Preparation of Platelet Cytosol

Human platelets outdated for transfusion are provided by the Kyoto Red Cross Blood Center. All steps of the preparation are performed at 4°. Platelet pellet (~4 ml prepared from approximately 4 liters of blood) is resuspended in 40 ml Buffer A containing protease inhibitor cocktail (Sigma) and sonicated five times each for 10 s with a Branson probe sonicator at an output level 5 under cooling. The cell lysate is centrifuged for 60 min at 50,000 rpm in a Beckman 60 Ti rotor. The supernatant is dialyzed three times against 1 liter Buffer A each for 2 h. Then the sample was centrifuged for 60 min at 50,000 rpm to remove potential aggregates. The supernatant is used as platelet cytosol. This procedure typically yields 40 ml of cytosol at a protein concentration of 5 mg/ml determined with the method of Bradford (Bio-Rad) using bovine serum albumin (BSA) as a standard. The cytosol is used for affinity purification immediately after the preparation since platelet cytosol is prone to aggregate. Part of the cytosol is stored at −80° and used for the reconstitution of the secretion in the functional assay described later in this chapter.

Preparation of GST-Rab27a

Rab27a cDNA is subcloned into the *Bam*HI site of pGEX-2T (Amersham Biosciences) to produce GST-tagged Rab27a. *Escherichia coli* strain BL21 (DE3) cells transformed with the plasmid are precultured in 100 ml Luria-Bertani (LB) medium containing ampicillin at 50 μg/ml (LB/ampicillin) and grown overnight. The next day, the confluent culture is diluted 10 times with LB/ampicillin (1 liter total) and grown to an OD_{600} of 0.6 at 37° with vigorous shaking. Protein expression is induced with 0.3 m*M* isopropylthiogalactopyranoside (IPTG). After 3 h induction, cells are harvested by centrifugation at 5000 rpm for 10 min in a Beckman J2–21 rotor. The bacterial pellet is washed once with cold phosphate-buffered saline and stored at −80°.

Frozen cells are thawed, resuspended in 40 ml Buffer A containing protease inhibitor cocktail, and disrupted by sonication five times each for 30 s. The cell lysate is centrifuged at 50,000 rpm for 1 h at 4° in a Beckman 60 Ti rotor. The supernatant is collected and incubated with 0.5 ml glutathione-Sepharose beads (Amersham Biosciences) in a 50-ml conical tube for 1 h at 4° on a rotator. The beads are then collected by centrifugation and washed with 40 ml Buffer A four times in the same tube. The beads are transferred into a 10-ml Poly-Prep chromatography column (Bio-Rad), washed with 10 ml Buffer A, and bound GST-Rab27a is eluted with 2.5 ml Buffer A containing 10 m*M* glutathione. The eluate is dialyzed against 1 liter Buffer A, followed by centrifugation at 100,000 rpm

for 10 min in a Beckman 100.2 rotor. The supernatant is recovered, aliquoted, and stored at −80° until use. The purity of GST-Rab27a is examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining, and the concentration determined with the method of Bradford using bovine serum albumin (BSA) as a standard. This procedure typically yields 5 mg GST-Rab27a at a purity >90%.

Affinity Chromatography

To prepare GTP-bound active and GDP-bound inactive Rab27a-coated beads, 50 μg of GST-Rab27a is bound to 50 μl glutathione-Sepharose beads in each 1.5-ml tube. GST-Rab27a-coated beads are then resuspended in 500 μl Buffer A containing 10 m*M* EDTA and 1 m*M* GTPγS, a slowly hydrolyzable analogue of GTP, or 1 m*M* GDP and incubated for 90 min at 25° under gentle rotation. Chelating Mg^{2+} with EDTA serves to accelerate the rate of nucleotide exchange. The nucleotide exchange reaction is stopped by the addition of $MgCl_2$ at a final concentration of 14 m*M*. Beads are further incubated for 20 min to stabilize the nucleotide state of Rab27a.

These GST-Rab27a:GTPγS- and GST-Rab27a:GDP-coated beads are washed once with ice-cold Buffer A, mixed with 10 ml platelet cytosol prepared as described earlier in 15-ml conical tubes, and incubated at 4° under gentle rotation. As a negative control, GST-coated beads are also prepared and treated identically. After 1 h incubation, the beads are collected by centrifugation at 740×*g* for 2 min and transferred to 1.5-ml tubes. The beads are quickly washed with 1 ml Buffer A five times and bound proteins are eluted by boiling in 100 μl SDS sample buffer. Samples (20 μl each) are analyzed on a 4–20% gradient SDS-polyacrylamide gel (Daiichi Chemical, Japan) with Coomassie blue staining. As shown in Fig. 1A, a protein band migrating at 120-kDa is specifically detected in the GST-Rab27a:GTPγS lane. Mass spectrometry analysis of the band reveals that the 120-kDa protein is Munc13-4. The identity of Munc13-4 is confirmed by Western blotting analysis using anti-Munc13-4 antibody (Fig. 1B).

Expression and Purification of Recombinant Munc13-4

For characterization of the role and significance of Munc13-4 in platelet granule secretion, recombinant Munc13-4 protein was first produced. Since Munc13-4 is a large protein (120-kDa) and hardly purified as a soluble protein in bacterial cells, we used a baculovirus expression system. Using recombinant Munc13-4, we confirmed the direct interaction with Rab27:

