of death in highly industrialized countries where much of the population has excessive blood cholesterol levels.

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations of the LDL receptor gene (3). FH heterozygotes cause approximately double the normal LDL cholesterol concentration in early childhood, and have increased rise of early myocardial infarction (4, 5). Serum total cholesterol concentrations in unaffected subjects, FH heterozygotes and FH homozygotes showed a distinct trimodal distribution (4). Thus, the bimodal frequency distribution of cholesterol concentration in unaffected and heterozygous FH patients can be used to separate the general population into normal and hypercholesterolemic groups because of increased low-density lipoprotein (LDL)-cholesterol concentrations.

The present study demonstrates that serum total cholesterol concentrations were distributed bimodally in FH families studied to determine LDL-receptor gene abnormalities (6). The observation of bimodality in cholesterol frequency distributions may provide an independent confirmation of a hypercholesterolemic subgroup within the general population.

Methods

Subjects

Subjects included 181 FH patients and 100 unaffected first- and second-degree relatives. No subjects were taking lipid-lowering drugs, and none had a disease affecting serum lipid concentrations. All FH patients were heterozygous and were diagnosed by abnormalities of the LDL-receptor gene, while unaffected family members showed no LDL receptor gene mutations (6).

Lipid measurements

Blood samples were drawn for assays after overnight fasting. Concentrations of serum total cholesterol, triglyceride (TG), and HDL-cholesterol were determined enzymatically. LDL-cholesterol concentrations were derived using the Friedewald formula (7).

Molecular analysis

Genomic DNA was isolated according to a standard method from the buffy coat of a centrifuged 5-ml blood sample anticoagulated with disodium EDTA. Techniques used for PCR-denaturing gradient gel electrophoresis (DGGE), DNA sequencing, and Southern blot analysis were reported in our previous paper (6). Briefly, fragments that showed a variant by DGGE were amplified by PCR, and the PCR products were sequenced by an ABI 310 automated sequencer.

Statistical analysis

Lipid concentrations and other parameters were compared between FH and non-FH groups using Student's *t*-test.

Receiver operating characteristics (ROC) analysis

Serum total cholesterol, HDL-cholesterol, and LDL-cholesterol concentrations showed a symmetric normal distribution, while serum triglyceride concentration showed a skewed distribution and was transformed to a logarithmic value (log TG) that showed a normal distribution. Bimodal distributions of total cholesterol and LDL-cholesterol concentrations suggested points that could discriminate FH and non-FH patients. ROC analyses and plotting were performed using a program (ROCKIT) for the Macintosh personal computer (8, 9). To yield a full spectrum of sensitivity/specificity pairs corresponding to all possible decision levels for total and LDL-cholesterol concentrations; these were tested for ability to discriminate FH from non-FH subjects. The ROC curve was constructed by plotting sensitivity on the y-axis against the false-positive fraction (1-specificity) on the x-axis. Areas under ROC curves ranged from 1.0, corresponding to perfect discrimination (upper left corner), to 0.5 (no discrimination) (10, 11).

Results

Clinical features

The number of patients with each LDL-receptor gene mutation is shown in Table 1. The most common was at exon 17 K790X (6). Numbers of male and female subjects were nearly equal. Serum total cholesterol and LDL-cholesterol concentrations in FH respectively were 1.8 and 2.3 times higher than those in non-FH subjects (Table 2). HDL-cholesterol concentrations in FH patients were 10 mg/dl lower than those in non-FH subjects (Table 2). The histogram of serum total cholesterol concentrations in FH and non-FH subjects showed a bimodal distribution (Fig. 1). As for distributions of serum total cholesterol, LDL-cholesterol, HDL-cholesterol, and log TG (Fig. 2), total cholesterol and LDL-cholesterol showed distinct bimodality, while HDL-cholesterol and log TG levels did not.

ROC analysis

Sensitivity and specificity of the criteria proposed above were tested by ROC analysis of a sample of 281 sequentially sampled first- and second-degree relatives in which diagnosis of FH was established using genetic markers (Fig. 3). The proposed total cholesterol criteria of 224 and 225 mg/dl were in agreement with DNA marker, resulting in an observed specificity of 98.5% and sensitivity of 99.4%. The area under the curve was 0.9826 ± 0.0058 for total-cholesterol. LDL-cholesterol cutoffs of 161 to 163 mg/dl produced an observed specificity of

Table 1. Numbers of patients and serum lipid concentrations for each LDL-receptor gene mutation.

LDL-receptor gene mutation	No.	TC		TG		HDL-C		LDL-C	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tonami-1	17	322	58	110	49	48.4	12.5	252	56
Tonami-2	14	316	61	122	100	47.8	12.9	244	60
Okayama	2	346	118	92	21	48.5	5.0	279	118
Exon 2 C25Y TGC-TAC	3	276	49	109	35	26.3	14.6	228	59
Exon 4 R94H CGC-CAC	2	300	1	124	2	54.5	10.6	221	12
Exon 9 D412H GAC-CAC	4	366	67	101	61	46.5	10.7	299	51
Exon 11 FsN543 Cins1689	2	305	59	207	152	57.0	21.0	224	54
Exon 13 1927 ATC 3bp deletion	8	335	42	139	42	42.3	9.4	265	44
Exon 14 P664L CCG-CTG	19	358	46	162	149	38.3	12.4	287	56
Intron 15 2312-3 C-A	17	334	64	126	65	42.0	6.9	267	61
Exon 17 K790X	85	335	56	148	107	48.0	28.0	257	62
Others	8	336	67	128	62	43.0	10.1	267	69

TC: total cholesterol concentration (mg/dl), TG: triglyceride concentration (mg/dl), HDL-C: HDL-cholesterol concentration (mg/dl), LDL-C: LDL-cholesterol concentration (mg/dl)

Table 2. Clinical features and lipid parameters in subjects with and without FH.

	F/M		T-CHOL (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	Age(yrs)
FH	90/91	Mean	331.9	233.3	44.2	260.8	41.9
		SD	57.7	97.6	13.4	58.6	16.7
Non-FH	49/51	Mean	184.8	97.9	53.7	114.8	35.1
		SD	24.6	58.5	13.3	23.8	17.7
		P value	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	

FH: familial hypercholesterolemia, F/M: female/male, T-CHOL: Total cholesterol, TG: triglyceride, HDL-C: HDL-cholesterol, LDL-C: LDL-cholesterol, SD: standard deviation

98.5% and a sensitivity of 98.3%. The area under the fitted curve ± SD was 0.9852 ± 0.0043 for LDL-cholesterol. One of 181 FH patients showed a total cholesterol level less than 225 mg/dl, and none of non-FH patients showed total cholesterol levels higher than 225 mg/dl. Three of 181 FH patients showed LDL-cholesterol levels less than 160 mg/dl and none of the non-FH patients showed LDL-cholesterol levels higher than 160 mg/dl. Thus, an LDL-cholesterol concentration of 160 mg/dl and a total cholesterol concentration of 225 mg/dl had the best ability to discriminate between subjects with and without FH.

Discussion

Premature CHD can result from elevated LDL-cholesterol in blood even in the absence of other risk factors. A striking example is provided by young patients who have

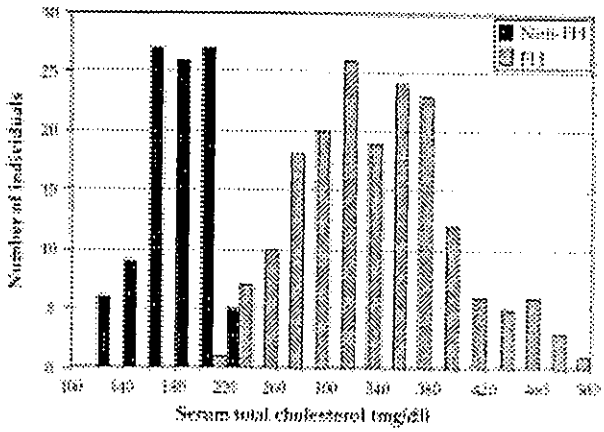

Fig. 1 Histogram of serum cholesterol concentrations in FH (n = 181) and non-FH subjects (n = 100)

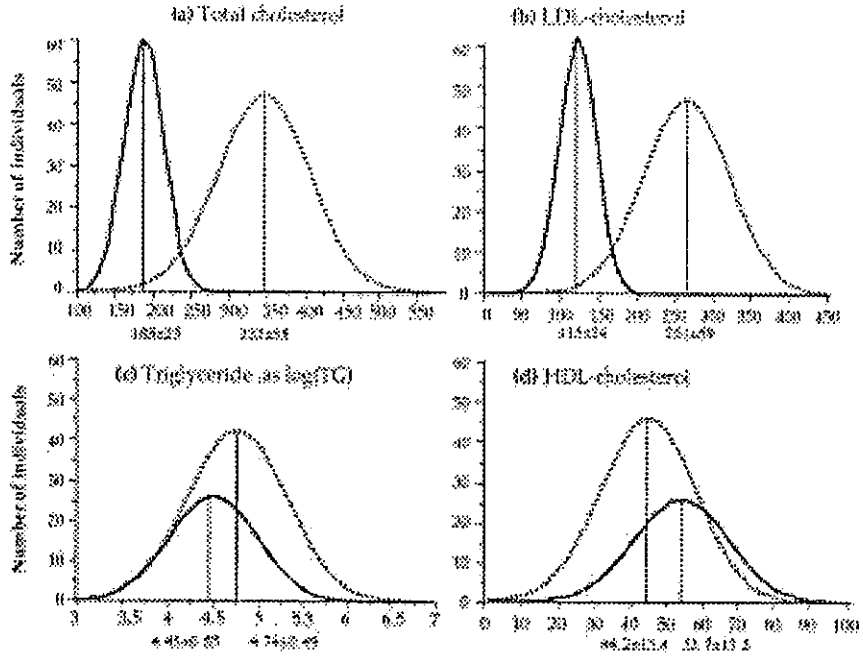

Fig. 2 Distributions of (a) serum total cholesterol, (b) LDL-cholesterol, (c) log triglyceride, and (d) HDL-cholesterol in normal subjects (solid line) and heterozygous familial hypercholesterolemia patients (dotted line). Numbers along the horizontal axis represent mg/dl. Mean ± SD are shown in each subject group.

