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Synthesis of Reconstituted High Density Lipoprotein (rHDL) Containing apoA-I and apoC-III: the Functional Role of apoC-III in rHDL

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Apolipoprotein (apo) C-III is a marker protein of triacylglycerol (TG)-rich lipoproteins and high-density lipoproteins (HDL), and has been proposed as a risk factor of coronary heart disease. To compare the physiologic role of reconstituted HDL (rHDL) with or without apoC-III, we synthesized rHDL with molar ratios of apoA-I:apoC-III of 1:0, 1:0.5, 1:1, and 1:2. Increasing the apoC-III content in rHDL produced smaller rHDL particles with a lower number of apoA-I molecules. Furthermore, increasing the molar ratio of apoC-III in rHDL enhanced the surfactant-like properties and the ability to lyse dimyristoyl phosphatidylcholine. Furthermore, rHDL containing apoC-III was found to be more resistant to particle rearrangement in the presence of low-density lipoprotein (LDL) than rHDL that contained apoA-I alone. In addition, the lecithin:cholesterol acyltransferase (LCAT) activation ability was reduced as the apoC-III content of the rHDL increased; however, the CE transfer ability was not decreased by the increase of apoC-III. Finally, rHDL containing apoC-III aggravated the production of MDA in cell culture media, which led to increased cellular uptake of LDL.

Thus, the addition of apoC-III to rHDL induced changes in the structural and functional properties of the rHDL, especially in particle size and rearrangement and LCAT activation. These alterations may lead to beneficial functions of HDL, which is involved in anti-atherogenic properties in the circulation.

INTRODUCTION

Apolipoprotein (apo) C-III is a protein comprised of 79 amino acids and is a constituent of very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL; Bruns et al., 1984). Although the physiologic role of apoC-III is still not fully understood, the results of many clinical studies indicate that apoC-III is a risk factor for atherogenesis and hypertriglyceridemia (Onat et al., 2003; Sacks et al., 2000). It has been reported that LDL containing apoC-III is an independent risk factor for coronary events in diabetic patients (Lee et al., 2003).

ApoC-III inhibits lipoprotein lipase (LPL; Wang et al., 1985) and causes an attenuated clearance of TG-rich lipoproteins, which is associated with increased coronary risk and the induction of myocardial ischemia (Ginsberg et al., 1995).

Even though apoC-III is a minor component of HDL, it can modify the functions of HDL, especially during the acute phase of infection and/or inflammation. For example, our research group recently reported that there is an abrupt increase in apoC-III during the acute oliguric phase of hemorrhagic fever renal syndrome (HFRS), which indicates the physiologic importance of apoC-III (Cho et al., 2008). In that study, as the apoC-III and inflammatory markers increased, serum amyloid A (SAA) and interleukin (IL)-6 were increased and the apoA-I level decreased simultaneously.

More recently, our research group reported that patients with myocardial infarction (MI) had unique serum and lipoprotein characteristics, including increased serum interleukin-6 and triacylglycerol, with increased apoC-III levels in the LDL and HDL fractions, as well as severely impaired antioxidant ability of HDL (Cho et al., 2009). These results strongly suggested that the composition of lipoprotein is altered in patients with MIs with increased inflammatory factors.

These reports indicate that there might be strong correlations between the levels of apoA-I and apoC-III with respect to HDL structure and function. It is well-known that HDL has antioxidant and anti-inflammatory activity (Barter et al., 2004; Lewis and Rader, 2005), and that such activities are exerted in accordance with the composition of essential apolipoproteins and associated enzymes, such as lecithin:cholesterol acyltransferase (LCAT) and paraoxonase (PON). Furthermore, we recently found that apoC-III was overexpressed in HDL, as well as SAA protein and attenuated serum LCAT and PON activity were observed during the oliguric phase in HFRS patients (Cho et al., 2008). Taken together, these findings demonstrate that structural, functional, and compositional changes in HDL are linked to increased apoC-III content in rHDL.

In spite of the clinical importance of apoC-III in acute inflammatory coronary events, relatively few studies have been conducted to evaluate the role of apoC-III in HDL (Jonas et al., 1984; Nishida et al., 1986). Almost all of the functional studies

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of rHDL have been conducted using reconstituted HDL (rHDL) containing apoA-I or apo-E. Therefore, this study was designed to elucidate the effect of apoC-III in rHDL containing apoA-I. To accomplish this, we synthesized rHDL containing different molar ratios of apoA-I and C-III and then compared their functional and structural characteristics.

