hol, 6 (6)%; and cholesterol, 385 (176) g. Compared with the 13 nonobese men, the 28 obese men had significantly increased plasma glucose, insulin, triglyceride, apoB-48, apoC-III, RLP-cholesterol, non-HDL cholesterol, VLDL-apoB, VLDL-triglycerides, and leptin concentrations and HOMA scores ($P < 0.01$), and significantly lower plasma HDL-cholesterol and adiponectin concentrations ($P < 0.01$), with no significant group differences in plasma resistin, IL-6, or TNF-α concentrations.

The univariate associations of plasma lipid and lipoprotein concentrations with plasma adipocytokine concentrations, measures of insulin resistance, and adipose tissue compartments are shown in Table 3. Plasma adiponectin concentration was significantly and negatively correlated with plasma apoB-48, apoC-III, RLP-cholesterol, triglyceride, total cholesterol, non-HDL cholesterol, VLDL-apoB, and VLDL-triglyceride concentrations and positively with HDL-cholesterol ($P < 0.05$ for both). By contrast, plasma leptin concentration was positively associated with plasma apoB-48, apoC-III, RLP-cholesterol, triglyceride, non-HDL cholesterol, VLDL-apoB, and VLDL-triglyceride concentrations and inversely with HDL-cholesterol (both $P < 0.05$). Plasma VLDL-apoB concentration was significantly and positively correlated with triglyceride ($r = 0.799$; $P < 0.001$), cholesterol ($r = 0.447$; $P < 0.01$), and non-HDL cholesterol ($r = 0.534$; $P < 0.001$) concentrations and negatively with HDL-cholesterol ($r = 0.402$; $P < 0.01$) concentration. The associations of apoB-48, apoC-III, RLP-cholesterol, and triglycerides with plasma adiponectin and leptin are shown in Figs. 1 and 2, respectively. Plasma resistin, IL-6, and TNF-α concentrations were not significantly associated with any of these lipid and lipoprotein variables except for a direct correlation between apoC-III and IL-6 ($r = 0.321$; $P < 0.05$). Plasma apoB-48, apoC-III, RLP-cholesterol, triglyceride, VLDL-apoB, and VLDL-triglyceride concentrations were positively associated with insulin, HOMA score, and the masses of all adipose tissue compartments except for total ATM in the case of VLDL-triglycerides. Plasma apoB-48 concentrations were also highly significant associated (both $P < 0.01$) with plasma concentrations of triglycerides ($r = 0.826$), RLP-cholesterol ($r = 0.732$), non-HDL cholesterol ($r = 0.517$), and VLDL-apoB ($r = 0.829$). Moreover, plasma adiponectin and leptin concentrations were significantly associated with insulin concentrations, HOMA score, and the masses of all adipose tissue compartments except for RPATM. Plasma resistin, IL-6, and TNF-α were not significantly associated with insulin concentration, HOMA score, and the masses of all adipose tissue compartments except for IPATM in the case of IL-6 and TNF-α (data not shown).

As shown in Table 4, plasma adiponectin concentration was a significant independent predictor of plasma apoB-48, apoC-III, RLP-cholesterol, and triglyceride concentrations ($P < 0.05$) in regression models including HOMA score, adipose tissue compartment, age, and NEFAs. Plasma adiponectin concentration was also a significant independent predictor of plasma VLDL-apoB (β coefficient = -0.377; $P = 0.016$) and VLDL-triglyceride (β coefficient = -0.364; $P = 0.042$) concentrations. In these models, IPATM was also an independent predictor of plasma apoC-III and VLDL-apoB concentrations, whereas total SAATM was an independent predictor of plasma triglyceride concentrations (Table 4). In contrast to adiponectin, plasma leptin was not an independent predictor of plasma apoB-48, apoC-III, RLP-cholesterol, and triglyceride concentrations in the same regression models (Table 4). In these models, total SAATM was an independent predictor of plasma apoB-48 and triglyceride concentrations, whereas HOMA score was an indepen-

Table 3. Associations (Pearson correlation coefficients) of plasma lipid and lipoprotein concentrations with plasma adipocytokine concentrations, measures of insulin resistance, and adipose tissue compartments.

	ApoB-48	ApoC-III	RLP-C[a]	TG	Cholesterol	HDL-C	Non-HDL C	LDL-C	VLDL-apoB	VLDL-TG
Adiponectin	−0.506[b]	−0.531[b]	−0.557[b]	−0.632[b]	−0.453[b]	0.474[b]	−0.564[b]	−0.172	−0.622[b]	−0.452[b]
Leptin	0.342[c]	0.400[b]	0.423[b]	0.548[b]	0.224	−0.414[b]	0.334[c]	0.050	0.463[b]	0.359[c]
Resistin	−0.113	0.027	−0.073	0.034	0.007	0.120	−0.014	−0.009	−0.010	−0.153
TNF-α	0.057	0.204	0.255	0.198	0.197	−0.105	0.227	0.119	0.187	0.197
IL-6	0.067	0.321[c]	0.166	0.169	0.132	−0.060	0.133	0.149	0.167	0.110
NEFAs	−0.207	−0.156	−0.200	−0.197	−0.078	0.163	−0.111	−0.063	−0.167	−0.042
Glucose	0.241	0.364[c]	0.387[c]	0.401[b]	0.016	−0.370[c]	0.108	−0.135	0.367[c]	0.335[c]
Insulin	0.483[b]	0.511[b]	0.613[b]	0.660[b]	0.423[b]	−0.439[b]	0.525[b]	0.119	0.572[b]	0.386[c]
HOMA score	0.343[c]	0.411[b]	0.512[b]	0.671[b]	0.395[b]	−0.459[b]	0.509[b]	0.092	0.440[b]	0.319[c]
IPATM	0.440[b]	0.508[b]	0.490[b]	0.565[b]	0.266	−0.366[c]	0.360[c]	0.022	0.553[b]	0.347[c]
RPATM	0.335[c]	0.382[c]	0.398[c]	0.438[b]	0.206	−0.285	0.280	0.025	0.310[c]	0.337[c]
Total SAATM	0.491[b]	0.401[b]	0.435[b]	0.591[b]	0.317[c]	−0.433[b]	0.431[b]	0.132	0.517[b]	0.417[c]
Anterior SAATM	0.458[b]	0.385[c]	0.386[c]	0.546[b]	0.255	−0.340[b]	0.342[c]	0.018	0.452[b]	0.420[c]
Posterior SAATM	0.462[b]	0.369[c]	0.424[b]	0.560[b]	0.328[c]	−0.453[b]	0.450[b]	0.195	0.508[b]	0.371[c]
Total ATM	0.386[c]	0.367[c]	0.396[c]	0.468[b]	0.301	−0.309[c]	0.381[c]	0.173	0.490[b]	0.254

[a] RLP-C, remnant-like particle-cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol; Non-HDL C, non-HDL cholesterol; LDL-C, LDL-cholesterol.
[b,c] Significant at [b] $P < 0.01$; [c] $P < 0.05$.

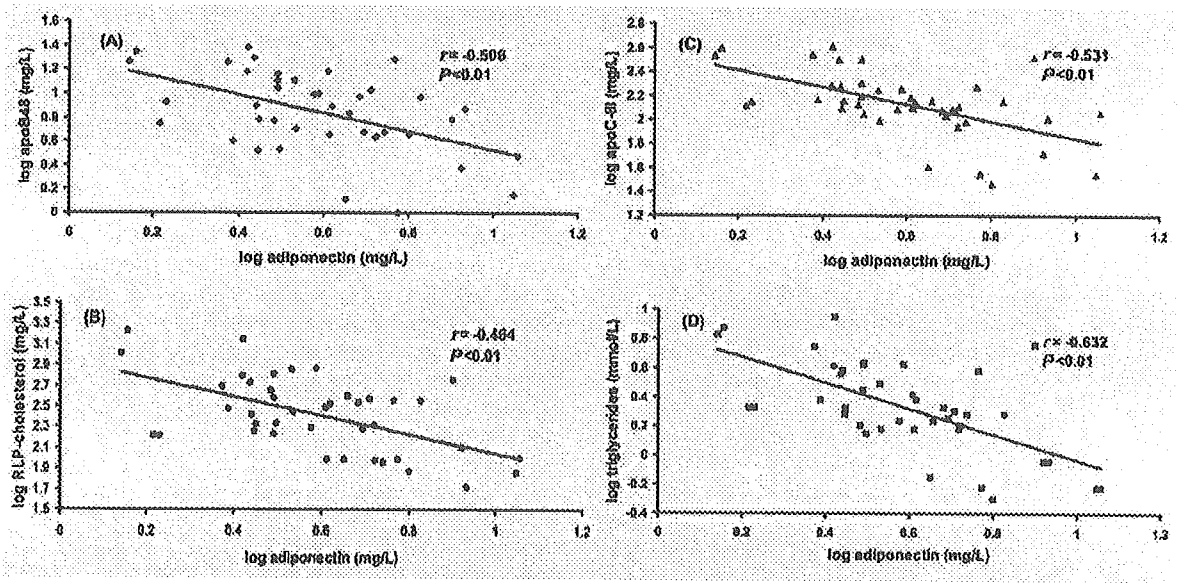

Fig. 1. Associations of plasma adiponectin concentrations with apoB-48 (*A*), apoC-III (*B*), RLP-cholesterol (*C*), and triglyceride (*D*) concentrations.

dent predictor of plasma RLP-cholesterol and triglyceride concentrations. Plasma IL-6 concentration was not an independent predictor of plasma apoC-III in the regression models including HOMA, age, NEFAs, and IPATM (data not shown).