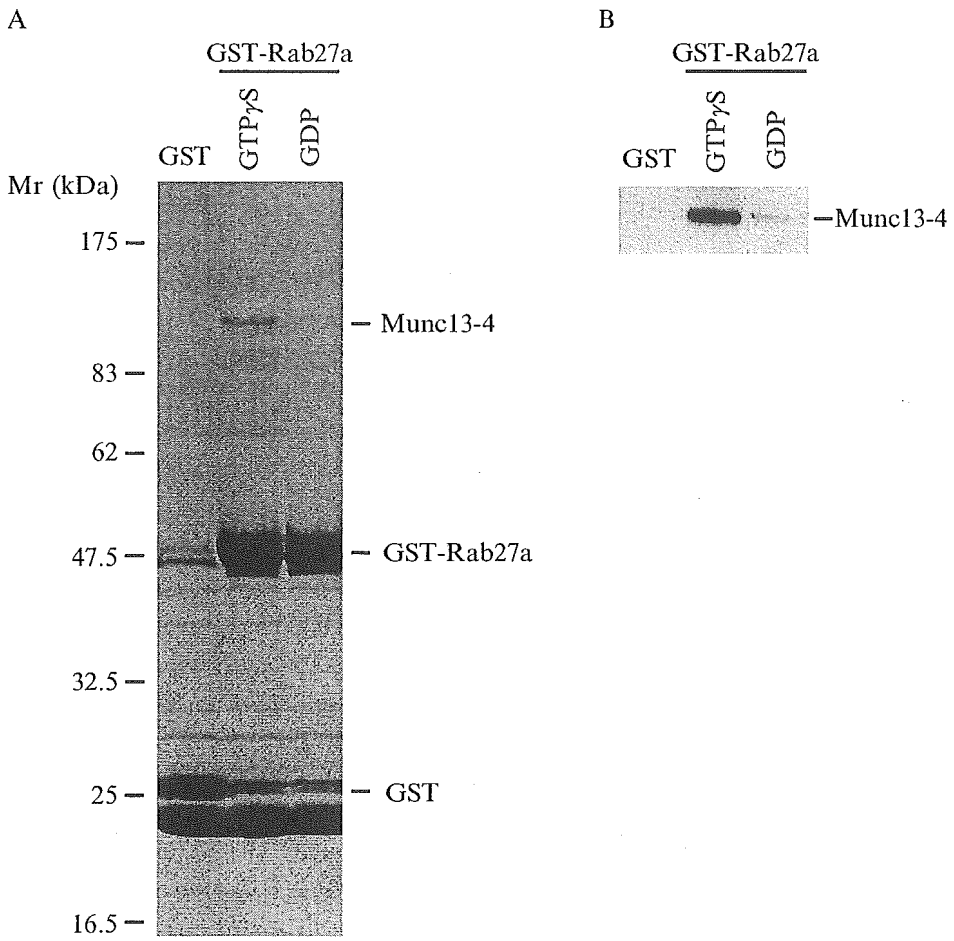

FIG. 1. Purification of Munc13-4 from platelet cytosol using Rab27a affinity chromatography. (A) GST-Rab27a was expressed in bacteria, bound to glutathione-Sepharose beads, and the corresponding nucleotide forms were prepared. After incubation with platelet cytosol, proteins bound to beads coated with either form of Rab27a and GST were eluted. The eluates (each 20 μl) were separated on a 4–20% gradient SDS-polyacrylamide gel and the gel was stained with Coomassie blue. Mass spectrometry analysis revealed that the 120-kDa protein is Munc13-4. (B) Western blotting analysis of the eluate (2 μl) obtained from the Rab27a affinity chromatography using anti-Munc13-4 antibody.

GTP and examined possible involvement of Munc13-4 in the regulation of dense-granule secretion in platelets (Shirakawa *et al.*, 2004). The method of recombinant Munc13-4 production is described in the following subsection.

Solution

Buffer B: 50 m*M* HEPES/KOH, pH 7.4, 150 m*M* KCl, 4 m*M* $MgCl_2$, 1 m*M* DTT

Expression of Recombinant Munc13-4 in Insect Cells

Hexahistidine (His_6)-tagged Munc13-4 is expressed in insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. Briefly, Munc13-4 cDNA is subcloned into a pDEST10 donor vector to produce recombinant viruses encoding His_6-Munc13-4. Next, DH10Bac competent cells are transformed with the plasmid and plated on a selection plate containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-Gal, and 50 μg/ml IPTG. After a 48-h incubation, a white colony harboring the recombinant bacmid is inoculated into a 2-ml culture, and the recombinant viral genome is extracted.

For virus propagation and recombinant protein expression, *Spodoptera frugiperda* (Sf9) insect cells are used. Sf9 cells are cultured in Grace's insect medium (Gibco) supplemented with 5% fetal calf serum and penicillin/streptomycin. Sf9 cells seeded on a 10-cm^2 dish are transfected with the recombinant bacmid by liposome-mediated transfection using CellFECTIN (Invitrogen). After a 72-h incubation, medium containing recombinant viruses (P1) is collected, and Sf9 cells in a 75-cm^2 flask are infected with the P1 virus stock to amplify the virus titer. After 2 days, the medium is collected (P2), and virus amplification is repeated to obtain high-titer virus stocks. For recombinant protein expression, Sf9 cells in twenty 175-cm^2 flasks are prepared and infected with the high-titer virus stock (usually 1 ml of P4 per flask). After a 3-day incubation, cells are harvested, washed, snap-frozen in liquid nitrogen, and stored at −80°. The titer of the virus is examined by Munc13-4 protein expression. The high-titer virus stocks can be stored at 4° with light-shielding.

Purification of His_6-Munc13-4

Frozen cells (~2 ml) are thawed on ice and resuspended in 20 ml Buffer B containing protease inhibitor cocktail. The cells are disrupted by sonication and centrifuged for 1 h at 50,000 rpm in a Beckman 60 Ti rotor. Approximately 70% of His_6-Munc13-4 is recovered in the supernatant. The supernatant is collected and added with imidazole at a final concentration of 20 m*M*. The addition of a low concentration of imidazole prevents nonspecific binding of proteins to Ni-NTA agarose beads (Qiagen). The supernatant is incubated in batch with 0.5 ml Ni-NTA agarose beads for 1 h on a rotator. The beads are collected by centrifugation, washed three times with 40 ml Buffer B containing 20 m*M* imidazole, and loaded into a 10-ml chromatography column. After washing the beads with 10 ml of Buffer B, bound His_6-Munc13-4 is eluted with 2 ml Buffer B containing

250 m*M* imidazole. Following dialysis three times against 1 liter Buffer A each for 2 h, the eluted sample is centrifuged at 100,000 rpm for 10 min at 4° in a Beckman 100.2 rotor. The supernatant is collected, snap-frozen, and stored at −80° in aliquots. This procedure typically yields 0.8 mg of His_6-Munc13-4.

Functional Analysis of Munc13-4 Using Permeabilized Platelets

The major obstacle to biochemical studies of regulated exocytosis is inaccessibility to the secretory apparatus in intact cells. Several permeabilization techniques, including mechanical disruption, electropermeabilization, and use of detergents, have been employed to access the intracellular environment of secretory cells. We have used Streptolysin-O (SLO) (Palmer *et al.*, 1998), a bacterial pore-forming toxin, to establish a semiintact assay system analyzing dense-granule secretion in platelets (Shirakawa *et al.*, 2000).

