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Apolipoprotein C-II and C-III preferably transfer to both high-density lipoprotein (HDL)₂ and the larger HDL₃ from very low-density lipoprotein (VLDL)

<https://doi.org/10.1515/hsz-2020-0288>

Received August 23, 2020; accepted November 28, 2020;

published online December 18, 2020

Abstract: Triglyceride hydrolysis by lipoprotein lipase (LPL), regulated by apolipoproteins C-II (apoC-II) and C-III (apoC-III), is essential for maintaining normal lipid homeostasis. During triglyceride lipolysis, the apoCs are known to be transferred from very low-density lipoprotein (VLDL) to high-density lipoprotein (HDL), but the detailed mechanisms of this transfer remain unclear. In this study, we investigated the extent of the apoC transfers and their distribution in HDL subfractions, HDL₂ and HDL₃. Each HDL subfraction was incubated with VLDL or biotin-labeled VLDL, and apolipoproteins and lipids in the re-isolated HDL were quantified using western blotting and high-performance liquid chromatography (HPLC). In consequence, incubation with VLDL showed the increase of net amount of apoC-II and apoC-III in the HDL. HPLC analysis revealed that the biotin-labeled apolipoproteins, including apoCs and apolipoprotein E, were preferably transferred to the larger HDL₃. No effect of cholesteryl ester transfer protein inhibitor on the apoC transfers was observed. Quantification of apoCs levels in HDL₂ and HDL₃ from healthy subjects ($n = 8$) showed large individual differences between apoC-II and apoC-III levels. These results suggest that both apoC-II

and apoC-III transfer disproportionately from VLDL to HDL₂ and the larger HDL₃, and these transfers might be involved in individual triglyceride metabolism.

Keywords: apolipoprotein transfer; cardiovascular disease; cholesteryl ester transfer protein; lipoprotein lipase; triglyceride.

Introduction

High triglyceride (TG) levels in blood are a prevalent risk factor for cardiovascular disease (CVD) (Miller et al. 2008; Toth 2016). In circulation, exogenous and endogenous TGs are mainly carried by TG-rich lipoproteins (TRL), including chylomicron (CM) and very low-density lipoprotein (VLDL), respectively. TG in lipoproteins is hydrolyzed to glycerol and fatty acids by lipoprotein lipase (LPL), a key player in TG metabolism that is attached to the luminal surface of endothelial cells (Wolska et al. 2017). Activation of LPL is regulated by several apolipoproteins, including apolipoproteins A-V (apoA-V), C-I (apoC-I), C-II (apoC-II), and C-III (apoC-III). ApoA-V and apoC-II are stimulators of LPL activity (Kei et al. 2012; Lookene et al. 2005), and apoC-I and apoC-III are involved in inhibition of LPL activity (Larsson et al. 2013). Particularly, apoC-II and apoC-III are well-known LPL co-factors (Krauss et al. 1973; Larsson et al. 2013; Lookene et al. 2005; Ooi et al. 2008; Yamamoto et al. 2003). ApoC-II deficiency induced extremely high TG levels in plasma, leading to CVD (Baggio et al. 1986; Breckenridge et al. 1978; Fellin et al. 1983; Kawano et al. 2002), and high apoC-III levels is significantly associated with the risk of a CVD incident (Scheffer et al. 2008; van Capelleveen et al. 2017). Further, mutation of apoC-III led to a reduction in CVD risk (Crosby et al. 2014; Jorgensen et al. 2014), and recent studies demonstrated that apoC-III also plays a role in plasma TG increment through an LPL-independent pathway (Gaudet et al. 2014; Gordts et al. 2016). Thus, the balance between apoC-II and apoC-III in TRL is important for regulating TG levels in

