Apolipoprotein E and clusterin inhibit the early phase of amyloid-β aggregation in an in vitro model of cerebral amyloid angiopathy

Yoshinori Endo, Kazuhiro Hasegawa, Ryo Nomura, Hidetaka Arishima, Ken-ichiro Kikuta, Taro Yamashita, Yasuteru Inoue, Mitsuharu Ueda, Yukio Ando, Mark R. Wilson, Tadanori Hamano, Yasunari Nakamoto and Hironobu Naiki

Abstract

Sporadic cerebral amyloid angiopathy (CAA) is characterized by cerebrovascular amyloid-β (Aβ) deposition, which leads to lobar hemorrhage and dementia. Biological molecules affecting the development of CAA have not been fully characterized. In this study, we performed proteome analysis of biopsied leptomeningeal and cortical vessels obtained from 6 CAA patients and 5 non-CAA patients who underwent surgery for large lobar hemorrhages. We found that 6 proteins, including Aβ, apolipoprotein E (apoE), clusterin (CLU), albumin, complement C4 and vitronectin were significantly upregulated in the vessels of CAA patients as compared to non-CAA patients. ApoE and CLU were found in all CAA patients. We next examined the effects of apoE and CLU on the early phase of Aβ aggregation, using a simple yet powerful in vitro model of CAA, which recapitulates the intramural periarterial drainage pathway model. We found that physiological concentrations of apoE and CLU delayed the initiation time of amyloid growth kinetics in a concentration-dependent manner. These data indicate that apoE and CLU may act as extracellular chaperones to inhibit Aβ amyloid deposition in CAA.

Keywords: Cerebral amyloid angiopathy, Amyloid-β, Proteome analysis, Apolipoprotein E, Clusterin, Intramural periarterial drainage, Extracellular chaperone
Materials and methods

Materials
Aβ(1–40) (code 4307-v, trifluoroacetic salt, lyophilized from dimethyl sulfoxide solution) was purchased from Peptide Institute Inc. (Osaka, Japan). Human serum albumin (HSA) (code 70024–90-7, A8763) was purchased from Sigma. Matrigel (Phenol Red free, code 356237) was purchased from Becton-Dickinson and Co. (NJ, USA). NHS-activated Sepharose 4 Fast Flow (code 010–20261) and apoE4 (code 017–20271) were purchased from Wako (Osaka, Japan). Human CLU was purified from human serum as described previously [29].

Patients and specimens for proteome analysis
We recently reported the prevalence of CAA in patients who underwent surgery in our hospital for large lobar hemorrhages, i.e., supratentorial bleeding expanding from the cerebral cortex to subcortical white matter [18]. To diagnose CAA, we examined biopsied cortical tissues around hematomas with Congo-red and anti-Aβ staining. The same biopsied tissue was used for a series of histopathological and immunohistochemical staining to diagnose CAA and for the subsequent proteome analysis. We examined a cohort of 24 CAA patients and 5 non-CAA patients. Of 24 CAA patients, 16 patients (66.7%) had severe (Grade 4) CAA based on the pathological grading system for CAA developed by Greenberg et al. [9].

From this cohort, we selected 6 CAA patients (all Grade 4) and 4 non-CAA patients for which sufficient amounts of pathological specimens were available for the subsequent proteome analysis (Table 1). To increase the patient number, we added one additional non-CAA patient who underwent surgery in our hospital (B-1 in Table 1).

Protein extraction and proteome analysis
Protein extraction and proteome analysis were performed with liquid chromatography-tandem mass spectrometry (LC-MS/MS), essentially as described elsewhere [17]. Briefly, 4 μm thick slices of formalin-fixed and paraffin-embedded brain biopsy samples were placed on membrane slides (Leica Microsystems, Wetzlar, Germany). Sections were air-dried and then melted, deparaffinized, and stained with Congo red combined with nuclear counterstaining with hematoxylin. In the CAA group, Congo red-positive leptomeningeal and cortical vessels, which were identified using the bright-field setting, were isolated via laser capture microdissection (LCM) (LMD7000; Leica Microsystems, Wetzlar, Germany) (Table 1), then analysed using nano-flow reversed-phase LC–MS/MS (LTQ Velos Pro; Thermo Fisher Scientific). In the non-CAA group, leptomeningeal and cortical vessels, which were identified using the bright-field setting, were isolated via LCM. In both groups, we didn’t discriminate arteries from veins. The relative