This assay essentially involves four steps (Fig. 2): (1) isolation of platelets and labeling of dense granules with [^{3}H]serotonin, (2) permeabilization of the labeled platelets with SLO, (3) addition of platelet cytosol and ATP, and (4) stimulation with Ca^{2+} and counting of released [^{3}H]serotonin. It is well known that agonists induce platelet granule secretion by increasing the intracellular calcium ion concentration. Because [Ca^{2+}] inside the platelets would be the same as that outside following permeabilization, we used calcium chloride as a stimulus. Therefore, we analyzed the secretion mechanism triggered by increased [Ca^{2+}]. In this assay, secretion appears to be physiological with a time course and Ca^{2+} sensitivity similar to those in

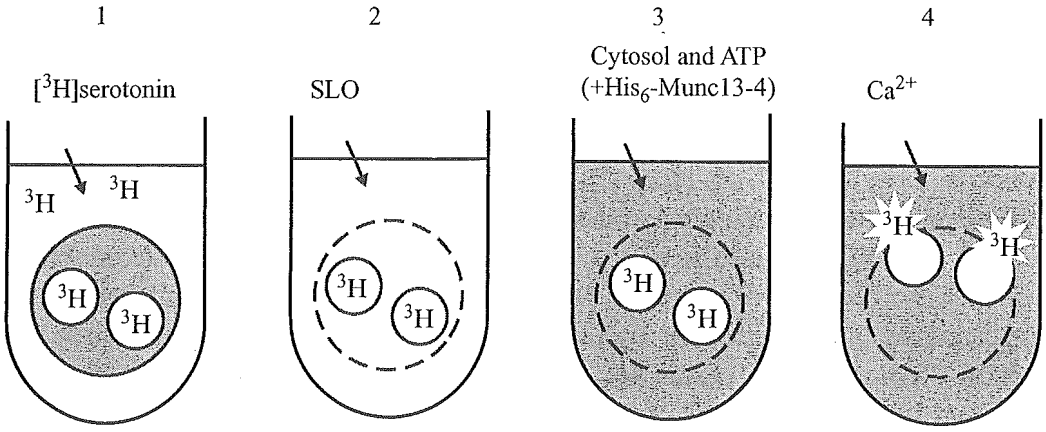

FIG. 2. Schematic representation of semiintact dense-granule secretion assay. (1) Isolate platelets from blood and label dense granules with [^{3}H]serotonin. (2) Permeabilize labeled platelets with SLO. (3) Add platelet cytosol and ATP. (4) Add Ca^{2+} and count released [^{3}H] serotonin.

intact platelets. The activity of Munc13-4 is tested in this assay system using the recombinant protein purified as described earlier.

Solutions

ACD buffer: 41.6 m*M* citric acid, 75 m*M* sodium citrate, 136 m*M* dextrose

Isotonic citrate (IC) buffer: 50 m*M* sodium citrate, 100 m*M* NaCl, 138 m*M* dextrose, pH 6.2

Stimulation buffer: 50 m*M* HEPES/KOH, pH 7.4, 78 m*M* KCl, 4 m*M* $MgCl_2$, 2 m*M* EGTA, 18 m*M* $CaCl_2$, 1 m*M* DTT

Stop buffer: 50 m*M* HEPES/KOH, pH 7.4, 78 m*M* KCl, 4 m*M* $MgCl_2$, 9 m*M* EGTA, 0.2 m*M* $CaCl_2$, 1 m*M* DTT

Isolation of Platelets

All the isolation steps are carried out at room temperature. Blood from a healthy donor is collected by venipuncture into one-sixth volume of ACD buffer. Citrate-anticoagulated blood is centrifuged at 200×*g* for 15 min to prepare platelet-rich plasma. Then, platelet-rich plasma is centrifuged at 1160×*g* for 10 min to sediment the platelets. The supernatant is discarded and the platelet pellet is gently resuspended in 10 ml IC Buffer containing 20 n*M* prostaglandin E_1 (PGE_1) (Sigma). Treatment with PGE_1 prevents activation of platelets through the preparation. After a 10-min incubation, platelets are centrifuged at 1160×*g* for 10 min and resuspended in 1 ml IC buffer.

Labeling of Dense Granules with [^{3}H]serotonin

To label dense granules with [^{3}H]serotonin, the platelet suspension (1 ml) is incubated with 5 μCi of [^{3}H]serotonin (10–20 Ci/mmol, Amersham Biosciences) at 30° for 5 min. Since platelets actively incorporate extracellular serotonin into dense granules through serotonin transporters that reside in the plasma membrane and dense granule membrane, this short incubation time enables efficient labeling of the organelle. Labeled platelets are centrifuged at 600×*g* for 5 min and resuspended in 1 ml ice-cold Buffer A containing 4 mg/ml BSA (Buffer A/BSA) and 0.6 μg/ml SLO obtained from Dr. S. Bhakdi (Mainz University, Germany) (Palmer *et al.*, 1998). The mixture is incubated for 10 min at 4°. At this temperature, SLO binds to the plasma membrane as monomers but does not oligomerize to form pores. Then, platelets are centrifuged at 600×*g* for 5 min to remove unbound SLO and resuspended in 1 ml Buffer A/BSA. Next, SLO-bound platelets are warmed to 30° and incubated for 5 min. This step allows SLO to oligomerize into ring-shaped structures surrounding pores of ~35 nm

diameter (Palmer *et al.*, 1998). This diameter permits free access of macromolecules. Permeabilized platelets are transferred onto ice and further incubated for 15 min to release cytosolic proteins and small molecules, such as ATP. This step results in more than 90% leakage of cytosol as determined by the release of lactate dehydrogenase, a cytosolic protein marker. Following incubation, platelets are centrifuged at 600×*g* for 5 min to remove the leaked cytosol and resuspended again in Buffer A/BSA. At this stage, platelets are rendered unresponsive to Ca^{2+} stimulation due to the leakage of ATP and cytosolic factors (Yoshioka *et al.*, 2001b). In spite of the high degree of permeability achieved, the intracellular ultrastructure is well preserved (Yoshioka *et al.*, 2001a).