MATERIALS AND METHODS

Apolipoproteins A-I and C-III

Wild type proapoA-I (WT) was expressed and purified as described previously (Cho and Jonas, 2000) using the pET30 expression system and BL21 (DE3) *E. coli* host cells (Novagen, USA). Human apoC-III was purchased from Millipore Korea (Cat# ALP-60, Korea).

Synthesis of reconstituted HDL

Discoidal rHDL was prepared by the sodium cholate dialysis method (Matz and Jonas, 1982) using initial molar ratios of palmitoyloleoyl phosphatidylcholine (POPC):cholesterol:apoA-I:apoC-III: sodium cholate of 95:5:1:x:150, where x represents 0, 0.5, 1, or 2. The (1:0.5)-rHDL indicates the molar ratio of 95:5:1:0.5:150 for POPC:cholesterol:apoA-I: apoC-III: sodium cholate. The rHDL particles were used without further purification because they showed high homogeneity. Their sizes were determined using 8-25% native polyacrylamide gradient gel electrophoresis (PAGGE, Pharmacia Phast system, GE healthcare, Sweden) to compare them with standard globular proteins (AmershamPharmacia, Sweden). Particle sizes of rHDLs were determined by native 8-25% PAGGE comparison with standard globular proteins (Cat# 17-0445-01, AmershamPharmacia). The relative migrations were compared via densitometric scanning analysis using a Gel Doc® XR (Bio-Rad, USA) with Quantity One software, version 4.5.2. The number of apoA-I molecules per rHDL particle, as well as the self-association properties of lipid-free proteins, were determined by crosslinking with BS_3 using the method described by Staros (1982) and then analyzing the products of the reaction by sodium dodecyl sulfate (SDS)-PAGGE on precasted 8-25% gradient gels (AmershamPharmacia).

DMPC-clearance assay

Interactions of the mutant proteins with dimyristoyl phosphatidylcholine (DMPC) were monitored by the method described by Pownall et al. (1978) with slight modification. The mass ratio of DMPC to protein was 2:1 (w/w) in 0.76 ml of total reaction volume. The measurements were initiated after addition of DMPC and monitored at 325 nm every 2 min using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Germany) equipped with a thermocontrolled cuvette holder adjusted to 24.5°C.

HDL-particle rearrangement assay

To examine the conformational adaptability of the wild type and mutant proteins in POPC-rHDL particles, changes in particle size were observed upon incubation with LDL, as well as the loss of phospholipid (Jonas et al., 1989). To observe changes in the particle sizes, 100 μg of (protein) POPC-rHDL (0.1 ml) were incubated with 120 μg (protein) of human LDL (0.1 ml) at 37°C for the designated time interval up to 24 h. After incubation, aliquots of the samples were collected and stored at 4°C. The products were separated on 8-25% native polyacrylamide gradient gels, using the Pharmacia PhastSystem.

Lecithin:cholesterol acyltransferase assay

The lecithin:cholesterol acyltransferase (LCAT) assay was carried out as described in detail previously (Matz and Jonas, 1982) using the apoA-I and mutants in POPC-rHDL and human lipoprotein deficient serum (LPDS, d < 1.21 bottom fraction) as a substrate and an enzyme source, respectively. The reaction mixture contained the radiolabeled cholesterol (1 μCi of [^{14}C]-cholesterol/69 μg cholesterol/1 mg of apoA-I) in the POPC-rHDL with 4% defatted bovine serum albumin and 4 mM β-mercaptoethanol. The reaction was initiated by the addition of 25 μl of the lipoprotein deficient serum (LPDS, 5.4 mg/ml) and incubated for 1 h at 37°C. The POPC-rHDLs were present in various concentrations, ranging from 1.0×10^{-6} to 2.5×10^{-7} M of apolipoprotein. The reaction was performed in duplicate and background values were determined by omitting only the LPDS from the reaction tubes at each substrate concentration. Initial reaction velocities at each substrate concentration were determined by thin layer chromatography (TLC) analysis of the cholesterol and cholesterol esters, and Lineweaver-Burk plots were used to obtain the apparent K_m and V_{max} values by linear regression.

Cholesteryl ester transfer protein assay

An rHDL containing apoA-I and cholesteryl oleate was synthesized in accordance with the method described by Cho et al. (1998) with trace amounts of [^{3}H]-cholesteryl oleate (TRK886, 3.5 μCi/mg of apoA-I; GE Healthcare). The rHDL was then immobilized using CNBr-activated Sepharose 4B (Amersham Biosciences) in accordance with the manufacturer's instructions, in order to facilitate separation from the CE-acceptor. In 300 μl reaction mixtures, human LPDS samples (20 μl) were used as a cholestryl ester transfer protein (CETP) source, and the rHDL-agarose (50 μl, 0.25 mg/ml) and human LDL (50 μl, 0.25 mg/ml) were used as a CE-donor and CE-acceptor, respectively. After incubation at 37°C, the reaction was halted via brief centrifugation (10,000 × *g*) for 3 min at 4°C. The supernatant (150 μl) was subjected to scintillation counting, and the percentage transfer of [^{3}H]-CE from agarose-rHDL to LDL was calculated.