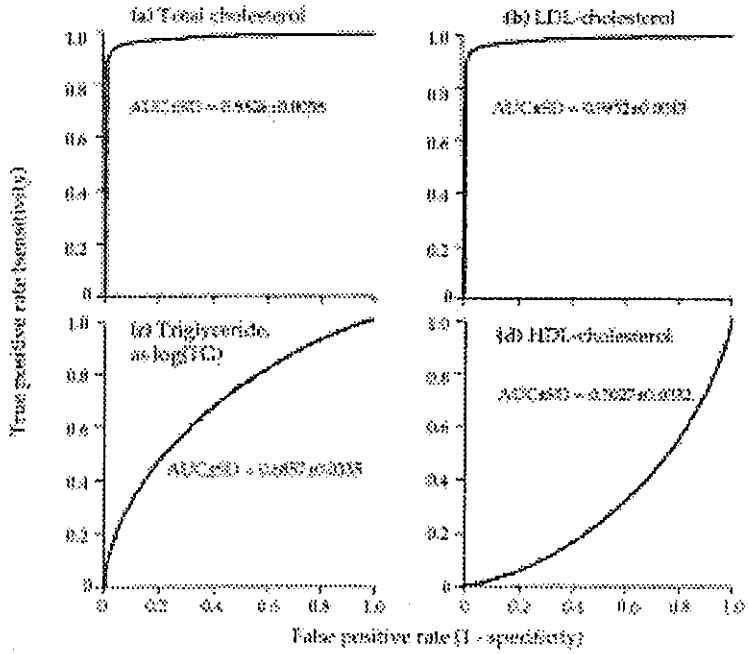

Fig. 3 Receiver operating characteristic (ROC) curves showing discrimination between FH and non-FH subjects by (a) serum total cholesterol, (b) LDL-cholesterol, (c) log triglyceride, and (d) HDL-cholesterol. Areas under ROC curves (AUC) are shown as mean ± SD.

the homozygous form of FH, a rare disorder characterized by essentially complete absence of specific cell-surface receptors that normally remove LDL from the circulation (3). The consequence is an increase in blood cholesterol occurring predominantly in the LDL fraction. As a result LDL cholesterol concentrations are extremely high (500 to 1,000 mg/dl), and severe atherosclerosis and CHD often develop during the first decades of life (12). Patients with the more common heterozygous form of FH have half the normal number of functioning LDL receptors; they have approximately twice-normal LDL-cholesterol concentrations and commonly develop CHD in the middle decades of life (5). To determine the molecular basis of FH in Japan, 200 unrelated patients with clinically diagnosed heterozygous FH were screened for mutations in the coding and promoter regions of the LDL receptor gene (6). Thirty-seven different mutations in the LDL receptor gene were identified in 125 of the patients (62.5%) (6). In the present study the family members of heterozygous FH patients with definite LDL receptor gene mutations were examined. Although most unselected populations show a unimodal distribution of serum cholesterol skewed toward higher concentrations, a bimodal pattern was observed in FH families. The lower component characterized the distribution in individuals with normocholesterolemia, while the upper component characterized the distribution in individuals with hypercholesterolemia. A blood cholesterol concentration of approximately 225 mg/dl divided lower and upper components in the present study. Several reports have outlined methods of screening for FH patients. Umans-Eckenhausen et al. found that the best available cutoff point to diagnose FH by total cholesterol concentration in relatives of known FH patients was the 90th percentile (13, 14).

Clinical accuracy, defined as the ability to discriminate between states of health, is the fundamental property required for any diagnostic test or system, being readily expressed as clinical sensitivity and specificity and represented in an elegant manner by the ROC curve (8). The area under the curve represents the discriminating ability of the particular screening method. Swets suggested the following guidelines for interpreting areas: 0.5 to 0.7, rather low accuracy; 0.7 to 0.9, accuracy useful for some purposes; and greater than 0.9, rather high accuracy (10). The MedPed group compared sensitivity and specificity of differing cutoff values of total serum cholesterol in both a general population sample and in close relatives of confirmed FH patients (15). Wiegman *et al.* (16) reported that by ROC curve analysis of plasma LDL-cholesterol, the largest area was found under the curve of plasma LDL-cholesterol, and the best LDL-cholesterol value for diagnosis of FH in children was 135 mg/dl. To demonstrate the use of ROC curves, Zweig *et al.* reexamined a study of the ability of serum lipid and apolipoprotein measures to discriminate among degrees of CHD in patients undergoing coronary angiography (11). In this study, one of 181 FH patients (0.6%) showed a false negative study by the total cholesterol concentration criteria of 225 mg/dl, and three of 181 FH patients (1.7%) showed false negative study in LDL-cholesterol criteria of 160 mg/dl.

Few diagnostic criteria of hypercholesterolemia have been established, although target concentrations of total cholesterol and LDL-cholesterol have been defined for primary and secondary prevention of CHD in the NCEP ATP III (17) and recommendations of the Second Joint Task Force of European and other Societies on Coronary Prevention (18). In NCEP ATP III as well as in ATP II, total cholesterol concentrations below 200 mg/dl are classified as desirable, those from 200 to 239 mg/dl as borderline-high, and those over 240 mg/dl as high. Because the relationship between serum cholesterol and CHD risk shows a continuous, steadily increasing curve, these cutoff points are somewhat arbitrary (like those for blood pressure) (1). At the cutoff point of 240 mg/dl for total cholesterol, CHD risk is roughly double that at 200 mg/dl, and continues to rise steeply (1).

Current diagnostic criteria for diabetes are based on the mean plus 2SD of glucose concentrations after an oral glucose load is given to healthy subjects. Epidemiologic surveys of populations with a high prevalence of type 2 diabetes such as Pima Indians have demonstrated bimodality of 2-h postprandial plasma glucose concentrations (19). Bimodal curve for 2-h plasma glucose after oral glucose loading is used for diagnosis of diabetes with the cutoff at 200 mg/dl (20). Similarly, the bimodality of genetic hypercholesterolemia can be used for diagnosis of hypercholesterolemia. Plasma cholesterol concentrations are distributed over a continuum in a general population, but an approximate threshold separates subjects at substantially increased risk for certain adverse outcomes caused by hypercholesterolemia (e.g. CHD) from those who are not. The relationship between these concentrations and risk of CHD is continuous with no single threshold value separating "normal" from "abnormal" (1). Ideally, diagnostic criteria should not be based on artificial dichotomization of a continuous variable. Serum cholesterol in FH patients correlates highly with LDL-cholesterol resulting from lack of functional LDL receptors. FH, therefore, is a model of pathologically abnormal LDL metabolism, resulting in frequent occurrence of CHD. Discovery of a bimodal distribution of plasma cholesterol concentrations in populations or FH family members strengthened the concept of the hypercholesterolemic state as a distinct clinical entity (4). As serum cholesterol is determined by both genetic and environmental factors, we can use serum cholesterol data determined by genetic factors interacting with the same environmental circumstances underlying diagnostic cri-

teria for hypercholesterolemia in the general population.

In conclusion, we define total cholesterol and LDL-cholesterol concentrations of 225 and 160 mg/dl respectively as cutoff points between normal subjects and FH patients. These cutoff points can be used as diagnostic criteria for hypercholesterolemia in a general Japanese population.

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Low-Density Lipoprotein Particle Size and Its Regulatory Factors in School Children

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Small low-density lipoprotein (LDL) particles are more atherogenic than larger LDL particles. To help prevent atherosclerotic coronary heart diseases, it may be useful to understand risk factors during childhood. In the present study, we evaluated LDL size and its relationship to other risk factors for atherosclerotic coronary heart disease. LDL size was measured by 2–15% gradient gel electrophoresis in 586 Japanese children (316 boys and 270 girls). Plasma lipids, apolipoproteins (apo), glucose, and insulin were also determined by conventional methods.

Pattern B (LDL size < 25.5 nm) was found in 10.8% of boys and 4.4% of girls. Children with pattern B had a higher body mass index (BMI) and insulin resistance and a more atherogenic lipoprotein profile [higher triglycerides, higher apoB, and lower high-density lipoprotein cholesterol (HDL-C)] than children with pattern A (LDL size ≥ 25.5 nm). BMI, insulin resistance, and plasma concentrations of triglycerides, glucose, and insulin decreased and plasma concentrations of HDL-C and apoA-I increased as LDL size increased. HDL-C and insulin in boys, and BMI, HDL-C, and apoA-I in girls predicted 22.9 and 28.1% of the variability of LDL size, respectively.

LDL size was correlated with BMI and plasma concentrations of HDL-C, apoA-I, and insulin. Although the contribution of these parameters to LDL size in children was less than that in adults, improvement of these parameters by changes in lifestyle might be important for preventing the development of atherosclerosis even in children. (*J Clin Endocrinol Metab* 89: 2923–2927, 2004)

PLASMA LEVELS OF low-density lipoprotein (LDL) cholesterol (LDL-C) are positively associated with the risk for coronary heart disease (CHD) (1, 2). However, several studies have shown that 30–40% of CHD patients have normal LDL-C levels (< 130 mg/dl) (3–5). LDL particles vary in size and hydration density (6, 7). Several LDL subfractions can be identified on gradient gel electrophoresis. Among these LDL subfractions, small LDL particles possess a lower binding affinity for cellular LDL receptor and are more easily oxidized *in vitro* (8–11). These data suggest that small LDL particles are atherogenic because their lower binding affinity for LDL receptor reflects a longer plasma residence time for them to be oxidized and taken up by macrophages in extravascular spaces. In accordance with these *in vitro* data, several case-control studies have indicated that smaller LDL particles are predominant in a high proportion of CHD patients, even those with normal LDL-C levels (12–15). Subjects with small, dense LDL have a 3-fold greater CHD risk (16). LDL particle size is associated with plasma triglyceride (TG), high-density lipoprotein (HDL) cholesterol (HDL-C), and fractional esterification rate in HDL (17, 18). In addition, age, sex, body fat, insulin resistance, and environmental (diet *etc.*) and genetic factors have been reported to affect LDL particle size (14, 19–21).

As previously mentioned, the relationships between LDL size and other risk factors for CHD have been extensively studied in adults. However, the relationships in children are not well understood. The initial stage of atherosclerosis begins in childhood and progresses from fatty streaks to raised lesions in adolescence and young adulthood (22, 23). Therefore, it is rational to investigate LDL size and factors influencing LDL size in children. In the present study, the relationships between LDL size and other parameters that are known as risk factors for CHD in adults were investigated in 586 Japanese children.

Abbreviations: apo, Apolipoprotein; BMI, body mass index; CHD, coronary heart disease; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride.

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Subjects and Methods

Subjects

The present study was approved by the Review Board of the University of the Ryukyus. We studied 586 Japanese children (316 boys and 270 girls), between the ages of 7 and 12 yr, who underwent screening for lifestyle-related diseases in Okinawa, Japan. Body mass index (BMI) was calculated as weight (kilograms) divided by height squared (meters^2). None of the children who were studied were receiving therapy for weight reduction or drugs to affect lipid metabolism. Venous blood was drawn after an overnight fast. Informed consent was obtained from the parents of all of the children.

Laboratory measurements

LDL size was evaluated by electrophoresis in nondenaturing polyacrylamide gradient gels on precast MULTIGEL-LP (2–15%) according to the procedure specified by the manufacturer (Daichi Pure Chemicals CO., LTD, Tokyo, Japan). Standards used for size calibration included latex beads (37 nm; Dow Chemical Company, Midland, MI) and high-molecular-weight standards (Pharmacia, Uppsala, Sweden). The stained gels were scanned with a laser scanning densitometer to provide a quantitative measurement of the size of the peak and its distance from the origin. Particle diameter was calculated from a plot of the log of the known diameters of the standards (latex beads, 37 nm; thyroglobulin, 17.0 nm; apoferritin, 12.2 nm) on the y-axis against their positions from the origin of the gel on the x-axis. LDL size was classified as pattern A (size ≥ 25.5 nm) or pattern B (size < 25.5 nm). Plasma concentrations of

total cholesterol (TC), TG, and HDL-C were analyzed using an autoanalyzer and enzymatic methods or by selective precipitation using sodium phosphotungstate and magnesium chloride (24, 25). LDL-C was calculated as TC − (HDL-C + TG/5). Apolipoproteins (apo; A-I, A-II, and B) were measured by the turbidity immunoassay method (26). Insulin resistance (homeostasis model of assessment ratio) was calculated using the homeostasis model approximation index, which correlates well with the results of both hyperinsulinemic euglycemic clamp and the intravenous glucose tolerance test, by the following formula: insulin resistance = fasting glucose (mg/dl) × insulin (μU/ml)/405 (27).

Statistical evaluation

Differences between two groups were determined by the Mann-Whitney *U* test. Differences among subjects with small, medium, and large LDL (tertiles of LDL size) were determined by the Kruskal-Wallis test. Tertiles of LDL size were compared with Scheffé's multiple comparison test. A stepwise multiple regression analysis was performed by entering the independent variable with the highest partial correlation coefficient at each step until no variable remained with an F value of ≥ 4. Group differences or correlations with $P < 0.05$ were considered to be statistically significant. All statistical analyses were performed using Stat View J-5.0 software (SAS Institute Inc., Cary, NC).

