,3] suggest that the assessment of IMT is an important predictor for the development of CHD in FH subjects. It appears to be essential to conduct IMT evaluation by B-mode ultrasound in the carotid arteries, especially for FH individuals with positive family history for CHD and/or 'midband lipoproteins' in the plasma.

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Increased Circulating Malondialdehyde-Modified LDL Levels in Patients With Coronary Artery Diseases and Their Association With Peak Sizes of LDL Particles

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Abstract—Recent establishment of a sensitive ELISA system using antibodies against malondialdehyde-modified low density lipoprotein (MDA-LDL) made it possible to determine the circulating oxidized lipoprotein levels. Here, we investigated the serum levels of MDA-LDL in 62 patients with coronary artery disease (CAD) compared with the levels in 42 patients without CAD [groups CAD(+) and CAD(−), respectively], which are adjusted for age, serum total cholesterol, LDL and high density lipoprotein cholesterol, and triglyceride levels. Serum MDA-LDL levels were 113.4±49.1 IU/L in CAD(+), which were significantly higher than the levels in CAD(−) (85.2±22.5 IU/L, $P<0.0005$). The ratio of MDA-LDL/LDL cholesterol was 0.95±0.32 in CAD(+), indicating a significant increase compared with the ratio in CAD(−) (0.68±0.19, $P<0.0005$). The positive correlation of MDA-LDL level and the ratio of MDA-LDL/LDL cholesterol with intima-media thickness in carotid arteries was observed. Age was not clearly associated with the MDA-LDL level ($P=0.865$). The serum MDA level was positively correlated with LDL cholesterol ($P<0.0001$) and with triglycerides ($P<0.001$) and negatively correlated with high density lipoprotein cholesterol ($P<0.05$). Furthermore, the MDA-LDL level was negatively correlated with the peak size of the LDL particle ($P<0.01$). The LDL subclasses that were identified by using the sera collected from the subjects by sequential ultracentrifugation showed that the ratios of MDA-LDL/apolipoprotein B in LDL3 and LDL4 were nearly 3-fold higher than those in LDL1 and LDL2 for CAD(+) and CAD(−). These results indicate that the circulating MDA-LDL level is increased in CAD(+), independent of the serum LDL cholesterol level but in association with the peak size of LDL particles. The measurement of serum MDA-LDL level may be useful for the identification of patients with advanced atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2002;22:662-666.)

Key Words: malondialdehyde-modified LDL ■ coronary artery disease ■ ELISA ■ atherosclerosis

Oxidized LDLs, as modified LDLs, are thought to play key roles in the progression of atherosclerosis (see reviews[1-3]). The modification of LDL by oxidation alters its native properties: oxidized LDL becomes incorporated into macrophages by scavenger receptors[4] and modulates the gene expression involved in the cellular function of endothelial cells and smooth muscle cells in the vessel walls.[5-8] Increasing evidence of atherosclerosis as an inflammatory disease raised the possibility of detection of circulating markers in the serum: increased levels of C-reactive protein and of cytokines, such as interleukins.[9,10] In this context, the detection and quantification of circulating oxidized LDL might reflect the severity and phases of atherosclerosis.

Until now, some new methods to measure the levels of circulating oxidized LDL have been established, and their clinical relevance has been suggested by use of the detection system for some parts of oxidized LDL.[11-14] The ELISA method for measurements of serum oxidized and malondialdehyde (MDA)-modified LDL (MDA-LDL) has been suggested as being useful for the identification of patients with acute coronary syndrome.[15-17] Toshima et al[15] have suggested that the sensitivity of oxidized LDL, which does not include MDA-LDL, is a better biochemical risk marker for coronary artery disease (CAD).[15] Furthermore, the circulating oxidized LDL has been proven to exist in macrophages in human atheroma.[15] We have recently reported a sensitive method to measure circulating MDA-LDL,[11] and we have revealed that its level is increased in patients with diabetes or hypertriglyceridemia.[18] The MDA-LDL level was highly correlated with the particle size of LDLs in their sera.[18] In the present study, therefore, we measured the serum levels of MDA-LDL in the patients with CAD [CAD(+)] and compared these levels with