Serotonin Secretion Assay

Standard assay is performed as described later in the chapter. Permeabilized platelets (approximately 10^8 platelets, 50,000 cpm/assay) in Buffer A/BSA (calculated free [Ca^{2+}] at 20 n*M* [Fabiato and Fabiato, 1979]) are

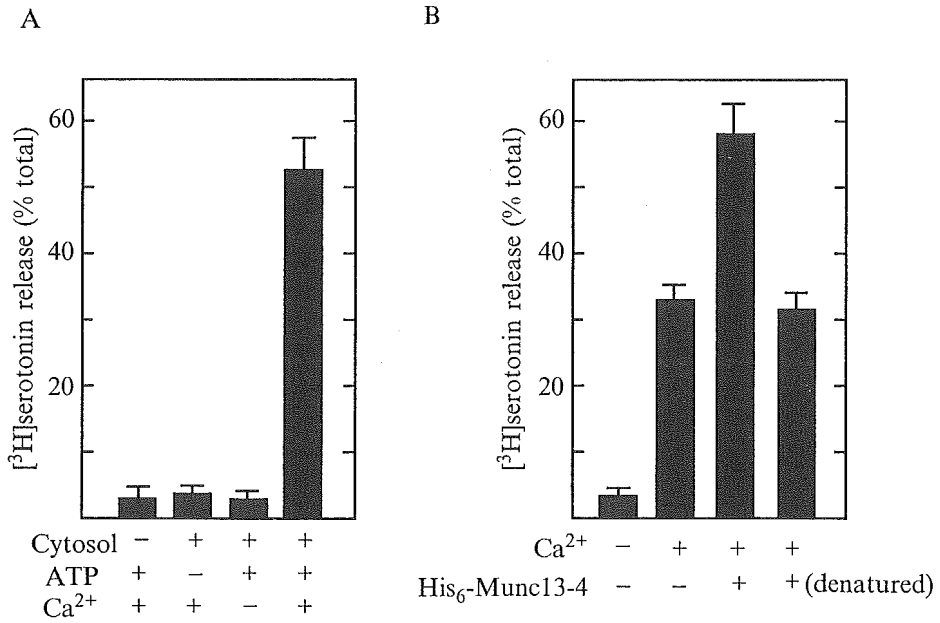

FIG. 3. Characterization of dense-granule secretion assay and functional analysis of Munc13-4 in the assay. (A) Permeabilized platelets are incubated with or without ATP and cytosol at 1.5 mg protein/ml, and stimulated with 20 μM free [Ca^{2+}] or 20 *nM* [Ca^{2+}] as indicated on the figure. After 1 min incubation, released [^{3}H]serotonin is counted. (B) Permeabilized platelets are incubated with ATP and cytosol (0.8 mg protein/ml) in the absence or presence of His_6-Munc13-4 (0.5 μM) and heat-denatured His_6-Munc13-4 (0.5 μM) as indicated on the figure. Released [^{3}H]serotonin after 1 min stimulation with 20 μM [Ca^{2+}] is measured. The extent of secretion is expressed as percentage of total. The data shown are expressed as means ± SE ($n = 3$).

mixed with platelet cytosol at 1.5 mg protein/ml, ATP regeneration system (8 m*M* creatine phosphate, 50 μg/ml creatine phosphokinase, and 1 m*M* ATP), and other regents, and incubated on ice for 15 min in a total volume of 90 μl. Then, the reaction mixture is incubated at 30° for 5 min, and finally stimulated with Ca^{2+} by addition of 10 μl Stimulation Buffer, which gives 20 μM free Ca^{2+} (Fabiato and Fabiato, 1979). After 1 min stimulation, 400 μl of ice-cold Stop Buffer is added to quench the reaction. Platelets are removed by centrifugation at 2000×*g* for 5 min and the released [^{3}H] serotonin is recovered in the supernatant. The radioactivity of [^{3}H]serotonin in 100 μl of the supernatant is counted in 3 ml Clear-sol II (Nacalai Tesque, Japan) using a liquid scintillation counter (Beckman). In this system, dense-granule secretion depends on exogenously added cytosol and ATP (Fig. 3A). The maximum extent of released serotonin under optimal conditions (with platelet cytosol at 1.5 mg protein/ml) represents 50–60% of the total intracellular serotonin pool, whereas the extent of the control (without Ca^{2+} stimulation) is ~5% (Fig. 3A).

The activity of Munc13-4 is tested in this assay system. As shown in Fig. 3B, addition of recombinant His_6-Munc13-4 in the reaction mixture significantly enhances (Shirakawa *et al.*, 2004) [^{3}H]serotonin secretion, whereas heat-denatured His_6-Munc13-4 does not.

Acknowledgments

We thank Dr. Y. Nozawa for Rab27a cDNA, the Kyoto Red Cross Blood Center for providing outdated platelets, and T. Matsubara for excellent technical assistance. R.S. is a recipient of Fellowship of Japan Society for the Promotion of Science. This work was supported by the Japan Ministry of Education, Culture, Sports, Science, and Technology Research Grants 16–1244 (to R.S.), 15590740 (to H. H.), and 16209031 (to T. K.), by Health and Labour Sciences Research Grant H14-Chouju-012 from the Ministry of Health Labour and Welfare (to T. K. and H. H.), and in part by grants from the Takeda Science Foundation (to H.H).

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Journal of Atherosclerosis and Thrombosis Vol. 12, No. 5
Original Article

Polymorphisms in Four Genes Related to Triglyceride and HDL-cholesterol Levels in the General Japanese Population in 2000

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We studied the association of six common polymorphisms of four genes related to lipid metabolism with serum lipid levels. We selected single-nucleotide polymorphisms (SNPs) in the genes for cholesteryl ester transfer protein (*CETP*), lipoprotein lipase (*LPL*), hepatic lipase (*LIPC*), and apolipoprotein CIII (*APOC3*), and studied 2267 individuals randomly selected from the participants of Serum Lipid Survey 2000. There was a significant association of *CETP* polymorphism (D442G, Int14 +1 G → A, and TaqIB), *LPL* polymorphism (S447X), and *LIPC* polymorphism (-514 → CT) with HDL-cholesterol levels. We also found a significant association of *LPL* polymorphism (S447X) and *APOC3* polymorphism (SstI) with triglyceride levels. This is the largest database showing the association of common genetic variants in lipid metabolism with serum lipid levels in the general Japanese population. Further study is necessary to elucidate the role of these gene polymorphisms in cardiovascular events. *J Atheroscler Thromb, 2005; 12: 240-250.*

Key words; Hyperlipidemia, Polymorphism, Cholesterol ester transfer protein, Lipoprotein lipase, Triglyceride lipase, Apolipoprotein CIII

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Received April 1, 2005
Accepted for publication July 1, 2005

Introduction

Hyperlipidemia is a major risk factor for coronary artery disease (CAD) (1). In contrast to the sharp decline in both serum cholesterol levels and mortality from CAD in the United States and Western Europe, remarkable increases

in serum cholesterol levels as well as CAD mortality have been anticipated in the Asian-Pacific area due to industrialization and the modernization of lifestyle (2). The importance of lifestyle is also proved by the fact that Japanese who migrated to Hawaii and California, for example, showed higher levels of serum cholesterol and a higher incidence of CAD than people in Japan (3). Thus, dietary habits and other environmental factors affect serum cholesterol levels and CAD mortality in the population. However, genetic traits are also an important determinant of serum lipid levels.

