Elevated HDL_2-paraoxonase and reduced CETP activity are associated with a dramatically lower ratio of LDL-cholesterol/total cholesterol in a hypercholesterolemic and hypertriglyceridemic patient

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Abstract. A female patient (64 years of age; body mass index, 26) had a markedly and relatively low low-density lipoprotein-cholesterol (LDL-C) level (97 mg/dl) despite high serum total cholesterol (TC) (331 mg/dl) and triacylglyceride levels (307 mg/dl). Since the expected LDL-C was 222 mg/dl, there was a significant difference between the calculation and measurement based on direct enzyme assay. Only 30% of serum cholesterol was associated with LDL-C in this patient. To determine the basis for the markedly low LDL-C/TC ratio, we isolated and analyzed lipoproteins from the patient as well as age- and gender-matched controls. The patient had lowered serum CETP activity and elevated paraoxonase activity with GOT and GPT values in the normal range. The very low-density lipoprotein particles from the patient were larger than those of the controls and enriched with lipid and protein, while the LDL from the patient (LDL-P) had a lower particle number and protein content than the controls. The LDL-P was more resistant to cupric ion-mediated oxidation. HDL_2 from the patient (HDL_2-P) had highly enhanced paraoxonase activity and antioxidant ability. The patient had a 1.5-fold higher level of apolipoprotein (apo) A-I expression in HDL_2. ApoA-I in HDL_2 and HDL_3 from the patient showed no fragmentation, while the control had fragmented bands (17 and 21 kDa) in the HDL. The HDL_2-P also had a larger particle size and greater protein content with less lipid content. HDL_3-associated cholesteryl ester transfer protein was reduced in the patient, although the particle size was similar to the controls. In conclusion, a patient who had a markedly lower LDL-C/TC ratio despite hyperlipidemia associated with higher paraoxonase activity, higher apoA-I level and lower CETP activity without fragmentation of apoA-I in the HDL fraction is presented. The enhanced antioxidant and anti-inflammatory activity of HDL might contribute to the low LDL-C/TC ratio in this patient.

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Introduction

Dyslipidemia is an important risk factor for coronary heart disease (CHD), and low-density lipoprotein-cholesterol (LDL-C) is a strong biomarker to predict the risk of CHD (1). Patients with diabetes and metabolic syndrome have a strong association with atherogenic dyslipidemia, which includes high levels of triacylglycerides (TG) and elevated levels of small dense LDL (2). The most widely used method to calculate LDL-C is the Friedewald equation (3), although several methods of direct measurement are currently used. However, the estimated LDL-C using the Friedewald equation does not produce an accurate number when the plasma TG is >400 mg/dl or when plasma is not collected in the fasting state (4). Otherwise, a discrepancy in the number of measured LDL-C and the Friedewald LDL-C might indicate a modified lipoprotein metabolism. The National Cholesterol Education Program (NCEP) guidelines recommend development of direct assays to measure LDL-C (5) since LDL-C can be influenced by the dynamic interaction of apolipoprotein and several lipoprotein-associated enzymes.

Apolipoprotein (apo) A-I and apo-B are the primary protein constituents of high-density lipoprotein (HDL) and LDL, respectively. In the healthy state, HDL exerts many beneficial effects for the maintenance of a healthy physiologic system, including antioxidant, anti-inflammatory and anti-thrombotic effects (6). LDL is the major cholesterol carrier in the plasma with heterogeneous particle size and composition. Small dense LDL, which is easily oxidized and glycated via interaction with reactive oxygen species and carbohydrate in the blood, are increased with aging and the

incidence of diabetes mellitus and coronary artery disease (7). The distribution of cholesterol and TG among lipoproteins is highly influenced by the dynamic interaction of apolipoproteins and relevant enzymes in lipoproteins via the reverse cholesterol transport pathway (8).

The patient presented herein exhibited a marked difference in LDL-C between the calculated number (222 mg/dl) and the measured number (97 mg/dl). Although the patient had hypercholesterolemia and hypertriglyceridemia, the LDL-C level was within the normal range (<100 mg/dl). To determine the fundamental basis for the markedly decreased ratio of LDL-C/total cholesterol (TC) in the patient presented, we analyzed the lipoprotein fractions in terms of antioxidant ability and associated enzyme activity.