Discussion

We report on the relationships between a wide spectrum of plasma adipocytokines and markers of TRL metabolism in humans. Our principal result was that low plasma adiponectin concentrations were highly predictive of increased plasma apoB-48, apoC-III, RLP-cholesterol, and triglyceride concentrations and that this was independent of both insulin resistance and size of adipose tissue compartments. Another new finding was that, in these men, other adipocytokines (resistin, IL-6, and TNF-α) were not significantly associated with these markers except for a direct association between plasma apoC-III and IL-6 concentrations. In the case of leptin, significant associations were not independent of body fat compartments and insulin resistance. We also showed good agreement across a wide range of plasma triglyceride concentrations

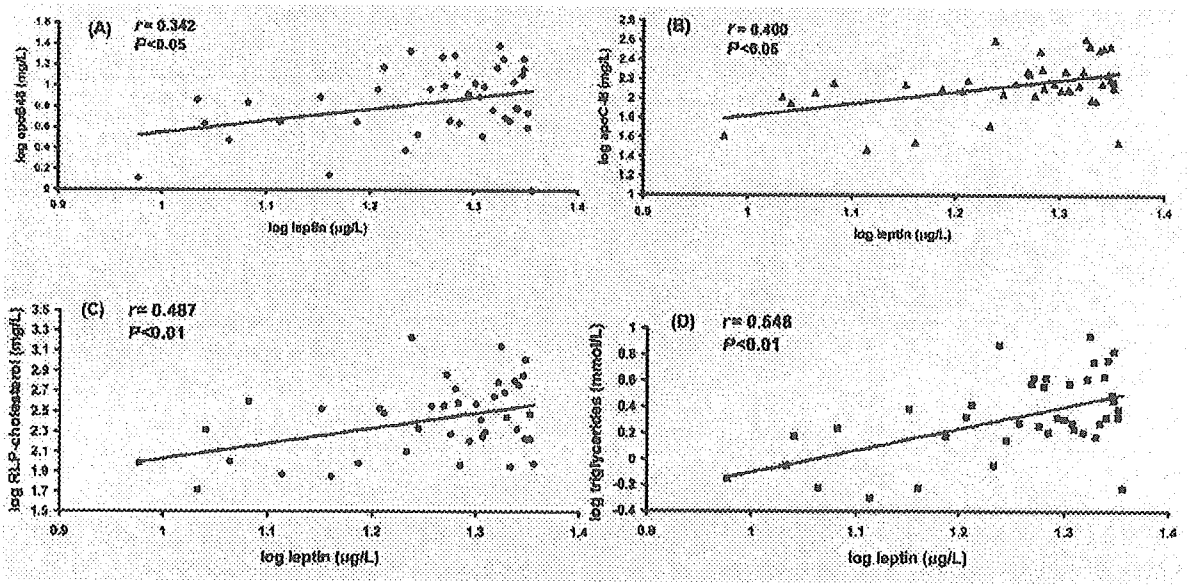

Fig. 2. Associations of plasma leptin concentrations with apoB-48 (*A*), apoC-III (*B*), RLP-cholesterol (*C*), and triglyceride (*D*) concentrations.

Table 4. Multiple regression analyses of the relationships between markers of TRL metabolism, plasma adiponectin or leptin, HOMA, and adipose tissue compartment.[a]

	Predictor	β coefficient	P	Adjusted R^2
ApoB-48	Adiponectin	−0.354	0.044	
	HOMA	0.019	0.909	
	Total SAATM	0.297	0.072	
				28% (P = 0.005)
	Leptin	−0.200	0.386	
	HOMA	0.187	0.259	
	Total SAATM	0.576	0.013	
				21% (P = 0.019)
ApoC-III	Adiponectin	−0.406	0.012	
	HOMA	0.038	0.820	
	IPATM	0.322	0.047	
				33% (P = 0.002)
	Leptin	0.093	0.618	
	HOMA	0.19	0.274	
	IPATM	0.354	0.077	
				20% (P = 0.025)
RLP-cholesterol				
	Adiponectin	−0.377	0.016	
	HOMA	0.203	0.219	
	IPATM	0.236	0.130	
				36% (P = 0.001)
	Leptin	0.133	0.461	
	HOMA	0.336	0.049	
	IPATM	0.241	0.207	
				26% (P = 0.008)
Triglycerides				
	Adiponectin	−0.374	0.013	
	HOMA	0.213	0.129	
	Total SAATM	0.325	0.021	
				49% (P = 0.001)
	Leptin	0.099	0.623	
	HOMA	0.335	0.024	
	Total SAATM	0.403	0.044	
				40% (P = 0.001)

[a] The adipose tissue compartment most closely correlated with the corresponding dependent variable in stepwise regression analysis was included in the models, and statistical models were also adjusted for age and NEFAs.

between a new direct ELISA for apoB-48 and a previously published method based on SDS-PAGE coupled with immunoblotting and enhanced chemiluminescence *(19)*.

Our data are consistent with previous findings that low adiponectin concentrations are associated with an atherogenic lipid profile, including increased triglycerides and low HDL-cholesterol *(13, 14, 21)*. We have extended these studies by investigating the association of plasma adiponectin concentrations with markers of TRL metabolism as measured by plasma apoB-48, apoC-III, and RLP-cholesterol concentrations and demonstrating that low adiponectin concentrations are most closely correlated with accumulation of TRLs independent of insulin resistance and body fat distribution. We also provide new data, based on comprehensive investigation of body fat compartments by MRI, that plasma concentrations of apoB-48, apoC-III, and RLP-cholesterol are strongly associated with adipose tissue compartments, including IPATM, RPATM, anterior SAATM, posterior SAATM, and total ATM.

Dyslipidemia in obesity and insulin resistance is fundamentally related to expansion in the plasma pool of TRLs *(6, 22)*. Accumulation of adipose fat, particularly in the abdominal region, leads to a markedly increased flux of NEFAs to the liver *(6, 23)*, which stimulates triglyceride synthesis *(24)*. Insulin resistance increases hepatic synthesis of lipid substrates and the secretion of VLDL apoB-100; it also down-regulates LDL receptors *(22, 25)*. These effects potentially increase the plasma concentrations of remnant lipoproteins containing apoB-100 and increase competition for hepatic uptake between chylomicron and VLDL remnants *(26)*. However, the lack of a significant correlation of plasma NEFAs with VLDL-triglyceride and/or total triglyceride concentration in our study suggests that measurement of circulating NEFAs in plasma may not simply reflect portal flow of NEFAs to the liver. We have previously reported that, in obese men, accumulation of TRL remnants is attributable to defective lipolysis and impaired clearance of chylomicron remnants, as reflected by increased apoC-III concentrations and a reduced catabolic rate of a remnant-like emulsion *(3)*. The metabolic differences between obese and nonobese men in this study were consistent with our previous data *(3)*. We also demonstrated that insulin resistance and body fat distribution were strongly and independently predictive of plasma apoB-48, apoC-III, RLP-cholesterol, triglyceride, and VLDL-apoB concentrations.

The effect of adiponectin on TRL metabolism may principally involve intrinsic changes in skeletal muscle lipid metabolism and effects on lipoprotein lipase activity in both skeletal muscle and adipocytes *(8, 27, 28)*. Adiponectin may decrease accumulation of triglycerides in skeletal muscle by enhancing fatty acid oxidation through activation of acetyl-CoA oxidase, carnitine palmitoyltransferase-1, and AMP kinase *(27)*. Adiponectin may also stimulate both lipoprotein lipase *(29)*, the lipolytic enzyme that catabolizes VLDL, and apoC-III by increasing the expression of peroxisome proliferator-activated receptor-α in the liver and adipocytes *(30)*. At the hepatic level, adiponectin may decrease the supply of NEFAs to the liver for gluconeogenesis, hence decreasing triglyceride synthesis. Taken together, low circulating adiponectin concentrations could lead to delayed removal of TRLs by the liver and peripheral tissue by increasing competition between chylomicrons and VLDL for LPL lipolysis, and between chylomicron remnants and VLDL remnants for LDL-receptor-mediated clearance *(26)*. Because resistin, IL-6, and TNF-α were not associated with insulin resistance and total body fat in the present study, it was not surprising that we found no significant association of these peptides with markers of TRLs. Our findings also suggest that plasma leptin may not per se have a direct

impact on the metabolism of TRLs and may simply reflect changes in body fat stores *(31)*.

Several methods have been used for the measurement of apoB-48 in plasma *(18, 19, 32)*. The Western blotting method is time-consuming and is less quantitative than the standard ELISA technique; the specificity of polyclonal antibodies in the competitive ELISA is also questionable. In the present study, we used a novel ELISA system that incorporates monoclonal antibodies against apoB-48 to measure apoB-48 in plasma *(18)*. This method enhances the specificity and sensitivity of apoB-48 measurements in plasma without the need for time-consuming isolation of TRLs. Differences in fasting apoB-48 values reported by different methods reflect differences in standardization *(32)*. Despite the analytical shortcomings listed above, we found that the apoB-48 values obtained by our ELISA and the SDS-PAGE methods were well correlated.

We used a surrogate estimate of insulin resistance, the HOMA score, which is well correlated with the hyperinsulinemic, euglycemic clamp technique *(17)*. Measurements of apoB-48 may not differentiate between the nascent chylomicron and its remnant. However, because participants were fasted for at least 12 h to ensure minimal intestinal secretion of nascent chylomicrons, the apoB-48 concentration was probably indicative of small, dense chylomicrons and their remnants. In addition, fasting RLP-cholesterol is not a specific marker of chylomicron and VLDL remnants because it quantifies apoE-rich lipoproteins of intestinal origin as well as some hepatic lipoproteins *(20, 33)*. The association of plasma adipocytokines with apoC-III kinetics also requires further investigation. Future studies should examine the effect of adiponectin genotypes on TRL metabolism *(34, 35)*. In addition, the individual effects of the full-length peptide as well as the low- and high-molecular-weight forms of adiponectin on TRL metabolism also merit further investigation *(36)*.

Several studies have clearly demonstrated the close relationship between the impaired metabolism of TRLs and the development of CVD and type 2 diabetes *(33, 37–39)*. Clinical and experimental data have also recently demonstrated that adiponectin is a strongly protective predictor of CVD, having several antiatherogenic properties *(28, 40, 41)*. Our study therefore suggests that the relationship between low adiponectin concentrations and CVD may in part be mediated by the accumulation of TRLs in plasma. However, definitive evidence of the role of adiponectin in regulating TRL metabolism will require further investigation using adiponectin-knockout animals and recombinant adiponectin replacement therapy *(29)*.

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Probucol Enhances the Expression of Human Hepatic Scavenger Receptor Class B Type I, Possibly Through a Species-Specific Mechanism

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Objective—Scavenger receptor class B type I (SR-BI) is a major receptor for high-density lipoproteins (HDL) in the liver, which is the terminus of reverse cholesterol transport. Overexpression of SR-BI attenuated experimental atherosclerosis in murine models, concomitant with a reduction in plasma HDL-cholesterol levels. Probucol is known to be a potent hypolipidemic drug to regress xanthoma formation and carotid atherosclerosis in conjunction with a marked reduction in HDL-cholesterol levels. The aim of the present study was to know the effect of probucol on the expression of SR-BI and the underlying mechanism.

Methods and Results—We found that probucol increased the expression of SR-BI proteins in in vitro human liver cells and an in vivo rabbit model, but not in wild-type C57Bl6 mice. The decay curve of SR-BI protein was markedly retarded in probucol-treated HepG2 cells in the presence of cycloheximide, indicating that probucol may stabilize human SR-BI protein. To determine the underlying mechanism for the observed species-specific effect, we conducted the following host-swap experiments, in which SR-BI was transfected or expressed in heterologous cells or hosts. Probucol did not increase human SR-BI protein in the liver of transgenic mice carrying the entire human SR-BI genome. Although probucol could stabilize even murine SR-BI, when transfected into a human cell line, HepG2, human SR-BI was not stabilized in a mouse hepatoma cell line, Hepa 1-6, treated with probucol.

