A distinct brain beta amyloid signature in cerebral amyloid angiopathy compared to Alzheimer´s disease

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Abstract

Cerebral amyloid angiopathy (CAA) is a type of vascular disease present in more than 50% of demented elderly and more than 80% of Alzheimer's disease (AD) patients. Both CAA and AD are characterized by extracellular Aβ deposits with the distinction that CAA has vascular deposits while AD has amyloid plaques. In this study, we used immunoprecipitation (IP) in combination with mass spectrometry (MS) to test the hypothesis that the Aβ peptide pattern differs between subjects having Aβ plaque pathology only or Aβ plaque pathology together with CAA pathology. Occipital lobes from 12 AD brains, ranging from no CAA to severe CAA, were extracted using 70% formic acid followed by IP-MS analysis. The Aβ peptide pattern differed greatly between subjects with no CAA compared to subjects with CAA. In cases with CAA, the most abundant Aβ peptides ended at amino acid 40 including Aβ1-40 (P=.048) and Aβ 2-40 (P=.0253) which were significantly increased compared to cases with no CAA. This was in contrast to subjects with no CAA where the most abundant Aβ peptides ended at amino acid 42 of which Aβ1-42 (P=.0101) and Aβ2-42 (P=.0051) as well as the pyroglutamate (pGlu)-modified peptides pGlu Aβ3-42 (P=.0177), and pGlu Aβ11-42 (P=.0088) were significantly increased compared to CAA subjects. The results are in line with earlier immunohistochemistry data and show that the molecular composition of the Aβ deposits found in blood vessels are different to the parenchymal deposits, suggesting they arise from distinct pathogenic pathways. This information may be useful in the development of pathology-specific biomarkers.

Key Words: Alzheimer's disease, cerebrovascular amyloid angiopathy, amyloid beta, brain, immunoprecipitation, mass spectrometry

[1]Highlights

- Distinct difference in Aβ peptide pattern in subjects with and without CAA
- Aβ peptides ending at amino acid 42 are more abundant in AD subjects with no CAA
- Aβ peptides ending at amino acid 40 are more abundant in CAA subjects

Introduction

Cerebral amyloid angiopathy (CAA) is a disease characterized by the deposition of amyloid beta (Aβ) peptides in the walls of cerebral, leptomeningeal and parenchymal arteries and small-to-medium-sized blood vessels [1, 2]. Neocortical regions are primarily affected [3], with the occipital lobe particularly vulnerable [4-6]. There is a close correlation between CAA and dementia since CAA is present in more than 80% of patients with Alzheimer's disease (AD) and up to 40% of cognitively unimpaired elderly individuals [7, 8]. It has previously been shown that CAA can directly contribute to cognitive decline and dementia by causing vascular lesions, such as (micro) haemorrhage and cerebral ischaemia, and inflammatory changes. Pathologically, CAA can be divided into two major sub-types [9]: type 1, where Aβ is present in both the capillaries and non-capillary blood vessels, and type 2, where there is no Aβ deposition in the capillaries.

In the mid-1980s the deposits in CAA were identified to be composed mainly of Aβ [10]. Aβ in the vessel walls may originate from the peripheral blood, from the direct production by the vessel smooth muscle or endothelial cells, or from the perivascular drainage of neuronal Aβ from the brain parenchyma [1, 11]. One hypothesis is that failure of eliminating neuronal-derived Aβ by the perivascular drainage pathway results in an increase of Aβ, which in turn may lead to CAA and cognitive decline [13, 14].

CAA has both hereditary (missense mutations in the *APP* gene, like *HCHWA-D* [15] and *BRI2 [16]* gene related dementias, and mutations that interfere with the enzymatic degradation of amyloid precursor protein (APP), like *PSEN1* and *PSEN2* [17]) and more common sporadic forms. One of the main genetic risk factors for both sporadic AD and CAA is the *APOE* gene. The ε4 allele is associated with increased Aβ deposition in both plaques and vessels [18-20]. In CAA, it is thought that the ε4 isoform of ApoE contributes to less efficient clearance of Aβ from the brain parenchyma [21-23] by causing changes in the structure and function of the capillary and arterial membranes. Moreover, *APOE* ε2, which is protective against AD [24], appears to be disease-promoting in CAA [25, 26]. *APOE* ε4 carriers are more common in CAA-type 1 while *APOE* ε2 carriers are more common in CAA-type 2 [9].