Table 1 Profiles of cases of CAA and non-CAA patients analysed by LMD-LC-MS/MS

| Number | Group | Age | Sex | Lesion of brain hemorrhage | Amyloid grading scale a | Hypertension and medication | Anticoagulants or antiplatelets | Microbleeding at MRI (T2*) | Patient number in Table 3 of [18] |
|--------|-------|-----|-----|---------------------------|------------------------|-----------------------------|-------------------------------|---------------------------|----------------------------------|
| A-1    | CAA   | 66  | F   | R temporoparietal         | 4                      | No                          | No                            | negative                  | 3                               |
| A-2    | CAA   | 80  | F   | L temporoparietal         | 4                      | Yes, medication             | No                            | NA                        | 4                               |
| A-3    | CAA   | 79  | F   | L frontal                 | 4                      | No                          | No                            | NA                        | 5                               |
| A-4    | CAA   | 74  | F   | R frontal                 | 4                      | Yes, medication             | No                            | positive                  | 6                               |
| A-5    | CAA   | 71  | F   | L frontal                 | 4                      | Yes, medication b            | No                            | positive                  | 7                               |
| A-6    | CAA   | 63  | F   | L parietal                | 4                      | No                          | No                            | negative                  | 12                              |
| B-1    | non-CAA | 83  | F   | R putamen and frontal     | 0                      | Yes, no medication          | No                            | negative                  | Not included                     |
| B-2    | non-CAA | 67  | M   | R fronto-parietal         | 0                      | Yes, no medication          | No                            | negative                  | 25                              |
| B-3    | non-CAA | 75  | M   | R temporoparietal         | 0                      | No                          | No                            | NA                        | 26                              |
| B-4    | non-CAA | 61  | M   | R frontal                 | 0                      | No                          | No                            | negative                  | 28                              |
| B-5    | non-CAA | 68  | M   | R frontal                 | 0                      | Yes, medication             | No                            | NA                        | 27                              |

M male, F female, R right, L left, NA not applicable

aPathological grading system for CAA by Greenberg SM et al. [9]
bSelf-withdrawal 2 years before onset
abundances of the identified molecules were obtained using the normalized spectral abundance factor (NSAF) [27] (Table 2).

Kinetic analysis of the seeded aggregation of Aβ(1–40) amyloid fibrils
In this paper, we used only Aβ(1–40) because Aβ(1–40) is the predominant Aβ species deposited in the vessels of CAA patients [40, 41]. Aβ(1–40) amyloid fibrils (fAβ(1–40)) were first formed by incubating 1.0 ml of the reaction mixture containing 50 μM Aβ(1–40), 50 mM sodium phosphate, pH 7.5, 100 mM NaCl phosphate buffered saline (PBS), and 0.03% NaN₃ at 37°C for 1 week. Subsequently, a reaction mixture containing 2.5 μg/ml fAβ(1–40), 5 μM Aβ(1–40), 0–0.5 μM apoE3 or 0–1.0 μM CLU, 0.3 mg/ml (4.5 μM) HSA, PBS, and 5 μM thioflavin T (ThT) was incubated at 37°C without shaking in a 96-well plate (code HSP9666, Bio Rad, USA) sealed with a sealing film (code 676070, Greiner Bio-One GmbH, Frickenhausen, Germany). ThT fluorescence was measured every 5 min for 2 h using a Safire2 microplate fluorometer (TECAN, Austria) with excitation at 445 nm and emission at 490 nm.

Analysis of the effects of ApoE and CLU on the length of the lag phase of Aβ(1–40) amyloid aggregation
In this paper, we utilized a previously established powerful in vitro model of CAA [10] to analyse the effects of apoE and CLU on the length of the lag phase of Aβ(1–40) amyloid aggregation, essentially as described in [10].

Table 2 Proteins in the cerebral blood vessels of CAA patients vs. non-CAA patients

| Accession number | Protein                        | CAA (% detection) | NSAF CAA | Non-CAA (% detection) | NSAF non-CAA | p value* |
|------------------|--------------------------------|-------------------|----------|-----------------------|--------------|----------|
| P02649           | Apolipoprotein E               | 100               | 0.1259   | 20                    | 0.0006       | 0.004    |
| P05067           | Amyloid beta A4 protein        | 100               | 0.0685   | 0                     | ND           | 0.004    |
| P10909           | Clusterin                      | 100               | 0.039    | 20                    | 0.0015       | 0.004    |
| P02768           | Serum albumin                  | 100               | 0.046    | 100                   | 0.0154       | 0.017    |
| P08123           | Collagen alpha-2(I) chain      | 100               | 0.0331   | 80                    | 0.0019       | 0.329    |
| P68871           | Hemoglobin subunit beta        | 100               | 0.1695   | 100                   | 0.249        | 0.662    |
| P69905           | Hemoglobin subunit alpha       | 100               | 0.0778   | 100                   | 0.0718       | 0.792    |
| P04004           | Vitronectin                    | 83                | 0.0085   | 20                    | 0.0004       | 0.03     |
| P00L4            | Complement C4-A                | 83                | 0.0032   | 20                    | 0.0001       | 0.03     |
| P08670           | Vimentin                       | 83                | 0.0207   | 80                    | 0.0159       | 0.247    |
| P41222           | Prostaglandin-H2 D-isomerase   | 67                | 0.013    | 40                    | 0.0021       | 0.177    |
| Q15149           | Plectin                        | 67                | 0.0006   | 40                    | 0.0001       | 0.177    |
| Q8IY6            | Cytoskeleton-associated protein 2-like | 67 | 0.0147 | 80 | 0.006 | 0.537 |
| P06727           | Apolipoprotein A-IV            | 50                | 0.0096   | 0                     | ND           | 0.177    |
| Q70EL1           | Inactive ubiquitin carboxyl-terminal hydrolase 54 | 50 | 0.0035 | 0 | ND | 0.177 |
| P11047           | Laminin subunit gamma-1        | 50                | 0.0014   | 0                     | ND           | 0.177    |
| P02042           | Hemoglobin subunit delta       | 50                | 0.0313   | 80                    | 0.0735       | 0.247    |
| Q8N413           | Solute carrier family 25 member 45 | 50 | 0.0102 | 20 | 0.0011 | 0.329 |
| P35625           | Metalloproteinase inhibitor 3  | 50                | 0.0084   | 20                    | 0.0009       | 0.329    |
| P07437           | Tubulin beta chain             | 50                | 0.0061   | 40                    | 0.0018       | 0.429    |
| P12814           | Alpha-actinin-1                | 50                | 0.0015   | 40                    | 0.0005       | 0.429    |
| P14136           | Gial fibrillary acidic protein  | 50                | 0.0265   | 60                    | 0.0116       | 0.662    |
| Q9BQE3           | Tubulin alpha-1C chain         | 50                | 0.0051   | 60                    | 0.0018       | 0.662    |
| A6NNT2           | Putative uncharacterized protein C16orf96 | 50 | 0.004 | 60 | 0.0011 | 0.662 |
| P0435O           | Tubulin beta-4A chain          | 50                | 0.0064   | 80                    | 0.0045       | 0.792    |
| P98160           | Basement membrane-specific heparan sulfate proteoglycan core protein | 50 | 0.0004 | 60 | 0.0004 | 1 |