Materials and methods

Patient. A 64-year-old female patient attended our outpatient lipid clinic. She had a history of hypertension for 20 years, hyperlipidemia, and diabetes for 11 years. The patient had been treated with ramipril (5 mg qd), amlodipine (5 mg qd), atenolol (25 mg qd), doxazocin (4 mg qd), metformin (500 mg qd), rosiglitazone (4 mg qd) and atorvastatin (20 mg qd) before she attended our clinic. She has been in stable condition for the past several years and appears to be in good health. The patient's height was 158 cm; weight, 65 kg; and the body mass index was 26.0 kg/m^2. The blood pressure was 110/65 mmHg and the heart rate was 70/min. To measure the lipid parameters without the effect of atorvastatin, this medication was discontinued, and blood tests were performed 8 weeks later.

A complete blood count showed a white blood cell count of 3,790/μl, a hemoglobin of 11.0 g/dl, and a platelet count of 189,000/μl. Blood chemistries were as follows: fasting glucose, 102 mg/dl; blood urea nitrogen, 13 mg/dl; creatinine, 0.7 mg/dl; aspartate aminotransferase, 18 IU/l; and alanine aminotransferase, 12 IU/l. Additional blood measurements were as follows: thyroid stimulating hormone, 0.58 mIU/ml (normal); apolipoprotein A1, 148 mg/dl; apolipoprotein B, 91 mg/dl; TC, 331 mg/dl; TG, 307 mg/dl; HDL-C, 47 mg/dl; calculated LDL-C, 222 mg/dl; LDL-C measured by a direct enzyme assay (7600 Clinical Analyzer, Hitachi Ltd., Tokyo, Japan), 97 mg/dl. After collection of the patient's blood following overnight fasting, fenofibrate (160 mg qd) was added to her prescription. She is being followed up at our outpatient clinic without specific problems.

We recruited age- and gender-matched adults (n=4; 62-65 years of age) as controls. Heavy alcohol consumers (>30 g EtOH/day) and those who had consumed any prescribed drugs to treat hyperlipidemia, diabetes mellitus, or hypertension were excluded. All subjects had unremarkable medical records without histories of endocrinologic disorders. Informed consent was obtained from all patients and subjects prior to enrollment in the study, and the Institutional Review Board of the Medical Center of Yonsei University (Seoul, Korea) approved the study protocol.

Isolation of lipoproteins. After overnight fasting, blood was collected using a vacutainer (BD Sciences, Franklin Lakes, NJ, USA) containing EDTA (final concentration, 1 mM). Plasma was isolated by low-speed centrifugation and stored at -80˚C until analysis.

Very low-density lipoproteins (VLDL, d<1.019 g/ml), LDL (1.019<d<1.063), HDL_2 (1.063<d<1.125) and HDL_3 (1.125<d<1.225) were isolated from individual patient and control sera via sequential ultracentrifugation (9), with the density adjusted by the addition of NaCl and NaBr in accordance with standard protocols. Samples were centrifuged for 24 h at 10˚C at 100,000 x g using a Himac CP-90α (Hitachi, Tokyo, Japan) at the Instrumental Analysis Center of Yeungnam University.

For each of the lipoproteins which were individually purified, TC and TG measurements were obtained using commercially available kits (cholesterol, T-CHO and TG; Cleantech TS-S; Wako Pure Chemical, Osaka, Japan). The protein concentrations of lipoproteins were determined via the Lowry protein assay as modified by Markwell *et al* (10) using the Bradford assay reagent (BioRad, Seoul, Korea) with bovine serum albumin (BSA) as a standard. To assess the degree of oxidation of individual LDL, the concentration of oxidized species in LDL was determined by the thiobarbituric acid reactive substances (TBARS) method using malondialdehyde as a standard (11).

Copper-mediated LDL-oxidation. To compare the susceptibility of copper-mediated LDL oxidation, 300 μg of LDL was incubated with 5 μM $CuSO_4$ for up to 3 h. During the incubation, the quantity of formed conjugated dienes was monitored by measuring the absorbance at 234 nm (Abs_{234}) at 37˚C (12) using a Beckman DU 800 spectrophotometer equipped with a MultiTemp III thermocirculator.

