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Soluble LR11 competes with amyloid β in binding to cerebrospinal fluid–high-density lipoprotein

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ABSTRACT

Background: LR11 is a member of the low-density lipoprotein (LDL) receptor family with high expression in neurons. Some cell surface LR11 is cleaved and secreted into the cerebrospinal fluid (CSF) as soluble LR11 (sLR11). Patients with Alzheimer's disease (AD), particularly apolipoprotein E4 carriers, have high CSF–sLR11 and low CSF–amyloid β (Aβ) concentrations. Therefore, we assessed whether sLR11 is bound to CSF–high-density lipoprotein (HDL) and whether sLR11 competes with Aβ in binding to apoE in CSF–HDL.

Methods: We measured CSF–sLR11 concentrations (50 controls and 16 patients with AD) using enzyme immunoassay. sLR11 and apoE distribution in the CSF was evaluated using non-denaturing two-dimensional gel electrophoresis (N–2DGE). ApoE bound to sLR11 or Aβ was identified using co-immunoprecipitation assay.

Results: CSF–sLR11 concentrations were higher in patients with AD than controls (adjusted for sLR11 using phospholipid). N–2DGE analysis showed that sLR11 and Aβ comigrated with a large apoE-containing CSF–HDL. Moreover, fewer apoE was bound to Aβ when a higher amount of apoE was bound to sLR11 in patients with AD who presented with ε4/4.

Conclusion: sLR11 binds to CSF–HDL and competes with Aβ in binding to apoE in CSF–HDL, indicating that sLR11 affects Aβ clearance via CSF–HDL.

1. Introduction

Alzheimer's disease (AD) is one of the most common types of dementia. The number of patients with AD has been increasing worldwide. According to the World Alzheimer Report in 2016, 47 million individuals present with dementia globally, and the number is projected to increase to > 131 million by 2050 [1]. Therefore, causal treatment for AD is eagerly anticipated. The principal features of the brain of patients with AD include the aggregation of amyloid β (Aβ), formation of senile plaques, excessive accumulation of phosphorylated tau, and appearance of neurofibrillary tangles [2–4]. Following these pathological changes, neurons, particularly those in the cerebral cortex, degenerate, leading to cortical atrophy. This series of events is considered as the pathogenesis of AD because it is closely related to a decline in cognitive function [2,3].

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β; apo, apolipoprotein; sLR11, soluble LR11; PL, phospholipid; N–2DGE, non-denaturing 2-dimensional gel electrophoresis; HRP, horse radish peroxidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TGN, trans-Golgi network; SAA, serum amyloid A

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Although several risk factors for AD have been proposed in numerous studies [5–9], the presence of the apolipoprotein E4 (apoE4) gene is the strongest risk factor of sporadic AD [10,11]. In the central nervous system, apolipoprotein E (apoE) is predominantly synthesized by astrocytes, which secrete high-density lipoprotein (HDL) into the cerebrospinal fluid (CSF). CSF–HDL accounts for a large proportion of lipoprotein [12], and it is enriched in the apoE, particularly during childhood [13,14]. Numerous studies have shown that low-density lipoprotein (LDL) receptor family members are abundantly expressed in the human brain [15]. These receptors have a high affinity for apoE, and they are presumed to modulate not only CSF–HDL metabolism but also neural functions, including neural signaling and synaptic plasticity [16].

LR11, which is also called SorLA1, is a membrane-bound lipoprotein receptor of the LDL receptor family. LR11 has a structure similar to that of the neural adhesion molecule [17–19], and it is expressed in neurons. A meta-analysis has suggested that the variants of the LR11 gene are associated with an elevated risk for AD [20]. LR11 is susceptible to proteolytic shedding, which yields a soluble form of LR11 (sLR11). In our previous study, we have shown that sLR11 concentration is elevated in the CSF of patients with AD, particularly those presenting with apoE4 [21]. However, the precise mechanism is not fully understood.

2. Materials and methods

2.1. CSF samples

We obtained CSF samples from the controls who did not present with AD (n = 50) and patients with AD (n = 16), who were diagnosed according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer Disease and Related Disorders Association [22]. The Mini-mental State Examination (MMSE) score was used to evaluate the cognitive function of patients with AD. All samples were centrifuged at 1500 × *g* for 5 min and CSF samples contaminated with serum were excluded. The samples were discarded on macroscopic detection of red blood cells at the bottom of the centrifuge tube. The aliquots of eligible CSF samples were frozen at −80 °C until the in vitro experiments were initiated. Either the patient or guardian signed the informed consent before CSF sampling. The experimental protocol was reviewed and approved by the ethics committee of Niigata University and Juntendo University.