TABLE 1. Clinical and chemical data

	Boys (n = 316)	*P*	Girls (n = 270)
Age (yr)	9.8 ± 0.1	NS	9.7 ± 0.1
LDL size (nm)	26.8 ± 0.1	<0.0001	27.1 ± 0.1
BMI (kg/m^2)	20.4 ± 0.2	<0.0001	18.8 ± 0.2
Glucose (mg/dl)[a]	87.5 ± 0.4	<0.0001	83.5 ± 0.4
Insulin (μU/ml)	10.2 ± 0.5	NS	9.7 ± 0.7
Insulin resistance (HOMA-R)[b]	2.3 ± 0.1	<0.05	2.1 ± 0.2
TC (mg/dl)[c]	180 ± 2	NS	180 ± 2
TG (mg/dl)[d]	88 ± 3	NS	86 ± 3
LDL-C (mg/dl)[c]	101 ± 2	NS	103 ± 2
HDL-C (mg/dl)[c]	62 ± 1	NS	61 ± 1
apoA-I (mg/dl)	144 ± 1	<0.0001	138 ± 1
apoA-II (mg/dl)	30.6 ± 0.2	<0.0001	27.7 ± 0.2
apoB (mg/dl)	75 ± 1	NS	75 ± 1

NS, Not significant; HOMA-R, homeostasis model of assessment ratio. Values are expressed as mean ± SEM.

[a] To convert to mmol/liter, divide by 18.

[b] HOMA-R = fasting glucose (mg/dl) × insulin (μU/ml)/405.

[c] To convert to mmol/liter, multiply by 0.0259.

[d] To convert to mmol/liter, multiply by 0.0113.

Results

The clinical and chemical characteristics of the 586 children are summarized in Table 1. Significant gender-related differences were found in LDL size, BMI, plasma concentrations of glucose, apoA-I, and apoA-II, and insulin resistance. Therefore, we separated the data for boys and girls in the following analysis.

Pattern B was seen in 10.8% of boys and 4.4% of girls. If obese children (BMI ≥ 21 kg/m^2) were omitted, the prevalence in nonobese children was 6.1% in boys and 2.1% in girls (a BMI of 21 kg/m^2 in our study subjects was equivalent to +20% of ideal body weight in Japanese school children). As shown in Table 2, plasma concentrations of glucose, insulin, TG, and apoB in children with pattern B were significantly higher than those in children with pattern A. Plasma concentrations of TC and LDL-C were higher in pattern B than in pattern A children, but these differences were significant only in boys. The plasma concentrations of HDL-C and apoA-I in pattern B children were significantly lower than those in pattern A children, but the difference of apoA-I was significant only in girls. BMI and insulin resistance were significantly higher in children with pattern B than in children with pattern A.

To understand the relation of LDL size to lipids and other parameters in more detail, subjects were divided into tertiles based on LDL size (boys: small tertile, LDL size < 26.3 nm; medium tertile, 26.3 nm ≤ LDL size ≤ 27.2 nm; and large tertile, LDL size > 27.2 nm; girls: small tertile, LDL size < 26.7 nm; medium tertile, 26.7 nm ≤ LDL size ≤ 27.6 nm; and large tertile, LDL size > 27.6 nm). As shown in Table 3, there were significant graded relationships between tertiles of LDL size and parameters (BMI, HDL-C, glucose, insulin, insulin resistance, TG, and apoA-I) in boys. However, significance of parameters other than BMI and HDL-C were found between small and medium and/or between small and large tertiles. In girls, there were significant graded relationships between tertiles of LDL size and parameters (BMI, glucose, insulin, insulin resistance, TG, HDL-C, and apoA-I), but significance was only found between small and medium and/or between small and large tertiles (Table 4). Because each of these parameters can potentially contribute directly to LDL size, we

TABLE 2. Clinical and chemical data on children with pattern A and pattern B

	Boy			Girl		
	Pattern A (n = 282)	*P*	Pattern B (n = 34)	Pattern A (n = 258)	*P*	Pattern B (n = 12)
LDL size (nm)	26.9 ± 0.1	<0.0001	25.1 ± 0.1	27.2 ± 0.1	<0.0001	25.1 ± 0.1
BMI (kg/m^2)	20.1 ± 0.3	<0.0001	23.4 ± 0.4	18.6 ± 0.2	<0.0001	23.4 ± 0.9
Glucose (mg/dl)[a]	87 ± 1	<0.01	91 ± 1	83 ± 1	<0.05	88 ± 2
Insulin (μU/ml)	9.5 ± 0.5	<0.0001	16.2 ± 1.6	8.8 ± 0.6	<0.0001	28.2 ± 4.9
Insulin resistance	2.1 ± 0.1	<0.0001	3.7 ± 0.2	1.9 ± 0.1	<0.0001	6.2 ± 1.1
TC (mg/dl)[b]	179 ± 2	<0.05	189 ± 5	179 ± 2	NS	192 ± 8
TG (mg/dl)[c]	82 ± 3	<0.01	137 ± 21	82 ± 3	<0.0001	166 ± 28
LDL-C (mg/dl)[b]	100 ± 2	<0.01	110 ± 4	102 ± 2	NS	116 ± 8
HDL-C (mg/dl)[b]	63 ± 1	<0.0001	54 ± 2	62 ± 1	<0.0001	49 ± 2
apoA-I (mg/dl)	145 ± 1	NS	138 ± 3	139 ± 1	<0.05	127 ± 4
apoA-II (mg/dl)	30.3 ± 0.2	NS	31.7 ± 0.7	27.7 ± 0.2	NS	29.1 ± 0.8
apoB (mg/dl)	74 ± 1	<0.001	86 ± 3	74 ± 1	<0.01	90 ± 6

NS, Not significant. Values are expressed as mean ± SEM.

[a] To convert to mmol/liter, divide by 18.

[b] To convert to mmol/liter, multiply by 0.0259.

[c] To convert to mmol/liter, multiply by 0.0113.

TABLE 3. Clinical and chemical data on boys with different LDL size particles (in tertiles)

	Small (n = 105)	P	Medium (n = 105)	P	Large (n = 106)
LDL size (nm)	25.64 ± 0.04	<0.0001	26.76 ± 0.03	<0.0001	27.82 ± 0.05[d]
BMI (kg/m^2)	22.2 ± 0.4	<0.01	20.4 ± 0.5	<0.05	18.9 ± 0.4[d]
Glucose (mg/dl)[a]	90 ± 1	<0.05	87 ± 1	NS	86 ± 1[e]
Insulin (μU/ml)	12.8 ± 0.9	NS	10.1 ± 0.9	NS	7.6 ± 0.9[d]
Insulin resistance	2.9 ± 0.2	NS	2.2 ± 0.2	NS	1.7 ± 0.2[f]
TC (mg/dl)[b]	181 ± 3	NS	176 ± 3	NS	183 ± 3
TG (mg/dl)[c]	103 ± 8	NS	84 ± 5	NS	76 ± 4[e]
LDL-C (mg/dl)[b]	105 ± 2	NS	97 ± 2	NS	101 ± 3
HDL-C (mg/dl)[b]	56 ± 1	<0.001	63 ± 1	<0.01	68 ± 1[d]
apoA-I (mg/dl)	138 ± 2	<0.05	145 ± 2	NS	151 ± 2[d]
apoA-II (mg/dl)	30.6 ± 0.4	NS	30.8 ± 0.4	NS	30.2 ± 0.4
apoB (mg/dl)	78 ± 2	<0.05	72 ± 2	NS	73 ± 2

NS, Not significant. Values are expressed as mean ± SEM.
[a] To convert to mmol/liter, divide by 18.
[b] To convert to mmol/liter, multiply by 0.0259.
[c] To convert to mmol/liter, multiply by 0.0113.
[d] $P < 0.0001$, significantly different from Small.
[e] $P < 0.01$, significantly different from Small.
[f] $P < 0.001$, significantly different from Small.

TABLE 4. Clinical and chemical data on girls with different LDL size particles (in tertiles)

	Small (n = 90)	P	Medium (n = 90)	P	Large (n = 90)
LDL size (nm)	26.02 ± 0.06	<0.0001	27.15 ± 0.03	<0.0001	28.20 ± 0.06[d]
BMI (kg/m^2)	21.0 ± 0.5	<0.0001	18.5 ± 0.4	NS	17.4 ± 0.3[d]
Glucose (mg/dl)[a]	85 ± 1	NS	83 ± 1	NS	82 ± 1[e]
Insulin (μU/ml)	15.0 ± 1.6	<0.0001	8.0 ± 0.8	NS	5.9 ± 0.5[d]
Insulin resistance	3.3 ± 0.4	<0.0001	1.7 ± 0.2	NS	1.2 ± 0.1[d]
TC (mg/dl)[b]	178 ± 3	NS	176 ± 3	NS	183 ± 3
TG (mg/dl)[c]	104 ± 7	<0.01	79 ± 4	NS	71 ± 3[d]
LDL-C (mg/dl)[b]	104 ± 3	NS	99 ± 3	NS	106 ± 3
HDL-C (mg/dl)[b]	56 ± 1	<0.0001	63 ± 1	NS	65 ± 1[d]
apoA-I (mg/dl)	132 ± 2	<0.01	139 ± 2	NS	143 ± 2[d]
apoA-II (mg/dl)	28.0 ± 0.3	NS	27.6 ± 0.4	NS	27.7 ± 0.4
apoB (mg/dl)	77 ± 2	NS	71 ± 2	NS	75 ± 2

NS, Not significant. Values are expressed as mean ± SEM.
[a] To convert to mmol/liter, divide by 18.
[b] To convert to mmol/liter, multiply by 0.0259.
[c] To convert to mmol/liter, multiply by 0.0113.
[d] $P < 0.0001$, significantly different from Small.
[e] $P < 0.05$, significantly different from Small.

performed a stepwise multiple regression analysis with LDL size as the dependent variable and the other parameters as independent variables (calculated parameters were excluded; model I: independent parameters exclude BMI, glucose, and insulin; model II: independent parameters include all uncalculated parameters listed in Table 2). In model I analysis (step 1), HDL-C was most significantly associated with LDL size and accounted for 16 and 18.4% of the variability of LDL size in boys and girls, respectively (Table 5). apoA-I in boys and TG in girls had a small additional effect (2.5% in boys and 2.6% in girls; step 2). In model II analysis, HDL-C in boys and BMI in girls had the most significant association with LDL size and accounted for 15.9 and 18.7% of the variability of LDL size in boys and girls, respectively. Insulin in boys and HDL-C and apoA-I in girls had additional effects. All of these parameters accounted for 22.9 and 28.1% of the variability of LDL size in boys and girls, respectively.

Discussion

The present study demonstrates the following: 1) the prevalence of pattern B is 10.8% in boys and 4.4% in girls; 2) children with pattern B have higher BMI and insulin resistance and a more atherogenic lipoprotein profile (higher TG, higher apoB, and lower HDL-C) than children with pattern A; 3) BMI, glucose, insulin, insulin resistance, HDL-C, apoA-I, and TG change as a function of the LDL size tertile; and 4) HDL-C and insulin in boys and BMI, HDL-C, and apoA-I in girls can predict 22.9 and 28.1% of the variability of LDL size, respectively.