Proteins which show the % detection to be ≥50% in CAA patients are listed. % Detection means the relative number of patients positive for each protein in both CAA and non-CAA patients

ND not detected

*The Mann-Whitney U test was used for comparisons between NSAF CAA and NSAF non-CAA values

Protein abundance values were estimated using NSAF (normalized spectral abundance factor) normalization

Boldface highlights the proteins which were significantly upregulated in the cerebral blood vessels of CAA patients as compared to non-CAA patients
Briefly, we reconstituted an artificial BM on the surface of NHS-activated Sepharose 4 Fast Flow beads by conjugating Matrigel to their surface (Fig. 1). Matrigel-coated beads were then incubated with 5 μM Aβ(1–40), 0.3 mg/ml (4.5 μM) HSA, PBS, 0.05% NaN₃ (PBS-NaN₃), 5 μM ThT, and 0–0.5 μM apoE3/E4 or 0–1.0 μM CLU at 37 °C in a clear microtiter plate module (Nunc, F8 Immuno module, Maxisorp, code: 468667) in which the air water interface was completely removed. The plate was gently rotated at 1 rpm. As these beads slowly sink from the top to the bottom of a well, their surfaces are exposed to the relative countercurrent of the reaction mixture, which mimics the IPAD flow in vitro.

The ThT-reactive aggregates in the microtiter wells were visualized with a fluorescence microscope (MVX10, Olympus Corporation, Tokyo, Japan) equipped with CFP filter sets (excitation 425–445 nm, emission 460–510 nm). Then the fluorescence images recorded for 1 s with a DP 72 digital camera (Olympus Corporation, Tokyo, Japan). We chose the first time point at which ThT-reactive aggregates were detected as the initiation time for fibril growth kinetics. The preceding lag phase is the time during which nuclei, on-pathway oligomers and protofibrils are successively formed prior to the subsequent rapid fibril growth phase. In most cases, one observer (Y.E.) mainly determined the initiation time by visual inspection of recorded images. In the specific cases of data shown in Figs. 4, 5 and 6, another observer (R.N.) re-determined the initiation time and similar results were obtained (data not shown).

To monitor Aβ(1–40) amyloid formation in a conventional way, we also measured the ThT fluorescence with a Safire2 microplate fluorometer (TECAN, Austria). For the kinetic analysis, data obtained with a fluorescence microscope was used because microscopic detection of fluorescent spots on the Sepharose-beads often preceded the increase in fluorescence measured by fluorometry by 12 to 24 h (data not shown).

We then measured the duration of the lag phase for each well using the Kaplan-Meier survival method and the initiation time of amyloid growth kinetics as the event of interest (Fig. 2). In this assay, the reaction mixture in which ThT-reactive aggregates have not yet been detected is considered as “surviving”. Thus, the survival rate corresponds to the percentage of the reaction mixtures in which ThT-reactive aggregates have not yet been detected. The statistical significance was compared by the log rank test, followed by pair to pair multiple comparisons using the Holm-Sidak method. For the calculation, we excluded any wells in which air bubbles emerged before the detection of ThT-reactive aggregates. The survival analysis was performed with SigmaPlot 12 (Systat Software, Inc. CA). The differences between the two groups were considered significant if P values were less than 0.05.

**Results**

**Profiles of CAA and non-CAA patients**

Table 1 shows the profiles of the CAA and non-CAA patients analysed by LCM-LC-MS/MS. The 6 CAA
patients were from 63 to 80 years old (mean ± SD 72.2 ± 6.9). Compatible with the female predominance in CAA patients [41], all 6 patients were female and had grade 4 CAA. Three of the 6 patients had hypertension and took antihypertensive agents. No patients took anticoagulant or antiplatelet drugs. Two patients were positive for strictly lobar microbleeding as evaluated by T2*-weighted magnetic resonance imaging (MRI). The ages of the 5 non-CAA patients were from 61 to 83 years old (mean ± SD 70.8 ± 8.4). Four patients were male, and 1 patient was female. We found no female predominance in non-CAA patients. Three of the 5 patients had hypertension and only 1 took antihypertensive agents. Thus, it is reasonable to consider that for these 3 patients, the etiology of hemorrhage may be hypertension. As for the remaining 2 patients, although we excluded CAA as an etiology of hemorrhage, we did not definitely identify the etiology of hemorrhage. No patients took anticoagulant or antiplatelet drugs. No patients were positive for microbleeding as evaluated by T2*-weighted MRI.

