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Context: Proprotein convertase subtilisin/kexin 9 (PCSK9) is known to be a good target to decrease LDL cholesterol (LDL-C) and two forms of PCSK9, mature and furin-cleaved PCSK9, circulate in blood. However, it has not been clarified whether and how the levels of each PCSK9 are affected by LDL-apheresis (LDL-A) treatment, a standard therapy in patients with severe forms of familial hypercholesterolemia (FH).

Objective: Our objective was to investigate the differences in LDL-A-induced reduction of mature and furin-cleaved PCSK9 between homozygous and heterozygous FH, and between dextran sulfate (DS) cellulose adsorption and double membrane (DM) columns and to clarify the mechanism of their removal.

Design: A sandwich ELISA to measure two forms of PCSK9s using monoclonal antibodies was developed. Using the ELISA, PCSK9 levels were quantified before and after LDL-A with DS columns in 7 homozygous and 11 heterozygous FH patients. A crossover study between the two column types was performed. The profiles of PCSK9s were analyzed after fractionation by gel filtration chromatography. Immunoprecipitation of apolipoprotein B (apoB) in FH plasma was performed.

Results: Both mature and furin-cleaved PCSK9s were significantly decreased by 55–56% in FH homozygotes after a single LDL-A treatment with DS columns, and by 46–48% or 48–56% in FH heterozygotes after treatment with DS or DM columns. The reduction ratios of LDL-C were strongly correlated with that of PCSK9 in both FH homozygotes and heterozygotes. In addition, more than 80% of plasma PCSK9s were in the apoB-deficient fraction and a significant portion of mature PCSK9 was bound to apoB, as shown by immunoprecipitation.

Conclusions: Both mature and furin-cleaved PCSK9s were removed by LDL-A in homozygous and heterozygous FH either by binding to apoB or by other mechanisms. The ELISA method to measure both forms of plasma PCSK9 would be useful for investigating physiological or pathological roles of PCSK9.

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Familial hypercholesterolemia (FH) is an inherited disorder caused by mutations in the low-density lipoprotein (LDL) receptor (LDLR), apolipoprotein B (apoB) or proprotein convertase subtilisin/kexin type 9 (PCSK9) (1, 2), and is characterized by high LDL cholesterol (LDL-C) levels leading to premature coronary artery disease (CAD). PCSK9, a serine protease, regulates plasma LDL-C levels by regulating degradation of LDLR (3, 4). It has also been reported that serum PCSK9 levels were significantly higher in FH patients than in controls (5), and were correlated with serum LDL-C levels (6).

PCSK9 encodes a 692-amino-acid protein composed of a signal peptide, a prodomain, catalytic, and C-terminal domains. It undergoes autocatalytic intramolecular processing to form a ~14-kDa prodomain and a ~60-kDa moiety with catalytic and C-terminal domains. Mature PCSK9 is composed of the prodomain, which is noncovalently attached to the catalytic domain. Another proprotein convertase, furin, cleaves PCSK9 at the Arg218-Gln219 peptide bond, and the cleaved PCSK9 includes a ~7-kDa domain, ~14-kDa prodomain, and ~53-kDa domain (furin-cleaved form) that lacks the Ser153-Arg218 segment (7, 8). It has been reported that furin-cleaved PCSK9 has no activity (7–9) to regulate LDLR and serum LDL-C or less activity than mature PCSK9 (10). Thus, it is important to measure both forms of PCSK9 separately, in order to clarify the significance of furin-cleaved PCSK9. However, no specific method has been reported for quantifying furin-cleaved PCSK9, and thus the association of the ratio of each form of PCSK9 with various pathological or physiological conditions, such as primary hyperlipidemia, hyperlipidemia in type II diabetes, obesity, etc., has not been clarified.

LDL-apheresis (LDL-A) treatment is a standard therapy in homozygous and severe forms of heterozygous FH. In order to selectively remove LDL, LDL adsorption techniques using dextran sulfate (DS) cellulose adsorption columns and double membrane (DM) filtration methods were developed (11, 12). We and other colleagues have reported that LDL-A reduces not only atherogenic lipoproteins, but also various proteins including coagulation factors and C-reactive protein (CRP), in serum (13–15).