Major mutations have been described coding for the low-density lipoprotein (LDL) receptor, apolipoprotein B, and so forth, affecting mainly serum LDL-cholesterol levels (4, 5). However, plasma triglyceride (TG) and high-density lipoprotein (HDL)-cholesterol levels are also considered established risk factors for CAD (6). Therefore, the association of common variants of candidate genes with changes in TG and HDL-cholesterol levels would be important determinants for CAD risk. Considering the recent prevalence of metabolic syndrome, it would be also intriguing to examine the effect of these genetic polymorphisms on the development of metabolic syndrome. So far in Japan, however, a large-scale analysis has not been performed on common gene variants related to lipid metabolism.

In 2000, we conducted a survey in the general Japanese population, involving 12,839 people from all over the country (7). We tried to examine the frequency of common polymorphisms of four genes related to lipid metabolism and show an association with serum lipid levels. Among the factors involved in lipid metabolism, we chose the following 4 genes because of the association with TG or HDL-cholesterol. Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl ester from HDL to apolipoprotein B-containing lipoproteins (8). CETP is a key protein in reverse cholesterol transport and its deficiency is associated with hyperalphalipoproteinemia (9–11). Among several polymorphisms of the *CETP* gene, a G to A substitution at the 5' splice donor site of intron 14 (Int14 +1 G → A) and a missense mutation in exon 15 (D442G) are common mutations of hyperalphalipoproteinemia in Japanese (12, 13). The Int14 +1 G → A mutation results in a null allele: homozygotes with the mutation have no CETP in plasma and markedly elevated levels of HDL-cholesterol (10). The D442G mutation is near the carboxy terminal region of CETP shown to be essential for its function (14, 15). The TaqIB polymorphism of the *CETP* gene is one of the most studied polymorphisms worldwide. The B2 allele of the TaqIB polymorphism in intron 1 was associated with decreased CETP levels and high HDL-cholesterol levels (16) and with coronary heart disease risk in the Framingham Study (17). Therefore, we selected these three polymorphisms for our analysis.

Lipoprotein lipase (LPL) is one of the key enzymes in the metabolism of TG-rich lipoproteins. Among several polymorphisms of the *LPL* gene we chose S447X, which is common, having an allele frequency of approximately 20% in healthy individuals, and whose mutation is associated with a favorable lipid profile (18–20). Hepatic lipase (LIPC) is also a member of the lipase superfamily and plays an important role in the metabolism and modeling of both pro- and anti-atherogenic lipoproteins (21). Among the several polymorphisms we selected, –514C → T, located in the promoter region of the *LIPC* gene, has been demonstrated to influence LIPC activity levels (22). Apolipoprotein CIII (apoCIII) can inhibit LPL and reduces the uptake of TG-rich remnant particles and the SstI polymorphism of the *APOC3* gene has been shown to be associated with hypertriglyceridemia and CAD in various human populations (23–27). Therefore, we also examined these polymorphisms in the general Japanese population.

The aim of this study was, therefore, to examine the incidence of these gene polymorphisms and their contribution to lipid concentrations in the general Japanese population.

Methods

Designs and data collection

This work is part of the Serum Lipid Survey 2000 from various areas around Japan. The Ethics committee, graduate school and faculty of Medicine, Kyoto University approved the study protocol and all subjects provided written informed consent for the genetic analysis. The DNA samples were handled according to the guidelines from the Ministry of Health, Labor, and Welfare. In the Serum Lipid Survey 2000, a total of 12,839 subjects were recruited at 36 hospitals across the country. The subjects in the present study were participants in the survey at 9 hospitals from whom informed content for genotyping was sought. Of 12,839 subjects, 2267 (17.7%) with no lipid-altering medication were randomly selected for the present study. In some institutes, information on gender was not disclosed.

Laboratory methods

All serum and blood samples were obtained in the fasting state. All lipid and other analyses were conducted with venous blood samples within one week of collection at BML (Saitama, Japan). Serum cholesterol and TG levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol levels were measured enzymatically with a kit from Daiichi Kagaku Co. Ltd. (Tokyo, Japan). The results of lipid analyses were indirectly standardized according to the criteria of the CDC Lipid Standardization Program (25). DNA was extracted with a QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection of gene mutations by Invader® assay

We used the Invader® assay to screen three known mutations of the *CETP* gene, one mutation of the *LIPC* gene, one mutation of the *LPL* gene, and one mutation of the *APOC3* gene, as previously described (26). In brief, the probe/Invader®/$MgCl_2$ mixture was prepared by combining 3 μl of primary probe/Invader® mix and 5 μl of 22.5 mM $MgCl_2$ per reaction. The primary probes/Invader® mixture contained 3.5 μmol/l wild primary probe, 3.5 μmol/l mutant primary probe, 0.35 μmol/l Invader® oligonucleotide, and 10 mmol/l MOPS. Eight microliters of primary probe/Invader®/$MgCl_2$ mixture as well was added into a 96-well plate. Seven microliters of 5 fmol/l synthetic target oligonucleotides, 10 μg/ml yeast tRNA (no target blank), and genomic DNA (15 ng/μl) were added, and denatured by incubation at 95°C for 10 min. After 15 μl of mineral oil (Sigma, St. Louis, MO, USA) was overlayed into each well, the plate was incubated isothermally at 63°C for 4 h in a DNA thermalcycler (PTC-200; MJ Research, Watertown, MA, USA) and then kept at 4°C until fluorescence was measured. The fluorescent intensities were measured using a fluorescence microtiter plate reader (Cytoflour 4000; Applied Biosystems) with excitation at 485 nm/20 nm (Wave length/Band width) and emission, at 530 nm/25 nm for FAM, and excitation at 560 nm/20 nm and emission, at 620 nm/40 nm for RED. The genotyping was based on calculations with the ratios of net counts with wild primary probe to net counts with mutant primary probe. The probes used in this study were designed and synthesized by Third Wave Technologies, Inc (Madison, WI).

Data analyses

Differences in means were evaluated using an analysis of variance. Multiple regression analysis was done to compare age- and sex-adjusted means. The χ^2-test was used to compare the incidence of each genotype. The analysis was performed with the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan).

Results

We investigated the frequency and phenotypic association of the common polymorphisms of *CETP*, *LPL*, *LIPC*, and *APOC3* genes at the population level in 2,267 subjects. Table 1 summarizes the mean serum lipid levels in the participants in this study. The mean age, and total cholesterol, TG, HDL-cholesterol, and LDL-cholesterol levels in this population were similar to the values for all 12,839 participants in Serum Lipid Survey 2000. We also found that the medians of total, LDL-, and HDL-cholesterol levels did not differ appreciably from the means, thereby excluding gross right-hand tailing of the distribution (data not shown). These results indicate that the participants in the gene analysis are representative of the general Japanese population.