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the levels in the patients without CAD [CAD(−)]; adjustments were made for age and for serum total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglyceride (TG) levels. We clearly found an increased serum level of MDA-LDL in CAD(+), and the level was positively correlated with the intima-media thickness (IMT) of the carotid arteries. Furthermore, the circulating level was negatively correlated with the peak size of LDL particles, and increased levels were observed in the LDL subfractions with increased densities, which are known to be a risk for coronary atherosclerosis.[19] Thus, the circulating MDA-LDL level is likely associated with advanced atherosclerosis, possibly related to the peak size of LDL particles.

Methods

Subjects

The present study included 62 patients with angiographically proven CAD [CAD(+)] and 42 healthy control subjects [CAD(−)]. Angiograms of CAD(+) showed at least 50% stenosis of 1, 2, or 3 coronary arteries. A total of 42 healthy volunteers matched for age and TC, LDL-C, and HDL-C levels served as CAD(−), ie, the control group. Among the CAD(−) group, none had diabetes mellitus or thyroid or endocrinological diseases. The CAD(−) group had no clinical evidence of CAD and an absence of significant atherosclerotic lesions, which was confirmed by ultrasound scanning of carotid arteries, as previously described (IMT <1.0 mm).[20] The frequency of smoking was not significantly different between 2 groups.

Measurement of IMT of the Common Carotid Artery

Examination of IMT was carried out with an ultrasound scanner (SSD-1200CV, Aloka) equipped with a linear 7.5-MHz transducer. IMT was defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface of the far wall. The measurement of IMT in the common carotid artery was made along a 10-mm-long section just proximal to the carotid bulb.[20] Average IMT was calculated from right and left IMTs of the common carotid arteries.

Measurement of MDA-LDL

The ELISA method used was based on the same principles as previous reported by Kotani et al.[11] Serum samples were drawn after an overnight fast, used within 4 days after the serum was separated by centrifugation, and stored at 4°C. Samples were diluted 2000-fold in a dilution buffer containing SDS. Duplicate 100-μL portions of diluted sample were then added to the wells of the plates, which were coated with monoclonal antibody against MDA-LDL (ML25).[11] ML25 has previously been shown to recognize MDA residue but has not been shown to be specific for MDA-LDL.[11] The reaction was allowed to stand for 2 hours at room temperature, and the plates were then washed. β-Galactosidase–conjugated monoclonal antibody against apoB (AB16) was then added. The combination of ML25 with AB16 has been shown to recognize MDA-LDL. LDL oxidized by Cu^{2+} has been shown to be detectable with this ELISA method.[11] Again, the reaction was allowed to stand for 1 hour at room temperature, and the plates were then washed. One hundred microliters of 10 mmol/L *o*-nitrophenyl-galactopyranoside as substrate was pipetted into the wells. After 2 hours, the reaction was stopped by adding 100 μL of 0.2 mol/L sodium carbonate (pH 12). Absorbance in the wells was determined at 415 nm with an MPR-4A microplate reader (Tosoh). Primary standard was used with preparative MDA-LDL, in which 15% of the total amino groups were modified. We tentatively defined 1 U/L MDA-LDL as the absorbance obtained with the primary standard at a concentration of 1 mg/L. A calibration curve was prepared by diluting a reference serum as a secondary standard from 300- to 9600-fold with a dilution buffer and calculating the amount of MDA-LDL in the samples. Reference sera were prepared from pooled sera from healthy volunteers.