Recently, it has been reported that PCSK9 was eliminated by LDL-A treatment in 6 FH patients (16). However, in FH homozygotes, the serum PCSK9 levels have been reported to be unaffected by LDL-A (17). Thus, the difference in the treatment-induced reduction of PCSK9 between FH homozygotes and heterozygotes has not been clarified. In addition, the differences in the PCSK9 reduction between DS and DM columns and between the mature and furin-cleaved forms have not been clarified. In the present study, we use a novel sandwich ELISA to measure the mature and furin-cleaved forms of PCSK9 and show that both forms were removed by LDL-A treatment in FH homozygotes and heterozygotes. Furthermore, the mechanism of their removal is also discussed.

Materials and Methods

Detailed materials and methods are shown in the Supplemental Materials and Methods.

Patient characteristics

The subjects were 18 FH patients, including 7 homozygotes and 11 heterozygotes, who were receiving either regular or an initial LDL-A treatment at either the National Cerebral and Cardiovascular Center Hospital or Kenporen Osaka Central Hospital from March 2009 to October 2013. They were diagnosed with homozygous or heterozygous FH using previously described criteria (18, 19). Among the patients who had undergone genetic testing (n = 12), the majority were found to have LDLR gene mutations (n = 6; 50%), and one had mutations of both the LDLR and PCSK9 genes. One patient had homozygous forms of LDL receptor adaptor protein 1 (LDLRAP1) gene mutation, and 5 patients had no mutation on either the LDLR, PCSK9, or LDLRAP1 genes (42%) (20). The backgrounds of the patients are summarized in Supplemental Table 1. The protocol of this study was approved by the Ethics Review Committee of the National Cerebral and Cardiovascular Center (M20–26). Each patient gave written informed consent to participate in the study. All clinical investigations were conducted in accordance with the principles of the Declaration of Helsinki.

LDL-apheresis

For LDL-A treatment, an instrument (MA-03®, Kaneka) with a plasma filter (Sulflex; Kaneka) and two DS columns (Liposorber LA-15®; Kaneka) to adsorb apoB-containing lipoproteins were used. A crossover study between DS and DM columns was performed in 5 FH heterozygotes (patients No. 8, 10, 11, 12, 14 in Supplemental Table 1). LDL-A by DM columns was performed using an instrument (KPS-8800Ce; Asahi Kasei Medical Co., Ltd.) with a plasma separator (Plasmaflow OP-05W; Asahi Kasei Medical Co., Ltd.) and a plasma fractionator (Cascadeflow EC-50W; Asahi Kasei Medical Co., Ltd.).

Plasma sample collections and assays

Peripheral blood was collected from the blood removal line immediately before and after a single LDL-A procedure. Plasma levels of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein (HDL-C) were measured using enzymatic methods (Sekisui Medical Co.) and an automated analyzer (Hitachi Labiospect 008, Hitachi-Hitec). Plasma Lipoprotein(a) (LP(a)) levels were measured using a latex agglutination method (Sekisui Medical Co.). LDL-C levels were calculated by the Friedewald formula. Apolipoproteins levels were determined by turbidimetric immunoassay (LSI Medience Coorporation).

Construction, expression, and purification of recombinant PCSK9 proteins

Human PCSK9 cDNA was obtained by RT-PCR from mRNA of HepG2 cells and a C-terminal His6 tag was added as described.
Production of monoclonal antibodies against PCSK9

Balb/c mice were immunized using a DNA-based or standard immunization method with 25 μg purified rhPCSK9 (21), and spleen cells from mice were fused with Sp2/0 myeloma cells. The supernatants of hybridoma cells were screened by ELISA using plates coated with purified rhPCSK9 (100 ng/well) and by immunoblotting. The specificities of each monoclonal antibody (Mab) obtained by standard immunization (1FB) and by DNA-based immunization (B1G, B12E, and G12D), respectively, were confirmed by ELISA and immunoblotting against purified rhPCSK9.

Measurement of plasma mature and furin-cleaved PCSK9 concentrations

Plasma mature and furin-cleaved PCSK9s were measured by an ELISA using a specific combination of Mabs as previously described (Supplemental Figure 1) (21). The absorbance was measured at 450 nm with a microplate reader.

Gel filtration chromatography

Gel filtration chromatography was performed on an AKTA purifier system (GE Healthcare). Plasma samples of 2 homozygous and 6 heterozygous FH patients were injected into two connected Superose 6 (1.0 × 30; GE Healthcare) columns (22). Cholesterol or Lp(a) was measured in the fractions using a BLO-Lis24 analyzer (Tokyo Boeki Medical System, Ltd.) or a Merckodia Lp(a) ELISA (Merckodia AB) with two Mabs against Apo(a) in accordance with the manufacturers’ instructions. PCSK9s in the collected fractions were measured using the ELISA as described above.