Proteome analysis
As shown in Table 2, 6 proteins, including Aβ, apoE, CLU, albumin, complement C4 and vitronectin were significantly upregulated in the cerebral blood vessels of CAA patients as compared to non-CAA patients. Aβ was found only in CAA patients. Albumin was found in all patients of CAA and non-CAA groups. ApoE and CLU were found in all patients of the CAA group but were found in only 1 patient of the non-CAA group (20%). Both apoE and CLU are representative amyloid signature proteins [2]. Thus, in the following study, we analysed the effects of apoE and CLU on Aβ amyloid formation in two different in vitro systems.

The effects of apoE and CLU on the seeded aggregation of Aβ(1–40) amyloid fibrils
We first used the conventional in vitro experimental system with an air-water interface to examine the effects of apoE and CLU on the seeded aggregation of Aβ(1–40) amyloid. In the absence of apoE and CLU, ThT fluorescence increased rapidly with no lag time to reach a plateau at around 2 h after initiation of the reaction (Fig. 3), consistent with the first-order kinetic model of amyloid fibril growth in vitro [25]. As shown in Fig. 3, both apoE and CLU concentration-dependently inhibited the formation of Aβ amyloid in this system.

The effects of apoE and CLU on the duration of the lag phase of Aβ aggregation in an in vitro model of CAA
As shown in Fig. 4, apoE3 delayed the initiation time of fibril growth kinetics in a concentration-dependent manner, indicating that apoE3 inhibited early phase Aβ aggregation. Importantly, apoE3 significantly inhibited the early phase aggregation of Aβ when at a concentration that is physiological in the cerebrospinal fluid (CSF) (1.8–4.0 μg/ml or 0.05–0.12 μM) [28]. CLU also concentration-dependently
delayed the initiation time of fibril growth kinetics (Fig. 5), significantly inhibiting the early phase of Aβ aggregation when at a concentration that is physiological in CSF (3.5–5.7 μg/ml or 0.07–0.11 μM) [33]. As shown in Fig. 6, the same concentrations of apoE3 and E4 exhibited no significant isoform-dependent difference in their ability to inhibit the early phase aggregation of Aβ.

We previously reported that under these same experimental conditions, 0.1–1.0 mg/ml (1.5–15.1 μM) HSA has no significant effect on the early phase of Aβ aggregation [10]. Thus, we conclude that apoE and CLU specifically and concentration-dependently inhibit the early phase of Aβ aggregation.

Discussion

In this study, we first performed proteome analysis of the Aβ-deposited leptomeningeal and cortical vessels (Tables 1 and 2). To the best of our knowledge, this is the first report of the proteome analysis of vessels biopsied from living symptomatic, clinical CAA patients. In previously published reports, proteome analysis was performed with leptomeningeal vessels obtained from post-mortem, autopsied cases, in which Aβ deposition was histopathologically confirmed [14, 17, 20]. Although CAA is known to be more severe in posterior brain regions compared to anterior brain regions [41] and post-mortem proteome analysis was performed using the vessels derived from occipital lobes [14, 17, 20], frontal lobes were involved in 3 CAA patients and parietal and temporal lobes were involved in other 3 CAA patients (Table 1). This difference in the brain regions from which the vessels were obtained may affect the results of our proteome analysis (Table 2 vs. [14, 17, 20]). For example, this may explain why we found no significant upregulation of tissue inhibitor of metalloproteinases-3 (Table 2), in contrast to the finding of Manousopoulou et al. [20]. Additionally, the discrepancy in male/female ratio between CAA and non-CAA patients (male/female: 0/6 and 4/1, respectively) may also affect the results of our proteome analysis. Both apoE and CLU
are representative signature proteins in various types of systemic amyloidosis [2]. Thus, it is worth noting that apoE and CLU are representative Aβ-associated proteins in the vessel walls of clinical CAA cases (Table 2), as well as in those of pathological CAA cases [14, 17, 20].

Carare and coworkers proposed the IPAD pathway model [1, 22, 34]. This model constitutes that instead of the conventional lymphatics, interstitial fluid and solutes are drained from the brain parenchyma to cervical lymph nodes along BMs in the walls of cerebral capillaries and tunica media of leptomeningeal arteries.

Fig. 4 Effect of apoE3 on the kinetics of early phase aggregation of Aβ(1–40). The reaction mixture containing 5 μM Aβ(1–40), 10 μl suspensions of Matrigel-coated beads, 0.3 mg/ml HSA, PBS-NaH₂, 5 μM ThT, and 0 to 0.5 μM apoE3 was incubated at 37 °C with rotation at 1 rpm in the absence of an air-water interface (30 replicate wells in each case). As a positive control, the reaction mixture containing 5 μM Aβ(1–40), 0.3 mg/ml HSA, PBS-NaH₂, and 5 μM ThT was also incubated at 37 °C with rotation at 1 rpm in the presence of an air-water interface (12 replicate wells) (Air). The kinetics of early phase aggregation was analysed by the Kaplan-Meier survival method using the initiation time of fibril growth kinetics as the event of interest (see Fig. 2). The P value was less than 0.05 between all combinations. This data is representative of three independent experiments.