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blood, as its imbalance could increase the risk of cardiovascular events. On the other hand, high-density lipoprotein (HDL) is also associated with TG metabolism in which HDL receives TG from VLDL instead of providing cholesteryl ester to VLDL by cholesteryl ester transfer protein (CETP). Moreover, apoCs are not only present in TRL, but also in HDL. It was reported that apoC-I in HDL has an inhibitory effect on CETP activity (Gautier et al. 2000). Recent epidemiological studies have shown that HDL subspecies with apoC-III had a contrasting relationship with those without apoC-III in the progression of atherosclerosis (Aroner et al. 2018; Jensen et al. 2018; Yamamoto et al. 2018) and other studies have demonstrated that apoC-III in HDL plays a role in HDL regulation (Cohn et al. 2003; Morton et al. 2018). Additionally, apoCs in HDL have been shown to be transferred from TRL during TG lipolysis (Glangeaud et al. 1976; Tall et al. 1978). Thus, the importance of apoCs in HDL has also been in the spotlight. Regarding the production of apoC-containing HDLs, a few studies have previously focused on the transfer of apoCs between TRL and HDL. Bukberg et al. (Bukberg et al. 1985) and Tornoci et al. (Tornoci et al. 1993) indicated that apoC-II and apoC-III were rapidly interchangeable between VLDL and HDL and there were non-equilibrating apoC pools in each lipoprotein. Another study showed, however, that the kinetics of apoC-III between VLDL and HDL were similar, indicating an equilibrium state (Boyle et al. 1999; Nguyen et al. 2006). Hence, although the phenomenon is well established, the factors and detailed mechanisms regarding apoC transfers between VLDL and HDL remain unclear (Kei et al. 2012; Ooi et al. 2008).

In this study, we investigated apoC-II and apoC-III transfer between VLDL and two main subfractions of HDL, HDL₂ and HDL₃, and their distribution in HDL subfractions. We further examined the relationship between the transfer of apoCs and CETP exchanging TG in VLDL, and hypothesized that the transfers might be proceeding simultaneously.

Results

Variations in apoC levels in HDL after incubation with VLDL

In order to investigate an effect of VLDL on HDL apoCs levels, HDL was incubated with various amounts of VLDL at protein ratio of 1:0, 1:0.2, or 1:1. After re-isolating the incubated HDL, the isolated HDL fraction was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB) using anti-apoC-II, anti-apoC-III and anti-apolipoprotein A-I (apoA-I) antibodies (Figure 1a). The band intensity of apoC-II in HDL was increased depending on the amount of reacting VLDL ($p < 0.05$, Figure 1c). Similarly, the intensity of apoC-III tended to be increased (Figure 1d), while changes of apoA-I levels in HDL was not observed (Figure 1b). To further investigate whether those increases of apoCs occur in both HDL subfractions, HDL₂ and HDL₃, each subfraction was incubated with or without VLDL at protein ratio of 1:0.2. After re-isolating HDL₂ or HDL₃ fraction by ultracentrifugation, the compositional profile of each HDL was

Figure 1: Change of apoC-II, apoC-III, and apoA-I levels in HDL after incubation with VLDL.

HDL (1.0 mg protein/mL) was incubated with VLDL (0.2 or 1.0 mg protein/mL) or without VLDL at 37 °C for 20 h and then HDL fraction was re-isolated by ultracentrifugation. The HDL was analyzed by SDS-PAGE and WB using anti-apoA-I, apoC-II and apoC-III antibodies (a) followed by quantification analysis of apoA-I (b), apoC-II (c), and apoC-III (d) bands. One set of the images are a representative of 3 separate experiments. Band intensities were semiquantified by densitometry. The values show relative intensity of each band in treated HDL to those in HDL incubated without VLDL defined as 1. * $p < 0.05$, one-way ANOVA with post-hoc Tukey HSD; ns, not significant.

analyzed via SDS-PAGE, followed by WB in the same manner (Figure 2a, b). The band intensity of the apoC-II in HDL₂ tended to be increased by incubation with VLDL (Figure 2c), while those in HDL₃ incubated with VLDL showed significantly higher intensity than without VLDL ($p < 0.05$, Figure 2d). Similarly with apoC-II, apoC-III was tended to be increased in HDL₂ by incubation with VLDL (Figure 2e), and significantly increased in HDL₃ ($p < 0.05$, Figure 2f). Similar results were shown (but not significant) when HDLs and VLDL were not incubated at 37 °C. No apparent difference was observed in the intensities of apoA-I bands under all test conditions. Based on these results, it was indicated that incubation of HDL₂ or HDL₃ with VLDL increased the amount of both apoC-II and apoC-III in each HDL depending on VLDL level regardless of the incubation temperature.