Co-immunoprecipitation of apoB from FH plasma

A 500 μL plasma sample was adjusted to a final concentration of 50 mM HEPES [pH 7.4], 2.5 mM magnesium chloride, 1% Triton X-100, 0.5% sodium deoxycholate and protease inhibitor cocktail in a final volume of 1 mL. Samples were rotated at 4°C for 30 min, then centrifuged at 15 000 rpm for 15 min. Co-immunoprecipitation experiments were performed using a Pierce Co-Immunoprecipitation Kit (Pierce) following the manufacturer’s instructions. The supernatants were applied to columns containing 20 μg of monoclonal anti-apoB antibody (Santa Cruz Biotechnology Inc.) or 20 μg of purified IgG from a nonimmunized mouse (Santa Cruz Biotechnology Inc.). The immunoprecipitates were separated by SDS-PAGE, followed by immunoblotting with monoclonal anti-apoB antibody (R&D Systems) or polyclonal PCSK9 antibody (R&D Systems). The bands were detected with ECL prime (GE Healthcare).

Statistical analysis

The statistical significance of differences between before and after LDL-A treatment was determined by the paired t-test. One-way ANOVA and Tukey’s test were used to assess differences between groups. Spearman correlation analysis and linear regression were used to examine the relationship between PCSK9 reduction and LDL-C or HDL-C reduction. Values of P < .05 were considered to be statistically significant. All statistical analyses were carried out using the JMP software package (SAS Institute Inc.).

Results

Characterization of anti-PCSK9 Mabs

Purified rhPCSK9 was confirmed by SDS-PAGE followed by silver staining or by immunoblotting (Figure 1, A and B). The reactivity of Mabs to rhPCSK9 (mature form) was examined by SDS-PAGE under a nonreducing or reducing condition and by immunoblotting; Mabs 1FB, B1G, and B12E reacted with the 60-kDa mature PCSK9, while Mab G12D reacted with a 14-kDa prodomain of PCSK9 (Figure 1C). These three Mabs did not react with the mature segment of rhPCSK9 under a reducing condition. Similarly, the reactivity of all Mabs with native PCSK9 in human plasma was examined by immunoprecipitation. The 60-kDa mature PCSK9 and the 14-kDa prodomain of PCSK9 were co-immunoprecipitated with Mabs 1FB, B12E, and G12D, while the 53-kDa furin-cleaved PCSK9 alone was precipitated with Mab B1G (Figure 1, D and E).

Standardization of ELISA for the mature and furin-cleaved PCSK9s in plasma

We have established three different sandwich ELISAs specific for plasma total, mature and furin-cleaved PCSK9s (Supplemental Figure 1). Each system showed a dose-dependent response to purified rhPCSK9 or cell lysate of rhΔ218PCSK9 as well as to plasma samples, and the reactivity profiles were equivalent with both recombinant and plasma PCSK9 (Supplemental Figure 2). Calibration curves in the ELISA for total and mature PCSK9, rhPCSK9 protein, as a primary calibrator and rhPCSK9 culture medium, as a secondary calibrator were obtained (Supplemental Figure 3). Similarly, the calibration curve was made using dilutions of the cell lysate of rhΔ218PCSK9 for furin-cleaved PCSK9.

Changes of plasma lipids and apolipoproteins between before and after LDL-A with DS columns in FH homozygotes or heterozygotes

In FH homozygotes, a single procedure of LDL-A treatment with DS columns produced 57–78% reduction in
levels of both PCSK9s in FH homozygotes before LDL-A treatment were not significantly different from those in the plasma of FH heterozygotes. As shown in Figure 3, there was a high degree of correlation between the reduction of plasma LDL-C and the reduction of mature PCSK9 in both FH homozygotes ($r = 0.79; P = 0.036$) and heterozygotes ($r = 0.79; P = 0.004$). In addition, there was a significant correlation between the reduction in plasma Lp(a) and that in mature PCSK9 in FH heterozygotes ($r = 0.74; P = 0.0098$; data not shown). On the other hand, there was no correlation between the reductions in plasma HDL-C and mature PCSK9 in FH homozygotes or heterozygotes (Supplemental Figure 4).