Fluorospectroscopy

The wavelengths of maximum fluorescence (WMF) of tryptophan residues in wild type and mutants were determined from uncorrected spectra obtained on LS55 spectrofluorometer (Perkin-Elmer, USA) using WinLab software package 4.00 (Perkin-Elmer) and a 1 cm path-length suprasil quartz cuvette (Fisher Scientific, USA). The samples were excited at 295 nm to avoid tyrosine fluorescence, and the emission spectra were scanned from 305-400 nm at room temperature.

LDL oxidation and cell culture

LDL (1.019 < d < 1.063) was isolated by ultracentrifugation from human plasma using a Himac CP90α (Hitachi, Japan) and then dialyzed against phosphate buffered saline (PBS). Oxidized LDL (oxLDL) was produced by incubation with $CuSO_4$ (final 10 μM) for 4 h at 37°C and then filtered (0.2 μm) prior to use. A thiobarbituric acid reacting substances (TBARS) assay was then used to evaluate the oxidation of oxLDL.

THP-1 cells, a human monocytic cell line, were obtained from the American Type Culture Collection (ATCC, #TIB-202™; USA) and maintained in RPMI1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) until needed. Cells that had undergone no more than 20 passages were incubated in medium containing phorbol 12-myristate 13-acetate (PMA; final concentration, 150 nM) in 24-well plates for 24 h at 37°C in a humidified incubator (5% CO_2, 95% air) to induce differentiation into macrophages. The differentiated and

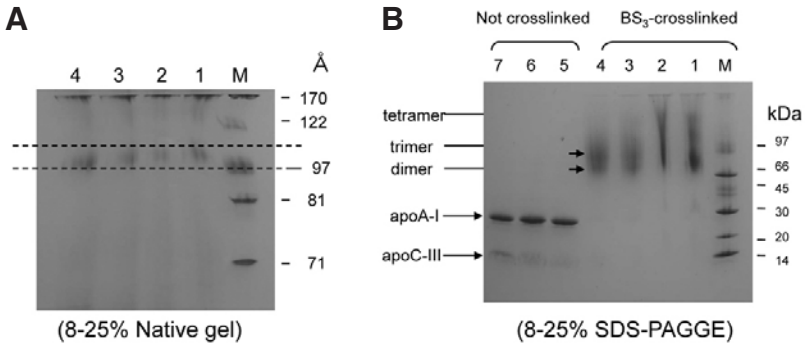

Fig. 1. Synthesis of rHDLs and protein composition analysis using BS_3-crosslinking. (A) Electrophoresis patterns of POPC-rHDL (8-25% native PAGGE). The rHDL particles were prepared following the sodium cholate dialysis method using various molar ratios of apoA-I:apoC-III. Particle sizes of rHDLs were determined by the PAGGE comparison with high molecular weight (HMW) markers (AmershamPharmacia). The relative migrations were compared via densitometric scanning analysis using a Gel Doc® XR (Bio-Rad) with Quantity One software, version 4.5.2. Lane 1, (1:0)-rHDL; lane 2, (1:0.5)-rHDL; lane 3, (1:1)-rHDL; lane 4, (1:2)-rHDL; and lane M, HMW markers (thyroglobulin, 170 Å; ferritin, 122 Å; catalase, 97 Å; lactate dehydrogenase, 81 Å; albumin, 71 Å). (B) Electrophoresis patterns of crosslinked (lanes 1-4) and intact (lanes 5-7) POPC-rHDL from panel A analyzed by 8-25% SDS-PAGGE. Lane 1, (1:0)-rHDL; lanes 2 and 5, (1:0.5)-rHDL; lanes 3 and 6, (1:1)-rHDL; lanes 4 and 7, (1:2)-rHDL; and lane M, low molecular weight marker (GE Healthcare).

adhered macrophages were then rinsed with warm PBS and incubated with 400 μl of fresh RPMI1640 medium containing 1% FBS, 50 μl of oxLDL (1 mg of protein/ml in PBS), and 50 μl of PBS or each rHDL (1.4 mg of protein/ml in PBS) for 48 h at 37°C in a humidified incubator. After incubation, the cells were washed with PBS three times, and then fixed in 4% paraformaldehyde for 10 min. Next, the fixed cells were rinsed with 100% polypropylene glycol, stained with oil-red O staining solution (0.67%), and then washed with distilled water. THP-1 macrophage-derived foam cells were then observed and photographed using a Nikon Eclipse TE2000 microscope (Japan) at 600× magnification. The mean area of the oil-red O-stained region per cell was then quantified via computer-assisted morphometry with 20 representative cells using the Image Proplus software (version 4.5.1.22, Media Cybernetics, USA). The cell media was then analyzed by TBARS assay and a LPO assay kit (Calbiochem) to determine change of oxidized species level.