In order to verify the spectroscopic data, the oxLDL samples were subjected to electrophoresis on a 0.5% agarose gel for an electromobility comparison (13). The post-oxidative electrophoretic mobility of LDL was compared via electrophoresis on a 0.5% agarose gel as there is some modification of amino acids in the apo-B by oxidation.

Ferric-reducing ability of the plasma assay. The ferric reducing-ability of plasma (FRAP) was determined using the method described by Benzie and Strain (14) with a slight modification as described recently by our research group (15). The antioxidant activities of the individual HDL fractions (20 μg each) were then estimated by measuring the increase in absorbance induced by the generated ferrous ions.

Cholesteryl ester transfer assay. A rHDL-containing apoA-I and cholesteryl oleate was synthesized in accordance with the method described by Cho *et al* (16) using trace amounts of [^{3}H]-cholesteryl oleate (TRK886, 3.5 μCi/mg of apoA-I; GE Healthcare). The CE-transfer reaction was allowed in 300-μl reaction mixtures that contained equal amounts of the individual lipoproteins (20 μl, 10-20 μg of protein) as a CETP source, and rHDL-agarose (50 μl, 0.25 mg/ml) and human LDL (50 μl, 0.25 mg/ml) as a CE-donor and CE-acceptor, respectively. After incubation at 37˚C, the reaction was halted via brief centrifugation (10,000 x g) for 3 min at 4˚C. The supernatant (150 μl) was then subjected to scintillation counting, and the percentage transfer of [^{3}H]-CE from rHDL to LDL was calculated.

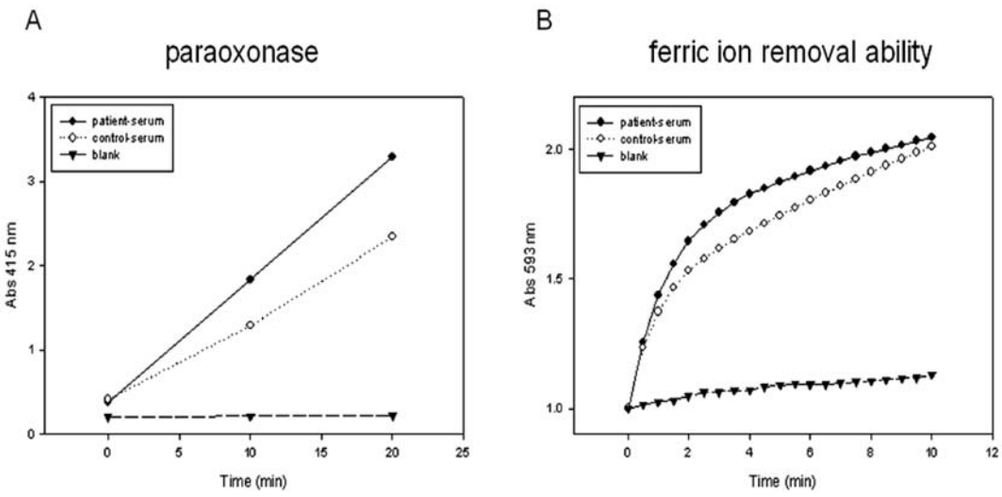

Figure 1. Comparison of the antioxidant activity based on paraoxonase activity (A) and ferric ion reduction ability (B). A representative graph from three measurements is shown. (A) Paraoxonase activity of serum. Twenty microliters of equally diluted serum (10 mg/ml) was added to 230 μl of the paraoxon-ethyl (Sigma catalog# D-9286) solution containing 90 mM Tris-HCl, 3.6 mM NaCl and 2 mM $CaCl_2$ (pH 8.5) for 30 min. (B) Ferric ion reduction assay. The more extensively reduced the ferric ion, the greater the increase in absorbance at 593 nm. The patient group showed a stronger reducing ability than the control during 10 min of incubation.

Paraoxonase assay. Paraoxonase-1 (PON-1) activity toward paraoxon was determined by evaluating the hydrolysis of paraoxon into *p*-nitrophenol and diethylphosphate, which was catalyzed by the enzyme (17). PON-1 activity was then determined by measuring the initial velocity of *p*-nitrophenol production at 37˚C, as determined by measuring the absorbance at 405 nm using a microplate reader (Bio-Rad model 680; Bio-Rad, Hercules, CA, USA), as described previously (18).