2.2. Measurements of phospholipid and sLR11

Because phospholipid (PL) is a major lipid component of CSF–HDL, we measured PL concentration in the CSF; this was determined via a slight modification of the enzymatic method using a commercial kit (Phospholipid C-test Wako; Wako Pure Chemical Industries) [13,14]. Briefly, reagents were dissolved in a buffer at 10-fold concentrations, and samples were mixed with the solution in a 2:1 ratio instead of 1:150 ratio, which was the original protocol. After 5 min of incubation at 37 °C, the products were measured at 600 nm and calculated with a calibration curve. The sLR11 concentration was determined via enzyme-linked immunosorbent assay using a commercial kit (Sekisui Medical) [21,23].

2.3. ApoE genotyping

DNA was extracted from potassium ethylenediaminetetraacetate acid-anticoagulated whole blood. The apoE gene, including residues 112 and 158, was amplified via polymerase chain reaction with primers, and apoE genotypes were determined by restricting fragment length polymorphism following *Hha* I digestion [24].

2.4. Non-denaturing 2-dimensional gel electrophoresis

To determine the distributions of apoE and sLR11 in the CSF, non-denaturing 2-dimensional gel electrophoresis (N–2DGE) was performed, as previously described [25]. Briefly, CSF samples (100 μl each) obtained from controls and patients with AD were applied to 0.75% 1.5–mm thick agarose gel strips (2.5 × 12.5 cm) at 200 V for 2 h and electrophoresed using a 2%–15% non-denaturing gradient polyacrylamide gel until 2000 Vh was achieved. Gels were electrically transferred into the nitrocellulose membranes at 30 V overnight, following which these membranes were incubated with anti-human apoE antibody (Merck Millipore) or anti-human LR11 antibody (A2-2-3), which was provided by Sekisui Medical, for the specific detection of sLR11 [23]. After incubation, the membranes were treated with horse radish peroxidase (HRP)-labeled secondary antibody and visualized using an ECL Advance Western Blotting Detection Kit (GE Healthcare). In addition, the apoE distribution between the plasma and CSF was compared by electrophoresing fasting plasma samples from healthy volunteers. As a reference, apolipoprotein A-I (apoA-I) distribution in the same plasma sample was visualized with anti-human apoA-I antibody (The Binding Site) and a secondary antibody.

2.5. Immunoprecipitation study

To determine whether sLR11 is bound to CSF–HDL, we conducted an immunoprecipitation study using CSF samples obtained from 6 controls and 11 patients with AD, including 3 patients presenting with ε4/4. First, magnetic beads coated with Protein G Mag Sepharose (GE Healthcare) were mixed with anti-human sLR11 antibody, anti-human Aβ antibody (IBL), or anti-human apoA-I antibody in a binding buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl; pH 7.5); this mixture was incubated at room temperature for 30 min. Subsequently, the treated beads were washed with the same solution as the binding buffer and were incubated with CSF samples at room temperature for 90 min to allow immune complex formation. The tubes were placed in a rack with a magnet and the unbound fraction in the supernatant was removed by decanting the solution; further the precipitate was washed five times with saline. Proteins bound to protein G beads were eluted with 2.5% acetic acid and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 4%–20% precast gel (Invitrogen) in the presence of β-mercaptoethanol. Separated proteins were transferred to a nitrocellulose membrane and incubated with the antibodies against the proteins of interest, which were visualized with HRP-conjugated secondary antibody using the ECL Advance Western Blotting Detection Kit.

2.6. Statistical analysis

We used an add-in software for Microsoft Excel (Statcel4, OMS) for statistical analyses. Depending on whether the data have a Gaussian distribution, we used single measured analyses of variance or Kruskal–Wallis test for multiple comparison among the groups, followed by Tukey–Kramer and Steel–Dwass as post hoc test, respectively. Differences in proportional variables between groups were analyzed using chi-square test. The Pearson correlation coefficient was used to determine the correlation between 2 variables. A $p < .05$ was considered statistically significant. Values are presented as mean ± SD or percentage.