Fig. 5 Effect of CLU on the kinetics of early phase aggregation of Aβ(1–40). The reaction mixture containing 5 μM Aβ(1–40), 10 μl suspensions of Matrigel-coated beads, 0.3 mg/ml HSA, PBS-NaH₂, 5 μM ThT, and 0 to 1.0 μM CLU was incubated at 37 °C with rotation at 1 rpm in the absence of an air-water interface (18 replicate wells in each case). As a positive control, the reaction mixture containing 5 μM Aβ(1–40), 0.3 mg/ml HSA, PBS-NaH₂, and 5 μM ThT was also incubated at 37 °C with rotation at 1 rpm in the presence of an air-water interface (18 replicate wells) (Air). The kinetics of early phase aggregation was analysed by the Kaplan-Meier survival method using the initiation time of fibril growth kinetics as the event of interest (see Fig. 2). The P value was less than 0.05 between all combinations except for between 0.25 and 0.5 μM CLU. This data is representative of three independent experiments.
Through this pathway, Aβ peptides, especially Aβ(1–40) produced by cortical neurons are carried away from the brain parenchyma [1, 22]. Reduced Aβ trafficking through this pathway may result in the aggregation of Aβ amyloid in the cerebrovascular BMs, leading to the manifestation of pathological as well as clinical CAA [41]. However, the driving mechanisms of this pathway and the molecules affecting the trafficking and aggregation of Aβ in this pathway are not fully understood [41, 43]. We previously established a simple yet powerful in vitro model of CAA, which recapitulates the IPAD flow draining Aβ and other solutes, as well as the vascular BM as a scaffold for Aβ aggregation in vitro [10]. To the best of our knowledge, this is the first, and the only in vitro system which recapitulates the pathogenesis of CAA in a physiologically relevant manner (Fig. 1). First, we completely removed the air-water interface, which is a hydrophobic-hydrophilic interface that potently induces protein denaturation and amyloid formation [21]. Complete removal of the air-water interface makes it possible to detect weak effects of BM molecules on the induction of amyloid formation in vitro. Second, we reduced Aβ concentration as low as possible (5 μM) to inhibit the spontaneous aggregation of Aβ in the reaction mixture. We also added HSA (4.5 μM) to mimic the CSF environment. Finally, we reproduced the IPAD flow in vitro by gently rotating the plate at 1 rpm. As Matrigel-coated beads slowly sink from the top to the bottom of a well, their surfaces are exposed to the relative countercurrent of the reaction mixture to induce the interaction of Aβ with BM molecules. Using this model, we have demonstrated that apoE and CLU inhibit the early phase aggregation of Aβ in vitro (Figs. 4 and 5).

Although apoE is considered as a key player in the pathogenesis of Alzheimer’s disease (AD) and CAA [16, 32], the effects of apoE on the aggregation of Aβ in vitro and in vivo are controversial. While some groups reported that apoE accelerates Aβ fibril formation in vitro [31, 35], we and other groups reported that apoE inhibits Aβ aggregation in vitro [6, 8, 15, 24, 37]. These opposite effects may be partly due to the difference in the concentrations of Aβ used in each experiment. We previously reported that apoE may inhibit or enhance Aβ amyloid fibril formation in a concentration-dependent manner [24]. When 50 μM Aβ(1–40) was incubated with 50–500 nM apoE, apoE dose-dependently inhibited Aβ amyloid fibril formation. In contrast, when 300 μM Aβ(1–40) was incubated with 3 μM apoE, apoE slightly enhanced Aβ aggregation. Similarly, while some groups reported that apoE promotes Aβ amyloid deposition in vivo [12], other groups showed that apoE delays Aβ amyloid deposition in vivo [4, 7, 13, 15, 32]. LaDu and coworkers produced EFAD mice, which are a tractable familial AD-transgenic (FAD-Tg) mouse model expressing human APOE rather than mouse APOE [32]. Consistent with our data (Fig. 4), they showed that introduction of human APOE to EFAD mice delays extracellular Aβ accumulation (not only plaque deposition but also CAA) from ~2 to 6 months compared with the control 5xFAD mice expressing mouse APOE.
They suggested that the mouse apoE is structurally and functionally distinct from human apoE.