Measurement of LDL Size

The diameter of the LDL in the major LDL peak was estimated by use of nondenaturing polyacrylamide gradient gel electrophoresis with a modified version of the technique described by Krauss and Burke.[21] Briefly, 5 μL of serum from each subject was diluted 2-fold with 400 g/L sucrose and electrophoresed for 24 hours at 10°C on 2% to 15% polyacrylamide gradient gels with buffer containing 90 mmol/L Tris, 80 mmol/L boric acid, and 3 mmol/L EDTA (pH 8.3). The gels were stained with oil red O. The lane containing the calibrators was stained with Coomassie blue-R250, and a calibration curve was constructed on the basis of the migration distance of 5 markers with known diameters as follows: ferritin (12.2 nm), thyroglobulin (17.0 nm), thyroglobulin dimer (23.6 nm, Pharmacia), and protein-coated gold particles (21.1 and 29.2 nm). A control serum was run as a reference on each gel. The locations of individual bands were compared with the control serum when each was scanned The predominant LDL size in each sample lane was calculated from the equation given below after the migration distance of major LDL peak was measured. Each gel lane was scanned with a densitometer interfaced with a PC computer. Control serum was drawn from 1 healthy volunteer and combined with 100 g/L sucrose, 37.5 mmol/L NaCl, and 0.25 mmol/L EDTA. This was divided into aliquots and stored at −80°C.

Lipid Analysis and Ultracentrifugation of Lipoproteins

TC, HDL-C, and TG levels were measured by enzymatic methods. ApoB concentration was determined by using commercially available immunoturbidimetric assay kits (Daiichi Pure Chemicals). LDL-C levels were calculated by the Friedewald formula: LDL-C=TC−HDL-C−(TG/5). LDL and its subspecies were isolated by sequential ultracentrifugation from human serum. The isolation techniques have been described previously.[18] The lipoproteins in the $1.022<d<1.032$, $1.032<d<1.038$, $1.038<d<1.050$, and $1.050<d<1.063$ kg/L fractions (where d indicates density) were defined as LDL1, LDL2, LDL3, and LDL4, respectively.

Statistical Analysis

Data are presented as mean±SD. The Student *t* test was used when means were compared. Simple regression was performed to study the association. A value of $P<0.05$ was considered statistically significant.

Results

Clinical and lipid profiles of CAD(+) in the present study are shown in the Table. The serum MDA-LDL levels in these 62 subjects were compared with the 42 control CAD(−), whose age, TC, LDL-C, HDL-C, and TG levels were adjusted to those of CAD(+). The lipid levels in males and females were not significantly different between CAD(+) and CAD(−). Serum MDA-LDL levels were 113.4±49.1 IU/L in CAD(+) and 85.2±22.5 IU/L in CAD(−) (Figure 1). There were statistically significant differences between CAD(+) and CAD(−) in circulating MDA-LDL levels ($P<0.0005$). Then, we compared the ratio of MDA-LDL/LDL-C in both groups. Figure 1D shows that the ratios of MDA-LDL/LDL-C were 0.95±0.32 in CAD(+) and 0.68±0.19 in CAD(−), indicating a significant difference between CAD(+) and CAD(−) ($P<0.0005$). We next compared the MDA-LDL levels or the ratios of MDA-LDL/LDL-C between CAD(+) and CAD(−) in males and females. As shown in Figure 1B, 1C, 1E, and 1F, the MDA-LDL levels and the ratios of MDA-LDL/LDL-C

Clinical and Lipid Profiles of the Subjects in Study

	CAD(+)	CAD(−)	P
Total			
n	62	42	
Sex (male/female), n	41/21	21/21	
Age, y	67.5±8.9	65.9±6.5	NS
BMI, kg/m²	24.1±3.0	23.4±3.0	NS
TC, mg/dL	202.4±36.5	208.6±23.4	NS
LDL-C, mg/dL	121.7±33.8	126.4±21.2	NS
TGs, mg/dL	138.6±63.4	142.1±94.3	NS
HDL-C, mg/dL	54.1±11.9	59.8±16.3	NS
Male			
n	41	21	
Age, y	65.5±8.9	67.3±6.3	NS
BMI, kg/m²	24.7±3.0	24.1±2.9	NS
TC, mg/dL	200.4±25.9	206.5±22.5	NS
LDL-C, mg/dL	120.0±22.0	128.8±21.8	NS
TGs, mg/dL	150.0±68.4	150.5±106.5	NS
HDL-C, mg/dL	50.7±9.6	55.9±14.5	NS
Female			
n	21	21	
Age, y	71.4±7.6	68.5±5.3	NS
BMI, kg/m²	23.0±2.7	22.7±2.9	NS
TC, mg/dL	206.2±50.7	210.7±24.1	NS
LDL-C, mg/dL	124.8±48.7	124.0±20.2	NS
TGs, mg/dL	116.4±43.9	133.8±80.1	NS
HDL-C, mg/dL	60.9±12.6	63.7±17.0	NS

BMI indicates body mass index. Quantitative data represent mean±SD.

were significantly higher in CAD(+) compared with CAD(−) in males and females.