Western blot analysis. The apolipoprotein/lipoprotein compositions were compared via sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) with identical protein loading quantities (5 μg of total protein per lane) from individual HDL_3, and the levels of expression of apolipoprotein were analyzed via immunodetection. Anti-human apoA-I antibody (ab7613) was purchased from Abcam (Cambridge, UK). The relative band intensity was compared via band scanning with Gel Doc® XR (Bio-Rad) using Quantity One software (version 4.5.2).

Electron microscopy. Transmitted electron microscopy (TEM) was performed with a Hitachi electron microscope (model H-7600; Ibaraki, Japan), operating at 80 kV as in our previous report (19). VLDL, LDL and HDL were negatively stained with 1% sodium phosphotungastate (PTA; pH 7.4) with a final apolipoprotein concentration of 0.3 mg/ml in the TBS. HDL suspension (5 μl) was blotted with filter paper and immediately replaced with a 5-μl droplet of 1% PTA. After a few seconds, the stained HDL fraction was blotted onto a Formvar carbon-coated 300 mesh copper grid and air-dried. The shape and size of HDL_2 were determined by TEM photography at a magnification of x40,000.

Data analysis. All data are expressed as the mean ± SD from at least three independent experiments with duplicate samples. In Fig. 5, data comparisons were assessed by the Student's t-test using the SPSS program (version 14.0; SPSS, Inc., Chicago, IL, USA).

Results

Serum profiles of the patient. The patient had a higher serum TC (331 mg/dl) and TG (307 mg/dl) with a markedly decreased ratio of LDL-C (97 mg/dl) in TC and a normal level of serum glucose (102 mg/dl). Although HDL-C was not high (47 mg/dl) in the serum, apo-B and apoA-I levels and serum GOT and GPT values were in the normal ranges. HDL-C and LDL-C corresponded to 14.1 and 29.3% of TC, respectively. CETP activity was decreased in the patient with 19.8% of CE-transfer during a 6-h incubation, while the control serum had 24.8% CE-transfer. However, as shown in Fig. 1, the patient had elevated antioxidant ability (1.4-fold higher serum paraoxonase activity and ferric ion removal ability).

Lipoprotein properties in the patient. As shown in Table I, the patient had well-developed VLDL particles and highly enriched protein content even though the serum was obtained after a 14-h fast. However, the LDL particle was not developed as much as the age- and gender-matched controls; specifically, there was less protein and lipid content. HDL_2 from the patient was protein-enriched; a 1.8-fold increase in protein content without an increase in TC and TG. HDL_3 from the patient had a similarly reduced content of protein and lipid.

LDL from the patient was more resistant to oxidation. As shown in Fig. 2, LDL from the patient was more resistant to cupric ion-mediated LDL-oxidation (up to 120 min). At 30 min of incubation with Cu^{2+}, LDL from the control had a 92% increase in the conjugated diene level (A_{234}), while the patient had a 49% increase in A_{234}. Agarose electrophoresis confirmed the different extent of oxidation. LDL from the patient

Table I. Lipid and protein distribution of lipoprotein classes between the patient and controls.

	F-case			F-controls		
	TC (mg/dl)	TG (mg/dl)	TP (mg/dl)	TC (mg/dl)	TG (mg/dl)	TP (mg/dl)
VLDL	97.7 (4.03)	111.7 (4.61)	0.2	50.7 (5.67)	62.9 (7.02)	0.1
LDL	144.5 (3.07)	43.4 (0.92)	0.5	192.4 (2.79)	40.8 (0.59)	0.7
HDL_2	58.1 (0.60)	12.4 (0.13)	1.0	66.6 (1.21)	15.3 (0.28)	0.5
HDL_3	58.6 (0.65)	20.1 (0.22)	0.9	78.3 (0.66)	14.4 (0.12)	1.2

TC, total cholesterol; TG, triacylglycerides. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