Electrophoresis and protein determination

The apolipoprotein/lipoprotein compositions were compared via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an equal amount of protein loading. The protein concentration was determined according to the Lowry protein assay, as modified by Markwell et al. (1978) or by using the Bradford assay reagent (BioRad, Korea) with bovine serum albumin as a standard.

RESULTS

Synthesis of rHDL containing apoA-I and apoC-III

Particle formation of all rHDLs containing apoA-I and apoC-III (molar ratios of 1:0.5, 1:1, and 1:2 designated as (1:0.5)-rHDL, (1:1)-rHDL, and (1:2)-rHDL, respectively) was conducted using the same molar ratio of POPC:cholesterol:apoA-I (95:5:1) following the sodium cholate dialysis method. Native polyacrylamide gradient gel electrophoretic analysis revealed that increasing the apoC-III content in rHDL caused formation of slightly smaller particle sizes (Fig. 1A). Specifically, the diameter of the (1:0)-rHDL was approximately 103 ± 1 Å (lane 1), while the diameter of (1:2)-rHDL was approximately 96 ± 1 Å (lane 4), based on a relative electrophoretic mobility calculation using the densitometric system (Gel-Doc XR).

BS_3-crosslinking analysis revealed that the molecule number of apoA-I in the particles decreased as the apoC-III content increased, as shown in Fig. 1B (lanes 1-4). In addition, rHDL containing only apoA-I, (1:0)-rHDL, contained 2, 3, or 4 molecules of apoA-I (lane 1, Fig. 1B). However, as the apoC-III content in rHDL increased, the number of apoA-I molecules in the rHDL was reduced to 2 or 3, and no tetramer band was detected (lanes 3 and 4). These results suggest that the addition of apoC-III during the preparation of rHDL hampers the formation of larger particles, which are required for apoA-I molecules in a particle (Jonas et al., 1990).

The final phospholipid molar ratio also decreased in response to increased apoC-III, and this decrease occurred in a dose-dependent manner. Specifically, nearly 30% of the phospholipid content was reduced in (1:2)-rHDL (Table 1). Taken together, these results indicate that increasing the level of apoC-III resulted in a reduction of the rHDL size (Fig 1A), the number of apoA-I molecules (Fig. 1B), and the phospholipid content (Table 1), all of which occurred in a dose-dependent manner.

Spectroscopic analysis

Wavelength maximum fluorescence (WMF) determination revealed that lipid-free apoA-I and apoC-III exhibited 347 and 352 nm, respectively, indicating that the Trp in apoC-III is more exposed to the aqueous environment in the lipid-free state (Table 1). There are three Trp moieties at the 42nd, 54th, and 65th positions of the primary sequence of apoC-III. In addition, the WMF of (1:0)-rHDL showed 4 nm blue-shifted WMF when compared to that of apoA-I in the lipid-free state. These findings indicate that the Trp molecules were exposed to a more non-polar phase as a result of lipid-binding. However, as the content of apoC-III increased, the WMF of the rHDL shifted towards red wavelengths. Specifically, (1:0)-rHDL and (1:2)-rHDL showed 1 and 2 nm red-shifted WMF, respectively (Table 1). This result indicates that the addition of apoC-III may cause movement of Trp108 of apoA-I, which is responsible for the majority of the fluorescence activity, into a more polar phase. These results are in close agreement with those of a previous report by Jonas et al. (1984), which showed that the WMF of eggPC-rHDL containing apoC-III was 333 nm, while that of eggPC-rHDL containing apoA-I was 330 nm.