3. Results

3.1. Constituents of CSF–HDL

CSF–HDL composition significantly differed between the controls and patients with AD. Mean age of the patients with AD was significantly higher than that of the controls (69.4 ± 11.2 vs. 40.2 ± 25.2 y, $p < .001$), and therefore, the control group was divided into 2 subgroups according to age (Table 1). In patients with AD, PL and sLR11 concentrations were both higher than those in the young control group. However, only the sLR11 but not PL concentration was higher than that in the elderly control group. Furthermore, sLR11 was divided according to PL concentration to compare CSF–HDL composition among the groups. Although sLR11/PL ratio did not differ in the young and elderly

Table 1
Characteristics and CSF components of the controls and patients with AD.

	Controls		Patients with AD
	Young (n = 26)	Elderly (n = 24)	(n = 16)
Age (y) (range)	17.9 ± 7.9 (10–39)	64.3 ± 10.6** (40–80)	69.4 ± 11.2** (47–86)
Male (%)	68.0	54.2	42.9
MMSE score	N/A	N/A	16.2 ± 9.4
PL (mg/dl)	0.47 ± 0.15	0.69 ± 0.12**	0.65 ± 0.21**
sLR11 (ng/ml)	6.7 ± 2.3	9.5 ± 3.4	13.3 ± 7.1**,†
sLR11/PL	15.4 ± 6.8	14.2 ± 6.2	21.7 ± 12.6†

Data are expressed as mean ± SD, range or percentage. AD, Alzheimer's disease; N/A, not applicable; MMSE, Mini-mental State Examination; PL, phospholipid; sLR11, soluble LR11.
** $p < .01$ vs. young control.
† $p < .05$ vs. elderly control.

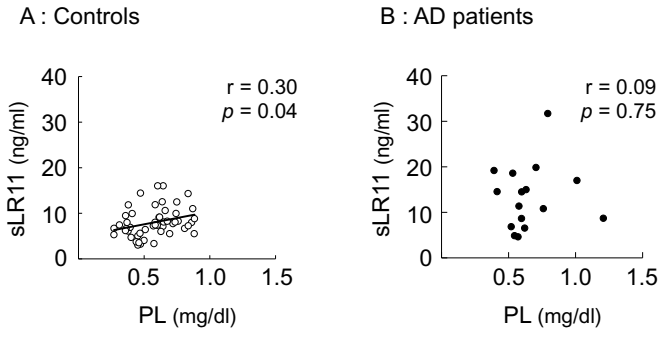

Fig. 1. Relationship between sLR11 and phospholipid concentrations in the cerebrospinal fluid. Cerebrospinal fluid samples were obtained from controls (A) and patients with Alzheimer's disease (B) via lumbar puncture.

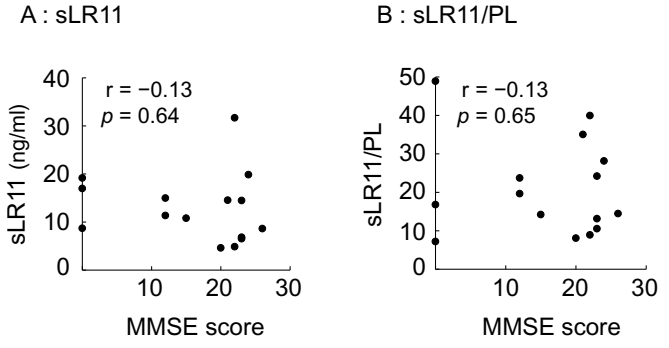

Fig. 2. Relationship between sLR11 concentration, sLR11/PL ratio, and MMSE score in patients with AD. The Mini-mental State Examination (MMSE) was used to assess patients with AD. The relationship between MMSE score and sLR11 concentration (A) and sLR11/phospholipid ratio (B) in the CSF was analyzed.

control groups, sLR11/PL ratios were 41% and 53% higher in patients with AD than in the young and elderly control groups, respectively. Scatter plots revealed a significant positive correlation between PL and sLR11 concentrations in controls but not in patients with AD (Fig. 1). No gender differences were observed in terms of CSF parameters in the controls and patients with AD (Supplementary Table 1).

Although patients with AD had a moderate cognitive loss based on the MMSE score (16.2 ± 9.4, Table 1) (normal: ≥28, mild cognitive impairment: 24–27, mild: 21–23, moderate: 11–20, and severe: ≤10), no correlation was observed between sLR11 concentration, sLR11/PL ratio, and MMSE score (Fig. 2).