The pathogenesis of AD and CAA is affected by apoE isoform-dependently [16, 32]. Robust data confirmed that ε4 allele of APOE is not only the risk factor of AD but also that of nonhemorrhagic-type CAA [3, 16, 32, 40]. In contrast, while the ε2 allele of APOE is protective for the manifestation of AD, it is a risk factor of hemorrhagic-type CAA [3, 16, 32, 40]. Tai et al. reported that the ability of human APOE to delay the extracellular Aβ accumulation in EFAD mice was in the order of 5xFAD < E4FAD < E3FAD ≤ E2FAD [32]. Consistent with this in vivo observation, Hori et al. reported that the in vitro conversion of Aβ protofibrils to fibrils progressed more slowly upon co-incubation with apoE2 or apoE3 compared to the case with apoE4 [15]. In contrast, we found that the inhibitory effect of apoE on the kinetics of early phase Aβ aggregation was not significantly different from that of apoE4 (Fig. 6). It is hypothesized that apoE affects the pathogenesis of AD and CAA through a variety of mechanisms, including the effects on Aβ aggregation, Aβ transport and clearance from the interstitial/cerebrospinal fluid, and cellular metabolism of Aβ [16]. Thus, the linkage of ε4 allele of APOE to the manifestation of nonhemorrhagic-type CAA might result from mechanisms other than the direct effects of apoE on Aβ aggregation. Future studies are eagerly awaited to resolve this issue.

The pathogenesis of AD and CAA is affected by CLU [11, 36, 38]. Wilson and coworkers reported that CLU inhibits Aβ aggregation in vitro [26, 39, 42]. Consistent with our data (Fig. 5), Yerbury et al. reported that CLU significantly inhibits Aβ amyloid fibril formation at a molar ratio of CLU:Aβ =1:100 [42]. The effects of CLU on the aggregation of Aβ in vivo are controversial. While DeMattos et al. reported that CLU promotes amyloid plaque formation in vivo [5], Qi et al. reported that CLU reduces Aβ plaques as well as the severity of CAA in vivo [30]. Interestingly, DeMattos et al. reported that apoE+/+/Clu−/− PDAPP mice had both earlier onset and marked increase of Aβ deposition, suggesting that apoE and CLU cooperatively lower the Aβ level and suppress deposition [4]. Importantly, Wojtas et al. found a marked decrease in cortical plaque deposition but an equally striking increase in CAA in the brains of APP/PS1/Clu−/− mice as compared to Clu+/+ mice. They proposed that CLU facilitates Aβ clearance along the IPAD pathway by preventing binding of Aβ to cerebrovascular BMs [36]. This model is consistent with our data indicating that CLU acts as an extracellular chaperone to prevent the manifestation of CAA.

Finally, we consider how apoE and CLU inhibit amyloid formation in vitro. Based on a nucleation-dependent polymerization model [19, 23], we confirmed that apoE and CLU inhibit the early phase of Aβ aggregation (Figs. 4 and 5) as well as the seeded aggregation of Aβ amyloid fibrils (Fig. 3). Hori et al. reported that apoE interacts with Aβ protofibrils in the order of apoE2 = E3 > E4, leading to the inhibition of the conversion of Aβ protofibrils to fibrils [15]. Narayan et al. reported that CLU binds Aβ oligomers formed during the aggregation of Aβ monomers, thereby inhibiting the further growth of these oligomers into mature amyloid fibrils [26]. These reports may indicate that apoE and CLU interact with Aβ nuclei/on-pathway oligomers formed on the Matrigel-coated beads, leading to the inhibition of the successive aggregation of these species into amyloid fibrils (Figs. 4 and 5). Additionally, we previously reported that, like CLU, apoE inhibits the in vitro growth of Aβ amyloid fibrils by binding and sequestering Aβ monomers [24] (Fig. 3).

Conclusions

We performed proteome analysis with the vessels biopsied from symptomatic, clinical CAA patients and confirmed that the expression of both apoE and CLU are significantly increased in the vessels of CAA patients as compared to non-CAA patients. Next, we used a unique in vitro model of CAA to confirm that apoE and CLU specifically inhibit the early phase of Aβ aggregation on the surface of BM-coated beads. The interaction of Aβ with vascular BMs may be a promising therapeutic target for CAA. Future studies are essential to develop the therapeutics for CAA.

Abbreviations

AD: Alzheimer’s disease; apoE: apolipoprotein E; Aβ: amyloid β; BM: Basement membrane; CAA: Cerebral amyloid angiopathy; CLU: Clusterin; CSF: Cerebrospinal fluid; fAβ(1–40): Aβ(1–40) amyloid fibrils; HSA: Human serum albumin; IPAD: Intramural perianterial drainage; LC:M: Laser capture microdissection; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; MRI: Magnetic resonance imaging; NSAF: Normalized spectral abundance factor; PBS: Phosphate buffered saline; SRPX1: Sushi repeat-containing protein 1; ThT: Thioflavin T

Acknowledgements

The authors thank N Takimoto and H Okada for excellent technical assistance.

Funding

This work was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (B) 16H05170 (HN. and KN).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

Study concept and design: YE, KH, HN. Acquisition analysis and interpretation of the data: YE, KH, RN, HA, KK, TY, YY, MU, YA, MWR, HN. Critical revision of the manuscript for intellectual content: KH, MWR, TH, YN, HN. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Fukui University Hospital.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1Second Department of Internal Medicine, University of Fukui, Fukui 910-1193, Japan. 2Department of Molecular Pathology, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan. 3Department of Neurosurgery, University of Fukui, Fukui 910-1193, Japan. 4Department of Neurology, Graduate School of Medical Sciences, University of Kumamoto, Kumamoto 860-8555, Japan. 5School of Chemistry and Molecular Bioscience, Illawarra Health and Medical Research Institute (IHMRI), and Molecular Horizons Research Institute, University of Wollongong, Wollongong, New South Wales 2522, Australia. 6Department of Aging and Dementia, University of Fukui, Fukui 910-1193, Japan.