Addition of apoC-III-facilitated phospholipid binding affinity

As shown in Fig. 2, all of the proteins readily solubilized a DMPC liposome. Interestingly, the DMPC-clearance speed was increased when the apoC-III content was increased in the reaction mixture, while the apoA-I concentration was fixed at 0.15 mg/ml. The observed clearance speeds were 28 ± 4, 8 ± 2, 6 ±

Table 1. Compositional properties and spectroscopic data of apoA-I in the lipid-free and rHDL state

State	rHDL	WMF average	Molar composition (POPC:cholesterol: apoA-I: apoC-III)		Number of apoA-I/ particle[2]
			Initial	Final[1]	
POPC-rHDL.	1:0	343.8 ± 0.2	95:5:1:0	101 ± 4: 5: 1:0	2, 3, 4
	1:0.5	344.9 ± 0.1	95:5:1:0.5	92 ± 4: 4: 1:0.5	2, 3, 4
	1:1	345.4 ± 0.2	95:5:1:1	84 ± 5: 5: 1:1	2, 3
	1:2	345.6 ± 0.2	95:5:1:2	71 ± 5: 4: 1:2	2, 3
Lipid-free	apoA-I	347.5 ± 0.1			
	apoC-III	352.1 ± 0.1			

[1]Phospholipid, cholesterol: protein (molar ratio)
[2]Determined from crosslinking and 8-25% SDS-PAGGE

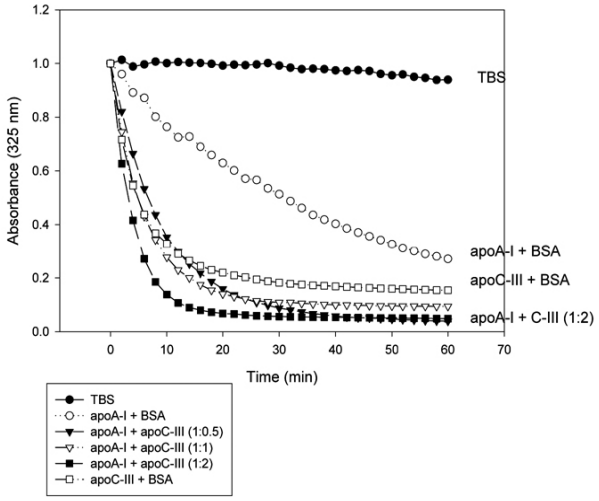

Fig. 2. Kinetics of the interaction of apolipoproteins with DMPC multilamellar liposomes. The reaction was initiated by the addition of 0.7 ml of apolipoproteins (an apoA-I and apoC-III mixture, 0.15 mg/ml) to the multilamellar DMPC liposomes (0.06 ml, 3.5 mg/ml) in TBS (pH 8.0). The mass ratio of DMPC to protein was 2:1 (w/w) in a total reaction volume of 0.76 ml. BSA was added to the apoA-I only and apoC-III only reaction tubes as controls to compensate for the total protein amount. The absorbance at 325 nm was monitored at 24.5°C at 2-min intervals for up to 1 h.

2, and 3 ± 1 min for (1:0, A-I:C-III), (1:0.5, A-I:C-III), (1:1, A-I:C-III), and (1:2, A-I:C-III), respectively. The fastest clearance activity occurred with (1:2, A-I:C-III), while apoC-III only showed a clearance activity 5 ± 2 min. Taken together, these results indicate that apoA-I and C-III exerted a synergistic effect on clearance.

HDL particle size rearrangement

It is well-known that rHDL containing only apoA-I (100-105 Å) undergoes particle rearrangement that results in the formation of smaller particles (78-84 Å) following overnight incubation at 37°C in the presence of LDL (Han et al., 2005; Fig. 3A). However, the (1:2)-rHDL was found to be resistant to this size rearrangement (Fig. 3B). This result indicates that the presence of apoC-III in rHDL hampers spontaneous phospholipid transfers and the movement of the putative hinge domain of apoA-I that results in the production of smaller HDL particles.

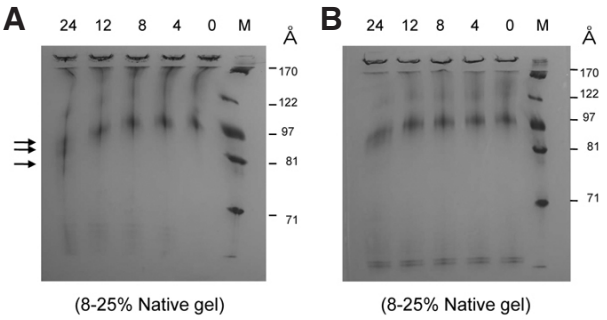

Fig. 3. Size rearrangements of the rHDL particles in the presence of LDL. The rHDLs were prepared as described in the text. The reaction was initiated by adding 120 μg of human LDL (protein) to 100 μg of rHDL (protein) at 37°C. An aliquot of the incubation mixture was then collected at the indicated time intervals and stored at 4°C with gel loading buffer without SDS. The aliquoted samples were then separated on 8-25% native gels, after which the protein bands were visualized by Coomassie blue staining. The rearrangement patterns of (1:0)-rHDL and (1:2)-rHDL are displayed in (A and B), respectively. Lane M, high molecular weight markers; the upper number indicates the incubation time at 37°C.