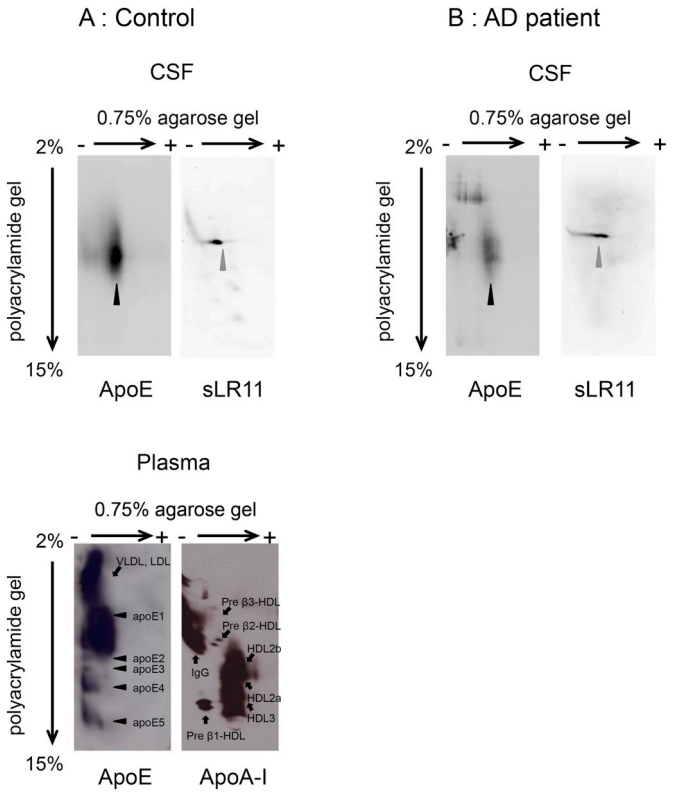

Fig. 3. Characteristics of CSF–lipoproteins using non-denaturing 2-dimensional gel electrophoresis. Lipoproteins in the CSF [A (upper panel), a control; B, patients with AD] and plasma [A (lower panel), a control] were separated via non-denaturing 2-dimensional gradient gel electrophoresis according to charge (the first dimension, horizontal arrows) and particle size (the second dimension, vertical arrows). Because it was challenging to detect the sLR11 spot with a short exposure time, we showed the data obtained by 50 min of exposure in the controls. ApoE, sLR11, and apoA-I were identified via Western blot analysis.

3.2. Distributions of apoE and sLR11 in CSF–HDL

To validate whether the proteins of interest were associated with CSF–HDL, N–2DGE was performed to determine the distributions of apoE and sLR11 in CSF samples. In the control group, we easily detected apoE on large α–migrating HDL (Fig. 3A, upper panel, black arrowhead). However, minimal sLR11 was observed in the CSF samples of the control group (Fig. 3A, upper panel, gray arrowhead; exposed for 50 min). By contrast, in patients with AD, we detected sLR11 as well as apoE on large α-migrating HDL (Fig. 3B, black and gray arrowhead; exposed for 10 min). It was noted that apoE colocalized with sLR11 in the controls