Received: 14 December 2018 Accepted: 18 January 2019
Published online: 28 January 2019

References
1. Albargothy NJ, Johnston DA, MacGregor-Sharp M, Weller RO, Verma A, Hawkes CA et al (2018) Convective influence/lymphatic system: tracers injected into the CSF enter and leave the brain along separate periallelal basement membrane pathways. Acta Neuropathol 136(1):139–152
2. Brambilla F, Lavatelli F, Di Silvestre D, Valentinii V, Palladini G, Merlini G et al (2013) Shotgun protein profile of human adipose tissue and its changes in relation to systemic amyloidoses. J Proteome Res 12(12):5642–5655
3. Chandranou A, Martinez-Ramirez S, Shaomanesh A, Olivera-Filo J, Frosch M, Vashkevich A et al (2015) Cerebral amyloid angiopathy with and without hemorrhage: evidence for different disease phenotypes. Neurology 84(12):1206–1212
4. DeMattos RB, Crito JR, Pasadanian M, May PC, O’Dell MA, Taylor JW et al (2004) ApoE and clusterin cooperatively suppress Aβ levels and deposition: evidence that apoE regulates extracellular Aβ metabolism in vivo. Neuron 41:2193–202
5. DeMattos RB, O’Dell MA, Pasadanian M, Taylor JW, Harmony JA, Bales KR et al (2002) Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer’s disease. Proc Natl Acad Sci U S A 99(16):10843–10848
6. Evans KC, Berger EP, Cho CG, Weisgraber KH, Lansbury PT Jr (1995) Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: implications for the pathogenesis and treatment of Alzheimer disease. Proc Natl Acad Sci U S A 92(3):763–767
7. Fagan AM, Watson M, Pasadanian M, Bales KR, Paul SM, Holtzman DM (2002) Human and murine apoE markedly alters Aβ metabolism before and after plaque formation in a mouse model of Alzheimer’s disease. Neurobiol Dis 9(3):305–318
8. Garai K, Verghese PB, Baban B, Holtzman DM, Frieden C (2014) The binding of apolipoprotein E to oligomers and fibrils of amyloid-β alters the kinetics of amyloid aggregation. Biochemistry 53(40):6323–6331
9. Greenberg SM, Vonsattel JP (1997) Diagnosis of cerebral amyloid angiopathy. Sensitivity and specificity of cortical biopsy. Stroke 28(7):1418–1422
10. Hasegawa K, Ozawa D, Oooski T, Naka H (2013) Surface-bound basement membrane components accelerate amyloid-β peptide nucleation in air-free wells: an in vitro model of cerebral amyloid angiopathy. Biochim Biophys Acta 1834(8):1624–1631
11. Holtzman DM (2004) In vivo effects of apoE and clusterin on amyloid-β metabolism and neuropathology. J Mol Neurosci 23(3):247–254
12. Holtzman DM, Bales KR, Tenkova T, Fagan AM, Pasadanian M, Sartorius LJ et al (2000) Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer’s disease. Proc Natl Acad Sci U S A 97(6):2892–2897
13. Holtzman DM, Bales KR, Wu S, Bhat P, Pasadanian M, Fagan AM et al (1999) Expression of human apolipoprotein E reduces amyloid-β deposition in a mouse model of Alzheimer’s disease. J Clin Invest 103(6):R15–R21
14. Hondius DC, Eigenhuis KN, Moerema TH, van der Schors RC, van Nierop P, Bugiani M et al (2018) Proteomics analysis identifies new markers associated with capillary cerebral amyloid angiopathy in Alzheimer’s disease. Acta Neuropathol Commun 6(1):46
15. Hori Y, Hashimoto T, Nomoto H, Hyman BT, Iwatsubo T (2015) Role of apolipoprotein E in β-amyloidogenesis: isoform-specific effects on protofibril to fibril conversion of Aβ in vitro and brain Aβ deposition in vivo. J Biol Chem 290(24):15163–15174
16. Huyhn TV, Davis AA, Ulrich JD, Holtzman DM (2017) Apolipoprotein E and Alzheimer’s disease: the influence of apolipoprotein E on amyloid-B and other amyloidogenic proteins. J Lipid Res 58(5):824–836
17. Inoue Y, Ueda M, Tasaki M, Takeshima A, Nagatoshi A, Masuda T et al (2017) Sushi repeat-containing protein 1: a novel disease-associated molecule in cerebral amyloid angiopathy. Acta Neuropathol 134(6):605–617
18. Lin CM, Arishima H, Kikuta KI, Naiki H, Kitali R, Kodera T et al (2018) Pathological examination of cerebral amyloid angiopathy in patients who underwent removal of lobar hemorrhages. J Neurol 265(3):567–577
19. Loamak A, Chung DS, Benedek GB, Kirschner DA, Teplow DB (1996) On the nucleation and growth of amyloid β-protein fibrils: detection of nuclei and quantitation of rate constants. Proc Natl Acad Sci U S A 93(3):1125–1129