Activation ability of LCAT and CETP

An increase of apoC-III content in rHDL weakened its LCAT activation activity, as shown in Fig. 4A and Table 2. For (1:2)-rHDL, the LCAT activation ability (expressed as V_{max}) was reduced by up to 62% and the substrate affinity (expressed as K_m) was decreased by up to 3.5-fold, when compared to (1:0)-rHDL. In addition, the calculated reactivity (V_{max}/K_m) of the (1:2)-rHDL was decreased to only 10% of that of the (1:0)-rHDL (Table 2).

In contrast to the change in LCAT activity, the CE-transfer activity mediated by CETP from tritiated rHDL (as a CE-donor) to LDL (as a CE-acceptor) was not decreased (Fig. 4B). Furthermore, regardless of the apoC-III content in each rHDL, all substrate rHDLs showed a similar activity of approximately 24-28% CE-transfer for 6 hr incubation, indicating that there might be different activation mechanisms involved in the CE-esterification and CE-transfer processes by apoA-I and C-III.

Cellular uptake of oxLDL was increased by treatment of rHDL containing apoC-III

After 48 h of incubation, the THP-1 cells were stained with oil

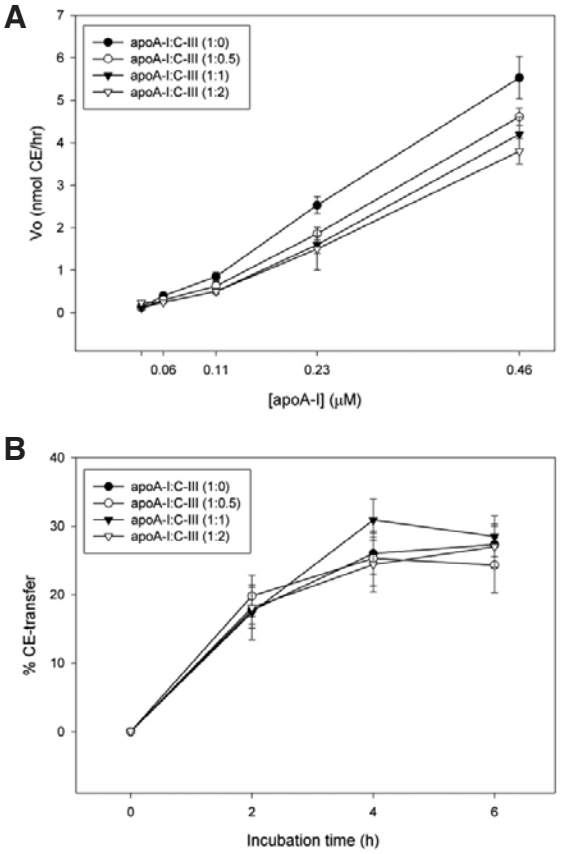

Fig. 4. Ability of the rHDLs to activate cholesterol esterification reaction of lecithin: cholesterol acyltransferase (A) and cholesteryl ester transfer reaction from rHDL to LDL (B). (A) Kinetics of the LCAT reaction with POPC-rHDL substrates. The rHDL particles for the LCAT reaction were prepared as described in the text with the addition of a trace amount of [^{14}C]-cholesterol. Reconstituted HDL (rHDL) was prepared by the sodium cholate dialysis method (Matz and Jonas, 1982) using initial molar ratios of POPC:cholesterol: apoA-I:apoC-III:sodium cholate of 95:5:1:x:150. The x was 0, 0.5, 1, or 2. The activity was expressed as formed cholesteryl ester (CE, nmol) per 1 h incubation. The error bars indicate the SD from three independent experiments with duplicate samples. (B) Cholesteryl ester transfer of POPC-rHDL-agarose. A human serum sample (20 μl) was used as a CETP source, and rHDL-agarose (50 μl, 0.25 mg/ml) and human LDL (50 μl, 0.25 mg/ml) were used as a CE-donor and CE-acceptor, respectively. Following incubation at 37°C, the reaction was halted via brief centrifugation (10,000 × *g*) for 3 min at 4°C. The supernatant (150 μl) was then subjected to scintillation counting, after which the percentage of [^{3}H]-CE transferred from the agarose-rHDL to the LDL was calculated. Error bars indicate the SD from three independent experiments with duplicate samples.