Determination of the apolipoproteins in HDL transferred from VLDL

To trace the apolipoproteins that originated from VLDL, HDL₂ or HDL₃ was incubated with biotinylated VLDL and re-isolated, then each HDL was analyzed via WB using the Streptavidin system. Five to six biotinylated apolipoproteins were observed in HDL₂ and HDL₃ (Figure 3a, b, 2nd lane). For further identification of the biotinylated apolipoproteins which transferred from VLDL to HDLs, immunoprecipitation assays were performed. The HDL₂ or HDL₃ fraction, including biotinylated apolipoproteins, was broken down with 2% Tween 20 and incubated with agarose conjugated with anti-apoC-II, -apoC-III, or -apolipoprotein E (apoE) antibodies. Each immunoprecipitated sample was analyzed via WB (Figure 3a, b). When HDL₂ or HDL₃ was immunoprecipitated with anti-apoE antibody, the biotin-labeled apolipoprotein was detected as a sole band at the same size of apoE. Similarly, immunoprecipitated samples with anti-apoC-II and apoC-III antibodies showed bands corresponding to the molecular mass of apoC-II and apoC-III, respectively, whereas biotin-labeled apolipoprotein was also detected at the apoE position in both samples. These results suggested that apoC-II, apoC-III, and apoE were transferred from VLDL to HDL under this experimental condition.

Distribution of transferred apolipoproteins

To investigate whether the transfers of these apoCs and apoE affect HDL₂ or HDL₃ particle sizes, size-exclusion chromatography was performed. Each HDL₂ or HDL₃

Figure 2: Detection of apoC-II, apoC-III, and apoA-I in HDLs after incubation with VLDL.

HDL₂ and HDL₃ (1.0 mg protein/mL) were incubated with or without VLDL (0.2 mg protein/mL) at 37 °C for 0 or 20 h and then re-isolated by ultracentrifugation. Each HDL sample was separated by SDS-PAGE, apoC-II (a) and apoC-III (b) were detected by WB using anti-apoC-II and -apoC-III antibodies. The bands of apoA-I were stained on the same membranes by anti-apoA-I antibody to make a relative comparison possible. One set of the images is a representative of 3 separate experiments. Band intensities were semiquantified by densitometry. The intensities normalized by that of apoA-I (HDL loading control) for apoC-II in HDL₂ (c) and HDL₃ (d), and apoC-III in HDL₂ (e) and HDL₃ (f) are shown as the ratio to that without VLDL defined as 1. * $p < 0.05$, by unpaired Student's *t*-test; ns, not significant.

fraction incubated with or without biotinylated VLDL was injected to high-performance liquid chromatography (HPLC) and monitored by the absorbance at 280 nm which reflects the amount of the subdivided lipoprotein. The retention time of the peak in HDL₂ did not change after incubation with VLDL, while that of HDL₃ was slightly shortened (Figure 4a). Then, three drops of the HDL₂ and HDL₃ aliquots were collected. At first, the peak shape of HPLC chromatogram was consistent with the protein concentration in each collected fraction measured by Lowry method (data not shown). Next, the distribution of transferred apolipoproteins from VLDL to HDL₂ and HDL₃ was

Figure 3: Detection of biotinylated proteins in HDLs and immunoprecipitation assay.

HDL₂ and HDL₃ (1.0 mg protein/mL) were incubated with biotinylated VLDL (0.2 mg protein/mL) at 37 °C for 20 h and then each HDL subfraction was re-isolated from VLDL by ultracentrifugation. The HDL subfraction (37.5 µg protein) was broken down, and immunoprecipitation (IP) assay was performed. Whole precipitated sample from HDL₂ (a) or HDL₃ (b) was analyzed by SDS-PAGE followed by WB using Streptavidin. The biotinylated VLDL (1st lane) and the original HDLs incubated with the biotinylated VLDL and re-isolated (2nd lane) were also analyzed. As negative controls (NC), immunoprecipitation assay was performed using agarose without antibody. One set of the images is a representative of 3 separate experiments.

examined via WB. A comparison between the chromatogram and WB patterns of the fractions showed that the distribution of biotinylated apolipoproteins in HDL₂ roughly overlapped with the elution profile monitored by the absorbance at 280 nm, whereas the biotinylated proteins in HDL₃ were mainly observed in the fractions eluted at earlier retention times, indicating the relatively large particles (Figure 4b, c). Moreover, the apoE and apoCs bands were distributed differently in HDL₃. The stronger signal of biotinylated apoCs and apoE were observed at retention time 18.6–19.0 min and 18.6–18.8 min, respectively (Figure 4c). As a result of HPLC and WB profiles, both apoC-II and apoC-III in VLDL transfer preferably to the larger HDL₃ followed by a slight change of the HDL₃ particle size.