20. Manousopoulou A, Gatherer M, Smith C, Nicol JAR, Woellk CH, Johnson M et al (2017) Systems proteomic analysis reveals that clusterin and tissue inhibitor of metalloproteinases 3 increase in leptomeningeal arteries affected by cerebral amyloid angiopathy. Neuropathol Appl Neurobiol 43(6):492–504
21. Morinaga A, Hasegawa K, Nomura R, Oooski T, Ozawa D, Goto Y et al (2010) Critical role of interfaces and agitation on the nucleation of Aβ amyloid fibrils at low concentrations of Aβ monomers. Biochim Biophys Acta 1804(6):986–995
22. Morris AW, Sharp MM, Albargothy NJ, Fernandes R, Hawkes CA, Verma A et al (2016) Vascular basement membranes as pathways for the passage of fluid into and out of the brain. Acta Neuropathol 131(2):725–736
23. Naiki H, Gelyo F (1999) Kinetic analysis of amyloid fibril formation. Methods Enzymol 309:305–318
24. Naiki H, Gelyo F, Nakakuki K (1997) Concentration-dependent inhibitory effects of apolipoprotein E on Alzheimer’s β-amyloid fibril formation in vitro. Biochemistry 36(22):6434–6430
25. Naiki H, Nakakuki K (1996) First-order kinetic model of Alzheimer’s β-amyloid fibril extension in vitro. Lab Invest 74(2):374–383
26. Narayan P, Orte A, Clarke RW, Bolognesi B, Hook S, Ganzinger KA et al (2011) The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid-β(1-40) peptide. Nat Struct Mol Biol 19(1):79–83
27. Paolotti AC, Parmley TJ, Tomonori-Sato C, Sato Z, Zhu D, Conway RA et al (2006) Quantitative proteomic analysis of distinct mammalian mediator complexes using normalized spectral abundance factors. Proc Natl Acad Sci U S A 103(50):18928–18933
28. Pittas RE, Boyles JK, Lee SH, Hui D, Weisgraber KH (1987) Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B, E (LDL) receptors in the brain. J Biol Chem 262(29):14352–14360
29. Poon S, Rydchyn MS, Easterbrook-Smith SB, Carver JA, Pankhurst GL, Wilson MR (2002) Mildly acidic pH activates the extracellular molecular chaperone clusterin. J Biol Chem 277(42):39532–39540
30. Qian XM, Wang C, Chu XK, Li G, Ma JF (2018) Intraventricular infusion of clusterin ameliorated cognition and pathology in Tg6799 model of Alzheimer’s disease. BMC Neurosci 19(1):2
31. Sanan DA, Weisgraber KH, Russell SJ, Mahley RW, Huang D, Saunders A et al (1994) Apolipoprotein E associates with β amyloid peptide of Alzheimer’s disease to form novel monofibrils. Isoform apoE4 associates more efficiently than apoE3. J Clin Invest 94(2):860–869
32. Tai LM, Balu D, Avila-Munoz E, Abdullah I, Thomas R, Collins N et al (2017) EFAD transgenic mice as a human APOE relevant preclinical model of Alzheimer’s disease. J Lipid Res 58(9):1733–1755
33. van Dijk KO, Jongbloed W, Heijst JA, Teunissen CE, Groenewegen HJ, Berendse HW et al (2013) Cerebrospinal fluid and plasma clusterin levels in Parkinson’s disease. Parkinsonism Relat Disord 19(12):1079–1083
34. Welker RO, Preston SD, Subash M, Carare RO (2009) Cerebral amyloid angiopathy in the aetiology and immunotherapy of Alzheimer disease. Alzheimers Res Ther 1(2):6
35. Wisniewski T, Castaño EM, Golabek A, Vogel T, Frangione B (1994) Acceleration of Alzheimer’s fibril formation by apolipoprotein E in vitro. Am J Pathol 145(5):1030–1035
36. Wojtas AM, Kang SS, Olley BM, Gatherer M, Shinohara M, Lozano PA et al (2017) Loss of clusterin shifts amyloid deposition to the cerebrovasculature via disruption of perivascular drainage pathways. Proc Natl Acad Sci U S A 114(33):E6962–E6971
37. Wood SJ, Chan W, Wetzol R (1996) Seeding of Aβ fibril formation is inhibited by all three isotypes of apolipoprotein E. Biochemistry 35(38):12623–12628
38. Wyatt AR, Yerbury JJ, Dabbs RA, Wilson MR (2012) Roles of extracellular chaperones in amyloidosis. J Mol Biol 421(4–5):499–516
39. Wyatt AR, Yerbury JJ, Ecroyd H, Wilson MR (2013) Extracellular chaperones and proteostasis. Annu Rev Biochem 82:295–322
40. Yamada M (2015) Cerebral amyloid angiopathy: emerging concepts. J Stroke 17(1):17–30
41. Yamada M, Naiki H (2012) Cerebral amyloid angiopathy. Prog Mol Biol Transl Sci 107:1–78
42. Yerbury JJ, Poon S, Meehan S, Thompson B, Kumita JR, Dobson CM et al (2007) The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. FASEB J 21(10):2312–2322
43. Zekonyte J, Sakai K, Nicoll JA, Weller RO, Carare RO (2016) Quantification of molecular interactions between apoE, amyloid-β (Aβ) and laminin: relevance to accumulation of Aβ in Alzheimer’s disease. Biochim Biophys Acta 1862(5):1047–1053