To date, very limited information is available on LDL size in children (18, 28–32). With respect to gender differences of LDL size, Freedman *et al.* (31) also found gender differences in children. Studies in adults have reported that there is a significant gender difference in LDL size, demonstrating that women have larger LDL particles than men (32, 33). Although the underlying mechanism is not clear, differences in visceral fat accumulation and TG catabolism between men and women appear to be responsible for this gender difference (32, 33). In the present study, even after adjusting for BMI and the plasma concentration of TG, a gender difference is seen in our subjects. Thus, further studies are needed to understand the gender difference in children. Arisaka *et al.*

TABLE 5. Stepwise multiple regression analysis of correlates of LDL particle size

	Independent parameters			r	r^2
Model I analysis					
Boys					
Step 1	HDL-C			0.400	0.160
Step 2	HDL-C	apoA-I		0.430	0.185
Girls					
Step 1	HDL-C			0.428	0.184
Step 2	HDL-C	Log_{10} TG		0.558	0.210
Model II analysis					
Boys					
Step 1	HDL-C			0.399	0.159
Step 2	HDL-C	Log_{10} insulin		0.478	0.229
Girls					
Step 1	BMI			0.433	0.187
Step 2	BMI	HDL-C		0.518	0.268
Step 3	BMI	HDL-C	apoA-I	0.530	0.281

For model I, independent parameters exclude BMI, glucose, and insulin. For model II, independent parameters include all uncalculated parameters listed in Table 2. The r and r^2 values for each stepwise combination of independent variables are given above.

(29) reported that the prevalence of small dense LDL (pattern B) was 9.3% in Japanese school children aged 7–13 yr, and Steinbeck *et al.* (30) also reported that the prevalence of pattern B was 7.5% in Australian children aged 6.0–9.9 yr. These results are not so different from our present data. In adults, the prevalence of pattern B was 31–44% in the general population in the United States (13, 34), whereas the prevalence in Japan was 10–34.7% (35, 36). In either case, the prevalence of pattern B in children appears to be lower than that in adults. However, as in adults, children with pattern B had more risk factors (atherogenic lipoprotein profile, adiposity, and insulin resistance) for CHD than children with pattern A.

With respect to the correlates of LDL size in children, we previously reported positive associations with HDL-C, apoA-I, and fractional esterification rate in HDL, and a negative association with TG, although the number of subjects studied was small (18). In the present study, we confirmed the previous data and found that BMI, insulin resistance, and plasma concentrations of apoB, glucose, and insulin are also linked to LDL size in children. These findings are similar to those in adults (14, 17–21). In adults, LDL size and plasma concentrations of TG and HDL-C are closely interrelated, and 50–67% of the variance in plasma LDL size can be explained by plasma concentrations of TG and HDL-C (17, 37). Thus, it is generally accepted that the presence of small LDL could reflect metabolic changes in TG-rich lipoproteins or HDL. It is of interest that HDL-C and insulin for boys and BMI, HDL-C, and apoA-I for girls are independent predictors of LDL size in children. The plasma concentration of TG is not a significant predictor of LDL size in boys and a minor predictor in girls after excluding BMI, glucose, and insulin for analysis (model I). This may be attributable to a nonlinear association between the plasma concentration of TG and LDL size (38). LDL size was affected by plasma TG when the concentration of TG exceeded 1.5 mmol/liter (133 mg/dl) (39). The mean plasma TG concentration in our subjects was lower than this level. In contrast to plasma TG, HDL-C was an independent predictor of LDL size in children (analysis of models I and II). Taken together, these data suggest that correlates of LDL size in children are similar to those in adults, but the effects of each correlate on LDL size in children are much different from those in adults. Furthermore, in the present study, BMI and chemical parameters explained 22.9–28.1% of the variation in LDL size. These values are much lower than those in adults (50–67%). Based on twin studies, it has been reported that one third to half of the variation in LDL size can be attributed to genetic influences (40, 41). However, genetic factors are constant in children and adults. Thus, the lower predictive value suggests that different environmental factors between children and adults, such as body size and lifestyle, may have a great influence on LDL size (children are usually leaner than adults).

In conclusion, LDL size was correlated with BMI and many chemical parameters. Although the contribution of these parameters to LDL size in children was less than that in adults, improvement of these parameters by changes in lifestyle might be important for preventing the development of atherosclerosis in children.

Study limitation

As mentioned, LDL size is influenced by both genetic and environmental factors. Japanese children rarely drink alcohol or smoke and usually exercise regularly at school. Thus, the effects of environmental and genetic factors on LDL size and their implications in Japanese children may be different from those in other countries and other ethnic groups. Thus, our present results may not be generalized before similar studies are performed in other countries and other ethnic groups.

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Probucol Inactivates ABCA1 in the Plasma Membrane with Respect to Its Mediation of Apolipoprotein Binding and High Density Lipoprotein Assembly and to Its Proteolytic Degradation*

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Probucol has been shown to inhibit the release of cellular lipid by helical apolipoprotein and thereby to reduce plasma high density lipoprotein. We attempted to explore the underlying mechanism for this effect in human fibroblast WI-38. Probucol inhibited the apoA-I-mediated cellular lipid release and binding of apoA-I to the cells in a dose-dependent manner. It did not influence cellular uptake of low density lipoprotein, transport of cholesterol to the cell surface whether *de novo* synthesized or delivered as low density lipoprotein, and overall cellular content of cholesterol, although biosynthesis of lipids from acetate was somewhat increased. Probucol did not affect the mRNA level of ABCA1, and ABCA1 was recovered along with marker proteins for plasma membrane regardless of the presence of probucol. However, the protein level of ABCA1 increased, and the rate of its decay in the presence of cycloheximide was slower in the probucol-treated cells. ABCA1 in the probucol-treated cells was resistant to digestion by calpain but not by trypsin. We concluded that probucol inactivates ABCA1 in the plasma membrane with respect to its function in mediating binding of and lipid release by apolipoprotein and with respect to proteolytic degradation by calpain.

Cholesterol is an essential molecule for animal cells to maintain and regulate function and structure of the biomembrane. It is synthesized in most somatic cells, whereas its catabolic site is limited to the liver and to the steroidogenic cells except for partial hydroxylation in some somatic cells. Accordingly, cholesterol is removed from the cells and transported to the liver for its conversion to bile acids, and this is one of the essential events in cholesterol homeostasis for the body and for the cells (1). High density lipoprotein (HDL)[1] is believed to play a central role in this system, and this is thought to be one of the antiatherogenic characteristics of HDL. This reaction takes place through at least two distinct mechanisms: 1) physicochemical release of cholesterol from the cell surface, which is driven by cholesterol esterification on HDL, and 2) the apolipoprotein-mediated pathway to remove cellular cholesterol and phospholipid to generate new HDL particles (2). HDL thus plays a central role in both mechanisms.

Apolipoprotein-dependent cellular cholesterol release is absent in fibroblasts from patients with Tangier disease (3, 4), and mutations in the gene encoding the ATP-binding cassette transporter A1 (ABCA1) are the underlying cause of this disease (5–9). On the other hand, *in vitro* overexpression of functional ABCA1 in the cells (10, 11) and induction of ABCA1 expression by cyclic AMP analogues (12, 13) or by the ligands for the liver X receptor or retinoid X receptor (14, 15) enhanced the release of cellular cholesterol and phospholipid by apolipoprotein. The transgenic mice for ABCA1 had a significant increase in plasma HDL (16, 17). These results indicate that this protein is a regulating factor for the plasma HDL level through generation of HDL by the apolipoprotein-cell interaction.

Probucol has been clinically used as an antiatherogenic compound, not only because of its lipid-lowering effect but also because of the hypothesis that its antioxidative nature prevents atherogenic oxidative modification of low density lipoprotein (LDL) shown by *in vitro* (18, 19) and *in vivo* models (20, 21). However, probucol substantially reduces plasma HDL (22). We reported that probucol causes dramatic selective inhibition of the apolipoprotein-mediated cellular lipid release and its binding to cells (23) to cause reduction of HDL (24), which is analogous to the finding with Tangier disease (3, 4). Thus, this is another piece of evidence that the apolipoprotein-cell interaction to generate HDL is a major source of plasma HDL. Probucol can therefore be considered an inhibitor of the function of ABCA1.

Cellular ABCA1 undergoes both transcriptional and post-transcriptional regulations (25). Transcriptional regulation of ABCA1 is carried out by oxysterol through the liver X receptor/retinoid X receptor system, which seems relevant to the function of ABCA1 to expel an excess amount of cell cholesterol (25). Up-regulation of ABCA1 is also mediated by other factors such as cyclic AMP (13), of which the exact mechanism is unknown. On the other hand, stabilization of ABCA1 can be an alternative mechanism to regulate ABCA1 activity, such as protection of ABCA1 protein by helical apolipoprotein (26–28) and enhancement of its degradation by unsaturated fatty acid (29) or overloaded cholesterol (30). Previous studies have suggested that ABCA1 phosphorylation is involved in its stabilization (31, 32). A PDZ adaptor protein α1-syntrophin was shown to stabilize ABCA1 (33).

In the present study, we attempted to examine the underlying mechanism for inhibition of ABCA1 activity by probucol. Probucol did not influence the transcription and intracellular

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[1] The abbreviations used are: HDL, high density lipoprotein; ABCA1, ATP-binding cassette transporter A1; LDL, low density lipoprotein; PBS, phosphate-buffered saline; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; CE, cholesteryl ester; MEM, modified Eagle's essential medium; PMSF, phenylmethylsulfonyl fluoride.

distribution of ABCA1 but suppressed its degradation by calpain to increase inactive ABCA1 in the plasma membrane.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Probucol (4,4′-(isopropylidenedithio)bis [2,6-di-*tert*-butylphenol]) was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Calpain and calpeptin were from Calbiochem. [1,2-^{14}C]Cholesteryl oleate and [^{3}H]acetic acid were obtained from Amersham Biosciences, and iodine-125 (^{125}I) was from PerkinElmer Life Sciences.

Lipoprotein and Apolipoprotein—Lipoproteins were isolated from fresh human plasma by sequential ultracentrifugation in sodium bromide at a density of 1.006–1.063 g/ml for LDL and 1.125–1.21 g/ml for HDL. Lipoprotein-free plasma protein fraction was collected as a bottom fraction with a density of 1.21 g/ml. All plasma fractions were thoroughly dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS). Apolipoprotein A-I (apoA-I) was isolated from the human HDL fraction by delipidation followed by anion exchange column chromatography in 6 M urea as described previously (34). ApoA-I was dissolved in PBS before use in experiments according to the methods described previously (34).

Preparation of Probucol-containing LDL and Probucol-carrying Bovine Serum Albumin (BSA)—To deliver probucol to the cells, probucol-containing LDL was prepared according to the method described previously (23, 35). Lipid microemulsion was prepared by sonicating egg phosphatidylcholine (Avanti) and triolein (Wako Pure Chemicals) with and without probucol. LDL (15 mg of protein) was incubated at 37 °C for 48 h with the microemulsion containing or not containing probucol in the presence of lipid-free plasma fraction (1.5 g of protein), dithionitrobenzoic acid (2 mM), aprotinin (20 units/ml), gentamycin (0.1 mg/ml), EDTA (0.5 mM), and NaN_3 (0.1% w/v). The mixture was applied to a dextran sulfate-cellulose column to recover LDL as the bound fraction eluted with 0.5 M NaCl. LDL was further purified by ultracentrifugal floatation at a density of 1.063 g/ml and thoroughly dialyzed against PBS. To label cholesteryl ester (CE) in LDL, the emulsion was prepared with [^{14}C]cholesteryl oleate (30 μCi), and the same procedure was applied to prepare the labeled LDL containing probucol. Alternatively, probucol was conjugated with BSA (fatty acid-free, Sigma). 250 μg of probucol was solubilized in 125 μl of methanol and incubated with 10 ml of 10% BSA (w/v) for 1 h at 37 °C.