Table 1. Lipid profile and age of all the participants.

	All	Men	Women
T-Cho (mmol/l)	5.18 (0.021)	5.23 (0.046)	5.15 (0.046)
TG (mmol/l)	1.31 (0.024)	1.58 (0.050)*	1.11 (0.039)*
HDL-c (mmol/l)	1.53 (0.010)	1.38 (0.020)*	1.65 (0.017)*
LDL-c (mmol/l)	3.00 (0.020)	3.08 (0.044)*	2.93 (0.039)*
Age (years)	47.1 (0.58)	49.5 (0.87)*	45.3 (0.76)*
Men (%)	43		

Data are expressed as the mean (SEM).
* $p < 0.01$, men vs. women.

Table 2 summarizes the association of the gene polymorphisms with serum lipid levels in all the participants. Tables 3 and 4 show the analysis in male and female participants, respectively. Table 5 shows age- and sex-adjusted means with 95% CI. We found that Hardy-Weinberg equilibrium was the case for all the SNPs, supporting the assumptions of random mating in this population except *CETP* Int14 +1 G → A, for which no homozygote was found in this population.

The incidence of heterozygote mutations of D442G and Int14 +1 G → A of the *CETP* gene was 8.1 and 0.6 %, respectively. These mutations were associated with higher HDL-cholesterol levels. The heterozygous mutation of D442G was also associated with lower TG levels only in men. Although the incidence of the homozygous mutation of D442G and heterozygous mutation of Int14 +1 G → A was quite low and the difference was not significant, the TG levels tended to be higher. The incidence of B1B1, B1B2, and B2B2 genotypes of the *CETP* TaqIB polymorphism was 35.8, 48.4, and 15.8%, respectively. The B2 allele of the *CETP* TaqIB polymorphism was associated with higher HDL-cholesterol levels in all the participants, men, and women. Although the difference was not statistically significant, the participants with the B2 allele tended to have lower TG levels, which is different from the results with the homozygous mutation of D442G and heterozygous mutation of Int14 +1 G → A.

We then determined the polymorphisms of *LPL* S447X mutations in this population. The incidence of heterozygous and homozygous mutations in the *LPL* gene was 20.7 and 1.3%, respectively. The mutation of the LPL S447X site was associated with higher HDL-cholesterol and lower TG levels, although the difference in the level of HDL-cholesterol in men or of TG in women was not statistically significant, possibly due to the small sample number.

The incidence of the CC, CT, and TT genotypes of *LIPC* in the Japanese was 24.9, 50.4, and 24.7%, respectively. Overall, the T allele was associated with an increase in HDL-cholesterol levels. However, the difference was not

Table 2. Demographic and lipid profile of all the participants according to genotype.

CETP D442G (rs2303790)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	47	91.6	1.53 (0.001)	1.37 (0.025)	3.06 (0.021)
hetero	48.4	8.1	1.75 (0.004)	1.15 (0.061)	2.90 (0.075)
homo	46.5	0.2	1.81 (0.18)	1.60 (0.101)	3.19 (1.580)
			$p = 0.000$	$p = 0.071$	$p = 0.154$

CETP Int14 +1 G → A (rs5742907)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	47	99.4	1.54 (0.009)	1.36 (0.024)	3.06 (0.020)
hetero	58.7	0.6	2.12 (0.262)	1.72 (0.362)	3.08 (0.316)
			$p = 0.000$	$p = 0.241$	$p = 0.938$

CETP TaqIB (rs708272)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
B1B1	46.8	35.8	1.50 (0.016)	1.36 (0.036)	3.00 (0.033)
B1B2	48.4	48.4	1.54 (0.013)	1.38 (0.038)	3.08 (0.030)
B2B2	48.2	15.8	1.66 (0.024)	1.25 (0.043)	3.08 (0.051)
			$p = 0.000$	$p = 0.160$	$p = 0.362$

LPL S447X (rs328)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	47.3	78	1.53 (0.011)	1.37 (0.029)	3.06 (0.023)
hetero	46.2	20.7	1.60 (0.020)	1.24 (0.043)	3.06 (0.046)
homo	48	1.3	1.63 (0.101)	1.08 (0.125)	3.29 (0.189)
			$p = 0.004$	$p = 0.032$	$p = 0.487$

LIPC 514CT (rs1800588)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
CC	49.7	24.9	1.49 (0.018)	1.37 (0.046)	3.11 (0.040)
CT	45.6	50.4	1.53 (0.013)	1.33 (0.034)	3.03 (0.029)
TT	47.6	24.7	1.63 (0.020)	1.39 (0.050)	3.06 (0.040)
			$p = 0.000$	$p = 0.520$	$p = 0.255$

APOC3 SstI (rs5128)

Genotype	Age	%	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
S1S1	46.6	42	1.56 (0.015)	1.32 (0.039)	3.06 (0.032)
S1S2	47	45.8	1.54 (0.013)	1.34 (0.033)	3.03 (0.029)
S2S2	48.9	12.2	1.52 (0.025)	1.53 (0.070)	3.11 (0.060)
			$p = 0.413$	$p = 0.021$	$p = 0.434$

Data are expressed as the mean (SEM). Each p-value was based on an analysis of covariance.

significant in men. The TG levels do not seem to be affected by this SNP.

The incidence of the S1S1, S1S2, and S2S2 genotypes of the *APOC3* SstI polymorphism was 42.0, 45.8, and 12.2%, respectively. Although the HDL and LDL-cholesterol levels were similar for all the genotypes, the S2 allele was associated with higher TG levels in all the participants and in men, but not in women. Among the SNPs studied, no polymorphism was found to affect LDL-cholesterol levels. We also determined sex- and age-adjusted means in Table 5 by multiple regression analysis. Due to the limited sample number and large variability of data, a

Table 3. Demographic and lipid profile of male participants according to genotype.