Conclusion—Probucol enhances hepatic SR-BI protein expression, possibly through species-specific stabilization of the protein. (***Arterioscler Thromb Vasc Biol.*** 2005;25:0-0.)

Key Words: atherosclerosis ■ high-density lipoprotein ■ probucol ■ reverse cholesterol transport ■ scavenger receptor class B type I

Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which high-density lipoprotein (HDL) particles play a crucial role as shuttles carrying cholesterol derived from peripheral tissues.[1] We have continued to elucidate the molecular mechanism for RCT by analyzing the pathophysiology of patients with abnormal HDL metabolism. It is believed that, in human liver, the terminus of RCT, there are at least 2 distinct pathways for the uptake of HDL-cholesterol. One is the low-density lipoprotein (LDL) receptor pathway where HDL-cholesterol is transferred to LDL by cholesteryl ester transfer protein (CETP), and cholesterol in LDL is taken up by this receptor. The other is an HDL receptor(s)–mediated pathway.[2,3]

One of the important candidates for hepatic HDL receptor in human is scavenger receptor class B type I (SR-BI).[4,5] SR-BI, cloned by Krieger et al, is abundantly expressed in murine liver and mediates the selective uptake of HDL-lipids. Several lines of evidence indicate that this molecule is a physiologically relevant HDL receptor in mice, because its hepatic overexpression of this molecule attenuated experimental atherosclerosis in mice, concomitant with a reduction in HDL-C levels and the appearance of smaller sized HDL particles.[6,7] Conversely, SR-BI null mice had accelerated atherosclerosis in the apoE-negative background with the appearance of larger sized apo AI-containing particles.[8,9]

Probucol is a potent hypolipidemic drug, which can reduce Achilles tendon xanthoma in patients with homozygous familial hypercholesterolemia as well as attenuate atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits.[10,11] A unique aspect of this compound is its capability to induce hypoalphalipoproteinemia. We previously reported

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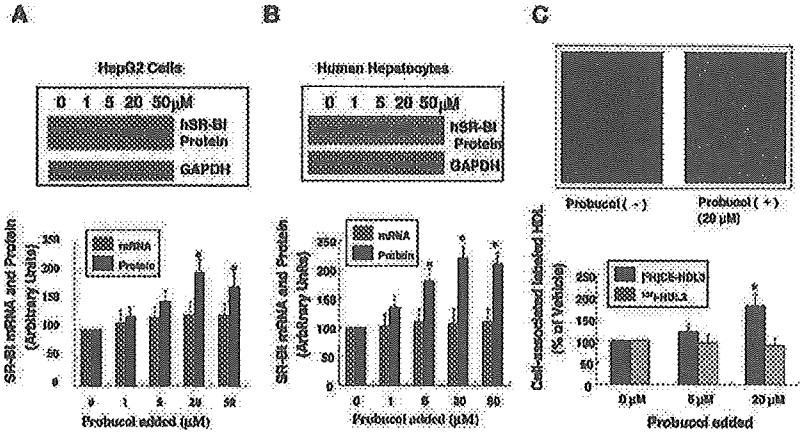

Figure 1. In vitro effect of probucol on the expression of SR-BI (A and B) and the uptake of HDL-lipids (C) in human liver cells. A and B, Protein and mRNA levels of SR-BI in HepG2 cells (A) and human cryopreserved hepatocytes (B). Probucol dissolved in ethanol was added at the indicated concentrations to the media. Forty-eight hours after incubation, proteins and mRNA were extracted and subjected to Western blotting and real-time PCR analyses. The effect of probucol on the expression of SR-BI was examined in the cryopreserved hepatocytes obtained from two different donors. Similar results were obtained from both lots of hepatocytes. Upper panels show representative films of Western blot data from a 33-year-old donor (Please see Materials and Methods). For each experiment, the liver cells with 0 μmol/L probucol values were designated as 100%. Relative abundance of SR-BI mRNA (hatched bars) and protein (solid bars) were graphed from 3 independent experiments (mean±SD), as shown in lower panels. *Statistically significant (at least $P<0.05$) compared with the value for cells treated without probucol. C, Uptake of HDL-lipids in probucol-treated HepG2 cells. Upper panel, After 48-hour incubation with probucol, DiI-HDL (50 μg/mL) was added. Four hours later, cells were washed by PBS and subjected to the fluorescence microscope (PROVIS AX80TR, OLYMPUS). Lower panel, Association of [^{3}H]CE-HDL3 (solid bars) and [^{125}I]-HDL3 (hatched bars) in probucol-treated HepG2 cells were determined as described in Materials and Methods. For each experiment, the HepG2 cells with 0 μmol/L probucol were designated as 100%. Relative radioactivity was plotted from 3 independent experiments (mean±SD). *Statistically significant (at least $P<0.05$) compared with the values for cells treated without probucol.

that probucol treatment induced not only a reduction in HDL-cholesterol levels but also the appearance of smaller-sized HDL particles,[12] which very actively promoted cholesterol efflux from the cells.[13] We also reported a positive correlation between the reduction in plasma HDL-cholesterol and the reduction rate of Achilles tendon xanthoma.[12] However, the mechanism for the reduction of HDL-cholesterol by probucol is not known yet.

The aim of the present study was to determine the effect of probucol on the expression of SR-BI. Because the effect of probucol on atherosclerosis seems to be different among species,[14-16] the experimental materials were obtained from different species including human, rabbits, and mice. We found that probucol increased hepatic SR-BI protein in a species-specific fashion. To gain further understanding of the underlying mechanism(s), we conducted the host-swap experiments, in which SR-BI was expressed in heterologous cells or hosts. Results of these experiments indicated that the upregulation of SR-BI by probucol may be attributable to a species- and host-specific stabilization of the protein.

Materials and Methods

Materials

HepG2 and Hepa 1-6 cells were obtained from ATCC and maintained by the standard protocol. Two different lots of cryopreserved hepatocytes were obtained from the hepatocyte bank maintained at In Vitro Technologies (Baltimore, MD). According to information from the company, one donor was a 33-year-old Caucasian male who had died of intracranial hemorrhage. The other was a 23-year-old Hispanic female who died of cerebrovascular accident. The hepatocytes were plated on collagen-coated plates and maintained in a complete medium from In Vitro Technologies. Aprotinin, ALLN, leupeptin, pepstatin, phosphoramidon, lactacystin, and MG125 were purchased from Sigma. The polyclonal antibody against the carboxy terminus of SR-BI was generated, as described previously.[17] The antibody against C-terminal linking and modulating proteins (CLAMP) was kindly provided by Dr Hiroyuki Arai (Tokyo University, Tokyo, Japan). Monoclonal antibodies against green fluorescence protein (GFP) (JL8) and glycelaldehyde-3-phosphate dehydrogenase (GAPDH) (6C5) were purchased from BD Biosciences and Research Diagnostic Inc, respectively.

In vitro treatment with probucol, isolation of lipoproteins and modification of HDL, Western blot analyses, synthesis of cDNA and real time polymerase chain reaction (PCR), lipoprotein cell association assay, generation of transgenic mice expressing human SR-BI, animal protocol, construction of GFP-tagged SR-BI plasmid, transfection of plasmid DNA, cycloheximide experiments, primers used, and statistical analyses are described in Materials and Methods section in the online data supplement available at http://atvb.ahajournals.org.

Results

Probucol Increased SR-BI Protein in Human Hepatoma Cell Line, HepG2, and Human Hepatocytes

We examined the in vitro effect of probucol on the expression of SR-BI proteins in the human hepatoma cell line, HepG2 (Figure 1A), and cryopreserved hepatocytes (Figure 1B). The addition of probucol to the media increased SR-BI protein in a dose-dependent manner (solid Bars in Figure 1A and 1B). The SR-BI mRNA levels were not apparently changed in either HepG2 cells or cryopreserved hepatocytes (hatched bars in Figure 1A and 1B). Figure 1C shows the effect of probucol on the uptake of DiI-labeled HDL (upper panel) and selective uptake of HDL-lipids (lower panel) in the HepG2 cells. Fluorescent microscopy showed an increased amount of

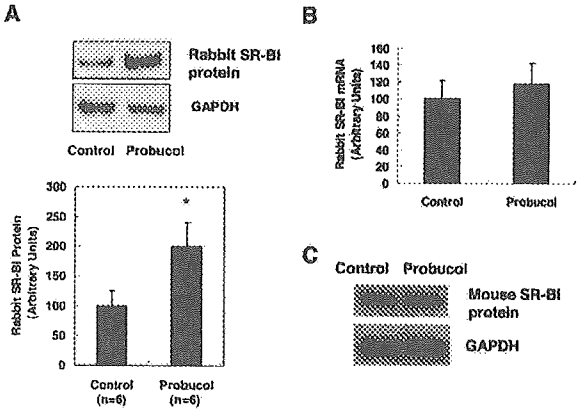

Figure 2. In vivo effect of probucol on the expression of SR-BI in Japanese white rabbits (A and B) and wild-type C57Bl6J mice (C). A, Expression of rabbit SR-BI (rSR-BI) protein in each group determined by Western blotting. Representative images of Western blotting are shown in the upper panel. Relative expression of rSR-BI was plotted (lower pane; n=6, each group). Values are expressed as relative abundance of rSR-BI to that of GAPDH. Bars are expressed as fold of controls (mean±SD of 3 independent experiments). *Statistically significant ($P<0.05$) compared with the value of control group. B, Expression of rSR-BI mRNA was determined with Rnase protection assay. C, Expression of mouse SR-BI (mSR-BI) protein in each group was determined with Western blotting. Pooled cellular protein extracts were made from each group.

uptake of DiI-HDL in cells treated with probucol, whereas the uptake of ^{3}H cholesteryl oleate was significantly increased by the addition of probucol to the media. In contrast, the amount of cell-associated ^{125}I-HDL was not changed. These findings suggest that probucol increased the selective uptake of HDL-lipids, which is known to be mediated by SR-BI, and this increase appeared consistent with that reported previously by Pfeuffer et al.[18]

In Vivo Effect of Probucol on the Expression of SR-BI in Rabbit and Mouse Models

We examined the in vivo effect of probucol on the expression of SR-BI in Japanese white rabbits and wild type C57Bl6J mice. Before the analyses of rabbit SR-BI (rSR-BI), its tissue distribution was investigated with a RNase protection assay. mRNA expression of rSR-BI was abundant in the liver and adrenal glands and very similar to the patterns of tissue distribution reported Ritsch et al[19] (data not shown). Rabbits were kept for 1 month on normal chow diet either with or without 1% probucol. In the animals treated with probucol, plasma HDL-cholesterol levels were significantly reduced during the treatment (15.8±1.4 versus 12.2±2.3 mg/dL, $P<0.05$, paired t test). The expression of rSR-BI protein was markedly elevated ($P<0.01$), as shown in Figure 2A. The mRNA expression of rSR-BI tended to increase, but not to a statistically significant extent. (Figure 2B). Next, we tested the in vivo effect of probucol on the expression of SR-BI in mice. After a 2-week treatment with 5% probucol, the expression levels of murine SR-BI protein had not changed (Figure 2C), which was consistent with the findings reported by Rinninger et al.[20]

These results indicate that probucol may increase hepatic SR-BI protein without apparent changes in its mRNA expression levels in humans or rabbits. On the other hand, this effect was not observed in probucol-treated wild-type mice. These effects may therefore be species-specific.