Cell Culture and Loading with LDL—WI-38 human fibroblast cells (RIKEN Cell Bank) (36) were grown at 37 °C in Eagle's minimum essential medium (Sigma) with 10% fetal calf serum (HyClone Laboratories, Inc.), 5 units/ml penicillin, and 5 μg/ml streptomycin (Invitrogen). Cells were seeded into a 35-, 60-, or 100-mm dish at a density of 1.5×10^5 cells/ml. When the cells were grown to 80% of a confluent stage, the probucol-containing LDL (0–50 μg as LDL protein/ml) or probucol-carrying BSA (0.2%, w/v) was added for 24-h incubation to load probucol in the cells.

Cellular Lipid Release Assay—The probucol-loaded cells prepared as described above were washed and maintained in the lipoprotein-free medium for the next 24 h prior to any further experiments. The cells were incubated for 24 h with various amounts of apoA-I in the medium containing 0.02% BSA. Lipid was extracted from the medium with chloroform:methanol (2:1, v/v) and cells with *n*-hexane:2-propanol (3:2, v/v), and total cholesterol, free cholesterol, and choline-phospholipid were determined by colorimetric enzymatic assay system (Kyowa Medics) (13).

Determination of Probucol in the Cells—Probucol in the cell lipid extracts was measured by reverse-phase high performance liquid chromatography according to the method described by Satonin and Coutant (37). The cell extracts from the medium of a 60-mm dish were dissolved in acetonitrile, hexane, 0.1 M ammonium acetate (90:6.5:3.5, v/v/v) and injected into a Deltapak C_{18} reversed-phase column (150 × 3.9 mm, 300 A; Waters). The mobile phase was acetonitrile:water (85:15, v/v) with the flow rate at 1.5 ml/min, and the probucol was detected by absorbance at 240 nm.

Evaluation of the Synthesis of Lipids—WI-38 cells at 80% of confluent stage in 35-mm dishes were washed with PBS and cultured in 1 ml of modified Eagle's essential medium (MEM) containing 0.2% BSA with or without probucol. After replacement with 1 ml of fresh medium, the cellular lipid was labeled by incubating for 2 h with 20 μCi/ml [^{3}H]acetic acid. Lipid was extracted with *n*-hexane:2-propanol (3:2, v/v), and radioactivity was determined in cholesterol, phosphatidylcholine, sphingomyelin, and cholesteryl ester after separation by thin layer chromatography.

Determination of Cholesterol Distribution to the Plasma Membrane—To determine traffic of newly synthesized cellular cholesterol moved to the cell surface, the cells were treated with 0.2% BSA carrying and not carrying probucol for 24 h and were labeled with [^{3}H]acetic acid for 2 h. To determine distribution of LDL-derived cholesterol to the cell surface, the cells were incubated for 24 h with [^{14}C]CE-LDL (50 μg of protein/ml) containing or not containing probucol, washed with PBS, and incubated with MEM containing heparin (1400 IU/ml) for 45 min at 4 °C to remove surface-bound LDL. The labeled cells were quickly washed with PBS, fixed with 1% glutaldehyde at room temperature for 10 min, and washed with PBS to remove the fixative reagent. The cells were then incubated with cholesterol oxidase (1 unit/ml) in MEM for 1 h to allow the conversion of cholesterol to cholestenone in the plasma membrane (38). After the cells were washed with PBS, cellular lipids were extracted, and radioactivity was determined for cholesterol, cholestenone, and cholesteryl ester separated by thin layer chromatography.

Subcellular Membrane Fractionation—Bulk membrane fraction was prepared as follows. Cells in 100-mm dishes were harvested and treated with 5 mM Tris-HCl (pH 7.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM benzamidine) for 30 min on ice with vortexing every 10 min. The cell debris and nuclei were removed by centrifugation at 650 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 444,000 × *g* for 40 min at 4 °C. Subcellular membrane subfractions were prepared as follows. The cell pellet after centrifugation at 600 × *g* for 10 min was lysed with cold extract solution (0.02 M boric acid, 0.3 mM EDTA, 1 mM PMSF, 1 mM benzamidine, and protease inhibitor cocktails (Sigma), pH 10) for 15 min on ice with vortexing every 5 min. The cell debris and nuclei were discarded by centrifugation at 650 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 12,000 × *g* for 1 h at 4 °C. The pellet was harvested, and the supernatant was further centrifuged at 290,000 × *g* for 30 min at 4 °C. The second pellet was harvested, and the supernatant was treated with 10% trichloroacetic acid to precipitate the protein.

Western Blotting—The membrane fraction and cellular subfractions were resuspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 10 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1% Triton X-100, and 1% protease inhibitor cocktails (Sigma) and were sonicated for 5 s. After determination of the protein content by a BCA method (Pierce), the fractions were dissolved in 9 M urea, 2% Triton X-100, 1% dithiothreitol and were developed in 6 or 15% (w/v) polyacrylamide gel electrophoresis in the presence of 10% SDS, respectively, and the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) by semidry blotter in blotting buffer (25 mM Tris-HCl, 0.2 M glycine, and 10% methanol (v/v)) for 3.5 h. The membrane was blocked with 5% skim milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 and was probed with the rabbit antiserum against the C-terminal peptide of human ABCA1 (26, 39), rabbit anticaveolin-1 (N-20) (Santa Cruz Biotechnology), anti-GLUT-1, mouse anti-integrin β_1 (CHEMICON International, Inc.), anti-GM130, and anti-Bip/GRP78 (BD Transduction Laboratories, BD Biosciences), respectively. The immunoreactive proteins were visualized by ECL or the ECL Plus system (Amersham Biosciences).

ABCA1 Degradation Rate—WI-38 cells in 100-mm dishes were incubated in 6 ml of the medium with LDL (300 μg of protein) containing or not containing probucol as described above. Cells were washed three times with PBS and incubated at 37 °C in MEM containing 20 ng/ml cycloheximide (Wako Pure Chemicals). ABCA1 in the cell membrane fraction was detected by immunoblot analysis as described above. For studying the calpain-mediated proteolysis of ABCA1, the experiment was performed according to the method described by Wang *et al.* (27). Cells were washed three times with PBS and placed on ice for 10 min. Then cells were permeabilized by incubating on ice for 15 min with 80 μg/ml digitonin in MEM. The cells were washed twice with PBS and incubated for 20 min at room temperature with μ-calpain (0.1 μM) in MEM containing 2 mM $CaCl_2$. The cells were lysed with 3 ml of buffer (5 mM Tris-HCl, pH 7.5, 1 mM PMSF, and 1 mM benzamidine) containing 40 μg/ml calpeptin. The ABCA1 in the cell membrane fraction was analyzed by using immunoblot analysis.

RNA Extraction and Real Time Quantitative PCR—Total RNA was extracted from cells by using RNA extraction reagent (Isogen, Nippon Gene). After contaminated genomic DNA was digested with DNase I (Takara Shuzo Co.), first standard cDNA was synthesized by a SuperScript™ preamplification system (Invitrogen) from 2 μg of the total RNA. PCR was performed by using primers (sense and antisense) for cDNA 5′-GAA CTG GCT GTG TTC CAT GAT-3′ and 5′-GAT GAG CCA GAC TTC TGT TGC-3′ (for ABCA1) and 5′-ATG GTG GGA ATG GGT CAG AAG-3′ and 5′-CAC GCA GCT CAT TGT AGA AGG-3′ (for β-actin)

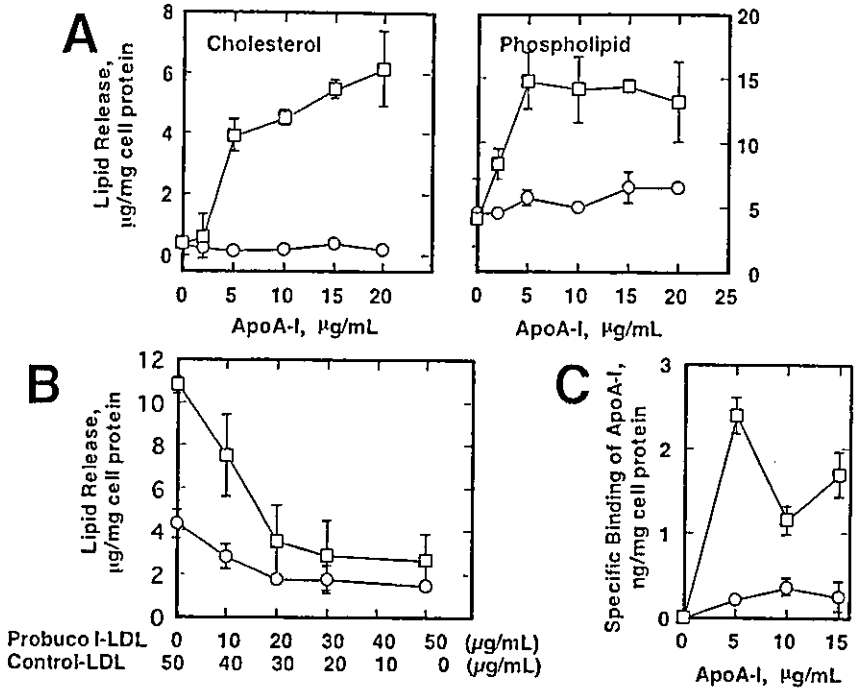

FIG. 1. **Effect of probucol on the apoA-I-mediated cellular lipid release.** WI-38 cells were incubated with LDL containing probucol or not containing probucol (50 μg of LDL protein/35 mm-dish) at 37 °C for 24 h and equilibrated for distribution of cholesterol and probucol by further incubation in 0.2% BSA for 24 h. Lipid-free apoA-I was added to the cells and incubated for 24 h. *A*, the *left-hand panel* shows release of cholesterol into the medium from the control cells (*squares*) and the probucol-treated cells (*circles*). The *right-hand panel* shows the release of choline-phospholipid from the control and probucol-treated cells (*squares* and *circles*, respectively). *B*, dose-dependent effect of probucol. The dose of probucol-containing LDL was increased (0, 10, 20, 30, 40, and 50 μg of LDL protein/35-mm dish) as the control LDL was decreased to maintain the total LDL protein at 50 μg. The release of cholesterol (*circles*) and choline-phospholipid (*squares*) was measured as described above. *C*, the ^{125}I-labeled apoA-I was incubated at 0 °C for 2 h with the WI-38 cells pretreated with LDL containing probucol or not as described above. Specific binding of ^{125}I-apoA-I was calculated by subtracting the binding after displacement with cold apoA-I from the total binding of the labeled apoA-I as described in the text for the control-LDL-treated (*squares*) and probucol-LDL-treated (*circles*) cells. The *data points* represent the average ± S.E. of an assay performed in triplicate.

(synthesized by Sawady Technology Co., Ltd.). The quantification of ABCA1 and β-actin mRNA was achieved using SYBR Green PCR master mix reagent in an ABI PRISM 7700 sequence detection system (Applied Biosystems Japan).

^{125}I-Apolipoprotein A-I Binding to the Cell Surface—Specific binding of ^{125}I-labeled apoA-I was estimated as displaceable binding by excessive cold apoA-I (23). ApoA-I was dissolved as 1 mg in 20 ml of the 0.1 M PBS (pH 6.5) and incubated at 37 °C for 1 h. ^{125}I (2 mCi) and 15 beads of IODO-BEAD® iodination reagent (Piece) were added to the solution, and the labeling reaction was carried out with stirring for 15 min at room temperature. The solution was concentrated by using the Ultrafree-15 centrifugal filter device (Millipore) with centrifugation at 700 × *g* for 1.5 h at 4 °C. The analysis of product by polyacrylamide gel electrophoresis showed that 94% of the total radioactivity was recovered in apoA-I and that the specific radioactivity was 2367, 608 cpm/μg of protein. After treatment with LDL containing or not containing probucol, cells in 35-mm dishes were incubated with various concentrations of ^{125}I-apoA-I at 0 °C for 2 h in 500 μl of MEM (pH 7.4, containing 25 mM HEPES, 0.2% BSA). The cells were chased twice by the medium with or without 50 μg/ml non-labeled apoA-I at 0 °C for 4 h and washed with cold PBS. The cell-bound radioactivity was recovered in 1 ml of 0.5 M NaOH.