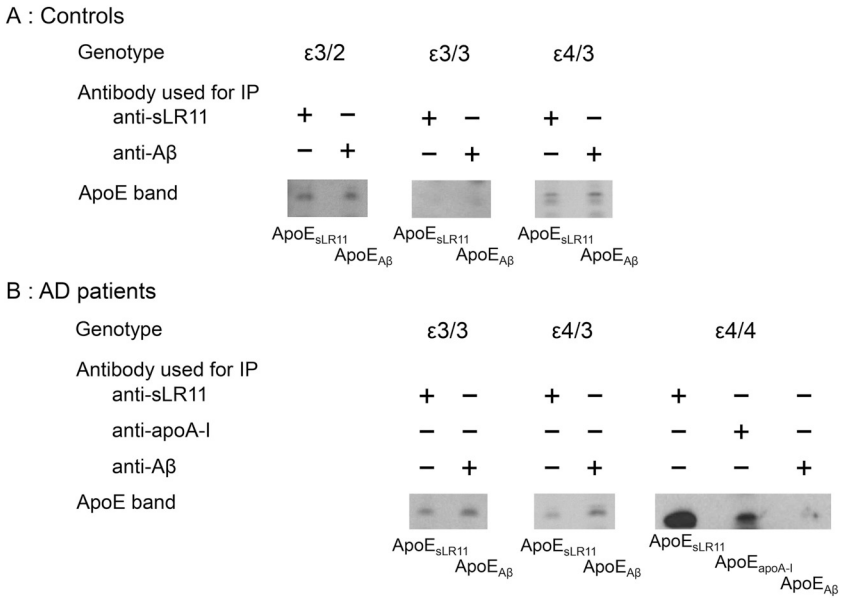

Fig. 4. Co-immunoprecipitation compositional analysis of CSF–HDL. We mixed CSF samples obtained from the controls (A) and patients with AD (B) with a specific antibody and detected the apoE band of the immunoprecipitates via Western blot analysis. ApoE$_{sLR11}$, ApoE$_{Aβ}$, and ApoE$_{apoA-I}$ indicate that apoE was associated with sLR11, Aβ, and apoA-I, respectively. IP, immunoprecipitation.

and in patients with AD. CSF samples had no smaller apoE-containing particles, which were detectable in normal plasma (Fig. 3A, lower panel). CSF–HDL particles in both the controls and patients with AD were larger than the plasma HDL particles associated with apoA-I (Fig. 3).

3.3. Co-immunoprecipitation of CSF–apoE with sLR11 or Aβ

We isolated CSF–apoE associated with either sLR11 or Aβ using immunoprecipitation assay and analyzed it using SDS–PAGE. Western blot analysis of the samples obtained from the controls revealed that the apoE band intensity was similar between the anti-sLR11 antibody-induced and anti-Aβ antibody-induced precipitates (ApoE$_{sLR11}$, ApoE$_{Aβ}$) in any apoE genotypes (Fig. 4A). Contrastingly, in patients with AD, the apoE band intensity of the precipitate varied widely among the samples. In patients with AD who presented with ε3/3, the apoE band exhibited similar intensity between the anti-sLR11 antibody-induced and anti-Aβ antibody-induced immune complexes (Fig. 4B, left). Furthermore, in patients with AD who presented with ε4/3, the apoE band intensity was stronger in anti-Aβ antibody-induced precipitates (ApoE$_{Aβ}$) than in anti-sLR11 antibody-induced precipitates (ApoE$_{sLR11}$) (Fig. 4B, middle). Notably, patients with AD who presented with ε4/4 had extremely stronger apoE band intensity in anti-sLR11 antibody-induced precipitates (ApoE$_{sLR11}$) than in anti-apoA-I antibody-induced or anti-Aβ antibody-induced precipitates (ApoE$_{apoA-I}$, ApoE$_{Aβ}$) (Fig. 4B, right).

4. Discussion

The present study indicated that sLR11 binds to CSF–HDL and competes with Aβ in binding to apoE in CSF–HDL. Moreover, sLR11 comigrated with apoE in large CSF–HDL particles (Fig. 3). Immunoprecipitation studies have confirmed that apoE was present in the immune complex induced by anti-sLR11 antibody (Fig. 4). Furthermore, in patients with AD who presented with ε4/4, more sLR11 and less Aβ binding to CSF–HDL were observed.

At the neuronal surface, membrane-bound LR11 is presumed to follow distinct metabolic pathways. In the first pathway, membrane-bound LR11 is digested by several proteases, such as tumor necrosis factor receptor-α converting enzyme [26,27], resulting in the release of sLR11 to the CSF. In the second pathway, which is perhaps the more likely pathway, neuronal surface LR11 is subjected to clathrin-mediated endocytosis with the aid of clathrin adaptor protein 2, followed by endosome formation [28]. Most LR11s in the endosomal compartment are guided to the trans-Golgi network (TGN) for continuous shuttling between the endosome and TGN. Certain endosomal LR11s are guided to the lysosome for degradation, whereas the rest are transported back to the neuronal surface for receptor recycling. These metabolic destinations are determined using associated adaptor proteins. As reported in our previous study, sLR11 increased in the CSF of patients with AD. In the present study, similar findings were observed [young control group vs. elderly control group vs. patients with AD, 6.7 ± 2.3 vs. 9.5 ± 3.4 vs. 13.3 ± 7.1 ng/ml ($p < .001$)] (Table 1). The elevation of sLR11 concentration in the CSF was more evident in apoE4 carriers than in non-carriers [21]. These observations indicated that the cleavage of LR11 is enhanced at the cell surface in patients with AD. In the control group, sLR11 concentrations were significantly correlated with the concentrations of PL, which is the major lipid component of CSF–HDL (Fig. 1A). However, in patients with AD, sLR11 concentrations were remarkably high than PL concentrations (Fig. 1B). These observations were in accordance with our hypothesis. Despite certain earlier studies that showed a reduced LR11 expression in the brain of patients with AD [29], a recent study has reported that LR11 expression in the brain tissue does not differ among participants with mild cognitive impairment and mild/moderate AD or those without cognitive impairment [30]. However, it remains to be elucidated whether the release of sLR11 is enhanced in patients with AD.