Probucol May Stabilize SR-BI Protein in HepG2 Cells

We next used HepG2 cells as a model to investigate the underlying mechanism for upregulation of SR-BI by probucol. Because SR-BI mRNA levels were not apparently altered in the HepG2 cells, human cryopreserved hepatocytes, or rabbit liver, we focused on the posttranscriptional regulation of SR-BI by probucol. To determine whether SR-BI protein is regulated by its proteolytic and proteasomal degradation, we tested the effect of various kinds of protease and proteasome inhibitors on the basal levels of SR-BI protein in HepG2 cells. As shown in Figure 3A (left panel), some of the inhibitors, including aprotinin, leupeptin, and pepstatin, seemed to increase the protein levels of SR-BI, but N-acetyl-leucyl-leucyl-norleucinal (ALLN) did not increase the SR-BI protein in the experiment. Proteasome inhibitors such as lactacystin and MG132 reduced SR-BI protein levels. We confirmed that apoB protein was increased by ALLN (data not shown). On the basis of these findings, we tested the hypothesis that probucol may stabilize the SR-BI protein by analyzing SR-BI with immunoblotting in the presence of cycloheximide. A decrease in SR-BI was apparent at 4 hours, and SR-BI continued to decay up to 8 hours (Figure 3B, left panel). The treatment with probucol clearly slowed down the rate of SR-BI degradation (Figure 3B, right panel). It is noted that GAPDH proteins were not decreased up to 8 hours in these experiments.

Figures 1 through 3 demonstrate that probucol increased hepatic SR-BI protein, which may be species-specific. In the HepG2 cells treated with probucol, the degradation of SR-BI was apparently delayed, which may account for the increased levels of SR-BI proteins. This finding led to the question whether or not probucol directly affects the SR-BI genome or protein itself, or some related genes and proteins, or both. To address this issue, we have conducted the following host-swap experiments, in which SR-BI was transfected or expressed in heterologous cells or hosts.

Host-Swap Experiment 1

In Vivo Effect of Probucol on Human SR-BI Expressed in Mice

The first experiment was designed to test the in vivo effect of probucol on human SR-BI in mouse. For that purpose, we generated mouse lines expressing the entire human SR-BI genome in the murine SR-BI$^{-/-}$ background (Human SR-BI BAC Tg/mSR-BI$^{-/-}$ mice). In this model, the expression of human SR-BI was regulated by its own promoter. The mice were treated for 2 weeks with diets containing 5% probucol. As shown in Figure IA (available online at http://atvb.ahajournal.org), human SR-BI mRNA was clearly detected with the hSR-BI riboprobe by RNase protection assay and showed no difference of SR-BI mRNA between the probucol and control groups. Figure IB and IC shows the expression of

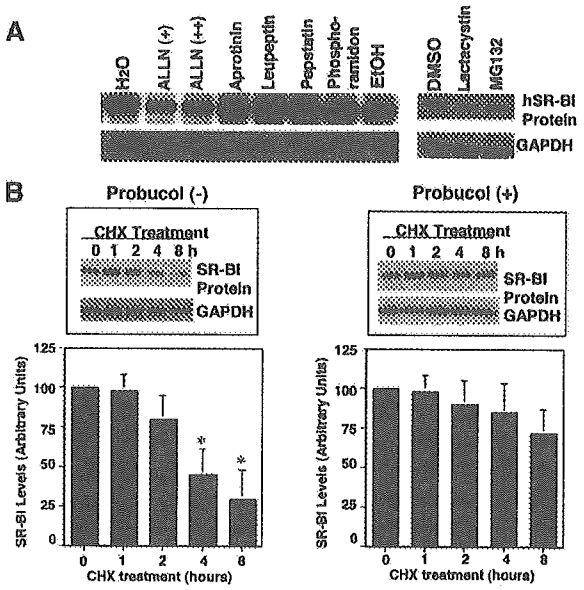

Figure 3. Effect of protease and proteasome inhibitors on SR-BI expression (A) and that of probucol on SR-BI degradation in the presence of cycloheximide (B) A, Western blot analyses of SR-BI in HepG2 cells treated with various proteases and proteasome inhibitors. Cells treated with vehicle alone (H2O, ethanol, and DMSO) are also shown. HepG2 cells were incubated in media containing protease inhibitors for 12 hours. Protein levels of SR-BI and GAPDH were analyzed by means of immunoblotting. Aprotinin, leupeptin, and phosphoramidon were dissolved in water, and pepstatin and ALLN in ethanol. Lactacystin and MG125 were dissolved in dimethyl sulfoxide (DMSO). These proteases and proteasome inhibitors were added to media at the following final concentrations: ALLN (10, 50 μmol/L), aprotinin (10 μmol/L), leupeptin (500 μg/mL), pepstatin (20 μmol/L), phosphoramidon (250 μg/mL), lactacystin (10 μmol/L), and MG125 (3 μmol/L). B, HepG2 cells were pretreated for 48 hours with control and probucol-containing media. The cells were incubated in the presence of cycloheximide (CHX; 20 ng/mL) for the indicated times and whole cell lysates were analyzed with immunoblotting. Relative abundance of SR-BI was graphed from 3 independent experiments (mean±SD). Bars show multiples of cells treated with 0-hour incubation of CHX (mean±SD of 3 independent experiments). *Statistically significant (at least $P<0.05$) compared with the values of cells with 0 minutes treatment of CHX.

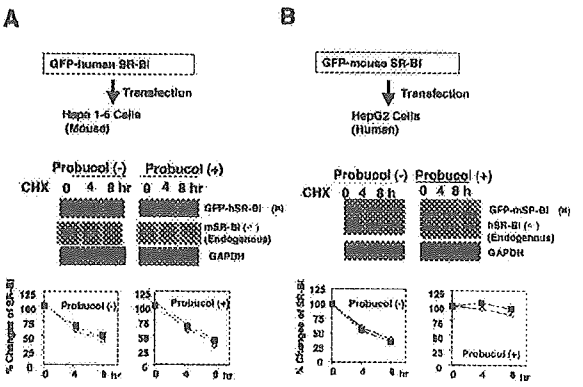

Figure 4. Host swap experiment 2: the expression and degradation of GFP-tagged human and mouse SR-BI proteins in heterologous cells. The cells were transfected with the indicated plasmids using the Nucleofector device and plated onto 12-well dishes. Six hours later, probucol or control medium was added. Forty eight hours after nucleofection, CHX was added and the cells were incubated for the indicated times and whole cell lysates were subjected to Western blotting. These experiments were repeated 3 times with similar results, and the representative data are shown in the figures. A, GFP-human SR-BI was transfected into Hepa 1-6 cells with the Nucleofector device. Neither GFP-human SR-BI nor endogenous mouse SR-BI were stabilized when the cells were treated with probucol. The expression levels of endogenous mouse SR-BI were very low, compared with those of GFP-human SR-BI. The data for endogenous mouse SR-BI were obtained with a longer exposure. B, GFP-mouse SR-BI was transfected into the human hepatoma cell line HepG2. Probucol stabilized both GFP-mouse SR-BI and human endogenous SR-BI.

human SR-BI protein in whole cell lysates as well as the cytoplasmic and membrane fractions, indicating no apparent difference of SR-BI protein levels between the probucol and control groups. These results indicated that probucol did not increase human SR-BI protein levels in mice, suggesting that the increase in SR-BI protein observed in the probucol-treated HepG2 cells and human cryopreserved hepatocytes may not have been caused by the direct or sole effect of probucol on the human SR-BI genome or the protein itself.

Host-Swap Experiment 2

Cycloheximide Experiments for Human and Mouse SR-BI Expressed in Heterologous Cells

We next examined the in vitro effect of probucol on SR-BI expressed in the heterologous cells. For this experiment, we generated the GFP-tagged constructs for human and murine SR-BI. As shown in Figure IIA (available online at http://atvb.ahajournal.org), we tested the expression of GFP-human SR-BI and GFP-murine SR-BI constructs which were transfected into HepG2 cells. Western blot analyses clearly indicated that GFP-tagged proteins were successfully expressed in HepG2 cells. We confirmed the expression of these constructs in the murine hepatoma cell lines, Hepa 1-6 (data not shown). As shown in Figure IIB, DiI-labeled HDL was significantly taken up by cells expressing GFP-tagged human and murine SR-BI. Figure IIC illustrates the uptake of radiolabeled HDL in cells transfected with GFP-hSR-BI and mSR-BI, showing that the cells expressing GFP-SR-BI chimeric proteins achieved significant selective uptake of HDL-lipids.

We finally tested the degradation of SR-BI proteins expressed in heterologous cells. The human or mouse GFP-SR-BI constructs were transfected into either of HepG2 or Hepa 1-6 cells, the latter is a murine hepatoma cell line, and the cycloheximide experiments were performed in cells treated with or without probucol. GFP-human SR-BI expression was not stabilized in the probucol-treated Hepa 1-6 cells (Figure 4A). On the other hand, when murine SR-BI was expressed in the human hepatoma cell line HepG2, probucol clearly slowed down the decay of endogenous human SR-BI and GFP-murine SR-BI proteins (Figure 4B). We also confirmed that GFP-mouse SR-BI was not stabilized in Hepa 1-6 cells, whereas GFP-human SR-BI was stabilized in Hep G2 cells (data not shown).

The results of these 2 host-swap experiments led us to conclude that probucol may stabilize the hepatic SR-BI

protein, possibly through host-specific or species-specific mechanism(s). The probucol-induced upregulation did not seem to be caused by the direct effect of probucol on the human SR-BI genome or protein itself, so that it is more likely that probucol may affect some factors existing in human liver cells, which regulate the protein levels of SR-BI. Because it was recently reported that CLAMP/PDZK1 is one of the crucial regulators for the expression of SR-BI,[21,22] we examined the effect of probucol on the expression of CLAMP/PDZK1 in HepG2 cells, showing no significant changes in either the mRNA or protein levels of this molecule (data not shown).