RESULTS

Consistent with our previous reports (23, 24), probucol inhibited the apoA-I-mediated cellular lipid release (Fig. 1*A*). This effect was dose-dependent with respect to the dose of probucol-containing LDL (Fig. 1*B*). Probucol also inhibited displaceable binding of ^{125}I-apoA-I (Fig. 1*C*). Dose-dependent increase of cellular probucol content was observed within the range of the dosage of the probucol-containing LDL employed in the present experiment (Fig. 2*A*). Loading of cholesterol to the cells via LDL was not influenced by the presence of probucol in LDL (Fig. 2*B*).

The effect of probucol on lipid biosynthesis was examined. To avoid the effect of lipid loading via LDL, probucol was given to the cells as a BSA-probucol conjugate. Probucol also inhibited the apoA-I-mediated lipid release even when it was given directly by this procedure (Fig. 3*A*). In this condition, incorporation of acetate was somewhat increased by probucol in all of the lipid fractions tested, including free cholesterol, cholesteryl ester, phosphatidylcholine, and sphingomyelin (Fig. 3*B*).

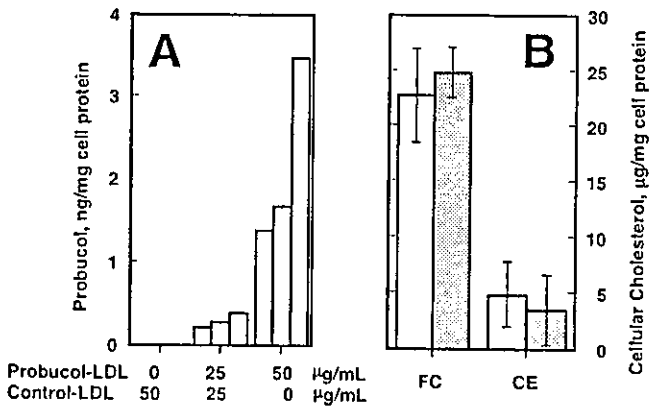

FIG. 2. **Accumulation of LDL-derived cholesterol and probucol in WI-38 cells.** *A*, WI-38 cells were incubated with probucol-free or probucol-containing LDL (total of 50 μg of protein) in 3 ml of medium within a 60-mm dish for 24 h. Probucol accumulated in the cells was measured using reverse-phase high pressure liquid chromatography as described under "Experimental Procedures." Each *bar* represents an individual experimental data point. *B*, the accumulation of cholesterol in WI-38 by incubating with probucol-free LDL (*open bar*) and probucol-carrying LDL (*dot-filled bar*) was measured. LDL (50 μg of LDL protein/35-mm dish) was incubated with cells at 37 °C for 24 h. The cellular cholesterol was determined as described under "Experimental Procedures." The values are the average and S.E. of triplicate assays. *FC*, free cholesterol.

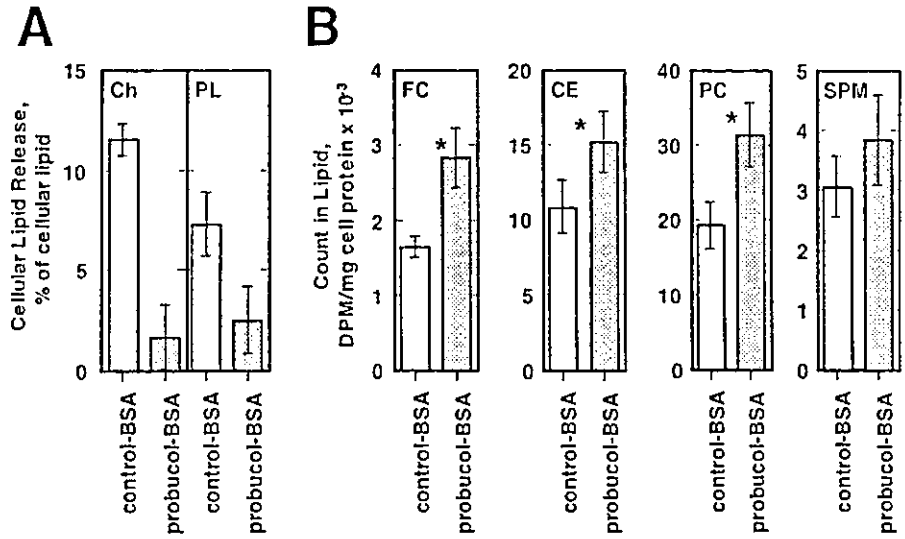

FIG. 3. **Effect of probucol on cellular lipid synthesis.** *A*, probucol-carrying or probucol-free 10% BSA was added to the medium to make the final BSA concentration 0.2% and incubated for 24 h at 37 °C. ApoA-I was added to the cells and incubated for another 24 h. The cholesterol (*Ch*) and phospholipid (*PL*) in the medium were measured as described under "Experimental Procedures." *B*, WI-38 cells were loaded with probucol as described above, followed by labeling with [^{3}H]acetic acid (20 μCi/ml) for 2 h. The cellular lipids were extracted and separated by thin layer chromatography, and radioactivity was determined in the fractions of free cholesterol (*FC*), cholesteryl ester (*CE*), phosphatidylcholine (*PC*), and sphingomyelin (*SPM*). Each *bar* represents the average and S.E. of three data points. *Dot-filled columns* and *open columns* indicate the data for probucol-treated and probucol-free cells, respectively. *Asterisks* indicate a significant increase from the control ($p < 0.05$).

Cellular cholesterol is distributed throughout 60–80% of the plasma membrane (40), and cholesterol accounts for as much as 30–40% of lipid molecules in the plasma membrane (41). Transport of cholesterol to the plasma membrane is differentially regulated for the newly synthesized and the LDL-derived cholesterol molecules. Therefore, distribution of cholesterol from these different sources was examined with respect to the effect of probucol. Cellular cholesterol was labeled by incubating with [^{3}H]acetic acid or by incorporating [^{14}C]CE-LDL, and cell surface cholesterol was probed by extracellular cholesterol oxidase. As shown in Fig. 4, probucol caused a small decrease in relative distribution of *de novo* synthesized cholesterol to the surface, probably because of the apparent increase of the synthesis shown in Fig. 3*B*, but did not cause any significant difference in the LDL-derived cholesterol. Based on the results above, it is unlikely that the inhibitory effect of probucol on the HDL assembly reaction is related to alteration of cellular cholesterol metabolism.

We analyzed the expression of ABCA1. The message of ABCA1 was not influenced by probucol at all (Fig. 5*A*). However, probucol increased ABCA1 as analyzed by immunoblotting of the membrane fraction, whereas there was no change in integrin β_1 and GLUT-1 (Fig. 5*B*). The initial immunocytochemical studies suggested that endogenously expressed human ABCA1 was localized in the plasma membrane (7, 42, 43), so the effect of probucol on intracellular distribution of ABCA1 was investigated. The cell membrane was fractionated as described under "Experimental Procedures," and each fraction was analyzed by immunoblotting for ABCA1 and membrane marker proteins. As demonstrated in Fig. 5*C*, ABCA1 was detected along with a plasma membrane marker, integrin β_1, predominantly in the pellet fraction that was centrifuged at 12,000 × *g* for 60 min (*lanes 1* and *5*). Caveolin-1 was also recovered mainly in this fraction. In contrast, a significant amount of a marker for endoplasmic reticulum, Bip/GRP78, was found in the supernatant fraction centrifuged at 290,000 × *g* for 30 min (Fig. 5*C*, *lanes 3* and *4* and *lanes 7* and *8*). A Golgi marker, GM130, was also distributed to this fraction. Probucol did not influence this pattern of distribution of ABCA1 and marker proteins. Thus, ABCA1 is predominantly present in the plasma membrane, and probucol does not significantly alter

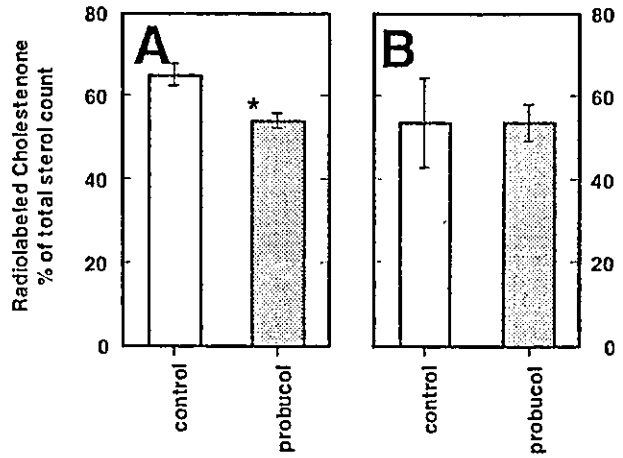

FIG. 4. **Determination of cholesterol content in plasma membrane by cholesterol oxidase.** WI-38 cells were incubated with 0.2% probucol-free or probucol-carrying BSA for 24 h and then were radiolabeled with [^{3}H]acetic acid (20 μCi/ml) for 2 h (*A*) or were incubated with LDL (50 μg protein/ml) containing [^{14}C]cholesteryl ester and probucol for 24 h at 37 °C (*B*). The cholesterol on the cell surface was probed by extracellular cholesterol oxidase as described under "Experimental Procedures." The results are displayed as the percentage of radioactive cholestenone within total cellular sterol. Results represent the mean ± S.D. of triplicate determinations of a representative experiment. An *asterisk* indicates a significant difference from the control ($p < 0.05$).

the traffic of ABCA1. ABCA1 and caveolin-1 may also be somewhat recovered with the Golgi-endoplasmic reticulum fractions, which is consistent with previous findings (44, 45).

To study the underlying mechanism for the increase of ABCA1 without changing its message, degradation of ABCA1 in the cell was examined. ABCA1 was analyzed by immunoblotting in the presence of cycloheximide. A decrease in ABCA1 was apparent at 30 min, and ABCA1 decayed throughout the incubation up to 120 min (Figs. 6*A* and 7*A*). Probucol apparently slowed the rate of the decay of ABCA1 (Figs. 6*A* and 7*A*).

To examine the effect of probucol on proteolytic degradation of ABCA1, the susceptibility of ABCA1 to protease was observed after the cells were permeabilized by digitonin. To exclude a possibility that probucol may inhibit the membrane

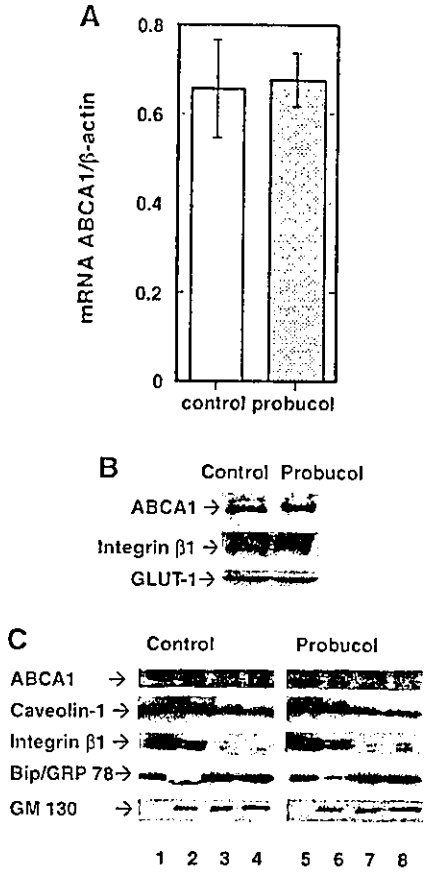

FIG. 5. **Effect of probucol on ABCA1.** WI-38 cells were incubated with LDL containing probucol or not for 24 h. *A*, specific messages of ABCA1 and β-actin were quantified by real time PCR. The ABCA1 message is standardized for the β-actin mRNA in the figure. The data represent the average ± S.E. of an assay performed in triplicate. *B*, total membrane fraction was prepared from the probucol-treated and control cells as described in the text. Fifty μg of the membrane protein was analyzed by immunoblotting for ABCA1, GLUT-1, and integrin β_1. *C*, the subcellular membrane fractions were prepared as described under "Experimental Procedures" as the pellet of the first centrifugation (12,000 × *g* for 60 min), the pellet of the second centrifugation (290,000 × *g* for 30 min), the supernatant of the first centrifugation, and the supernatant of the second centrifugation. Each fraction was analyzed by immunoblotting for ABCA1, caveolin-1, integrin β_1 (plasma membrane marker), Bip/GRP78 (endoplasmic reticulum marker), and GM130 (Golgi marker). *Lanes 1* and *5*, 55 μg of protein of the pellet fraction of the first centrifugation. *Lanes 2* and *6*, 55 μg of protein of the pellet fraction of the second centrifugation. *Lanes 3* and *4* and *lanes 7* and *8*, 55 μg of protein precipitated with 10% trichloroacetic acid from the supernatant of the second centrifugation.