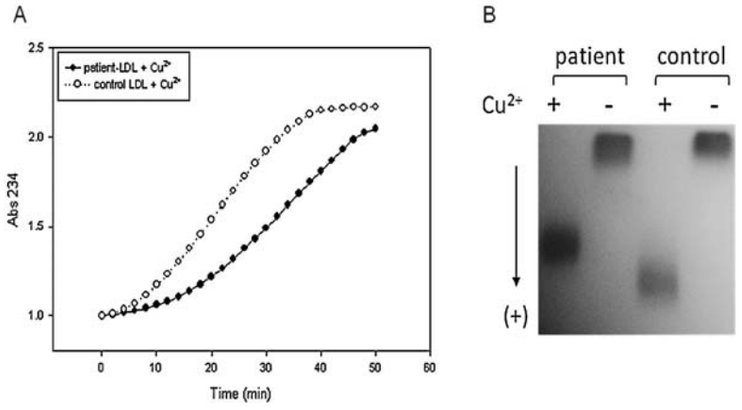

Figure 2. Susceptibility of cupric ion-mediated LDL oxidation. (A) Monitoring of conjugate diene level (Abs_{234} nm) of LDL (300 μg) in the presence of $CuSO_4$ (final concentration, 10 μM) at 37˚C. The patient showed much less sensitivity to the oxidation in up to 50 min of incubation. (B) Electromobility of LDL from the patient and controls, with or without $CuSO_4$ (final concentration, 10 μM) treatment. The more oxidized the LDL, the faster the front migrated to the bottom.

migrated slower than the LDL of the controls under the same treatment of cupric ion (final concentration, 10 μM). The more extensively oxidized LDL had a faster electrophoretic mobility on the agarose gel.

HDL-associated antioxidant activity is highly elevated in the patient. HDL_2-associated PON activity was markedly elevated in the patient as shown in Fig. 3A, while HDL_2 from the controls showed no activity at the same level as the blank. The HDL_2 of the patient showed a 2.7-fold more increase in PON activity compared to the control for up to 120 min of incubation. The HDL_3 from the patient also showed much stronger activity than the control HDL_3 (Fig. 3B), which exhibited adequate activity (166% or activity), indicating that the enhanced serum antioxidant activity of the patient (Fig. 1) might have originated from the HDL_2- and HDL_3-associated PON activity.

HDL_2 (0.24 mg/ml) from the patient exhibited a 1.3-fold stronger FRA activity (up to 216% increase in A_{593}), while the control showed a 166% increase. However, there was no marked difference between the patient and the control when HDL_3 (0.91 mg/ml) was used as an antioxidant source. This is a significant finding since HDL_2 in the general population has less antioxidant activity than HDL_3. This patient had a higher antioxidant activity in the HDL_2 fraction than that in an identical HDL_3 fraction.

Level of expression of apoA-I in HDL. As shown in Fig. 4, Western blot analysis revealed that the patient had a 1.5-fold increased apoA-I level in the HDL_2 fraction under the same amount of protein loaded (5 μg of protein/lane). In the patient and control, apoA-I was detected as multimerized bands up to a tetramer. However, the fragment of apoA-I (arrow head) was detected in the control HDL_2 and HDL_3, while the patient did not show the fragment of apoA-I in HDL_2 and HDL_3. This result indicates that the patient was more resistant to the aging-associated proteolytic degradation. Similarly, we recently reported that apoA-I is susceptible to proteolytic degradation with aging-associated glycation; there is a loss in antioxidant activity, anti-atherosclerosis, and anti-senescence activity with cleavage of apoA-I (20).

CETP activity. As shown in Fig. 5, there were no marked differences in CETP activity in VLDL, LDL and HDL_2 between the patient and the controls. However, HDL_3 from the patient had significantly lower CETP activity (7±1% of CE-transfer) than the controls (11±0.1% of CE-transfer). This result correlates well with the 20% lower serum CETP activity of the patient.