We next analyzed the IMT of carotid arteries in all subjects to determine the clinical significance of the increased circulating MDA-LDL levels in CAD(+). Figure 2A shows that the MDA-LDL levels are significantly positively correlated with IMT ($P<0.0001$). Furthermore, the ratio of MDA-LDL/LDL-C was significantly positively correlated with IMT ($P<0.0001$). These results together with the above results in Figure 1 indicate that circulating MDA-LDL levels are increased in the subjects with advanced atherosclerosis.

Figure 3 shows the correlation of serum MDA-LDL levels with age (panel A), LDL-C (panel B), HDL-C (panel C), and TGs (panel D). Age did not show a clear relationship with the MDA-LDL level ($P=0865$, Figure 2A). LDL-C was positively correlated with the MDA-LDL level ($P<0.0001$). On the other hand, HDL-C was inversely correlated with MDA-LDL ($P<0.05$). The plasma TG level was positively correlated with MDA-LDL ($P<0.001$). These results indicate that the circulating MDA-LDL levels are associated with the TG and HDL-C levels as well as with the LDL-C levels.

We next assessed the relationship between the LDL particle size and serum MDA-LDL level by measuring the peak size of LDL by gradient gel electrophoresis. Figure 4 shows that the MDA-LDL level is negatively correlated with the LDL size ($P<0.01$). Thus, the MDA-LDL level was associated with the

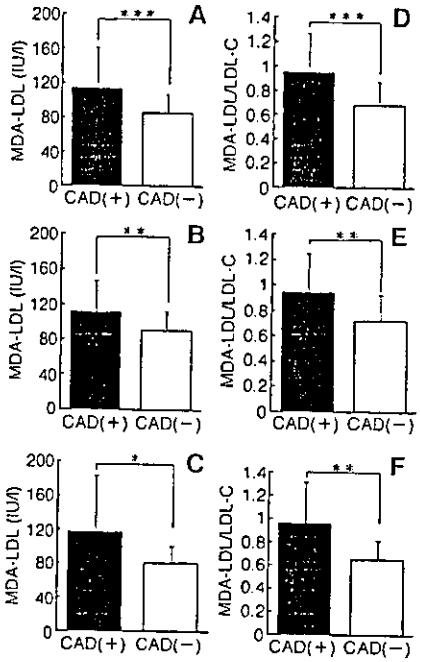

Figure 1. Comparison of plasma MDA-LDL levels between CAD(+) and CAD(−). Bar graph shows the plasma MDA-LDL levels (A, B, and C) or MDA-LDL/LDL-C ratios (D, E, and F) of CAD(+) and CAD(−) in all subjects (A and D), men (B and E), and women (C and F), respectively. Data are presented as mean±SD. ***$P<0.0005$, **$P<0.001$, and *$P<0.05$.

peak size of LDL in the subjects, probably suggesting its metabolic relationship with the smaller particle of LDL.

To further address the possibility of an association of the serum MDA-LDL level and the LDL particle size, we measured the MDA-LDL levels in the LDL subclasses identified by ultracentrifugation with the use of sera from all subjects. Figure 5 shows the serum apoB and MDA-LDL levels and the ratios of MDA-LDL/apoB in the LDL subclasses prepared from CAD(+) or CAD(−). The apoB and MDA-LDL levels showed similar distribution in the subfractions between CAD(+) and CAD(−). ApoB was most abundant in LDL2 fractions, followed by LDL3 fractions in CAD(+) and CAD(−). Nearly 50% of MDA-LDL was localized in LDL3 fractions in both groups. The ratios of MDA-LDL/apoB in LDL3 and LDL4 were increased nearly 3-fold compared with those in LDL1 and LDL2 in both groups. In CAD(+), the ratios of MDA-LDL/apoB in LDL3 and LDL4 were significantly higher than those in CAD(−). These results indicate that the increased circulating MDA-LDL particles are localized mostly in the dense LDL fractions.