Discussion

The present study for the first time demonstrates that probucol increases the expression of SR-BI proteins in human liver cells and rabbit liver. This effect seems to be species-specific, because probucol did not increase SR-BI protein in wild-type mice. Our data also indicate that the probucol-induced increase in the SR-BI protein may be caused by the slow decay of the protein. Growing evidence has established that overexpression of SR-BI attenuated atherosclerosis in many murine models.[6-9] It is obvious that SR-BI is an essential molecule that determines plasma HDL-cholesterol levels and atherogenicity in these species. Therefore, this molecule is an important target for the enhancement of reverse cholesterol transport in humans. The present data indicates that the stabilization of SR-BI may be a potentially important strategy to be considered.

The initial part of this study clearly indicated that the upregulation of SR-BI by probucol may occur at posttranscriptional levels. Many literatures reported that the SR-BI protein seems to be tightly regulated at posttranscriptional levels, with an underlying mechanism that seems very complicated. SR-BI protein levels were altered without changes of its mRNA in apoE-knockout mice,[23] vitamin E–fed rodents,[24] and nephrotic rats.[25] CLAMP/PDZK1 was reported to bind with SR-BI and regulate its cell surface expression.[21,26] Gene targeting of CLAMP/PDZK1 diminished hepatic expression of SR-BI in mice.[22] The small PDZK1-associated protein (SPAP/DD96/MAP17) which binds with CLAMP/PDZK1 was reported to regulate SR-BI protein expression in mice.[27] As mentioned in the text, probucol treatment did not alter the mRNA and protein levels of CLAMP/PDZK1 in the HepG2 cells. Because Kodama and Noguchi et al reported that probucol regulated some proteasome gene and proteins in human endothelial cells,[28] we tested the effect of some proteasome inhibitors on the expression of SR-BI in HepG2 cells. Because the proteasome inhibitors used did not increase the SR-BI protein levels, the upregulation of SR-BI by probucol may be independent of proteasomes. Although we could not clarify the precise mechanism, our study raised the following questions. Does it involve the generation of species-specific biologically active probucol derivatives or the existence of species-specific molecular and/or biochemical targets for probucol? The answers to these questions should be of great importance.

Recently, it has been reported that SR-BI is expressed in other tissues than the liver. We and others reported that SR-BI is expressed in foam cells in the human atherosclerotic lesions[17,29] as well as smooth muscle cells in vitro.[30] Yuhanna et al reported that SR-BI expressed in endothelial cells may play some roles in regulating nitric oxides.[31] We and others reported that SR-BI is expressed in the human central nervous system.[32,33] It would be of interest to know the effect of probucol on the expression of SR-BI in these kinds of cells and tissues.

Administration of probucol to both humans and animals has been shown to lower HDL-cholesterol levels. However, various different mechanisms could be responsible for probucol-mediated reduction of HDL-cholesterol. It was found that the particle size of HDL particles becomes smaller in patients and animals treated with probucol.[12,34] Such small HDL particles are very active for cholesterol efflux from the cells,[13] which may lead to the regression of foam cells and xanthomas.[10] On the other hand, Yokoyama and others reported that probucol inhibited apoAI-mediated cholesterol efflux at least in vitro without the alteration of ABCA1 protein levels,[35-37] which may lead to a reduction in the production of nascent HDL particles in vivo. In the present study, we clearly demonstrated that probucol may increase the expression of SR-BI protein in liver cells, possibly in a species-specific fashion. Recently, we demonstrated that SR-BI is expressed in human hepatic parenchymal cells by means of immunohistochemical analyses.[38] We therefore concluded that the overexpression of SR-BI produced by probucol may at least partially explain the low HDL-cholesterol levels observed in patients treated with this drug.

Acknowledgments

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Identification of Unique Lipoprotein Subclasses for Visceral Obesity by Component Analysis of Cholesterol Profile in High-Performance Liquid Chromatography

Mitsuyo Okazaki, Shinichi Usui, Masato Ishigami, Naohiko Sakai, Tadashi Nakamura, Yuji Matsuzawa, Shizuya Yamashita

Objective—The contribution of visceral fat accumulation to the development of coronary heart disease was previously reported, but the relation between visceral fat accumulation and serum lipoprotein subclasses was unknown.

Methods and Results—We examined the relation of lipoprotein subclasses with visceral fat accumulation in 62 male subjects (aged 22 to 67 years) with visceral fat syndrome or obesity. Cholesterol levels in very low–density, low-density, and high-density lipoprotein subclasses (VLDL, LDL, and HDL) were determined by computer-assisted high-performance liquid chromatography. Subcutaneous fat area and visceral fat area were measured by computed tomographic scanning. There was no significant correlation between the subcutaneous fat area and the cholesterol levels in all lipoprotein subclasses. In contrast, the visceral fat area was correlated positively ($P<0.002$) with VLDL and LDL subclasses, except for large LDL, but negatively ($P<0.001$) with those in large and medium HDL subclasses. The observed positive correlations of small and very small LDL subclasses remained significant ($P<0.005$) after adjustment for serum cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol, respectively, but a significant negative correlation ($P<0.005$) of large LDL was obtained after adjustment for LDL cholesterol.

Conclusion—These findings indicate that this simple high-performance liquid chromatography method may be applied for easy detection and evaluation of abnormal distribution of lipoprotein subclasses. (***Arterioscler Thromb Vasc Biol.* 2005; 25:578-584.**)

Key Words: obesity ■ risk factors ■ particle size ■ triglyceride

Lipoprotein profiles are well accepted as predictors of risk for coronary heart disease (CHD). The important relationship between elevated low-density lipoprotein cholesterol (LDL-C) or decreased high-density lipoprotein cholesterol (HDL-C) and the increased risk of CHD is definitely established in many epidemiological studies.[1,2] Another established risk factor for cardiovascular disease is obesity, which was confirmed to be a strong positive predicting factor of CHD in the Framingham Heart Study, and the risk for CHD is particularly increased when abdominal obesity is present.[3] Although measurement of LDL-C and HDL-C has been recommended by the U.S. National Cholesterol Education Program for initial classification of CHD risk status,[4] obesity is not considered to be a major risk factor, because the incremental risk imparted by obesity independently of accompanying risk factors is uncertain. Atherogenic dyslipidemia frequently found in abdominal obesity is a combination of high serum triglycerides (TG) and low HDL-C, which is a strong correlate of the small dense LDL phenotype.[5] Cholesterol synthesis is increased in men with visceral obesity, and this may be partly related to insulin resistance, and the reduction in visceral fat is associated with a decrease in the hepatic secretion of VLDL apolipoprotein B (apoB).[6] We also previously reported that the contribution of visceral fat accumulation to the development of CHD is partially through progression of insulin resistance in nonobese men.[7] The visceral fat area (VFA) had significant positive correlations with serum total cholesterol (TC), serum TG, apoB, and apoE levels and the concentrations of VLDL-C, intermediate density lipoprotein cholesterol (IDL-C), and LDL-C.

Many studies showed that LDL subclasses, characterized by variations in their density, size, and chemical composition, might be clinically significant.[8,9] Although earlier studies for lipoprotein subclass analysis have been done by analytical ultracentrifugation,[10] density analysis by various preparative ultracentrifugation methods have also been used: sequential separation at various density,[11] rate zonal ultracentrifugation,[12] and density gradient ultracentrifugation.[13] The particle

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size analysis by nondenaturing gradient gel electrophoresis has been used,[14,15] and recently the Lipoprint LDL system (Quantimetrix) using nongradient (3%) polyacrylamide gel electrophoretic method has been developed.[16] More recently, a rapid and convenient method using nuclear magnetic resonance (NMR) has been developed[17,18] and used widely in clinical subjects.[19,20] Many methods for detection and quantification of LDL subclasses based on their particle size, density, shape, and charge were reported, but it is unclear which method is clinically useful.[21]

High-performance liquid chromatography (HPLC) with gel permeation columns is an alternative method for classifying and quantifying lipoproteins on the basis of differences of particle size.[22,23] We successfully applied this technique to compare the effects of bezafibrate and pravastatin on lipoprotein subclasses in type 2 diabetes.[24] The HPLC method, similar to the NMR, measures all lipoprotein subclasses at a single analysis from very small amount of whole serum or plasma in a very short time. The HPLC, however, may be superior because of direct cholesterol determination available in major lipoproteins and their subclasses.

In this study, a new simple and fully automated method for analyzing lipoprotein subclasses by HPLC with gel permeation columns followed by mathematical treatment on chromatograms was applied to examine the relationship of cholesterol levels in lipoprotein subclasses with visceral fat accumulation in men with obesity or nonobese subjects with accumulation of visceral fat. The clinical significance for measurements of the cholesterol levels in all lipoprotein subclasses by HPLC method will be evaluated on the basis of visceral fat accumulation, which plays an important role in the occurrence of CHD associated with a cluster of multiple risk factors.

Methods

Subjects

Sixty-two men (aged 22 to 67 years) were enrolled in this study, which included 15 healthy volunteers and 47 hospitalized patients in Osaka University Hospital. All of the subjects gave their informed consent before entering the study according to the Osaka University Hospital ethics committees. All patients had no severe hepatic or renal diseases, and none of them had any medication known to affect insulin action or serum lipoprotein levels. Venous blood was drawn after an overnight fasting. Serum samples were kept in a refrigerator and analyzed within 7 days after blood collection.

HPLC Method

Serum lipoproteins were analyzed by HPLC, as previously described.[23,25] In brief, 5 μL whole serum sample was injected into 2 connected columns (300×7.8 mm) of TSKgel LipopropakXL (Tosoh) and eluted by TSKeluent Lp-1 (Tosoh). The effluent from the columns was continuously monitored at 550 nm after an online enzymatic reaction with a commercial kit, Determiner L TC (Kyowa Medex). The cholesterol concentration in major lipoproteins and their subclasses was calculated by our own computer program, which was designed to process the complex chromatograms with the modified Gaussian curve fitting for resolving the overlapping peaks by mathematical treatment.