The structure of apoE may play a crucial role in the genotype-dependent binding preference to sLR11. ApoE consists of 299 amino acids, and its three common isoforms differ in the amino acids at positions 112 and 158 [31]. At these positions, the amino acids are cysteine–cysteine in apolipoprotein E2 (apoE2), cysteine–arginine in apolipoprotein E3 (apoE3), and arginine–arginine in apoE4. Because apolipoprotein A-II (apoA-II), which is a minor component of CSF–HDL [32], also contains cysteine, apoE2 and apoE3, but not apoE4, can form heterodimers with apoA-II via disulfide bonds. In fact, both free apoE and apoE–apoA-II heterodimers are detected in apoE2 and apoE3 carriers, whereas only free apoE is detected in apoE4 homozygotes. In our previous study, apoA-II comigrated with apoE in CSF samples assessed via N–2DGE [32]. The receptor binding domain of apoE is located between positions 136 and 150, which are close to the arginine–cysteine site at positions 112 and 158 [31]. LR11 is an apoE receptor, and sLR11

preserves its ligand-binding domain. Therefore, the formation of apoE–apoA-II heterodimers could cause structural rearrangement, which disturbs the access of sLR11 to the receptor binding site of apoE in CSF–HDL. Conversely, the receptor binding domain must be fully exposed in apoE4 because apoE4 has no cysteine residues.

Although the association between Aβ and CSF–HDL remains controversial, our data strongly suggest that the binding of Aβ to CSF–HDL is disturbed by the association between sLR11 and CSF–HDL. In patients with AD, Aβ was detected with apoE, which is associated with a large CSF–HDL subfraction (Fig. 3). Our previous study has also showed that Aβ was associated with a large apoE-containing CSF–HDL particle when free Aβ was added to CSF [32]. Patients with AD, particularly those carrying apoE4, had low Aβ and high sLR11 concentration [21]. In the present study, immunoprecipitation analysis revealed that Aβ was bound less to CSF–apoE when the sLR11 concentration was higher (Fig. 4), indicating the competitive inhibition of apoE binding to Aβ.

ApoE4 may modulate Aβ metabolism via the following mechanisms. First, apoE4 may impair Aβ clearance via CSF–HDL. CSF–HDL coupled with Aβ could be taken up by apoE receptors, such as lipoprotein receptor-related protein, and degraded in the glial cells [33]. Compared with others, CSF–HDL of apoE4 carriers (particularly apoE4 homozygotes) is associated with less Aβ (Fig. 4). In addition, CSF–HDL of apoE4 carriers has more sLR11 than the others, and sLR11 masks the ligand-binding domain of apoE. Therefore, sLR11-associated apoE on CSF–HDL particles cannot bind to apoE receptors. Second, apoE4 is more susceptible to dissociation from CSF–HDL than apoE2 and apoE3, which is induced by serum amyloid A (SAA) [32], which is an acute phase protein that is found in the CSF. SAA replaces apolipoproteins during inflammation. Third, the low Aβ-binding capacity of CSF–HDL in apoE4 carriers may promote the formation of small oligomeric Aβ in the CSF. Recent studies have suggested that small Aβ oligomers are present in the early phase of AD and are more toxic than larger insoluble aggregates [34]. Instead of measuring CSF–Aβ concentration, we performed N–2DGE and immunoprecipitation analyses to confirm the association between apoE and Aβ in CSF–HDL particles. However, because a large sample volume was required for these analyses, we were unable to conduct numerous experiments. Therefore, future experimental and clinical studies must be conducted to confirm our results.

In conclusion, sLR11 binds to CSF–HDL and competes with Aβ in binding to apoE in CSF–HDL. We speculate that high CSF–sLR11 concentration relative to CSF–apoE concentration is a marker of impaired Aβ metabolism in patients with AD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.024.

Acknowledgments

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