Crossover study of LDL-A treatment with DM columns in FH heterozygotes

A crossover study comparing the treatment efficacy between DS and DM columns was performed in 5 FH heterozygotes. A single LDL-A treatment with DM columns produced a 49–68% reduction in plasma TC, LDL-C, TG, ApoB, ApoC-II, ApoC-III, ApoE, and Lp(a), while the plasma levels of HDL-C, ApoA-I, and ApoA-II decreased by 13–16% (Table 1 and Supplemental Table 2). In FH heterozygotes, a similar reduction was shown.

Removal of PCSK9s in FH homozygotes or heterozygotes by LDL-A with DS columns

In FH homozygotes, the plasma levels of mature and furin-cleaved PCSK9 averaged 490 ± 173 ng/mL and 74 ± 23 ng/mL, respectively, before LDL-A treatment. The two forms of PCSK9 were, respectively, reduced by 56% and 55% in FH homozygotes by a single LDL-A procedure (Figure 2A). Furin-cleaved PCSK9 constituted approximately 15% of circulating PCSK9 in the plasma of FH patients. In FH heterozygotes, the plasma levels of the two forms of PCSK9 averaged 443 ± 128 ng/mL and 55 ± 26 ng/mL, respectively, before LDL-A treatment. The two forms of PCSK9 were reduced by 46% and 48% by a single LDL-A procedure in FH heterozygotes (Figure 2B). Thus, there were no significant differences in the reduction rate of either form of plasma PCSK9 after LDL-A between FH homozygotes and heterozygotes. In addition, plasma
served in the apoB-fraction (data not shown). The distribution pattern of furin-cleaved PCSK9 was similar to that of mature PCSK9. Both forms of PCSK9s in the apoB-deficient fraction were reduced by 52–54%, while those in the apoB-containing fraction were reduced by 92–97%.

Coimmunoprecipitation of apoB in plasma of FH
To examine the association of apoB with PCSK9, plasma samples of FH were immunoprecipitated with monoclonal anti-apoB antibody. The control samples that were incubated in nonimmune serum instead of apoB antibody and negative control samples that were incubated in only resin showed no bands reactive to anti-apoB antibody (Figure 5A). Based on the coimmunoprecipitates of apoB, a mature PCSK9 band was detected by polyclonal PCSK9 antibody, confirming an association between mature PCSK9 and apoB in the plasma of FH. The band of furin-cleaved PCSK9 could not be detected in coimmunoprecipitation of apoB, because it overlapped that of IgG (data not shown).

Profile of Lp(a) by gel filtration chromatography
To examine the association of Lp(a) with PCSK9 in the plasma of FH, Lp(a) was measured by ELISA in the collected fractions obtained by gel filtration analysis. Lp(a) was recovered predominantly in apoB-containing fraction, and was not recovered in the apoB-deficient fraction which contains the highest levels of both PCSK9s (Figure 5B).

Discussion
In the present study, we demonstrated that the two forms of plasma PCSK9 were removed by LDL-A treatment with either DS or DM columns in both FH homozygotes and heterozygotes based on measurements using a new sandwich ELISA. The two forms of PCSK9 were significantly decreased by 55–56% in FH homozygotes after a single LDL-A treatment with DS columns, and were decreased to a similar extent in FH heterozygotes after the treatment with DS or DM columns. The removal of two forms of PCSK9 would have contributed to some extent to the control of LDL-C medi-

Table 1. Larry Data in FH Homozygotes and Heterozygotes Before and After a Single LDL-A Treatment With DS Columns

<table>
<thead>
<tr>
<th></th>
<th>FH homozygotes (n = 7)</th>
<th></th>
<th>FH heterozygotes (n = 11)</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Reduction (%)</td>
<td>Before</td>
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<tr>
<td>TC (mg/dL)</td>
<td>288±63</td>
<td>86±24</td>
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<td>LDL-C (mg/dL)</td>
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<td>55±22</td>
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<td>HDL-C (mg/dL)</td>
<td>31±12</td>
<td>26±9</td>
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<td>TG (mg/dL)</td>
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<td>27±21</td>
<td>74</td>
<td>97±43</td>
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<tr>
<td>Apo A-I (mg/dL)</td>
<td>81±22</td>
<td>71±20</td>
<td>13</td>
<td>99±39</td>
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<tr>
<td>Apo A-II (mg/dL)</td>
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<td>25±8</td>
</tr>
<tr>
<td>Apo B (mg/dL)</td>
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<td>40±21</td>
<td>78</td>
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<tr>
<td>Apo C-II (mg/dL)</td>
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<td>3.6±1.8</td>
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<tr>
<td>Apo C-III (mg/dL)</td>
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<td>3.5±1.6</td>
<td>57</td>
<td>8.5±2.8</td>
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<tr>
<td>Apo E (mg/dL)</td>
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<td>76</td>
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<tr>
<td>Lp(a) (ng/mL)</td>
<td>27±22</td>
<td>8.1±5.8</td>
<td>67</td>
<td>55±27</td>
</tr>
</tbody>
</table>