Changes in HDL lipid levels after incubation with VLDL

To confirm whether regular lipids exchange occurs under this experimental condition (mixing HDL and VLDL at protein ratio of 5:1), changes in TG and total cholesterol (TC) levels in the isolated HDL fraction after incubation with or without VLDL were also determined. Following VLDL incubation, the TG levels in HDL₃ increased significantly ($p = 0.007$) by $28.7 \pm 10.5\%$ while TG levels in HDL₂ increased by $15.3 \pm 11.0\%$ (Figure 5a), implying a TG transfer from VLDL to HDL. However, no significant change was observed in the TC levels of both HDLs (Figure 5b).

Effect of CETP inhibition on the transfer of apoCs

CETP in HDLs was inhibited by Torcetrapib so as to investigate whether CETP is involved in the apoCs transfer. TG levels in HDL separated via polyethylene glycol (PEG) precipitation increased significantly following the incubation of serum (incubation (–) vs (+), $p < 0.05$, Figure 6a); however, Torcetrapib counterbalanced this increase (torcetrapib (–) vs (+) with incubation, $p < 0.05$, Figure 6a) indicating the effective inhibition of CETP. In contrast, no significant change was observed in TC levels after incubation with or without Torcetrapib treatment (Figure 6b). Despite the inhibition in TG transfer, Torcetrapib did not affect the apparent amounts of biotinylated apolipoproteins in both HDL₂ and HDL₃ transferred from biotinylated VLDL (Figure 6c). These results indicated that CETP is not involved in the apoCs transfers.

Variation in apoC-II and apoC-III levels in HDL₂ and HDL₃ obtained from healthy subjects

Since the apolipoproteins from VLDL were preferably transferred to larger HDL, we investigated potential individual differences in apoC distribution in HDL₂ and HDL₃ among healthy subjects. The basic parameters and measured values of apolipoproteins and lipids in isolated HDL₂ and HDL₃ by ultracentrifugation are shown in the

Figure 4: HPLC analysis.

HDL₂ and HDL₃ (2.0 mg protein/mL) were incubated with or without biotinylated VLDL (0.4 mg protein/mL) at 37 °C for 20 h. Fifty microliter of the mixture was injected to HPLC and the elution profiles of HDL were monitored at 280 nm (a). HDL fractions separated by HPLC were collected at regular intervals (3 drops; 9.6 s) and the same volumes of the collected fractions from HDL₂ (b) and HDL₃ (c) were applied to SDS-PAGE followed by WB using Streptavidin system. One set of the images is a representative of 2 separate experiments.

supplementary Table 1. The average relative amounts of both apoC-II and apoC-III to total proteins in HDL₂ were about four folds higher than those in HDL₃, consistent with the preference of apoC-II and apoC-III for large HDLs as shown in Figures 4b, c, while the relative amounts of apoA-I in HDL₂ and HDL₃ are similar (0.754 and 0.691) (Table 1). As a result, individual differences in the amount of apoC-II and apoC-III in HDL₂ and HDL₃ were observed. The relative amounts of apoC-II and apoC-III to total

protein in HDL₂ ranged from 0.010 to 0.026 and from 0.035 to 0.057, respectively (Table 1). In HDL₃, they ranged from 0.002 to 0.005 and from 0.006 to 0.015, respectively. Regarding the lipid profile, TC, phospholipids (PL), and TG were about two folds higher in HDL₂ than in HDL₃. These lipids and apolipoproteins profile showed that there was individual difference of apoC-II and apoC-III levels in HDL₂ and HDL₃ among healthy subjects.

Figure 5: Determination of lipids in HDLs.

TG (a) and TC (b) in HDL₂ and HDL₃ (1.0 mg protein/mL) re-isolated by ultracentrifugation after incubation with or without VLDL (0.2 mg protein/mL) were determined. The data were corrected per protein ($n = 3$). * $p < 0.05$, by unpaired Student's t -test; ns, not significant.

Figure 6: Effect of CETP inhibition.

To confirm the effectiveness of Torcetrapib, serum was incubated with or without Torcetrapib (final 5 μ M) at 37 °C for 0 or 20 h. TG (a) and TC (b) were measured in HDL containing fraction obtained by PEG precipitation method. The value of serum without any treatment was defined as 1 ($n = 3$). * $p < 0.05$, by unpaired Student's t -test; ns, not significant. HDL₂ and HDL₃ (final 3.0 mg protein/mL) were incubated with or without torcetrapib (final 5 μ M) at 37 °C for 1 h, followed by a further incubation with biotinylated VLDL at 37 °C for 20 h. HDLs re-isolated by ultracentrifugation ($1.063 < d < 1.210$ g/mL) were determined by WB using Streptavidin system (c).