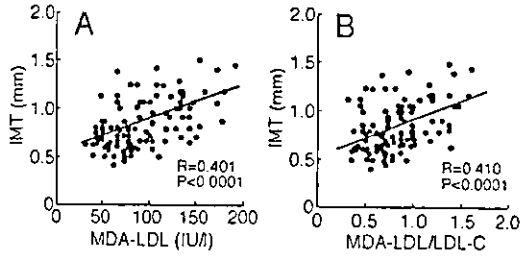

Figure 2. Correlation of IMT with serum MDA-LDL level (A) or MDA-LDL/LDL-C (B).

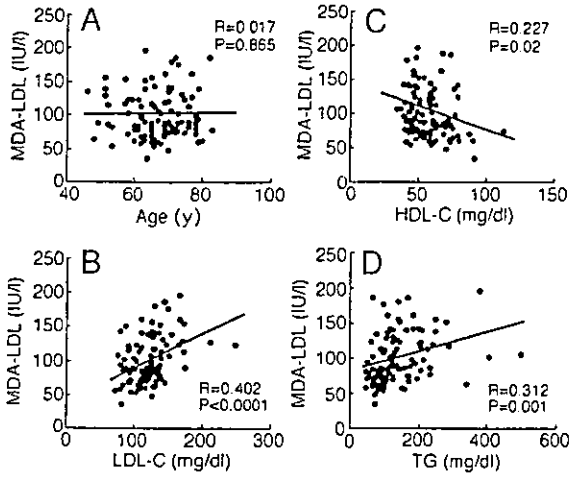

Figure 3. Correlation of serum MDA-LDL level with age (A), serum LDL-C (B), HDL-C (C), or TGs (D).

Discussion

The oxidative modification of LDL is believed to play a key role in the progression of atherosclerosis (see reviews[1-3]). One of the oxidized forms, MDA-LDL, has been isolated from the sera of patients with CAD.[21] We have recently established a new sensitive method to measure MDA-LDL in the serum by using ELISA.[11] In the present study, we could show the increased serum levels of immunoreactive MDA-LDL in CAD(+) compared with CAD(−). The ratio of MDA-LDL and LDL was obviously higher in the CAD(+) for males and females. Furthermore, the level and ratio were positively correlated with the progression of IMT in carotid arteries. These results clarified the clinical significance of immunoreactive serum MDA-LDL level as a factor in association with the progression of atherosclerosis. Next, an analysis of the relationship between age and serum lipid levels showed a positive correlation with LDL-C and TGs but no significant correlation with age. These clear relationships of MDA-LDL with other lipids suggest a biochemical and pathological connection with the functional properties of LDL particles. As a consequence, the MDA-LDL level was negatively correlated with the peak sizes of LDL particles and was mostly distributed in the heavier LDL subfractions separated by ultracentrifugation. Taken together, the circulating MDA-LDL levels are probably related to the densities of LDL particles, which are known to be affected by serum TG levels as well as LDL-C levels.[19]

Previous experiments using the measurements of circulating autoantibodies to MDA-LDL have shown the importance

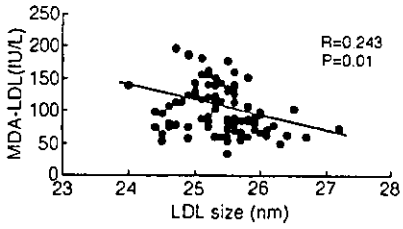

Figure 4. Correlation between the peak size of LDL particles and serum MDA-LDL level.