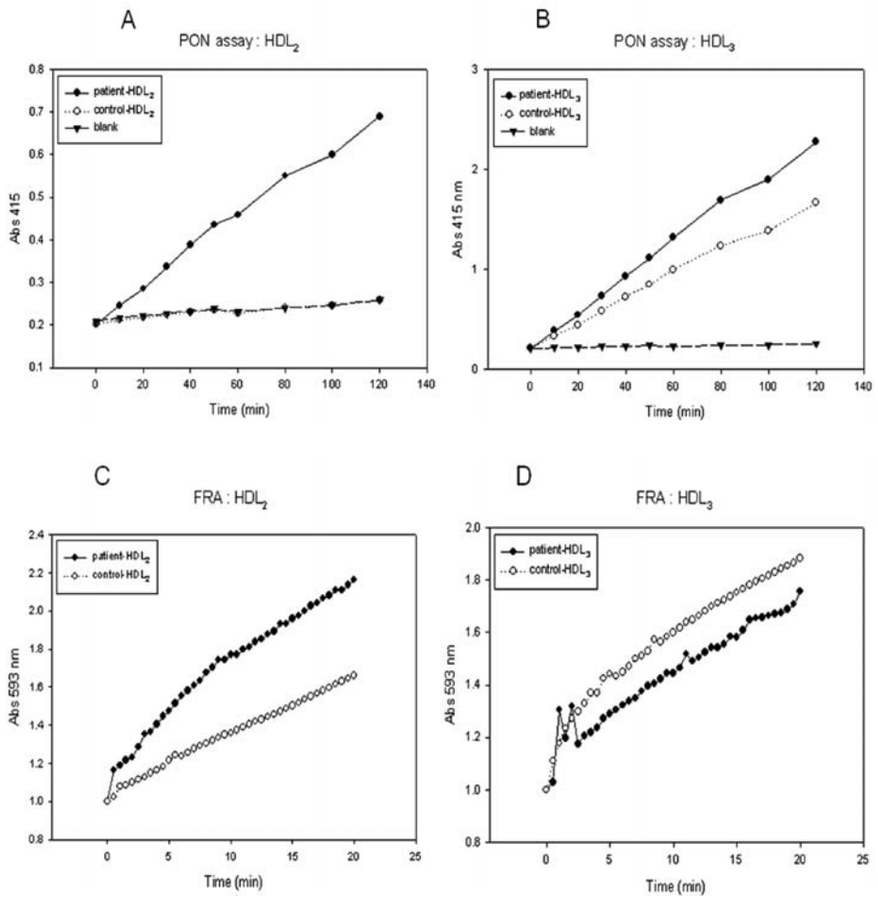

Figure 3. Antioxidant ability of HDL_2 and HDL_3 with paraoxonase (A and B) and ferric ion removal ability (C and D). Twenty microliters of equally diluted HDL_2 (0.24 mg/ml of protein) and HDL_3 (0.91 mg/ml of protein) was used as antioxidant sources.

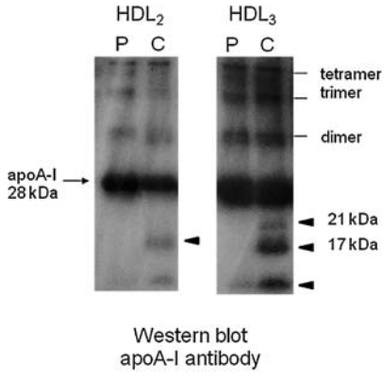

Figure 4. Western blot analysis of HDL_2 and HDL_3 using the apoA-I antibody. The same amount of protein (5 μg/lane) was loaded on the gel (15% SDS-PAGE).

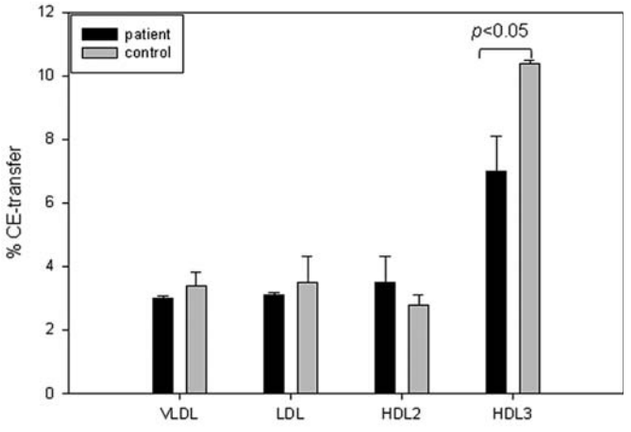

Figure 5. Comparison of the CETP activity using lipoproteins between the patient and the controls. The same amount of protein in the individual fraction (100 μg of total protein) was utilized as a source of CETP. Human LDL (0.25 mg/ml of protein) and rHDL containing [^{3}H]-cholesteryl oleate were used as a CE-acceptor and CE-donor, respectively. Error bars indicate the SD from three independent experiments with duplicate samples.