Abbreviations: FH, familial hypercholesterolemia; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; Lp(a), lipoprotein (a); TC, total cholesterol; TG, triglyceride. All values are shown as mean ± SD.

n = 6 in Lp(a) of FH homozygotes.

aP < 0.01.
bP < 0.05 vs the respective values before LDL-A.
cP < 0.01.
dP < 0.05 vs the respective values in FH heterozygotes.
ated by LDLR in heterozygous FH or receptor-defective homozygous FH patients undergoing LDL-A treatment. However, it would not have contributed to the control of LDL-C in receptor-negative homozygous FH patients. In addition, DM columns whose treated volumes are limited are not usually used for patients who need a high volume of treated plasma. The use of DS columns is contraindicated for patients taking angiotensin-converting enzyme (ACE) inhibitors. Thus, we cannot decide whether DS or DM columns are superior, but we need to decide an appropriate application for each case.

Statins, the most effective commercially available medication for lowering serum LDL-C, decrease cholesterol synthesis, and increase LDLR activity in the liver. Meanwhile, they also stimulate expression of PCSK9, thereby reducing their own effects (23, 24). Thus, antisense oligonucleotides, RNA-mediated interference and Mabs that target PCSK9 have been developed as new treatment strategies for lowering LDL-C (25–29). The use of PCSK9-MAb could reduce the frequency of LDL-A and control LDL-C in heterozygous FH or homozygous FH patients with the LDLR defective type. In addition, the combination of PCSK-Mab and LDL-A treatment may improve the control of LDL-C synergistically or additively.

Recently, Dubic et al has developed an ELISA for the measurement of total PCSK9 using polyclonal antibodies (5). In the present study, we developed a new sandwich ELISA using Mabs for plasma mature and furin-cleaved PCSK9s, respectively, for the first time. This ELISA method could clarify association of the ratio of each form of PCSK9 with the effects of medication in hyperlipidemia patients with gain- or loss-of-function PCSK9 mutations and those taking cholesterol-lowering drugs (30), and with various conditions of hyperlipidemia concomitant with type II diabetes, obesity, and so on. In addition, it has been reported that furin-cleaved PCSK9 represents up to 40% of the total PCSK9 in normal subjects (9), whereas it represented 15% of the total PCSK9 in FH patients in the present study. We thus formed a hypothesis that FH shows high LDL-C levels due to not only LDLR mutations but also higher activity of LDLR degradation. The association

Figure 3. Correlation between plasma LDL-C reduction and mature PCSK9 reduction in FH homozygotes and FH heterozygotes. (A) Correlation between plasma LDL-C reduction (Y-axis) and mature PCSK9 reduction (X-axis) in FH homozygotes after a single LDL-A treatment with DS columns (N = 7). (B) Correlation between plasma LDL-C reduction (Y-axis) and mature PCSK9 reduction (X-axis) in FH heterozygotes after a single LDL-A treatment with DS columns (N = 11).

Figure 4. Typical gel filtration chromatography of mature and furin-cleaved PCSK9s and cholesterol in FH plasma before and after LDL-A treatment. Profiles of cholesterol (A, closed circles: before; open circles: after), mature (B, closed triangles: before; open triangles: after), and furin-cleaved PCSK9s (C, closed diamonds: before; open diamonds: after) were analyzed in FH plasma before and after a single LDL-A treatment with DS columns after fractionation by gel filtration chromatography as described in Materials and Methods.
of the ratio of each form of PCSK9 with the regulation of LDL-C metabolism would be validated by the present method.