red O to evaluate the degree of lipid or LDL uptake into cells that occurred in the presence of each rHDL. As shown in Fig. 5A, (1:0)-rHDL-treated cells (Fig. 5A-b) showed slightly smaller lipid-stained areas than PBS-treated cells (Fig. 5A-a), indicating that rHDL containing apoA-I can inhibit cellular uptake of oxLDL. However, a stronger red color intensity was observed in (1:1)-rHDL-treated cells (Fig. 5A-c) and (1:2)-rHDL-treated cells (Fig. 5A-d), indicating that more apoC-III existing in rHDL cause more oxLDL uptake in the THP-1 cells. The strongest red color

Table 2. Reaction of rHDL particles with lecithin:cholesterol acyltransferase (LCAT)[1]

Ratio of apoA-I: C-III in rHDL	Apparent[2] V_{max} (nmol CE/h)	Apparent K_m (μM)	Apparent V_{max}/ Apparent K_m (nmol CE/h · M × 10^{-6})
1:0	3.1 ± 0.3	1.1 ± 0.1	2.8 ± 0.2
1:0.5	2.7 ± 0.2	2.1 ± 0.2	1.3 ± 0.2
1:1	1.7 ± 0.1	3.3 ± 0.2	0.5 ± 0.1
1:2	1.2 ± 0.1	3.9 ± 0.3	0.3 ± 0.1

[1]Values were expressed as the mean ± SD from three independent LCAT assays.
[2]The apparent kinetic parameters were determined by linear regression analysis using a Lineweaver-Burk plot of the reciprocals of the reaction velocity versus the substrate concentrations.

intensity (Photo d) suggests that apoC-III did not prevent the uptake of oxLDL into macrophages. This result correlated well with the results of a TBARS assay of the culture media and lipid stained area measurements as shown in Fig. 5B. The media used to culture (1:0)-rHDL-treated cells contained less MDA than the media used to culture PBS-treated cells. Furthermore, (1:2)-rHDL-treated cell media contained 2-fold higher MDA than that of media used to culture the PBS-treated cells. The calculated percentage of lipid-stained area per cell was highest in the (1:2)-rHDL-treated cells (46 ± 5%), as compared to PBS-treated cells (19 ± 5%), as shown in panel B. Taken together, these results suggest that rHDL containing apoC-III aggravated the production of MDA in cell culture media as a result of the increased cellular uptake of LDL. These results indicate that the antioxidant activity of rHDL was weakened by the increase of apoC-III content in the particle.

DISCUSSION

ApoC-III is a primary component of triglyceride-rich lipoproteins (VLDL and chylomicrons), and its concentration is both highly and positively correlated with the concentration of serum TG (Shachter, 2001). ApoC-III is often considered to be an independent risk factor for coronary syndrome with inflammatory properties (Ooi et al., 2008). In addition, the presence of apoC-III in the LDL or VLDL subfractions has also been found to be significantly correlated with the progression of coronary artery lesions (Hodis et al., 1994). The linkage of apoC-III and TG-rich lipoproteins (TGRL) is based on the inhibition of lipoprotein lipase (LPL) by apoC-III. In addition, Ginsberg et al. (1986) showed that individuals lacking apoC-III have very low levels of TGRL accompanied by very efficient lipolysis of TG. Furthermore, increased TGRL in the blood is strongly associated with progression of proinflammatory mechanisms in vascular cells and plaques (Ting et al., 2007).

Recently, we demonstrated that apoC-III was increased in the acute inflammatory phase, such as the oliguric phase of HFRS, and that this increase was associated with an abrupt increase of serum TG (Cho et al., 2009). The oliguric phase of HFRS is a typical inflammatory stage during acute renal failure that is characterized by an increase of pro-inflammatory factors. ApoC-III was found to be over-expressed during the acute inflammatory phase, especially in VLDL and HDL. Furthermore, this increase was found to be associated with a co-increase in apoSAA, attenuation of serum LCAT and PON activities, and a reduction of serum apoA-I (Cho et al., 2009). Based on these findings, it is possible that an increase of apoC-III alters benefi-