Discussion

LPL is attached to the endothelial cell surface via glycosylphosphatidylinositol-anchored HDL-binding protein 1 (Mysling et al. 2016). Apolipoproteins on the TRL need to form lipolytic complexes with LPL for lipolysis (Wolska et al. 2017). Therefore, each apoC level in TRL, rather than total apoC levels in plasma, might be essential for regulating TG metabolism. Moreover, another role of apoCs in HDL has been unveiled. For instance, the cholesterol efflux capacity of HDL enriched in apoC-III was impaired (Luo et al. 2017). A higher apoC-III level was observed in HDL obtained from patients with coronary artery disease, and these apoC-III-containing HDL also activated endothelial apoptosis than those from healthy subjects (Riwanto et al. 2013). Hence, investigating apoC-II and apoC-III transfers between VLDL and HDL to determine their distribution in each lipoprotein, might yield crucial information regarding CVD risk.

In this study, when HDL and VLDL were mixed at physiological condition (protein rate, 5:1) (Edelstein 1986; Havel and Kane 1995), the net amount of apoC-II and apoC-III in HDL increased and the higher amount of VLDL showed the higher increase of apoCs. This increase happened in both HDL₂ and HDL₃. Although apoC-II and apoC-III might be transferred bidirectionally between VLDL and HDL, the increase in apoCs in HDL indicates that apoCs are dominantly transferred from VLDL to HDL in these mixing ratio (HDL:VLDL, 5:1 or 1:1, protein ratio). Moreover, these transfers do not require 37 °C incubation and are caused by only mix of HDL and VLDL. A previous study showed that apoCs were released from VLDL in the presence of HDL depending on HDL concentration (Chung et al. 2000). In another study, these transfers of apoCs to HDL were also observed in mixtures of HDLs and VLDL without incubation at 37 °C, indicating that apoCs are rapidly transferred from VLDL to HDLs (Boyle et al. 1999; Bukberg et al. 1985). An experiment involving the incubation of VLDL with HDL at different rates would be required to further understand this process.

To trace these apoC transfers, surface apolipoproteins in VLDL were labeled with biotin before incubation with HDL because we need to confirm that increased apoC-II and apoC-III were not an augmentation of reactivity with antibodies, such as that induced by apolipoprotein oxidation, which is occasionally recognized by WB. Several apolipoproteins with similar profiles derived from VLDL were observed in both HDL₂ and HDL₃, indicating that besides apoC-II, apoC-III, and apoE, which were also confirmed by immunoprecipitation assay, other apolipoproteins were

Table 1: Profile of apolipoproteins and lipids in HDL₂ and HDL₃ from healthy subjects.

Subjects	apoA-I/TP		apoC-II/TP		apoC-III/TP	
	HDL ₂	HDL ₃	HDL ₂	HDL ₃	HDL ₂	HDL ₃
1	0.709	0.667	0.024	0.004	0.051	0.011
2	0.770	0.675	0.013	0.002	0.040	0.009
3	0.704	0.723	0.026	0.005	0.057	0.015
4	0.760	0.729	0.015	0.004	0.053	0.009
5	0.814	0.692	0.010	0.003	0.036	0.011
6	0.808	0.677	0.013	0.003	0.035	0.012
7	0.775	0.665	0.013	0.003	0.038	0.011
8	0.693	0.702	0.014	0.005	0.036	0.006
Mean ± SD	0.754 ± 0.047*	0.691 ± 0.025	0.016 ± 0.006**	0.004 ± 0.001	0.043 ± 0.009**	0.011 ± 0.003

Subjects	TC/TP		PL/TP		TG/TP	
	HDL ₂	HDL ₃	HDL ₂	HDL ₃	HDL ₂	HDL ₃
1	0.51	0.25	0.91	0.52	0.08	0.03
2	0.56	0.26	0.94	0.55	0.06	0.03
3	0.49	0.24	0.96	0.61	0.08	0.04
4	0.58	0.28	0.95	0.55	0.10	0.04
5	0.48	0.24	0.91	0.57	0.11	0.05
6	0.54	0.28	0.89	0.62	0.11	0.05
7	0.45	0.27	0.88	0.53	0.06	0.03
8	0.47	0.31	0.91	0.53	0.04	0.02
Mean ± SD	0.51 ± 0.05**	0.26 ± 0.02	0.92 ± 0.03**	0.56 ± 0.04	0.08 ± 0.02**	0.04 ± 0.01

Plasma samples were collected from eight healthy subjects. HDL₂ and HDL₃ were isolated from the plasmas by ultracentrifugation. ApoA-I, apoC-II, apoC-III, TC, PL, TG and total protein (TP) concentrations in each HDL₂ and HDL₃ were measured and normalized per protein content.