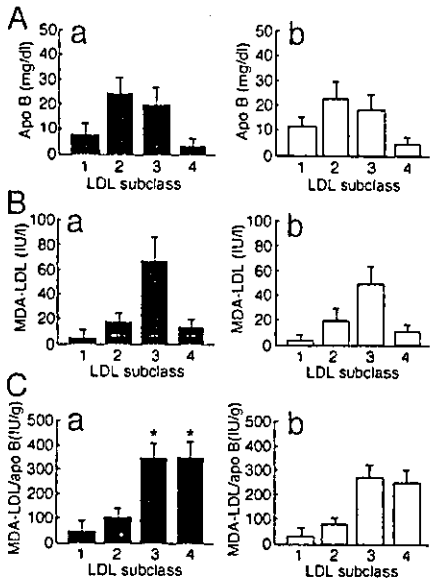

Figure 5. Distribution of serum levels of apoB (A) and MDA-LDL (B) and the ratio of MDA-LDL/apoB (C) in the LDL subclasses isolated from CAD(+) (a) or CAD(−) (b). Data are presented as mean±SD. *$P<0.05$ vs the same LDL fraction in CAD(−).

of oxidized product, MDA-LDL, in the vessel wall in the process and regression of atherosclerosis.[22,23] Furthermore, a positive association of autoantibodies against MDA-LDL with carotid atherosclerosis has been suggested in a case-control study.[24] Recently, it has become possible to detect immunoreactive MDA-LDL in the serum.[11,16] Holvoet et al[25] have shown that plasma levels of MDA-LDL were significantly higher in patients with acute coronary syndromes than in patients with stable CAD. However, the histological distribution in atheromatous plaques in humans revealed that compared with other immunoreactive oxidized LDL, immunoreactive MDA-LDL was not much observed in macrophages in the shoulder areas.[25] Thus, the clinical significance of immunoreactive MDA-LDL in the serum has not been fully established in association with CAD. To approach this topic, we measured the serum levels of MDA-LDL in CAD(+) (all of whom were diagnosed by coronary angiography) by using a sensitive ELISA method recently established by us. As a result, we found significantly higher plasma levels of MDA-LDL in CAD(+) compared with CAD(−) whose levels of LDL-C were adjusted.

Another important finding in the present study is the relationship between serum levels of MDA-LDL and TGs. So far, it is known that small dense LDL, in association with an increased serum TG level, is a risk factor for coronary atherosclerosis.[19] It has been also become clear that the dense LDL subpopulation is more susceptible to oxidative modification and, therefore, may contribute more to foam cell formation than the large LDL.[26,27] Our results using LDL subfractions showed the higher concentrations of MDA-LDL in the dense fractions of molecules. These findings suggest that the atherogenic properties of MDA-LDL identified in the present study are partly caused by the higher production of modified LDL from the dense LDL particles, although the latter mechanism should be studied in detail. In agreement

with our results, the increased atherogenic risk associated with the pattern B phenotype has been suggested to result from increased concentrations of lipoprotein subpopulations that are relatively susceptible to oxidative modification.[28] LDL subclass pattern B, characterized by a preponderance of small dense LDL particles, is associated with an increased risk of myocardial infarction, independent of age and sex.[19] The close relationships between the circulating MDA-LDL and TG levels in addition to the LDL-C level seem indicative of common pathways for atherogenic oxidized lipoproteins derived from TG-rich lipoproteins. The obvious difference in the ratio of MDA-LDL/apoB in dense LDL fractions that is shown between CAD(+) and CAD(−) in Figure 5 suggests that the production pathways of MDA-LDL from dense LDL fractions play a key role in the progression of atherosclerosis.

In conclusion, we clarified the clinical significance of increased serum levels of MDA-LDL for the presence of CAD and the progression of carotid atherosclerosis by using the recently established sensitive ELISA method, independent of the serum LDL-C level. This level in serum was closely related to TG level and the LDL particle size, thus suggesting a pathological connection of TG-rich lipoproteins, small dense LDL, and oxidized LDL. The circulating level of MDA-LDL may reflect the local levels of oxidized lipoproteins and the following progression of atherosclerosis. Further histochemical and metabolic analyses are needed to address these important issues.