CETP D442G (rs2303790)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	351	1.36 (0.020)	1.60 (0.052)	3.11 (0.045)
hetero	26	1.60 (0.105)	1.19 (0.176)	2.98 (0.194)
		$p = 0.003$	$p = 0.035$	$p = 0.453$

CETP TaqIB (rs708272)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
B1B1	121	1.33 (0.034)	1.64 (0.087)	3.06 (0.073)
B1B2	203	1.36 (0.026)	1.55 (0.068)	3.11 (0.064)
B2B2	53	1.56 (0.063)	1.53 (0.147)	3.13 (0.107)
		$p = 0.001$	$p = 0.664$	$p = 0.758$

LPL S447X (rs328)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	292	1.36 (0.022)	1.65 (0.060)	3.08 (0.047)
hetero	81	1.43 (0.048)	1.36 (0.082)	3.16 (0.112)
homo	4	1.51 (0.386)	0.95 (0.295)	2.80 (0.513)
		$p = 0.278$	$p = 0.029$	$p = 0.617$

LIPC 514CT (rs1800588)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
CC	99	1.32 (0.032)	1.66 (0.094)	3.08 (0.072)
CT	188	1.40 (0.032)	1.51 (0.075)	3.08 (0.069)
TT	90	1.40 (0.041)	1.60 (0.095)	3.08 (0.085)
		$p = 0.266$	$p = 0.499$	$p = 0.996$

APOC3 SstI (rs5128)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
S1S1	165	1.37 (0.031)	1.50 (0.073)	3.16 (0.072)
S1S2	173	1.40 (0.031)	1.58 (0.076)	3.00 (0.060)
S2S2	39	1.31 (0.054)	1.92 (0.162)	3.13 (0.138)
		$p = 0.473$	$p = 0.041$	$p = 0.196$

Data are expressed as the mean (SEM). Each *p*-value was based on an analysis of covariance.

Table 4. Demographic and lipid profile of female participants according to genotype.

CETP D442G (rs2303790)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	440	1.58 (0.018)	1.128 (0.0412)	2.93 (0.041)
hetero	34	1.67 (0.074)	1.15 (0.092)	2.98 (0.140)
		$p = 0.002$	$p = 0.590$	$p = 0.306$

CETP TaqIB (rs708272)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
B1B1	183	1.58 (0.028)	1.13 (0.057)	2.93 (0.062)
B1B2	220	1.67 (0.026)	1.15 (0.066)	2.98 (0.059)
B2B2	72	1.75 (0.043)	0.92 (0.057)	2.85 (0.105)
		$p = 0.004$	$p = 0.127$	$p = 0.461$

LPL S447X (rs328)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
wt	369	1.62 (0.020)	1.14 (0.046)	2.95 (0.046)
hetero	102	1.73 (0.038)	0.99 (0.065)	2.85 (0.081)
homo	4	1.97 (0.164)	0.72 (0.177)	3.89 (0.321)
		$p = 0.010$	$p = 0.185$	$p = 0.054$

LIPC 514CT (rs1800588)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
CC	102	1.59 (0.041)	1.15 (0.089)	2.93 (0.086)
CT	249	1.63 (0.022)	1.04 (0.046)	2.90 (0.050)
TT	124	1.73 (0.037)	1.20 (0.091)	3.03 (0.090)
		$p = 0.014$	$p = 0.210$	$p = 0.406$

APOC3 SstI (rs5128)

Genotype	*n*	HDL-c (mmol/l)	TG (mmol/l)	LDL-c (mmo/l)
S1S1	207	1.65 (0.028)	1.05 (0.054)	2.90 (0.062)
S1S2	208	1.62 (0.026)	1.18 (0.067)	2.93 (0.059)
S2S2	60	1.75 (0.045)	1.08 (0.079)	3.03 (0.106)
		$p = 0.078$	$p = 0.272$	$p = 0.608$

Data are expressed as the mean (SEM). Each *p*-value was based on an analysis of covariance.

significant difference was not found in TG levels in *LPL* or *APOC3* polymorphisms.

To determine the contribution of *CETP* and *LPL* gene polymorphisms to hyperalphacholesterolemia (2.58 mmol/l or over) and hypoalphacholesterolemia (1 mmol/l or under), we divided all the participants into 3 groups according to HDL-cholesterol levels; 1 mmol/l or under, 1 to 2.58 mmol/l, and 2.58 mmol/l or over. We then assessed the incidence of each genotype. The incidence of hyper- and hypoalphacholesterolemia was 1.8 and 8.3%, respectively. Among the genes studied, we found 3 gene polymorphisms to be associated with the incidence of high HDL-cholesterol (2.58 mmol/l or over) (Table 6). Participants with the B2B2 genotype of *CETP* TaqIB had a higher incidence of high HDL-cholesterol levels than the others. Heterozygotes of the *CETP* D442G polymorphism had a higher incidence of higher HDL-cholesterol levels than individuals with the wild type. Homozygotes of the *LPL* S447X polymorphism had a higher incidence of higher HDL-cholesterol levels than the others.

Discussion

In this study we have demonstrated the frequency of six common polymorphisms of four genes related to lipid

Table 5. Age- and sex-adjusted means of all the participants according to genotype.

CETP D442G (rs2303790)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
wt	1.53	1.49	1.56	1.37	1.29	1.45	3.05	2.98	3.11
hetero	1.72	1.62	1.83	1.18	0.90	1.46	2.90	2.68	3.12
homo	1.91	1.70	2.13	1.00	0.42	1.55	2.75	2.30	3.20
	$p = 0.0005$			$p = 0.200$			$p = 0.210$		

CETP Int14 +1 G → A (rs5742907)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
wt	1.54	1.51	1.57	1.35	1.27	1.43	3.04	2.97	3.10
hetero	2.13	1.72	2.54	1.70	0.63	2.79	2.97	2.11	3.83
	$p = 0.0048$			$p = 0.514$			$p = 0.877$		

CETP TaqIB (rs708272)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
B1B1	1.47	1.42	1.51	1.41	1.30	1.54	3.03	2.93	3.13
B1B2	1.56	1.53	1.59	1.34	1.25	1.42	3.04	2.97	3.10
B2B2	1.65	1.59	1.71	1.26	1.09	1.41	3.05	3.00	3.12
	$p = 0.0001$			$p = 0.154$			$p = 0.873$		

LPL S447X (rs328)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
wt	1.53	1.49	1.56	1.38	1.30	1.47	3.03	2.96	3.10
hetero	1.60	1.54	1.65	1.24	1.09	1.39	3.07	2.95	3.19
homo	1.66	1.55	1.78	1.11	0.80	1.40	3.11	2.87	3.35
	$p = 0.033$			$p = 0.090$			$p = 0.546$		

LIPC 514CT (rs1800588)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
CC	1.48	1.46	1.51	1.33	1.26	1.40	3.05	3.00	3.10
CT	1.54	1.52	1.56	1.35	1.31	1.39	3.04	3.01	3.07
TT	1.59	1.57	1.62	1.37	1.30	1.44	3.02	2.97	3.07
	$p = 0.0076$			$p = 0.770$			$p = 0.530$		

APOC3 SstI (rs5128)

Genotype	HDL-c (mmol/l)			TG (mmol/l)			LDL-c (mmo/l)		
	mean	low	upper	mean	low	upper	mean	low	upper
S1S1	1.55	1.51	1.60	1.30	1.18	1.41	3.04	2.95	3.13
S1S2	1.54	1.50	1.57	1.38	1.29	1.46	3.03	2.96	3.10
S2S2	1.52	1.45	1.58	1.45	1.29	1.63	3.02	2.89	3.16
	$p = 0.4223$			$p = 0.180$			$p = 0.816$		

Data are expressed as the mean (95% confidence interval). Each *p*-value was based on an analysis of covariance.

metabolism and its incidence and association with serum lipid levels in the general Japanese population. Because this is the largest Japanese population ever analyzed, these data would be useful for future analyses on the general Japanese population.