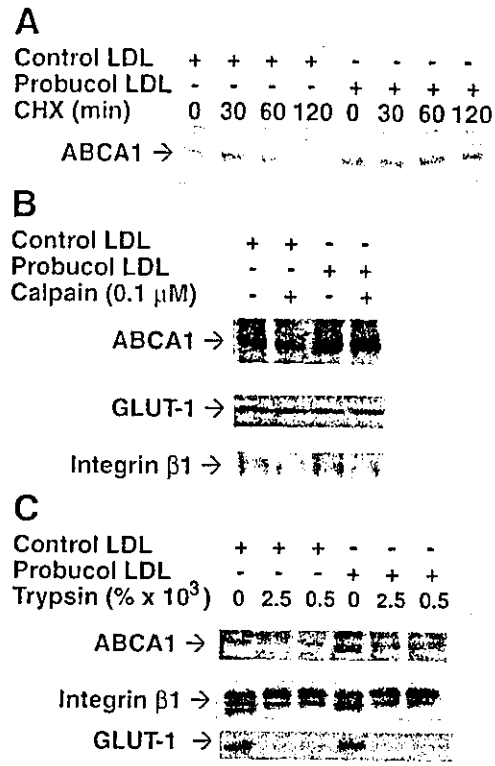

FIG. 6. **Effect of probucol on ABCA1 degradation.** *A*, WI-38 cells were pretreated with control or probucol-containing LDL. The cells were then incubated in the presence of cycloheximide (*CHX*) (20 ng/ml) for the indicated times, and membrane ABCA1 was analyzed by immunoblotting. 55 μg of the membrane protein was applied to each *lane*. *B* and *C*, WI-38 cells pretreated with control or probucol-containing LDL were permeabilized by incubating with 80 μg/ml digitonin on ice for 15 min. The cells were then incubated with μ-calpain (0.1 μM) in MEM containing 2 mM $CaCl_2$ for 20 min at room temperature (*B*) or with trypsin at the indicated concentration for 3 min at room temperature (*C*). Membrane ABCA1, GLUT-1, and integrin β_1 were analyzed by immunoblotting assay.

permeabilization by digitonin, cells were examined with immunostaining for β-tubulin after the permeabilization treatment. There was no difference between the cells treated with LDL containing probucol and no probucol (data not shown). ABCA1 in the cells treated with probucol became resistant to μ-calpain (a ubiquitously expressed subtype of calpain) (Figs. 6*B* and 7*B*). In contrast, probucol did not influence degradation of ABCA1 by trypsin (Figs. 6*C* and 7*C*). GLUT-1 was insensitive to calpain, whereas integrin β_1 was susceptible (Figs. 6*B* and 7*B*). Probucol did not influence digestion of integrin β_1 and GLUT-1, either by calpain or by trypsin (Fig. 6, *B* and *C*, and Fig. 7, *B* and *C*). The data provided direct evidence that physiological degradation of ABCA1 by calpain is inhibited by probucol.

DISCUSSION

The underlying mechanism for the inhibitory effect of probucol on the function of ABCA1 was investigated. The results of the study are summarized as follows. 1) Probucol inhibited the events mediated by ABCA1 such as apolipoprotein-mediated cellular lipid release and apolipoprotein binding to the cells. 2) Probucol did not interfere with transcription and intracellular trafficking of ABCA1, and ABCA1 was predominantly found in the plasma membrane even in the probucol-treated cells as judged by biochemical parameters. 3) Probucol made ABCA1 resistant to calpain-mediated degradation and consequently increased its cellular level. 4) Probucol did not influence cellular lipid accumulation via LDL and cholesterol distribution to the cell surface but rather enhanced lipid biosynthesis. We thereby concluded that probucol inactivated ABCA1 in the plasma membrane with respect to its functions and its susceptibility to proteolysis.

Probucol was used as a lipid-lowering drug for years before statins became available. This drug was known for its apparent clinical effect of regression of cutaneous and tendinous xanthomas more than expected from the reduction of plasma LDL (46). Because probucol is characterized for its strong antioxidative nature, it was expected to work as an antioxidant against oxidative modification of plasma lipoprotein to prevent development of atherosclerotic vascular lesions and even to cure them. This hypothesis was indeed strongly supported by several experimental approaches using animal models for atherosclerosis (20, 21). However, this drug has also been known for its strong effect of lowering plasma HDL. What is more puzzling is that reduction of HDL by probucol is sometimes seriously aggravated by fibrates, which are otherwise expected to raise HDL in plasma (22). Thus, probucol has been a very

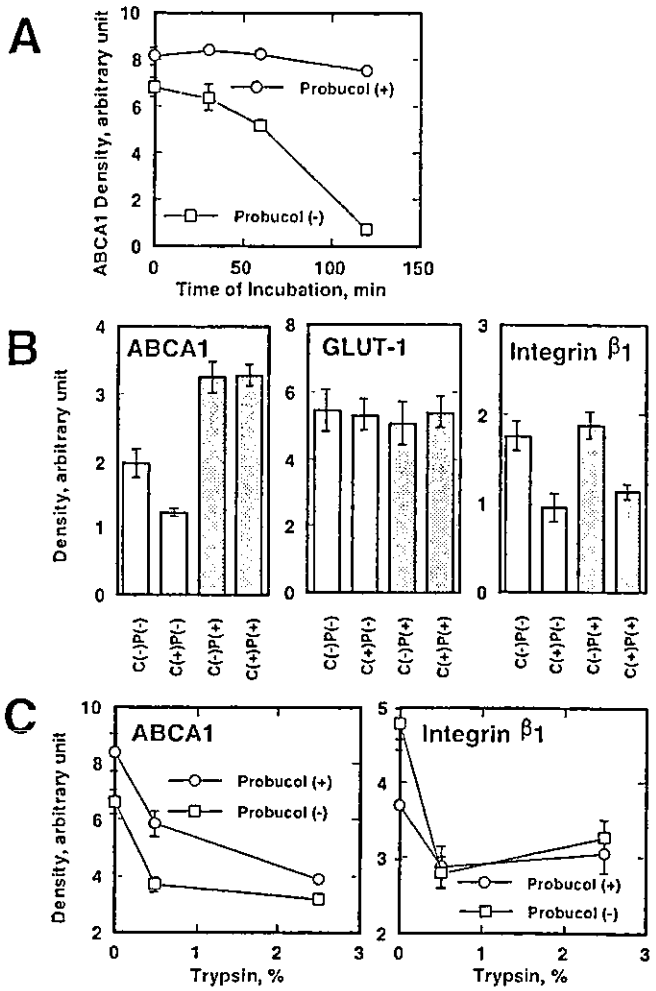

FIG. 7. **The results of density scanning of the Western blotting bands in the experiments represented by Fig. 6.** *Panels A, B,* and *C* correspond with *A, B,* and *C* of Fig. 6, respectively. In *B, C* and *P* indicate calpain and probucol, respectively. The density of each band was quantitated by digital scanning in an Epson GT9500. Data points represent mean ± S.E. of three experiments.

controversial drug, and it lost its popularity in the market as statins took over.

We discovered that probucol inhibits the apolipoprotein-mediated cellular lipid removal and generation of HDL as well as the binding of apolipoprotein to the cells (23). This finding is very similar to the observation with the cells of patients with Tangier disease, a familial HDL deficiency (3, 4). We thereby speculated that this is a reason for probucol to reduce HDL, and accordingly the apolipoprotein-mediated generation of HDL with cellular lipid is a main source of plasma HDL. We further investigated an *in vivo* effect of probucol by using mouse models. Probucol inhibited the production of HDL in mice, and the kinetic analysis of plasma HDL revealed the enhanced clearance of HDL apoprotein by probucol but no difference in the HDL-lipid clearance (24), an exact analogy to the behavior of HDL in Tangier disease patients (47), indicating that probucol inhibits the reaction to generate HDL that is lacking in patients with Tangier disease. The use of probucol in the lecithin:cholesterol acyltransferase-deficient mice showed that cholesterol accumulated only in the liver, suggesting that the liver is the major organ where generation of HDL by this mechanism takes place (48). ABCA1 was identified as a protein essentially responsible for this reaction, and the action of probucol on ABCA1 has become a subject of study.

In the current study, we focused on the effects of probucol on ABCA1 with respect to its transcription and trafficking as well as its role in mediating apoA-I binding and cellular lipid release by apolipoprotein. We also investigated its effect on proteolytic degradation of ABCA1, another main mechanism for regulating the level of ABCA1 in cells. Interestingly, there was no effect of probucol either on transcription or on trafficking of ABCA1 as judged by biochemical analysis. The cellular ABCA1 was rather significantly increased, and this was apparently because of the decrease of susceptibility of ABCA1 to proteolysis by calpain, a physiological regulation pathway for ABCA1. Thus, probucol inactivated not only functional aspects of ABCA1 but also its clearance system. Probucol is a very hydrophobic compound, and accordingly it is almost water-insoluble. Therefore, it is almost exclusively carried by lipoprotein in plasma and distributes in the membrane when cleaved to cells (37). Because almost all ABCA1 is recovered in plasma membrane, probucol is likely to act on ABCA1 in the plasma membrane. The decrease of protease susceptibility is not observed for trypsin, so the effects of probucol seem to induce a certain specific conformational alteration of ABCA1 to inactivate this protein against calpain, presumably at the membrane-spanning regions.

Probucol did not significantly influence intracellular lipid metabolism except that incorporation of [^{3}H]acetic acid into various lipid fractions increased somewhat, which may require further investigation. Thus, the effect of probucol on ABCA1 is unlikely to be a secondary phenomenon to its effect on cellular lipid metabolism.

There are no previous reports of any chemical compound acting in such a manner to inactivate membrane proteins. Further studies are required to elucidate the detail of the mechanism by which probucol inactivates ABCA1 in the membrane. This would provide important information about the reaction mechanism of ABCA1, cellular cholesterol homeostasis, and generation of HDL.