Particle size of lipoprotein. As shown in Fig. 6A, the patient had a much larger VLDL particle size than the controls based on electron microscopic observations (magnification, x30,000). This result had a good correlation with the development of particle components (~2-fold increased enriched protein and lipid in the patient's VLDL compared to the controls; Table I). However, there was no notable difference in the LDL particle size between the patient and the controls, except that the patient showed less density of the population (Fig. 6A).

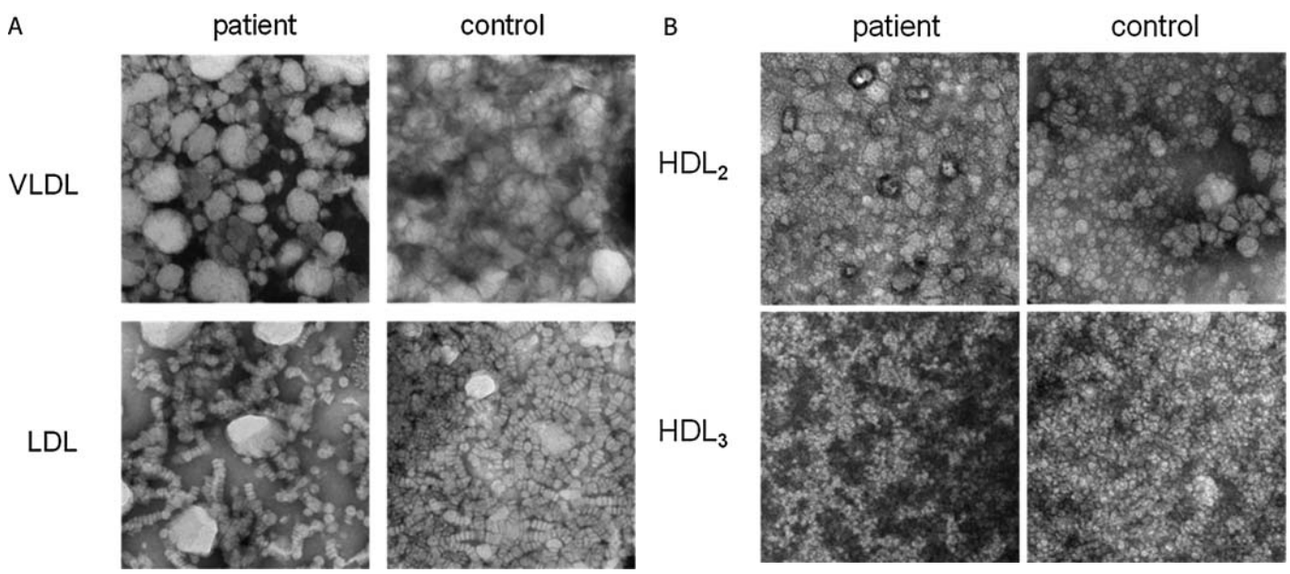

Figure 6. Electron microscopic observation of the lipoprotein after negative staining between the subject and controls. (A) Micrographs for VLDL (0.09 mg/ml of protein) and LDL (0.47 mg/ml of protein) are shown at a magnification of x30,000. (B) Micrographs for HDL_2 (0.3 mg/ml of protein) and HDL_3 (0.3 mg/ml of protein) are shown at a magnification of x40,000.

Based on the same electron microscopy (magnification, x40,000) as shown in Fig. 6B, HDL_2 from the patient had a slightly increased particle size (22±2 nm width and length) than that of the control HDL_2 (19±1 nm width and length). However, there was no marked difference between the patient and the controls in the particle size of HDL_3.