We determined the number, position, and width of each Gaussian component peak for subclass analysis to carry out a sufficient curve fitting analysis of various samples from animals and humans under the constant condition in which the peak width and position of each Gaussian curve were not changed. For this purpose, we first took priority to refer the mean particle size of VLDL and LDL of healthy normolipidemic men (n=28). Therefore, the positions of component peaks 6 and 9 corresponded to those of VLDL (36.8±2.5 nm) and LDL (25.5±0.4 nm) of healthy subjects, respectively. Similarly, the positions of peaks 5 and 10 were those of VLDL (44.5±2.1 nm) and LDL (23.0±0.5 nm) of extremely hypertriglyceridemic subjects >1000 mg/dL (n=7) with or without lipoprotein lipase (LPL), respectively. Peak 7 corresponded to LDL (or VLDL; 31.3±1.0 nm) of type III hyperlipidemia with apoE2/2 (n=5). Peak 15 was HDL (13.5±0.4 nm) of cholesterol ester transfer protein deficiency (n=6). Other component peaks (peaks 16 to 20) of HDL subclasses were based on the 5 subclasses determined by HPLC using a gel permeation column (G3000SW) with a separation range for only HDL.[23,26] In addition to the 11 component peaks determined by some experimental background as described above, 9 additional peaks (peaks 1 to 4, 8, and 11 to 14) were introduced to obtain the best curve fitting analysis by changing only peak height of each Gaussian curve. The position of peak 8 (28.6 nm) was determined as the middle point between peak 7 and peak 9, representing a transition component from TG-rich remnant lipoproteins to LDL. Four peaks (peaks 11 to 14) were regularly inserted between peak 10 and peak 15 to make similar intervals from peak 8 to peak 20. Moreover, 3 peaks (peaks 2 to 4) at least needed to be introduced between a void volume (peak 1) and peak 5 to perform the best curve fitting. Alternative setting of additional peaks resulted in the decrease of the degree of curve fitting analysis on the original chromatogram. The conversion of elution time to particle diameter was performed using a column calibration curve, a plot of logarithm of the particle diameter of standard samples, latex beads (Magsphere Inc) 25 and 37 nm in diameter, and a high molecular weight calibrator (Pharmacia Biotech) containing thyroglobulin (17 nm), ferritin (12.2 nm), catalase (9.2 nm), albumin (7.1 nm), and ovalbumin (6.1 nm) against their elution times.

Other Clinical and Lipid Parameter Analysis

Serum TC and TG were determined enzymatically using commercial kits (Kyowa Medex). HDL-C was quantified by the heparin-Ca^{2+} precipitation method.[27] LDL-C was calculated from the formula of Friedewald et al.[28] Uric acid (UA), fasting immunoreactive insulin (IRI), and plasminogen activator inhibitor (PAI)-1 were measured by enzymatic methods and by a double antibody radioimmunoassay, respectively.

Body fat distribution was determined by computed tomographic (CT) scanning (General Electric CT/T scanner, General Electric Co) in the supine position as described previously.[29] The fat layer to the

TABLE 1. Clinical Characteristics, Lipid, and Lipoprotein Profiles of 62 Men

	Mean±SD	Min/Max
Age, years	43.8±11.3	22/67
Height, cm	169.8±6.3	152/181
Weight, kg	77.5±15.1	56/135
BMI, kg/m^2	26.8±4.6	21.0/43.1
VFA, cm^2	129.2±50.4	24.0/255.0
SFA, cm^2	195.5±97.9	55.0/512.0
UA, mg/dL	6.4±1.7	3.8/12.5
IRI, μU/mL	9.3±7.7	2.0/42.8
PAI-1, ng/mL	25.4±18.6	5.0/75.7
TC, mg/dL	212.6±35.2	135.0/308.7
TG, mg/dL	146.3±88.3	41.5/416.0
HDL-C, mg/dL*	45.0±12.4	22.6/70.7
LDL-C, mg/dL†	138.3±34.5	61.1/211.5

*Determined by the precipitation method.
†Calculated by the Friedwald equation.

TABLE 2. Definition for Major and Subclasses of Serum Lipoproteins and Within-Day Precision (n=5) for Measurement of Their Cholesterol Levels

Component Peak No.	Particle Diameter (nm)	Major and Subclass Name	Pool 1 (mg/dL)			Pool 2 (mg/dL)		
			Mean	SD	CV%	Mean	SD	CV%
1	>90		NA	NA	NA	3.69	0.04	1.03
2	75		NA	NA	NA	2.06	0.06	3.13
3	64	large VLDL	0.06	0.01	20.69	1.95	0.04	2.30
4	53.6	large VLDL	1.16	0.04	3.07	1.65	0.06	3.86
5	44.5	large VLDL	2.37	0.09	3.76	7.58	0.24	3.14
6	36.8	medium VLDL	9.78	0.12	1.27	16.12	0.29	1.83
7	31.3	small VLDL	7.45	0.31	4.21	12.71	0.23	1.78
8	28.6	large LDL	27.92	0.98	3.51	18.56	0.46	2.46
9	25.5	medium LDL	38.95	0.14	0.37	23.28	0.23	0.97
10	23.0	small LDL	19.22	0.30	1.54	18.60	0.58	3.09
11	20.7	very small LDL	4.90	0.05	1.06	9.76	0.28	2.91
12	18.6	very small LDL	1.39	0.02	1.69	3.52	0.16	4.41
13	16.7	very small LDL	0.27	0.01	4.22	1.29	0.05	3.91
14	15.0	very large HDL	0.95	0.03	2.64	1.20	0.05	4.25
15	13.5	very large HDL	1.49	0.06	4.02	2.07	0.08	3.98
16	12.1	large HDL	20.27	0.68	3.35	11.51	0.27	2.37
17	10.9	medium HDL	24.64	0.27	1.09	14.30	0.20	1.38
18	9.8	small HDL	11.92	0.36	3.05	8.86	0.15	1.73
19	8.8	very small HDL	3.03	0.09	3.02	2.59	0.09	3.47
20	7.6	very small HDL	1.25	0.03	2.03	1.68	0.07	3.94
1–20		total	177.0	1.13	0.64	163.0	0.66	0.41
1–2	>80	CM	NA	NA	NA	5.75	0.10	1.75
3–7	30–80	VLDL	20.81	0.52	2.51	40.01	0.52	1.29
8–13	16–30	LDL	92.64	0.56	0.60	75.02	0.61	0.81
14–20	8–16	HDL	63.55	0.13	0.20	42.22	0.33	0.78

NA indicates not available; CV, coefficient of variation; CM, chylomicrons.
Pool 1, normolipidemic pooled serum (TG=56 mg/dL); Pool 2, hyperlipidemic pooled serum (TG=428mg/dL).

extraperitoneal region between skin and muscle was defined as subcutaneous fat area (SFA), with an attention range from −40 to −140 Hounsfield units. The intraperitoneal region, with the same density as SFA, was defined as the VFA. The SFA and VFA were measured at the level of the umbilicus.

Statistical Analysis

Data are expressed as mean±SD, unless stated otherwise. Correlations between various variables were presented as the Pearson correlation coefficient (r-value) with a P-value <0.05 considered to be statistically different.

Results

Clinical Characteristics and Lipid Levels of Studied Subjects

Clinical characteristics and lipid levels in 62 men in this study are shown in Table 1. A considerably wide range of anthropometric values was obtained, because they were recruited to cover a large spectrum of body fat values: body mass index (BMI) from 21 to 43 kg/cm^2, VFA from 24 to 255 cm^2, and SFA from 55 to 512 cm^2. Metabolic parameters showed a variation as compared reference values: UA from 3.8 to 12.5 mg/dL, IRI from 2 to 43 μU/mL, and PAI-1 from 5.0 to 75.7 ng/mL.

Analytical Performance of HPLC for Determination of Serum Cholesterol Levels in Major Lipoproteins and Their Subclasses

We defined 3 VLDL subclasses (large, medium, and small), 4 LDL subclasses (large, medium, small, and very small), and 5 HDL subclasses (very large, large, medium, small, and very small) on the basis of lipoprotein particle size (diameter), as shown in Table 2: chylomicrons (>80 nm, peaks 1 to 2), VLDL (30 to 80 nm, peaks 3 to 7), LDL (16 to 30 nm, peaks 8 to 13), and HDL (8 to 16 nm, peaks 14 to 20). The representative chromatograms for curve fitting analysis of normolipidemic (TC=131 mg/dL, TG=39 mg/dL) and hyperlipidemic subjects (LPL deficiency, TC=219 mg/dL, TG=1420 mg/dL) are presented in Figure 1.

Within-run reproducibility (n=5) for the cholesterol determination of 20 subclasses and major classes was determined on 2 different pooled samples (pool 1: TC=177 mg/dL, TG=56 mg/dL; pool 2: TC=163 mg/dL, TG=428 mg/dL) as shown in Table 2.

Within-run reproducibility (n=5) of LDL and HDL particle sizes was 25.20±0.07 nm (coefficient of variation [CV], 0.27%) and 11.25±0.04 nm (CV, 0.36%) for pool 1 and 25.63±0.14 nm (CV, 0.56%) and 11.03±0.05 nm (CV, 0.45%) for pool 2, respectively.

Sum area of the 20 Gaussian curves was 100.2±0.4% (99.1 to 101.7%, n=62) of the area under the original chromatogram. Sum of the peak area corresponding to HDL (peaks 14 to 20) was 99.7±1.1% (98.3 to 103.9%, n=62) of the HDL peak area under the original chromatogram.

A good correlation between HDL-C determined by the precipitation method (x) and total HDL (all HDL subclasses) by HPLC (y) was obtained: $y=0.975x+5.29$ ($r=0.973$, n=62, $P<0.0001$). Moreover, a good correlation between

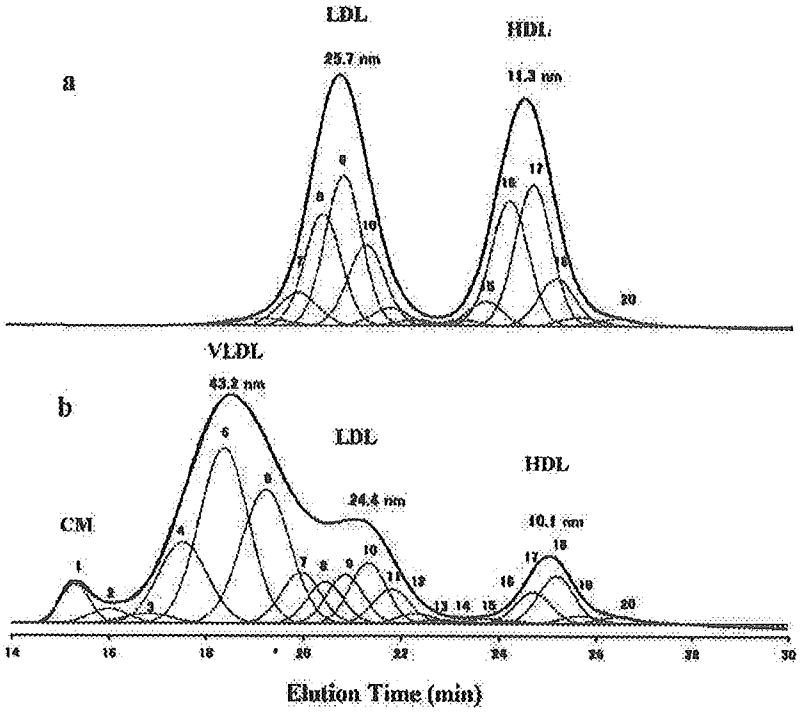

Figure 1. Representative HPLC patterns of (a) a healthy woman and (b) a patient with LPL deficiency. A 5-μL serum sample was injected onto 2 tandem gel permeation columns (TSKgel LipopropakXL) and eluted with TSKeluent LP-1 at a flow rate of 0.7 mL per min. Solid line is real HPLC pattern detected by online enzymatic reaction for TC reagent. Dashed lines are individual subclasses and their sum of Gaussian curves, which are determined curve fitting using Gaussian summation method. Serum TC and serum TG levels are 131 mg/dL and 39 mg/dL (a) and 219 mg/dL and 1420 mg/dL (b), respectively. Particle sizes (nm) determined from observed peak times are also presented.