* $p < 0.05$, ** $p < 0.001$ vs HDL₃ by paired Student's t-test.

transferred from VLDL to the HDLs. In addition to apoC-III, a protein with relatively high molecular mass was observed in the precipitates obtained from the immunoprecipitation assay using agarose conjugated with anti-apoC-III antibody. This protein is considered to be apoE contaminated via a nonspecific reaction, since a large amount of apoE was transferred to the HDLs. The band of apoE was slightly observed in the immunoprecipitation with anti-apoC-II antibody, this cause is the same as apoC-III. Two other unknown apolipoproteins with molecular masses of ~15.2 and ~19.5 kDa were also observed via WB. Although both bands showed equal or higher intensity than those of apoCs, quantitative comparison between these unknown apolipoproteins and apoCs cannot be applied, because the band intensity does not necessarily represent the amount of protein, but reflects the number of amino groups of each protein on the surface of VLDL to which Sulfo-NHS-LC-LC-biotin binds. The main apolipoproteins of VLDL are apolipoprotein B (apoB)-100, apoE, and apoCs, but VLDL also includes various minor apolipoproteins (Sun et al. 2010). The unknown protein bands on the WB pattern may be consistent with these minor apolipoproteins.

Some studies reported that apoCs transfers from VLDL to HDL (Boyle et al. 1999; Bukberg et al. 1985; Glangeaud

et al. 1976; Tornoci et al. 1993), however, detail distribution in HDL has not been fully understood. One of the important findings of our study is that those apolipoproteins were preferably transferred to the larger HDL₃ particles as indicated by WB analysis of the HPLC fractions. The HPLC profile by monitoring at absorbance at 280 nm represents the amount of lipoprotein. Since 70% of proteins contained in HDL is apoA-I, the higher absorption peak at retention time 19.0–19.2 min is due to apoA-I, while stronger signals of biotinylated apoCs and apoE at faster retention time than that of apoA-I. We also confirmed increased TG levels in both HDL₂ and HDL₃ under the same experimental condition for apoC transfer. In circulation, the particle size of HDL₃ becomes larger due to the esterification of surface-free cholesterol by lecithin-cholesterol acyltransferase and exchange of TG and esterified cholesterol between VLDL and HDL by CETP, and the larger HDL₃ eventually converts to HDL₂. Therefore, it is plausible that particles of the larger HDL₃ can obtain those apolipoproteins just before changing into HDL₂.

Taken together, as a potential candidate protein associated with both lipid and apoC transfers, CETP might dominate these transfers in this study. First, we confirmed CETP inhibition by Torcetrapib according to the

suppression of TG increase in both HDL₂ and HDL₃ incubated with VLDL. However, decreased TC levels in HDLs were not clearly observed regardless of the addition of Torcetrapib. Although the transfer of fluorescence or the isotope-labeled cholesteryl ester was easily detected as previously described (Clark et al. 2006; Ranalletta et al. 2010), the TC measured by the enzymatic method used in this study might be insufficient for detecting the transferred cholesterol level because of the relatively large amount of endogenous cholesterol compared to TGs. Unexpectedly, in spite of CETP inhibition, the amount of biotinylated proteins transferred from VLDL to HDLs did not change. These results suggest that CETP is not necessarily involved in apolipoprotein transfers. However, since the variation in cholesterol was similar, and it is difficult to quantitatively identify small differences via WB analysis, CETP involvement might be undeniable.

Interestingly, our study shows that apoC-II and apoC-III levels in HDL₂ and HDL₃ were quite different, even among healthy subjects (age range (year), 21 – 37, gender (male/female), 4/4). It is speculated that these differences are due to differences in transferred amounts of apoC-II and apoC-III and might be connected to the differences in TG metabolism in the individuals. In fact, in previous studies using triglyceride emulsion, the dynamics of these apolipoproteins were different between healthy and hypertriglyceridemic subjects (Tornoci et al. 1993). They demonstrated that when plasma obtained from healthy subjects were incubated with the emulsion, apoCs associated with HDL decreased while apoCs in VLDL decreased in the case of hypertriglyceridemic subjects. Furthermore, because of the inhibitory effect of apoE on LPL activity, apoCs and apoE concentrations and apoCs/apoE ratios are considered to be the key factors in the metabolism of TG-rich lipoproteins (Huang et al. 1998). Therefore, analysis of the apolipoprotein transfer might contribute to understanding of the mechanism of atherosclerosis progression.