Discussion

In spite of hypercholesterolemia and hypertriglyceridemia, the patient had potent serum PON and antioxidant activity (Fig. 1) with resistance against cupric ion-mediated LDL oxidation (Fig. 2). The enhanced PON and antioxidant activities were much higher in HDL_2 than in HDL_3 (Fig. 3). The patient had a 3-fold elevated HDL_2-PON activity compared to the control. The patient had a 1.5-fold higher level of expression of apoA-I in HDL_2 (Fig. 4). ApoA-I in HDL_2 and HDL_3 from the patient showed no fragmentation, while the control showed fragmented apoA-I (17 and 21 kDa) in HDL. However, the patient had lower CETP activity in the HDL_3 fraction (Fig. 5) with a larger HDL_2 particle size (Fig. 6B), although the HDL_3 particle size was not different.

Even though the patient was a postmenopausal female, she had higher serum apoA-I (148 mg/dl) and lower apo-B (91 mg/dl) than the average reported by Park *et al* (20); specifically, apoA-I and apo-B were 126±2 and 120±6 mg/dl, respectively. Furthermore, Zago *et al* (21) showed that HDL from postmenopausal women had a 19% increased CETP activity and 35% decreased PON activity compared to premenopausal women. Our patient had a 30% decreased HDL_3-CETP activity, while the LDL-CETP and HDL_2-CETP activities were similar.

In the VLDL and HDL_2 fractions, the patient had a 2-fold increased total protein content compared to the controls (Table I). The enrichment of VLDL and enhancement of PON activity might be associated, as in a previous study (22) in which VLDL was able to promote secretion of PON-1 from Chinese hamster ovary cells and stabilize the activity. They also showed that VLDL-associated PON-1 retains its antioxidant function.

The protein-enriched HDL_2 from the patient exhibited enhanced antioxidant activity and larger particle size. In general, the PON-1 enzyme is physically associated with the HDL_3 fraction, and most of the PON activity is found in the HDL_3 fraction as well as other antioxidant enzymes (23). It was noteworthy that the patient exhibited potent activity of PON in HDL_2 rather than HDL_3. PON-1 attenuates the oxidation of LDL via inhibition of the accumulation of lipid peroxides in LDL (24). The LDL from the patient showed potent resistance to cupric ion-mediated oxidation (Fig. 2).

CETP plays an important role in the re-distribution and equilibration of hydrophobic lipids packaged within the lipoprotein core (CE and TG) between HDL and LDL/VLDL (25). In CE and TG, re-distribution among lipoproteins, and lipoprotein particle composition and size were influenced by CETP activity. The reduced CETP activity of HDL_3 in the patient (Fig. 4) correlated well with the decrease in TC in LDL and HDL (Table I). The decreased CETP activity in the patient was associated with the enhanced antioxidant ability of serum (Fig. 1) and HDL (Fig. 3). These results are in good agreement with another report (26) that elevated serum CETP activity is associated with lowered PON-1 activity and the increased extent of oxidized species.

The patient showed a markedly elevated apoA-I without proteolytic fragments in HDL_2 and HDL_3. ApoA-I is a potent antioxidant protein that is capable of removing LDL lipid hydroperoxide *in vitro* and *in vivo* when infused into mice and humans (27). A potent antioxidant activity against LDL oxidation has been recognized as one of the primary beneficial

effects of HDL in the prevention or attenuation of the progression of atherosclerotic lesion formation. Modification of apoA-I, such as cleavage, oxidation, nitration and chlorination, can lead to the production of dysfunctional apoA-I and HDL (28). Truncation of apoA-I at the C-terminus, yielding apoA-I (1-192), results in decreased lipid-binding ability (29). Cho *et al* (30) demonstrated that the antioxidant activity of HDL derives, at least in part, from apoA-I itself, without any necessity for phospholipid interaction.

The patient had a markedly lower LDL-C/TC ratio with elevated apoA-I (148 mg/dl) in the serum, in spite of hyperlipidemia and a moderate level of HDL-C (47 mg/dl). The discrepancy between the measured LDL-C and calculated LDL-C (222 mg/dl) may have originated from different HDL_2- and HDL_3-associated enzyme activities and antioxidant abilities. It has been reported that small dense LDL is specifically associated with the development of coronary heart disease (7); however, the patient in the current study had a smaller population with similar particle size and distribution compared with the controls (Fig. 6A).

Conclusively, the atherogenic dyslipidemia of this patient might be overcome by the enhanced antioxidant properties of lipoprotein, such as robust apoA-I and enhanced PON activity in HDL, which contributes to make LDL less susceptible to oxidation.

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