LDL-C calculated by Friedewald equation (x) and total LDL (all LDL subclasses) by HPLC (y) was also obtained: $y=0.903x+6.28$ ($r=0.977$, n=62, $P<0.0001$).

Correlation of Cholesterol Profiles by HPLC With Clinical Parameters

Simple correlations of cholesterol levels in major lipoproteins and their subclasses with various clinical parameters (age, BMI, VFA, SFA, UA, IRI, and PAI-1) and serum TG levels are summarized in Table 3. Moreover, the correlations of LDL and HDL particle sizes are also presented in Table 3.

As for age, significant negative correlations ($P<0.01$) for medium and small HDL-C were obtained. As for BMI, significant negative correlations were observed only for HDL parameters: large HDL-C ($P<0.01$) and HDL particle size ($P<0.01$). Although no correlations were observed between SFA and all of the lipoprotein subclasses, there were significant positive correlations ($P<0.01$) of VFA with VLDL-C subclasses (large, medium, and small) and LDL-C subclasses (medium, small, and very small) and negative correlations ($P<0.01$) with large and medium HDL-C, LDL, and HDL particle sizes.

As for UA, positive correlations ($P<0.01$) for VLDL-C subclasses (medium and small) and negative correlations ($P<0.01$) for large HDL-C and HDL particle size were obtained. In the case of IRI, positive correlations ($P<0.01$) for small and very small LDL-C and negative correlations ($P<0.01$) for large HDL-C and HDL particle size were obtained. As for PAI-1, positive correlations ($P<0.01$) were observed for small HDL-C and very small HDL-C.

As for serum TG levels, there were significant positive correlations ($P<0.01$) of VFA with VLDL-C subclasses (large, medium, and small) and LDL-C subclasses (small and very small) and negative correlations ($P<0.01$) with large LDL-C, HDL-C subclasses (large and medium), LDL, and HDL particle sizes.

Influences of Traditional Lipid Parameters on the Correlation Between VFA and Lipoprotein Subclasses

Among the anthropometric values in Table 3, VFA levels showed most strong correlations with lipoprotein subclasses. Therefore, influences of traditional lipid parameters on the correlation between VFA and lipoprotein subclasses were examined by adjustment for serum TG, serum TC, HDL-C, and LDL-C levels, respectively (Table 4). Positive correlations of VFA with small LDL-C and very small LDL-C remained significant ($P<0.01$) after adjustment for TG, TC, HDL-C, and LDL-C, respectively.

As for VLDL subclasses, simple correlation analysis showed all VLDL subclasses were positively correlated with VFA, but large VLDL and small VLDL were correlated negatively and positively with VFA, respectively, after adjustment for serum TG level. In the case of LDL subclasses, adjustment for LDL-C gave a significant negative correlation ($P<0.01$) between large LDL and VFA but removed a significant positive correlation between medium LDL and VFA.

Effects of LDL-C on the Correlations Between VFA and LDL Subclasses

The studied subjects were divided into subgroups by the median value of total LDL-C levels (sum of all LDL subclasses) into the low LDL-C (n=31, LDL-C<130 mg/dL) and high LDL-C groups (n=31, LDL-C≥130 mg/dL). In the total population (n=62), a significant positive correlation

TABLE 3. Simple Correlations of Cholesterol Profiles by HPLC Method With Clinical Parameters (n=62)

Clinical Parameters	Age	BMI	VFA	SFA	UA	IRI	PAI-1	TG
Total VLDL	0.100	0.208	0.508†	0.055	0.368‡	0.283§	0.171	0.943†
Large VLDL	0.013	0.245	0.405‡	0.120	0.242	0.185	0.224	0.930†
Medium VLDL	0.126	0.210	0.488†	0.066	0.385‡	0.254§	0.192	0.951†
Small VLDL	0.119	0.070	0.434†	−0.063	0.327‡	0.318§	0.002	0.531†
Total LDL	0.229	0.146	0.431‡	0.090	0.220	0.349‡	0.010	0.057
Large LDL	0.079	0.009	0.115	0.016	0.083	0.183	−0.121	−0.329‡
Medium LDL	0.219	0.144	0.386‡	0.098	0.190	0.311§	0.006	−0.014
Small LDL	0.243	0.202	0.571†	0.111	0.268§	0.380‡	0.122	0.406‡
Very small LDL	0.240	0.219	0.556†	0.105	0.292§	0.375‡	0.164	0.577†
Total HDL	−0.230	−0.281§	−0.528†	−0.180	−0.269§	−0.295§	0.091	−0.418†
Very large HDL	0.200	−0.191	−0.095	−0.232	−0.139	−0.149	−0.180	−0.151
Large HDL	0.057	−0.334‡	−0.426†	−0.212	−0.356‡	−0.385‡	−0.277	−0.400‡
Medium HDL	−0.329‡	−0.251§	−0.502†	−0.159	−0.182	−0.220	0.203	−0.378‡
Small HDL	−0.382‡	0.240	−0.022	0.145	0.178	0.241	0.617†	0.126
Very small HDL	−0.303§	0.153	−0.029	0.238	0.031	0.010	0.369‡	0.045
LDL size*	−0.071	−0.246	−0.389‡	−0.188	−0.158	−0.170	−0.325§	−0.577†
HDL size*	0.166	−0.371‡	−0.368‡	−0.236	−0.354‡	−0.393‡	−0.325§	−0.364‡

Values are Pearson correlation coefficients.
*Average particle diameters (nm) obtained from LDL and HDL peak time by HPLC.
†$P<0.001$; ‡$P<0.01$; §$P<0.05$.

($r=0.431$, $P<0.001$) between VFA and total LDL-C was obtained as presented in Table 3. There was no correlation between VFA and total LDL-C in the low LDL-C group but a significant positive correlation ($r=0.546$, $P<0.002$) in the high LDL-C group.

Scattered plots between VFA and LDL subclasses are presented in Figure 2. Large LDL-C showed no significant correlations with VFA in total population and the high LDL-C group but showed a significant negative correlation ($r=-0.446$, $P<0.02$) in the low LDL-C group. Small LDL-C and very small LDL-C showed significant positive correlations with VFA in total population and both subgroups, except for very small LDL-C in high LDL-C group.

TABLE 4. Partial Correlations of Cholesterol Profiles by HPLC Method With VFA (n=62)

Controlling Factor	TG	TC	HDL-C	LDL-C
Total VLDL	0.009	0.362‡	0.363‡	0.495†
Large VLDL	−0.301§	0.278§	0.253§	0.427†
Medium VLDL	−0.084	0.386‡	0.336‡	0.522†
Small VLDL	0.257§	0.227	0.344‡	0.324§
Total LDL	0.476†	0.016	0.317§	0.141
Large LDL	0.366‡	−0.259§	0.155	−0.356‡
Medium LDL	0.466†	−0.026	0.266§	−0.035
Small LDL	0.458†	0.346‡	0.403†	0.437†
Very small LDL	−0.357‡	0.348‡	0.389‡	0.424†
Total HDL	−0.397‡	−0.514	−0.044	−0.460
Very large HDL	−0.016	−0.126	0.074	−0.106
Large HDL	−0.274§	−0.348‡	−0.082	−0.337‡
Medium HDL	−0.383‡	−0.497†	−0.089	−0.445†
Small HDL	−0.107	−0.157	0.134	−0.061
Very small HDL	−0.063	−0.047	0.292§	0.044
LDL size*	−0.116	−0.313§	−0.221	−0.379‡
HDL size*	−0.220	−0.277§	−0.081	−0.278§

Values are Pearson partial correlation coefficients. TC, HDL-C, and LDL-C are the values obtained by enzymatic method, precipitation method, and Friedewald equation.
*Average particle diameters (nm) obtained from LDL and HDL peak time by HPLC.
†$P<0.001$; ‡$P<0.01$; §$P<0.05$.

Discussion

It is well known that measurement of lipoprotein subclasses other than LDL-C and HDL-C is very important for prediction of risk for CHD. Recently, the Adult Treatment Panel (ATP)-III claimed that one component of atherogenic dyslipidemia is small LDL particles[4] but did not recommend a routine clinical measurement of small LDL particles because of lack of standard and inexpensive methodologies. We offer in this study another alternative method superior to NMR and other methods, giving cholesterol levels for all lipoprotein subclasses simultaneously. Size exclusion HPLC has been used for decades in lipoprotein research applications[23,30] but only recently has become sufficiently robust for consideration as a routine method.

Analytical precision of HPLC was demonstrated for the first time to be acceptable in the determination of 20 component peaks for 3 VLDL, 4 LDL, and 5 HDL subclasses as shown in Table 2, which is well comparable to major lipoprotein quantification reported previously.[25,30,31]

Our HPLC and the traditional methods gave a good agreement in LDL-C and HDL-C values ($r>0.97$) for 62 men in this study. Because Friedewald equation and precipitation methods were used for determination of LDL-C and HDL-C in large-scale epidemiological studies, our HPLC method could be used as an alternative technique in clinical studies. We already compared this method with ultracentrifugation, and very high correlations were obtained.[23] Each of the ultracentrifugally isolated fractions consisted of several sub-

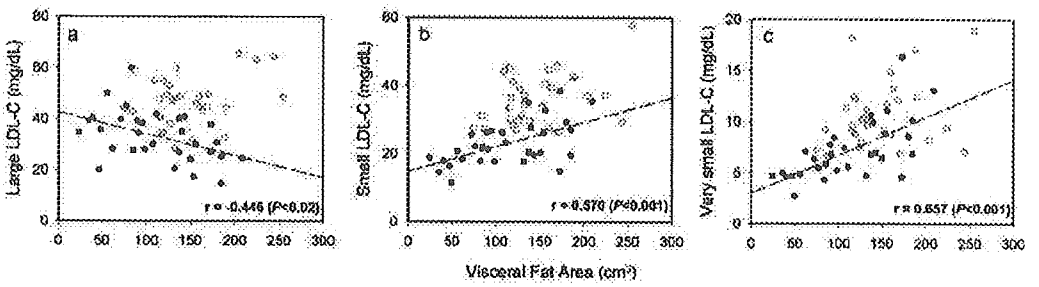

Figure 2. Scatter plots of VFA against (a) large LDL-C, (b) small LDL-C, and (c) very small LDL-C for high LDL-C group (○) and low LDL-C group (●). Dashed lines represent a linear regression for low LDL-C group. Correlation coefficients and *P*-values are also presented for low LDL-C group (n=31).

classes as follows: VLDL fraction, large VLDL (10% to 30%)+medium VLDL (45%)+small VLDL (10% to 25%); IDL fraction, small VLDL (40%)+large LDL (35%)+medium LDL (25%); LDL fraction, large LDL (10% to 30%)+medium LDL (35% to 40%)+small LDL (20% to 35%)+very small LDL (5% to 15%); HDL_2 fraction, very large HDL (5% to 10%)+large HDL (50%)+medium HDL (30% to 40%); and HDL_3 fraction, medium HDL (55%)+small HDL (35% to 40%).