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Reviews

Molecular Mechanisms of Cholesteryl Ester Transfer Protein Deficiency in Japanese

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Plasma cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl ester (CE) from high density lipoprotein (HDL) to apolipoprotein B-containing lipoproteins. Since CETP regulates the plasma levels of HDL cholesterol and the size of HDL particles, CETP is considered to be a key protein in reverse cholesterol transport (RCT), a protective system against atherosclerosis. The importance of plasma CETP in lipoprotein metabolism was demonstrated by the discovery of CETP-deficient subjects with marked hyperalphalipoproteinemia (HALP). Genetic CETP deficiency is the most important and common cause of HALP in the Japanese. Ten mutations of the CETP gene have been demonstrated as causes of HALP, including two common mutations: an intron 14 splicing defect (Int14 + 1 G → A) and an exon 15 missense mutation (D442G). The subjects with CETP deficiency show a variety of abnormalities in the concentration, composition, and function of both HDL and low density lipoprotein (LDL). CETP deficiency is considered a physiological state of impaired RCT, which may possibly lead to the development of atherosclerosis despite high HDL cholesterol levels. However, the pathophysiological significance of CETP in terms of atherosclerosis has been controversial. Epidemiological studies in Japanese-Americans living in Hawaii and Japanese in the Omagari area, where HALP subjects with an intron 14 splicing defect of the CETP gene are markedly frequent, have shown a relatively increased incidence of coronary atherosclerosis in CETP deficiency. On the other hand, the *Taq*IB polymorphism-B2 allele with low CETP mass and increased HDL cholesterol has been related to a decreased risk for coronary heart disease (CHD) in many studies, including the Framingham Offspring Study. The current review focused on the characterization of the Japanese subjects with CETP deficiency, including our recent findings. *J Atheroscler Thromb, 2004; 11: 110–121.*

Key words: Cholesteryl ester transfer protein deficiency, Hyperalphalipoproteinemia, HDL cholesterol, Atherosclerosis

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Introduction

Many epidemiological studies have demonstrated a negative correlation of serum high density lipoprotein (HDL) cholesterol levels with the incidence of coronary heart disease (CHD), and subjects with genetic HDL deficiency often have accompanying atherosclerotic car-

diovascular diseases (1, 2). Therefore, HDL plays a crucial role in the protection of blood vessels from atherosclerosis. HDL functions to deliver excess cholesterol from peripheral tissues to the liver for excretion into the bile, a pathway designated as 'reverse cholesterol transport' (3, 4). In this system, small HDL or free apolipoprotein (apo) A-I removes free cholesterol (FC) from the peripheral cells, and FC in HDL is esterified by lecithin: cholesterol acyltransferase (LCAT) to cholesteryl ester (CE), which is subsequently transferred to very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) by plasma cholesteryl ester transfer protein (CETP) (5–7). IDL and LDL are catabolized via the LDL receptor in the liver. Furthermore, the CE moiety of HDL is taken up selectively by the liver via scavenger receptor class B type I (SR-BI) (8). Thus, CETP is involved in the regulation of plasma HDL cholesterol levels, and facilitates the remodeling of HDL particles.

The significance of plasma CETP in lipoprotein metabolism was highlighted by the discovery of CETP deficiency with marked hyperalphalipoproteinemia (HALP). Genetic CETP deficiency is the most important and common cause of HALP in the Japanese. In the current review, we will focus on the findings obtained on Japanese CETP deficiency.

CETP gene mutations and their frequency in the Japanese

Human CETP cDNA, cloned by Drayna *et al.* (9), encodes a very hydrophobic glycoprotein with an *Mr* of 74,000, containing 476 amino acid residues (4, 10). The human CETP gene is mapped in chromosome 16 (16q13) near the LCAT gene and spans over 25k base pairs, including 16 exons (11). Since genetic deficiency of CETP activity was first described by Kurasawa *et al.* and Koizumi *et al.* in 1985 (12, 13), to date, genetic CETP deficiency has been the most important and frequent cause of HALP in the Japanese (14–19). Ten mutations in the CETP gene have so far been identified Japanese CETP deficiency, including 3 splicing site, 3 nonsense, 1 promoter, and 3 missense mutations (14, 17, 18, 20–25) (Table 1). Of the 10 mutations, it has been demonstrated that a G-to-A substitution at the 5' splice donor site of intron 14 (Int14 + 1 G → A) and a missense mutation of exon 15 (D442G) in the CETP gene are common mutations associated with HALP in the Japanese. Furthermore, the prevalences of the Int14 + 1 G → A and D442G mutations are extremely high in the general Japanese population, with heterozygote frequencies of 1% and 7%, respectively (15, 18, 26, 27). Other mutations are less frequent than the two common mutations in the CETP gene, but 4 mutations including −69 G → A, L151P, G181X and Int14 + 3 T ins have been found in unrelated Japanese subjects with HALP. Some studies including ours have demonstrated that CETP deficiency contributed to about half of HALP subjects in the Japanese (18, 20–22). By using the Invader® assay (28) for 9 CETP gene mutations, we studied their frequency among 466 unrelated Japanese subjects (163 men and 303 women) with HALP (HDL cholesterol ≥ 2.07 mmol/l = 80 mg/dl) excluding primary biliary cirrhosis, nephrotic syndrome, thyroid dysfunction and massive alcohol drinkers (≥ 80 g/day), and demonstrated that the frequency of CETP deficiency was 61.7% and 31.4% for marked HALP (HDL cholesterol ≥ 2.59 mmol/l = 100 mg/dl) and moderate HALP in the Japanese, respectively (Fig. 1) (20, 21).

CETP gene mutations and its functional impairment

Seven mutations including splicing and nonsense mutations are known to be null type mutations of the CETP

Table 1. Mutations of the CETP gene identified in Japanese subjects with hyperalphalipoproteinemia.

Exon/Intron	Mutation	Position	Nucleotide change	Codon	Amino acid change	Reference
Promoter	− 69 G → A	− 69	gccc(g/a)gaag	NA	NA	20
Exon 5	L151P	8030	CTG C(T/C)C CTG	151	Leu → Pro	21
Exon 6	G181X	9150	AAG (G/T)GA CAG	181	Gly → Stop	22
	Q182X	9153	GGA (C/T)AG GTG	182	Gln → Stop	23
Exon 9	R282C	11528	GGC (C/T)GC CTC	282	Arg → Cys	21
Exon 10	Q309X	13199	TTC (C/T)AA GAG	309	Gln → Stop	24
Intron 10	Int10 + 2 T → G	13206	GAGg(t/g)aact	NA	NA	25
Intron 14	Int14 + 1 G → A	20291	CTC(g/a)taagt	NA	NA	14
	Int14 + 3 T ins	20293	CTCgt(-/t)aagt	NA	NA	18
Exon 15	D442G	21433	TTC G(A/G)C ATC	442	Asp → Gly	17

The position is relative to the GenBank sequence AC010550 with the start of transcription denoted as position +1.
NA indicates not applicable.

gene in the Japanese. The homozygous subjects with an Int14 + 1 G → A, G181X, Q182X or G309X mutation had no detectable levels of CETP mass and activity in the plasma (14–16, 22–24). Furthermore, Int10 + 2 T → G and Int14 + 3 T ins mutations are also thought to be null type mutations, because compound heterozygotes with Int14 + 1 G → A had no CETP mass and activity in the plasma (18, 25). The Int14 + 1 G → A mutation causes exon 14 skipping of mRNA in monocyte-derived macrophages (29). The exon-skipping deletion causes a frameshift and introduces a premature termination codon downstream at codon 403. It decreases the stability of mRNA and produces a truncated protein that is rapidly degraded intracellularly in the transfected COS-1 cells. These observations clearly explain the molecular basis of the subjects with the common Int14 + 1 G → A splicing mutation but also explain the subjects with the other nonsense mutations that lead to a larger truncation of the carboxyl terminus of CETP. In the Int10 + 2 T → G mutation, which was identified in subjects with the Int14 + 1 G → A mutation, the sequence analysis of CETP mRNA in the proband's monocyte-derived macrophages demonstrated abnormal splicing with the deletion of exon 10 as well as alternative splicing at a native AG site located 31 nucleotides 5' of the normal splice acceptor in intron 13 (25). Thus, the Int10 + 2 T → G mutation causes exon 10 skipping and the insertion of a 31-bp fragment between exon 13 and exon 14, which contains an in-frame stop codon. On the other hand, the L151P mutation is the only missense mutation of the CETP gene, which has a null allelic effect (21). The CETP mass levels in two subjects with the L151P mutation were similar to those in subjects with the Int14 + 1 G → A mutation. Moreover, no CETP protein was detected in the media of the COS-7 cells transfected with the L151P mutant, although a comparable expression of CETP protein was detectable in the cells. The leucine at codon 151 is likely to be structurally important because this residue is homologously conserved among the lipid transfer/lipopolysaccharide binding protein family members, and located within one of the β-sheets (βB7) in the NH_2-terminal domain (30–32).

A promoter mutation and 2 other missense mutations identified in the Japanese HALP subjects are thought to cause partial CETP deficiency. The – 69 G → A mutation is a G-to-A substitution at the – 69 nucleotide of the promoter region, corresponding to the second nucleotide of the PEA3/ETS binding site (C*G*GAA) located upstream of the putative TATA box (20, 33). A reporter gene assay has shown that this mutation markedly reduced the transcriptional activities in HepG2 cells (8% of wild type). The importance of the PEA3/ETS binding site in transcriptional regulation has also been supported by Gaudet and Ginsburg (34). Plasma CETP levels in 6 subjects with the – 69 G → A mutation suggested that this mutation might have a null allelic effect rather than partial defi-

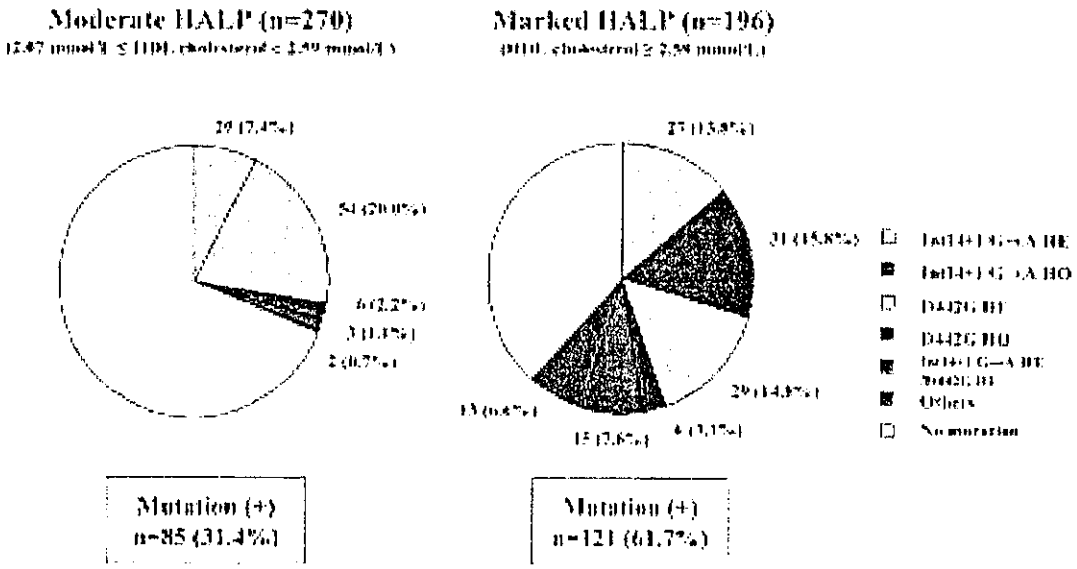

Fig. 1. Frequencies of CETP gene mutations in the 270 Japanese subjects with moderate hyperalphalipoproteinemia (HALP) (2.07 mmol/l ≤ HDL cholesterol < 2.59 mmol/l) (A) and the 196 subjects with marked HALP (HDL cholesterol ≥ 2.59 mmol/l) (B). HE: heterozygote, HO: homozygote, Int14 + 1 G → A HE/D442G HE, compound heterozygote for Int14 + 1 G → A and D442G. The others have at least one of the low prevalence mutations: – 69 G → A, L151P, G181X, R282C and Int14 + 3 T ins.