Gel filtration chromatography analysis showed that 20% of the total plasma PCSK9s existed in the apoB-containing fraction, which is a typical profile for plasma PCSK9 in FH patients (Figure 4). It has previously been reported that 35–39% or >40% of PCSK9 was associated with the LDL fraction in normolipidemic subjects by size exclusion chromatography or natural density gradient (31, 32). Thus, it was suggested that the amount of PCSK9 contained in the apoB-containing fraction in the plasma of FH patients was lower than that in normolipidemic subjects. Two forms of PCSK9 were reduced by 92–97% in the LDL fraction on gel filtration chromatography and the reduction in mature PCSK9 was strongly correlated with that in LDL-C after a single LDL-A treatment (Figure 3). By immunoprecipitation, plasma mature PCSK9 was confirmed to be bound to apoB (Figure 5A). Thus, it was suggested that a portion of plasma PCSK9 was removed in association with apoB by LDL-A. The distribution of Lp(a) was not overlapped like that of mature PCSK9, suggesting that mature PCSK9 was not associated with Lp(a) (Figure 5B). In addition, the reason why PCSK9 associated with LDL decreases more than LDL-C has not been clarified. Further studies are necessary to clarify the mechanism underlying the removal of the proportion of PCSK9 associated with LDL by LDL-A.

Recently, it has been reported that LDL-bound PCSK9 in human plasma exhibits diminished binding activity toward cell surface LDLR (31). However, it has not been clarified whether PCSK9-associated LDL is incorporated by LDLR, and further studies will be needed to examine the question. In addition, the interaction between apoB and PCSK9 has been reported to inhibit intracellular degradation of apoB and to result in increased secretion of apoB-containing lipoproteins (33). This secreted PCSK9-associated apoB may be derived from LDL associated with PCSK9 in plasma. A portion of PCSK9 may be bound to LDL extracellularly, thereby promoting cellular degradation of LDLR in the endosome.

In the present study, we found that the two forms of PCSK9 were reduced by 52–54% in the apoB-deficient fraction from gel filtration chromatography analysis. Circulating mature and furin-cleaved PCSK9s were mainly present in the apoB-deficient fraction. PCSK9s were removed by apoB-independent pathways based on the electric charge or nonspecific binding to the DS columns while they were removed based on particle size or nonspecific binding to the DM columns. The apoB-deficient fraction has been reported to contain PCSK9 that is mostly of a higher molecular weight, likely dimers and trimers (16, 32, 34). In addition, a previous study has shown that various proteins are present in this fraction, including albumin, globulin, serum amyloid-A, and more (35). LDL-A is thus suggested to remove circulating PCSK9 that is of high molecular weight, likely a dimer or trimer, in association with these proteins in the apoB-deficient fraction. Meanwhile, other lipoproteins such as HDL have been reported to affect the self-association of PCSK9 (35). It has been calculated that two forms of PCSK9 are negatively charged (mature PCSK9: pI = 6.6; furin-cleaved PCSK9: pI = 7.02) (16), so they are not likely to bind directly to the DS column which is also negatively charged. A future study will be required to investigate the forms of PCSK9 in the apoB-deficient fraction.

The result that PCSK9 was removed by LDL-A in homozygous FH is not consistent with the report by Cameron et al (17). Because the columns used in their study were not described, they may have been different from those used in our present study or the study by Tavori et al (16). Thus, the differences in columns, race, lifestyle, forms of PCSK9 in plasma and proteins associated with PCSK9 may affect the removal of PCSK9 by LDL-A.

This study has some limitations. The major limitation is that a small sample size of 5 may be insufficient to test subtle differences between the two methods of apheresis on PCSK9. However, we could not get enough subjects for a crossover study comparing the treatment efficacy between DS and DM columns. A second limitation is that we...
did not perform a time-dependent study of rebound trajectories in LDL-C, apoB, Lp(a), and PCSK9 in the interval between apheresis. That could provide greater insight into the mechanisms of the coordinated regulation of apoB, Lp(a), and PCSK9. A third limitation is that the number of gel filtration analyses was limited because there were not enough plasma residues for analysis.

In conclusion, our present study has shown that plasma mature and furin-cleaved PCSK9s were removed in FH homozygotes and heterozygotes by binding to apoB or other mechanisms. This report is also the first to demonstrate for an ELISA method to measure both forms of plasma PCSK9—mature and furin-cleaved form—and this technique is expected to be useful for investigating the effects of medications or the physiological or pathological roles of PCSK9.

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References

23. Dubuc G, Chamberland A, Wassef H, et al. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apop-