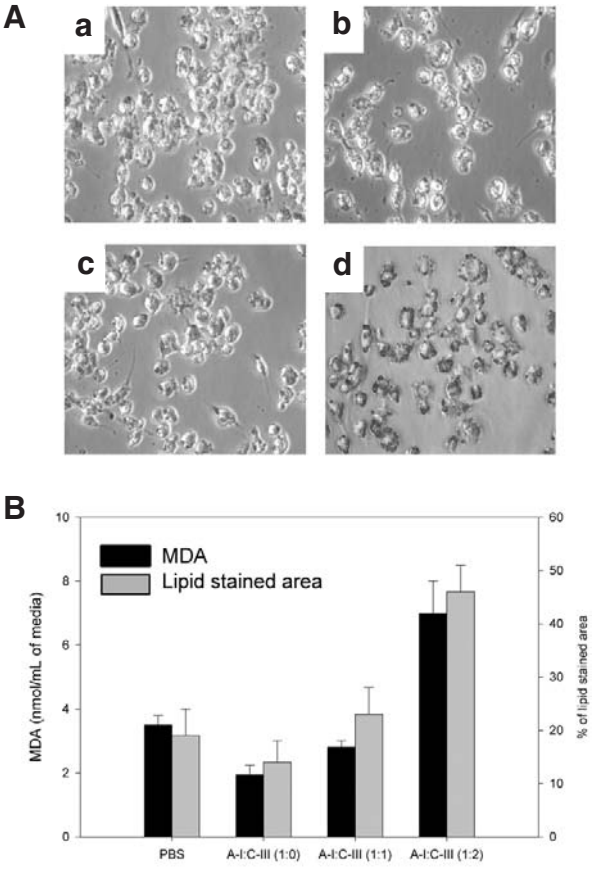

Fig. 5. Cellular uptake of oxLDL in the presence of rHDL containing apoC-III. PMA differentiated macrophages were incubated with 50 μl of oxLDL (1 mg/ml), 50 μl of rHDL (1.4 mg/ml), and 400 μl of RPMI1640 media. The extent of cellular uptake of lipids or LDL by macrophages was then compared by oil red O staining as described in the text. (A) The cells were then photographed using a Nikon Eclipse TE2000 microscope (Japan) at 600x magnification. a, PBS-treated; b, (1:0)-rHDL-treated; c, (1:1)-rHDL-treated; and d, (1:2)-rHDL-treated. (B) The graph shows the results of the thiobarbituric acid reactive substances (TBARS) assay and calculation of the lipid-stained area per cell. The cell media was analyzed by TBARS assay to determine the production of oxidized species. Malondialdehyde (MDA) was used as a standard and the extent of oxidation was expressed as the MDA concentration (nmol/ml of media). The mean area of oil-red O-stained region per cell was then quantified using computer-assisted morphometry for each well using the Image Proplus software (version 4.5.1.22, Media Cybernetics, USA).

cial functions of HDL. The beneficial roles of HDL include anti-inflammatory and anti-oxidant properties, which originate from apoA-I itself. Similarly, Breyer et al. (1999) showed that the addition of apoC-III to plasma displaces apoE from small VLDL particles, which raises the possibility that an interchange of the amphipathic helix occurs between apolipoproteins.

It has been suggested that apoC-III regulates LCAT activity indirectly through apolipoproteins that activate LCAT (Nishida et al., 1986). In the current study, we demonstrated that *in vitro* LCAT activity decreased in response to increased apoC-III content in rHDL, and that this occurred in a dose-dependent manner. This decreased ability of rHDL containing apoC-III to activate LCAT (Fig. 4A) is supported by the results of a previous study reported by the Jonas et al. (1984), which showed that apoC-III-rHDL exhibited 4-5.4% LCAT activation ability, while apoA-I-rHDL exhibited 100% reaction rates. Physiologically, LCAT is a key enzyme (E.C. 2.3.1.43) involved in reverse cholesterol transport (RCT). RCT is responsible for the esterification of FC into CE in HDL, which facilitates the maturation of nascent HDL into discoidal and spherical HDL. In this process, LCAT catalyzes the transfer of 2 acyl groups from lecithin to FC, yielding CE and lysolecithin. Although other apolipoproteins and synthetic peptides have been found to activate LCAT, apoA-I is the principal activating cofactor of LCAT (Jonas, 1991).

Preliminary results from other studies evaluating recombinant HDL particles suggest that apoC-III stimulates CETP activity (Sparks and Pritchard, 1989). Similarly, in this report, CETP activity did not decrease in response to an increase in apoC-III content of up to a 1:2 molar ratio (apoA-I:C-III). CETP promotes the redistribution and equilibration of hydrophobic lipids packaged within the lipoprotein core (CE and TG) between HDL and apo-B containing lipoproteins (LDL, IDL, VLDL, and chylomicrons), and CETP has been well-established as an atherogenic factor (Brousseau, 2005). Furthermore, studies with CETP-deficient patients have shown that they have remarkably high plasma levels of HDL-C and apoA-I (Brown et al., 1989).

In conclusion, an increase of apoC-III content in rHDL resulted in changes in several HDL properties, including decreased particle size, phospholipid content, and a decreased number of apoA-I molecules. Furthermore, the physiologic beneficial functions of HDL were almost completely deprived and the LCAT activation ability and antioxidant ability against LDL uptake by macrophages were hampered in a dose-dependent manner in response to increasing the apoC-III content of rHDL.

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