However, there were some limitations to this study. The quantification of transferred apoCs were performed via WB. Large change of TG and TC levels wasn't observed after CETP inhibition. To firmly support our hypothesis, assays with higher sensitivity would be required. Although we mainly focused on apoC-II and apoC-III variations in HDL after reacted with VLDL, the other apolipoproteins including apoE are also present in VLDL and HDL, and the other lipoproteins such as CM and LDL circulate in blood. Therefore, further studies focusing on the other apolipoproteins and lipoproteins would be required to clarify the detailed mechanism of apolipoprotein transfer as the next step. Moreover, it is known that apoC-II is also in macrophages and tissues such as the liver tissue (Hoffer et al. 1993).

Investigation of the interactions between these apoC-II expressing cells and lipoproteins should be conducted. Even so, our study is potentially useful for investigating apoC-II and apoC-III transfer rates between VLDL and HDL among individuals towards understanding individual differences in TG metabolism. In addition, evaluating the changes of VLDL-related LPL activity and the effects on HDL remodeling after transfers of apoC-II and apoC-III might reveal the significance of apolipoproteins transfer in lipids metabolism.

In conclusion, apoC-II and apoC-III transferred from VLDL to both HDL₂ and HDL₃. Especially as for HDL₃, these apolipoproteins were preferably transferred to the larger particles. In addition, apoC-II and apoC-III showed large differences in distribution between HDL subfractions and individuals. These results suggest that difference of the transfer of both apoC-II and apoC-III causes individual differences in the distribution of apoC-II and apoC-III in HDL, it might be involved in individual TG metabolism. Thus, analysis of apolipoprotein transfer might lead to an understanding of the mechanism of TG metabolism and atherosclerosis progression.

Materials and methods

Blood samples

Fasting blood samples were collected from healthy volunteers who gave their written informed consent to participate in this study, which was approved by our institutional research ethics committee (No.M2015-546).

Preparation of lipoproteins

Pooled plasma samples were centrifuged at 80,000 rpm for 30 min and the top surface layer, including CM, was removed by gentle pipetting. VLDL ($d < 1.006$ g/mL), HDL ($1.063 < d < 1.210$ g/mL), HDL₂ ($1.063 < d < 1.125$ g/mL), and HDL₃ ($1.125 < d < 1.210$ g/mL) were then isolated from CM-free pooled plasma by ultracentrifugation (80,000 rpm at 4 °C for 22 h) as previously described (Havel et al. 1955). Similarly, HDL₂ and HDL₃ were collected from individual plasma sample to investigate individual differences in distribution of apolipoproteins and lipids in each HDL. Isolated lipoproteins were dialyzed against phosphate-buffered saline (PBS) and stored at 4 °C until use within 24 h. The protein concentration in each lipoproteins was measured by the Lowry method (Lowry et al. 1951).

Biotinylation of VLDL

Amino groups of apolipoproteins on the surface of VLDL particles were labeled using EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Fisher scientific). VLDL (0.3–1.1 mg protein/mL) was mixed with the biotin-

labeling reagent in the ratio of 1:0.2 (mg protein: μmol) and incubated at 22 °C for 30 min. After incubation, the biotinylated VLDL was dialyzed against PBS to remove excess non-reacted biotin reagent.

Reaction between VLDL and HDL subfractions

HDL, HDL₂ or HDL₃ (1.0 mg protein/mL) mixed with or without VLDL (0.2 or 1.0 mg protein/mL) or biotinylated VLDL (0.2 mg protein/mL) was incubated at 37 °C for 20 h. Each HDL was re-isolated from the mixture as follows; VLDL was removed following the first ultracentrifugation ($d = 1.063 \text{ g/mL}$) and each HDL was isolated by the second ultracentrifugation ($d = 1.210 \text{ g/mL}$) as the top fraction. Both ultracentrifugation steps were performed at 100,000 rpm for 20 h. To prepare HDL₂ or HDL₃ fractions treated with or without VLDL in the absence of incubation, once HDL₂ or HDL₃ fraction was mixed with VLDL or PBS as control, each HDL subfraction was immediately re-isolated from the mixture by sequential ultracentrifugation as above (no incubation sample).