LDL subclass profiles were compared between HPLC and an electrophoretic method using 3% polyacrylamide gels (Lipoprint LDL system)[16] on non-insulin-dependent diabetes mellitus patients (n=87), and LDL-score values determined by the Lipoprint system were positively correlated with small LDL-C (r=0.356, P<0.001) and very small LDL-C (r=0.604, P<0.0001), respectively (M. Okazaki et al, unpublished data, 2004).

Obesity is a major cause of atherosclerotic vascular disease in industrial countries. Obesity is a heterogeneous phenotype, and there is some confusion in the fat distribution literature regarding measurements and indices used to assess regional fat distribution. Subcutaneous skinfolds, skinfold ratio, circumferences, or circumference ratios have been used, and more recently CT has been used to distinguish between measurements of subcutaneous and visceral fat accumulation at any site of the body.[29] We examined the relationship of cholesterol profiles in major lipoproteins and their subclasses by HPLC to various clinical parameter in 62 men with a wide range of anthropometric values to cover a large spectrum of body fat variation (Table 1).

Recent advances in the biology of adipose tissue have revealed that adipose tissue is not simply an energy storage organ, but it also secretes a variety of molecules which affect the metabolism of the whole body.[32] It has been clarified that adipose tissue development and the extent of subsequent fat accumulation are closely associated with the occurrence of advancement of the metabolic syndrome. The presence of obesity increases the risk of thrombotic vascular diseases. Plasma PAI-1 levels were closely correlated with VFA but not with SFA in human subjects.[33] Moreover, visceral fat accumulation is well known to be associated with insulin resistance through the increase of serum free fatty acid followed by the increase of VLDL production by liver.[34,35] In the subjects of this study, there was a weak correlation between VFA and PAI-1 (r=0.261, P<0.05; data not shown) but a strong positive correlation between VFA and IRI (r=0.443, P<0.001; data not shown). As presented in Table 3, the degree of correlations of major lipoproteins and their subclasses with IRI and PAI-1 were different. The strong positive correlations of small and very small HDL with PAI-1 were observed, although positive correlations of small LDL and very small LDL and negative correlation of large HDL with IRI were obtained. Similar correlations were obtained between subclasses and UA, which is increased under overnutrition state in obesity.

Visceral obesity causes various metabolic abnormalities including the increase of serum TG, and there was a strong positive correlation between VFA and serum TG levels in this study (r=0.536, n=62, P<0.0001; data not shown). The increase of serum TG was the result of the increased VLDL as shown by very strong positive correlations of TG with total VLDL-C and all VLDL-C subclasses (Table 3). The increased TG in VLDL results in its flow into LDL and HDL by cholesterol ester transfer protein (in exchange with cholesterol ester), and TG is kept hydrolyzed in LDL and HDL,[36,37] which clearly were demonstrated in our data by the results of negative correlations of serum TG levels with LDL and HDL particle size and by the positive correlation with small and very small LDL-C and negative correlations with large and medium HDL-C. Among anthropometric values, VFA showed a strong positive correlation with total VLDL, all VLDL subclasses, total LDL, and LDL subclasses, except for large LDL, and a negative correlation with total HDL and large and medium HDL as presented in Table 3. These statistical correlations between lipoprotein subclasses and VFA may be the consequence of the increase of TG. But in this study, these significant correlations remained after adjustment for serum TG level (Table 4). Therefore, we did not think the increase of small LDL or decrease of HDL might simply be the consequence of high serum TG in this study. Moreover, the large VLDL and small VLDL were correlated positively and negatively with VFA, respectively, after adjustment for serum TG levels. In our study, the influence of TG increase in fasting state could be evaluated, but the contributions of TG increase in postprandial state to lipoprotein subclass distributions need to be examined in future studies.

As shown in Table 4, partial correlation analysis by controlling LDL-C showed a different contribution of visceral fat accumulation to the cholesterol levels in LDL subclasses. In this study, large LDL-C was negatively associated with visceral fat accumulation in low LDL-C groups as presented in Figure 2. Increased CHD risk associated with

reductions of HDL could be related to reciprocal increases of IDL or smaller, denser LDL, or related considerably to parallel reduction of a component within the larger LDL subclasses. Different contribution of visceral fat accumulation to large LDL-C level demonstrated only by a new subclass analysis with HPLC might reflect the difference of atherogenicity of LDL subclasses. Our new approach demonstrating the presence of unique LDL particles by component analysis will help to discover new lipoprotein particles contributing to atherosclerotic diseases.

Our HPLC method can provide the cholesterol levels of major lipoproteins and their subclasses in a small amount of whole serum or plasma (<10 μL) within 16 minutes; this technique might be very useful in a large-scale clinical study.

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メタボリックシンドロームの遺伝的素因

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要　旨

メタボリックシンドロームに特徴的な異常はいずれも遺伝形質であり，メタボリックシンドローム自体も多因子遺伝性疾患の1つである．因子分析の結果，メタボリックシンドロームはそれぞれに高い遺伝度を有する3～4個以上の独立した病態を内包するものと考えられる．ゲノムワイド連鎖解析の結果を中心として，メタボリックシンドロームの疾患感受性を規定する幾つかの候補遺伝子と染色体領域が明らかになりつつある．メタボリックシンドロームの遺伝素因の解明は，その正確な診断と効果的な臨床介入に重要である．

はじめに

メタボリックシンドロームとは，何らかの共通の成因的基盤のもとに複数の冠血管危険因子が重複し，高率に心血管疾患を引き起こす病態と定義できる．近年，メタボリックシンドロームという臨床概念が新たに提唱されるに至った背景には，糖尿病や高血圧，高コレステロール血症などの既存の単独の危険因子を管理対象とした場合に漏れてしまうハイリスク集団のスクリーニングの必要性があった．ところが，現行の診断基準[1)2)]に従った場合のメタボリックシンドロームの心血管リスクの増大はせいぜい3倍程度[3)]と，既存の単独の危険因子によるリスク増大とほぼ同程度にとどまり，新たなハイリスク群の予測因子として十分とは言えない．その原因として，メタボリックシンドロームの成因が必ずしも明らかではないことに加えて，メタボリックシンドロームが単一の病態ではないことが挙げられる．本稿では，遺伝的な側面からメタボリックシンドロームの成因と病態に関して考察を加えたい．

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キーワード：メタボリックシンドローム，インスリン抵抗性，遺伝子，因子分析，連鎖解析

メタボリックシンドロームの遺伝因子の存在

双生児研究は疾患や表現型の遺伝度（heritability）を調べる最も基本的な方法であるが，メタボリックシンドロームに関する双生児研究の結果によると，メタボリックシンドロームに特徴的な糖，脂質，血圧，および体重の異常はいずれも高い遺伝度（0.52～0.80）を持つ遺伝形質であることが示されている[4)]．このことから，メタボリックシンドロームは生活習慣（環境因子）と複数の遺伝因子（疾患感受性遺伝子）がその発症に関与する，い

図1　因子分析によるメタボリックシンドロームの構成因子の抽出

Kaiser Permanente Women Twin Study	Framingham Offspring Study	Strong Heart Study of American Indians	Honolulu Heart Program of Japanese American	Japanese American Community Diabetes Study
体重	BMI	BMI	体重	中性脂肪
脂肪分布	W/H 比	グルコース	胴囲	HDL-C
	インスリン	インスリン	インスリン	LDL 粒子径
グルコース	中性脂肪			
インスリン	HDL-C	血圧	グルコース	胴囲
			インスリン	グルコース
血圧	グルコース	インスリン		インスリン
	インスリン	中性脂肪	血圧	CRP
中性脂肪		HDL-C		
HDL-C	BMI		中性脂肪	血圧
	血圧		HDL-C	

■：肥満関連因子，■：糖代謝関連因子，■：高血圧関連因子，■：脂質代謝関連因子
略語：巻末の「今月の略語」参照

わゆる多因子遺伝性疾患（複合遺伝形質）の1つであると考えられる．

一方，多因子遺伝性疾患としての観点からメタボリックシンドロームの遺伝的な構成因子を調べた報告が数多くなされている．因子分析（factor analysis）は，体重や血圧，インスリン値といった互いに関連の深い量的形質を幾つかの独立した因子のもとに抽出する多変量解析法の一種であるが，本法によってこれまでに 20 以上の大規模な疫学調査データが解析されている．その結果はいずれも，メタボリックシンドロームは少なくとも3～4個以上の互いに独立した構成因子（component）からなることを示している（図1）．すなわち，メタボリックシンドロームは遺伝的な観点から見て単一の病態ではなく，それぞれに高い遺伝度を有する少なくとも3～4個以上の独立した病態を内包するものと考えられる．このことは，遺伝的に日本人とほぼ同一である日系米国人の集団を対象とした研究（図1の右2つ）においても示されている．

因子分析の問題点

因子分析の結果では，インスリンが糖や脂質，肥満といった複数の因子と同時に抽出される（図1）ことから，やはりインスリン抵抗性が危険因子重複の背景に存在することが示唆される．また，各因子の中で血圧関連因子が単独で抽出される場合が多いことより，メタボリックシンドロームの中にあって血圧の異常は他の異常とは成因上やや異なった位置づけにあるとの主張もなされている[5]．しかし一方で，メタボリックシンドロームの成因を探る目的で因子分析が多用されている現状に対して，批判的な意見も出されている[6]．すなわち，因子分析は本来，生物学的な証拠に基づいて構築された仮説を検証する目的には非常に有用であるが，疫学データをもとに生物学的な成因や経路を探ったり，仮説を導く目的で用いるのは危険であるとの批判である．さらに血圧に関しても，収縮期血圧と拡張期血圧のように互いに非常に密接に関連す