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Increased circulating malondialdehyde-modified LDL in the patients with familial combined hyperlipidemia and its relation with the hepatic lipase activity

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Abstract

Familial combined hyperlipidemia (FCHL) is characterized by elevated levels of serum total cholesterol (TC), triglyceride (TG), or both. The increased incidence of coronary artery diseases (CAD) in the patients with FCHL is believed to be caused by circulating atherogenic lipoproteins associated with the complex phenotype. Recent establishment of sensitive detection system for malondialdehyde-modified (MDA)-LDL, which is one of oxidized lipoproteins, showed its increased circulating level in the patients with CAD. In order to know the atherogenic lipoproteins resulted from the dyslipidemia observed in FCHL, we measured the serum MDA-LDL level in the patients. The circulating MDA-LDL level and the ratio of MDA-LDL and LDL-C in FCHL were significantly higher ($P < 0.05$) than those in control, which are adjusted about the age, serum TC, LDL-C and HDL-C levels, respectively. Furthermore, the circulating MDA-LDL level and the ratio of MDA-LDL and LDL-C were negatively correlated ($R = -0.635$, $P < 0.01$ and $R = -0.702$, $P < 0.01$, respectively) with hepatic lipase (HL) activity in FCHL. The serum MDA-LDL level and the ratio of MDA-LDL and LDL-C were in the subjects with T/T genotypes in the HL C-514T polymorphism were significantly increased compared to those with C/C genotype, respectively. The subjects with T/T genotype showed the activities to 65 and 79% of those in the subjects with C/C genotype in male and female, respectively. The intima-media thickness (IMT) of carotid artery was significantly higher ($P < 0.05$) in the subjects with T/T genotype than those with C/C genotype in male. These findings indicate that the circulating MDA-LDL level is possibly contributing the atherogenic process in FCHL, and the common HL polymorphism might be a determinant of the serum level of oxidized LDL in the patients with FCHL.

Keywords: MDA-LDL; Oxidized; Familial combined hyperlipidemia; Hepatic lipase; Polymorphism

1. Introduction

Familial combined hyperlipidemia (FCHL) is the most frequent, genetic disorder of lipid metabolism, leading to the increased risk of premature CAD [1–3]. The high frequency of CAD in the FCHL patients emphasizes the importance of prediction of the advanced atherosclerosis. The dyslipidemia in FCHL is characterized with the elevated serum trigyleride (TG) level as well as total cholesterol (TC) level, accompanied with the existence of atherogenic lipoproteins, such as small, dense LDL in serum.

Until now, some new methods to measure the circulating oxidized LDL have been established, and their clinical relevance was investigated using the detection system for some parts of oxidized LDL in the subjects with hyperlipidemia or advanced atherosclerosis [4–7]. The accumulating evidence underlines the potential usefulness for the identification of patients with acute coronary syndrome [8–11]. Recent establishment of sensitive enzyme-linked immunosorbent assay (ELISA) system using antibodies against malondialdehyde-modified (MDA)-LDL, which is one of oxidized lipoproteins, made it possible to determine the circulating modified lipoprotein levels, and revealed

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that its level is increased in the patients with angiographically proven coronary artery diseases (CAD) [11,12]. Furthermore, using this system for the patients with CAD, we clarified that the serum level of MDA-LDL in the patients with CAD, was increased compared to those without CAD, which are adjusted about the age, serum TC, LDL-and HDL-cholesterol (C), and TG levels [12]. The MDA-LDL level was shown to be positively correlated with intima-media thickness (IMT) of catotid arteries. Thus, the circulating MDA-LDL level is likely associated with atherosclerosis.

Hepatic lipase (HL) is known to be one of the candidate genes underlying the familial dyslipidemia, FCHL, because of the key role in the hydrolysis of TG-rich lipoproteins and the formation and maturation of high-density lipoprotein (HDL) [13]. The polymorphism of HL promoter region is now much focused on the pathogenesis of CAD, with the evidence of the close association with dyslipidemia in subjects. Among the known mutations, the variant of C-514T is reported to link with dyslipidemia, such as increased HDL-C level and/or TG level in the serum, together with the decreased enzyme activities [14–20].