Electrophoresis and WB

SDS-PAGE was performed using 18% polyacrylamide gel under reducing condition. The separated proteins were transferred to PVDF membranes (Merck Millipore). After blocking the membrane with 5% skim milk, apoC-II, apoC-III, or apoA-I was primarily detected with goat anti-apoC-II, -apoC-III, or -apoA-I polyclonal antibody (Academy Bio-Medical Company), respectively, and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (Medical & Biological Laboratories) was the secondary antibody. Finally, the band containing apoC-II or apoC-III was visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare), and the apoA-I band was visualized using 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. In the case of biotinylated proteins, the detection was performed similarly except that 5% BSA was used as a blocking solution and detection was carried out using Streptavidin Protein, HRP (Thermo Fisher scientific), and ECL Prime Western Blotting Detection Reagent.

Immunoprecipitation assay

HDL₂ or HDL₃ which were re-isolated by ultracentrifugation after incubation with biotinylated VLDL were broken down with 2% Tween 20. The solutions containing HDL proteins were incubated with Protein G-Agarose (Sigma-Aldrich) conjugated with each of several types of antibodies: goat anti-apoC-II, -apoC-III or -apoE polyclonal antibody (Academy Bio-Medical Company). Unconjugated Protein G-Agarose was used as the negative control. After washing the agarose, a specifically combined protein against each antibody was resolved in the SDS sample buffer containing 2-mercaptoethanol and analyzed by SDS-PAGE followed by WB using Streptavidin.

HPLC

The mixture of HDL₂ or HDL₃ (2.0 mg protein/mL) with biotinylated VLDL (0.4 mg protein/mL) was analyzed using a HPLC system (Shimadzu Corporation) consisting of a pump LC-20AD, autosampler SIL-20AC, degassing unit DGU-20A, and ultraviolet detector SPD-20A.

Fifty microliters of the sample was injected into the serially connected size exclusion column (PROTEIN KW-803 and -804 (300 $\times$ 8.0 mm i.d., Shodex) and eluted with PBS at a flow rate of 1 mL/min. The HDL fraction incubated with biotinylated VLDL was separated and monitored by absorbance at 280 nm and the fractions were collected at regular intervals (3 drops; approx. 9.6 s). The biotinylated proteins of each fraction were detected via WB using the Streptavidin system.

CETP inhibition

HDL₂ or HDL₃ (3.0 mg protein/mL) was incubated with or without the CETP inhibitor, Torcetrapib (5 μM ; Cayman), at 37 °C for 1 h. These HDLs were incubated with biotinylated VLDL at 37 °C for 20 h and then re-isolated by ultracentrifugation ($1.063 < d < 1.210 \text{ g/mL}$). To confirm the effectiveness of Torcetrapib, serum incubated with Torcetrapib (20 h) was treated with PEG as previously described (Horiuchi et al. 2018) and centrifuged to obtain apoB-depleted serum (the supernatant, including HDL). TC and TG levels in the apoB-depleted serum were measured.

Determination of lipid and apolipoprotein levels

TC, TG, and PL were quantified using commercial enzymatic assay kits: Determinar L TC II, Determinar L TG II, and Determinar L PL (Kyowa Medex), respectively. ApoA-I, apoC-II, and apoC-III levels in HDL fractions were measured via immunoturbidimetric methods (SEKISUI MEDICAL, Tokyo, Japan).

Statistics

The results were expressed as the mean $\pm$ SD. All data were statistically analyzed using SPSS ver. 25.0 (Chicago, IL). The one-way ANOVA followed by Tukey HSD post-hoc test was used to compare the band intensities of apolipoproteins levels in HDL incubated with between various amounts of VLDL. Differences in lipids and apolipoproteins levels in between treated and untreated HDL, and individual HDL were evaluated using unpaired Student's *t*-test and paired Student's *t*-test, respectively. A $p < 0.05$ was considered statistically significant.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was supported in part by a Grant-in-Aid for Young Scientists (B) and Scientific Research (C) from the Japan Society for the Promotion of Science (grant number 17K15771 and 19K07932 to RO) and a grant from the Hokuto Foundation for Bioscience to R.O.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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Supplementary Material: The online version of this article offers supplementary material (<https://doi.org/10.1515/hsz-2020-0288>).