The prevalence of the D442G and Int14 +1 G → A mutations is very high in the general Japanese population, with heterozygote frequencies of 7 and 1%, respectively

Table 6. Incidence of CETP TaqIB, D442G, and LPL S447X genetypes according to HDL levels.

CETP TaqIB				
Genotype	HDL-c (mmol/l)			
	1.0> (8.3%)	1.0 ≤, 2.58 > (89.9%)	2.58 ≤ (1.8%)	†
B1B1	72 (9.9%)	644 (88.8%)	9 (1.2%)	$p = 0.009$
B1B2	79 (8.2%)	870 (90.2%)	16 (1.7%)	
B2B2	15 (4.8%)	284 (91.6%)	11 (3.5%)	
CETP D442G				
WT	161 (8.7%)	1671 (89.8%)	29 (1.6%)	$p = 0.011$
Hetero	5 (3.6%)	125 (91.2%)	7 (5.1%)	
Homo	0 (0%)	2 (100%)	0 (0%)	
LPL S447X				
WT	134 (8.9%)	1354 (89.4%)	26 (1.7%)	$p = 0.002$
Hetero	21 (5.0%)	390 (93.3%)	7 (1.7%)	
Homo	2 (8.0%)	21 (84.0%)	2 (8.0%)	

Column percentage is shown on top. Each box shows the number of participants in each category and its percentage in each genotype.
† The χ^2-test was used.

(10, 11, 27, 28). Our large-scaled study showed similar frequencies of these mutations, with 8.1 and 0.6%, respectively, indicating that our study population represents the general Japanese population and confirmed that the frequency of these mutations is quite high in Japanese. Because these mutations are associated with lower levels of CETP activity (27), the plasma level of HDL-cholesterol is higher in heterozygotes and homozygotes. We have also confirmed that the incidence of the mutation D442G is higher in people with hyperalphalipoproteinemia (2.58 mmol/l or over).

A genetic *CETP* deficiency is the most important and common cause of hyperalphalipoproteinemia in Japanese and contributes to 60% of hyperalphacholesterolemia (29). However, the role of CETP in atherogenesis is still under debate. A study in the Japanese Omagari area has shown a relatively increased incidence of coronary atherosclerosis in patients with CETP deficiency (30). In the Copenhagen City Heart Study, increased HDL-cholesterol levels caused by mutations in *CETP* were associated with an increased risk of CAD in caucasian females (31). In contrast, the B2 allele of the TaqIB polymorphism is associated with a low CETP mass, higher HDL-cholesterol levels, and a decreased risk of coronary artery disease (17). The reason for this discrepancy is unknown. Dose effects of CETP mass or another genetic abnormality may explain the difference in risk for CAD. Hirano *et al.* showed that people with weak LIPC activity had a higher incidence of CAD (32). Therefore, it is possible that LIPC activity is involved in these differences. More studies are needed to determine the role of CETP in CAD in various populations with different genetic backgrounds.

Our study is consistent with others in terms of the allele frequency of the S447X polymorphism of the *LPL* gene (19, 20, 33). Recent studies showed that the X447 mutation is associated with a favorable lipid profile, and lower TG and higher HDL-cholesterol levels, and that it may confer protection against coronary artery disease (19, 20, 33). We also found a similar tendency in men and women. However, a significant change in HDL-cholesterol levels was found in the total population and women, but not in men. Because the X447 mutation is associated with stronger LPL activity, the TG levels were lower in heterozygotes and homozygotes as expected, although the difference was not significant in women. Homozygotes seem to have lower TG levels than heterozygotes, which reflects the gene dosage effect. Because carriers of S447X have a favorable lipid profile in terms of HDL-cholesterol and TG, and a decreased risk of CAD (35, 36), we should examine whether carriers of S447X have fewer coronary artery events.

In terms of *LIPC* gene polymorphisms, our data clearly indicate that the frequency of the TT genotype is significantly higher in Japanese than in Caucasians (37, 38). However, a higher frequency of the TT genotype is also reported in Koreans and Japanese (39–41). Therefore, this difference might partly explain the higher HDL-cholesterol levels in Asians.

Our results on the allele frequency of the Sst1 polymor-

phism of the *APOC3* gene were almost comparable to the data on Asian Indians (42), but not on Caucasians (43). Caucasians seem to have a lower allele frequency of S2. Although a association of higher TG levels with the S2 allele has been reported in studies carried out in Caucasians (44–46) and Asians (47–49), our data show that such an association was found in the total population and in men, but not in women. Few other studies, however, have found any significant association between the Sst1 polymorphism and hypertriglyceridemia (50–52). The linkage disequilibrium between this polymorphism and the causative mutation might be weakened or absent in some populations (44).

Our data clearly showed that the heterozygotes of the D442G mutation, homozygotes of the LPL S447X mutation, and people with the TaqIB2B2 genotype had a higher incidence of hyperalphalipoproteinemia with HDL-cholesterol levels of 2.58 mmol/l or over. Alcohol consumption and smoking can also affect the levels of HDL-cholesterol. Corbex et al showed that the HDL levels of people with certain polymorphisms of the *CETP* gene are modulated by alcohol consumption (53). Therefore, it might be necessary to take into account environmental factors for the effect of gene polymorphisms on HDL-cholesterol levels as well as on the risk of cardiovascular events.

In summary, we have provided the largest ever database of gene polymorphisms related to lipid metabolism in the general Japanese population. A prospective study is now under way to determine the contribution of these gene polymorphisms to cardiovascular risk in Japanese.

Acknowledgements: We thank Shizuya Yamashita (Osaka University) and Hideaki Bujo (Chiba University) for critical reading of the manuscript. This study was supported by research grants for health sciences from the Japanese Ministry of Health and a grant from the Japan Atherosclerosis Society. We also thank the Osaka Pharmaceutical Manufacturer's Association for supporting our work.

Appendix

Research Group on Serum Lipid Survey 2000 in Japan

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