In this study, in order to know the circulating atherogenic lipoproteins resulted from the dyslipidemia observed in FCHL, we measured the serum levels of MDA-LDL in the patients with FCHL, and compared to those without FCHL, which are adjusted about the age, serum TC, LDL-C and HDL-C levels. As results, we clearly found the increased serum level of MDA-LDL in the patients with FCHL, and the level is negatively correlated with hepatic lipase activity in their sera. Furthermore, the circulating levels were significantly higher in the patients with T allele in the C-514 T polymorphism than those with C allele. The patients with T allele showed the advanced carotid intima-media thickness in male. Thus, the circulating MDA-LDL level is associated with the HL polymorphism in the patients, and possibly causes the progress of atherosclerosis in FCHL.

2. Methods

2.1. Subjects

Japanese FCHL families were ascertained using the criteria described by the Research Group for Primary Hyperlipidemia in the Japanese Ministry of health and Welfare, and exhibited remarkable similarity with the original description of FCHL [1]. The recruited FCHL families are of Japanese descent, and from a single geographic area in Japan. The probands met three criteria, as follows: (1) primary hyperlipidemia, with plasma TC > 220 mg/dl, TG > 200 mg/dl; (2) apoB concentrations exceeding by 2 S.D., the mean for age-adjusted levels; and (3) at least one first-degree relative with a different hyperlipidemic phenotype. Exclusion criteria for probands included diabetes, endocrine disorders, familial hypercholesterolemia (tendon xanthomas), and type III hyperlipidemia (apoE2/E2). Fifty-six male and 44 female control subjects, whose age, TC, LDL-C and HDL-C levels were adjusted to those of male and female FCHL subjects, respectively, were recruited. All subjects gave informed consent, and the protocol for gene analysis was approved by the Human Investigation Review Committee of the Chiba University Graduate School of Medicine.

2.2. Measurements of clinical markers

Venous blood was drawn after an overnight of 12–14 h. Serum was separated from blood cells by centrifugation, and immediately used for the measurement of TC, HDL-C, TG and apo B as described previously [21]. LDL-C was estimated with the equation of Friedewald et al. [22].

2.3. HL activity assay

The subjects fasted overnight, and then 30 units of heparin per kg body weight were injected and blood was collected 10 min later. The assay of total lipolytic activity was a modification of the method using [^{14}C] Triolein as a substrate described previously [23]. [^{14}C] Triolein emulsion was prepared by sonication on ice for 10 min. For measurement of lipolytic activity, the reaction mixture contained 0.33 mg of triolein (Sigma), 0.033 μCi of triolein carboxyl-^{14}C (New England Nuclear, 100 mCi/mmol), 5 mg of fatty acid free bovine serum albumin (Sigma, grade V), 0.02% Triton X-100, 1 M NaCl and an appropriate amount of postheparin plasma (5 μl) in the presence or absence of the 5D2 monoclonal antibody for lipoprotein lipase (LPL) in a final volume of 0.25 ml of 0.1 M Tris–HCl (pH 8.4). After incubation for 60 min at 37 °C, the enzyme reaction was terminated by addition of 3.25 ml of chloroform:methanol:heptane (1.41:1.25:1) and 1 ml of 0.1 N NaOH. The mixture was mixed vigorously and centrifuged for 10 min. The radioactivity of 1 ml of the resultant supernatant was counted in a scintillation counter. Hepatic lipase activity was calculated as the remaining activity in the presence of the 5D2 monoclonal antibody for LPL.

2.4. Measurement of MDA-LDL

The ELISA method used was based on the same principles as previous reported by Kotani et al. [4]. Samples were diluted 2000 -fold in a dilution buffer containing S.D.S. One hundred microliter of diluted sample were then added to the wells of plates which were coated with monoclonal antibody against MDA-LDL (ML25) [4]. The reaction was allowed to stand for 2 h at room temperature, and the plates were washed. β-Galactosidase-conjugated monoclonal antibody against apoB (AB16) was then added. Again, the reaction was allowed to stand for 1 h, and the plates were washed. One hundred microliters of 10 mmol/l *o*-nitrophenyl-galactopyransoide as substrate was added into the wells. After 2 h, the reaction was stopped by adding