Aβ peptides are produced by enzymatic processing of the transmembrane APP by β- and γ-secretase in a amyloidogenic pathway, generating Aβ peptides of different lengths of which Aβ

peptides ending at amino acid 42 (Aβ42) are most prone to aggregation [27]. APP can also undergo combined cleavage by α- and γ-secretases in a non-amyloidogenic pathway that precludes the formation of full-length Aβ [28-32]. Overproduction of amyloidogenic Aβ peptides and/or insufficient clearance leads to Aβ aggregation that, according to the amyloid cascade hypothesis, eventually causes AD dementia [33].

Aβ deposits in the brain can differ significantly between different conditions, such as pathological ageing [34] and AD [35]. Molecular subtypes of AD have also been reported, based on the relative abundance of observed Aβ peptides, each of which show different aggregation kinetics and resistance to degradation [36]. Hitherto, Aβ42 is one of the most well characterized diagnostic biomarkers for AD and it is well established that the concentration of Aβ42 is decreased in cerebrospinal fluid (CSF) from AD patients compared to healthy controls [37]. CSF Aβ40 may be used to normalize Aβ42 concentrations for inter-individual variation in the release of Aβ species into the CSF, making the CSF Aβ42/40 ratio an even better marker for Aβ plaque pathology than Aβ42 alone [38]. In CAA, it has previously been shown that the CSF levels of both Aβ40 and Aβ42 are decreased [39]. One potential explanation for this result is the high content of Aβ40 in blood vessel walls [11].

The aim of this study was to characterize the full spectrum of Aβ peptides present in individuals having an Aβ neuropathologic change qualifying them to be AD patients, and compare subjects also exhibiting CAA pathology (AD/CAA+ and CAA+) with subjects having no CAA pathology (AD/CAA-). To understand the molecular composition of these anatomically distinct pathologies, Aβ was extracted from occipital lobes of 12 AD subjects, ranging from no CAA to severe CAA, using formic acid (FA), immunoprecipitated (IP'd) and subsequently analysed by mass spectrometry (MS). We found distinct differences in the Aβ pattern between CAA negative and CAA positive groups, with Aβ4-40 being one of the most abundant peptides in CAA positive subjects and Aβ4-42 being the most abundant form in CAA negative subjects.

Material and Methods

Patient characteristics

Human post-mortem brain tissue was obtained through the brain donation program at Queen Square Brain Bank for Neurological Disorders (QSBB), Department of Clinical and Movement Neurosciences, Institute of Neurology, University College London (UCL). Standard diagnostic pathological criteria for AD and CAA were used [40-44]. AD cases were identified without CAA (AD/CAA-, n=5), with CAA (AD/CAA+, n=5) and severe CAA (CAA+, n=2); the CAA+ exhibited moderate to high Aβ pathology but were not diagnosed as AD since they did not fulfill the Braak score criteria for tau. The demographic and neuropathological classifications are shown in Table

1. The study followed the Helsinki declaration and was approved by the regional ethics committees at UCL and the University of Gothenburg.

Immunohistochemistry

Eight-µm thick sections were deparaffinized and rehydrated using xylene and graded ethanol respectively, as described previously [45]. Tissue sections were pre-treated in 100% FA for 10min, washed and further treated in citrate buffer (pH 6.0) for 10 min in a pressure cooker. Endogenous peroxidase activity was blocked by addition of 0.3% H_2O_2 in methanol for 10min and non-specific binding was blocked with 10% dried milk solution. Incubation with the primary antibody (anti-Aβ, epitope amino acids 8-17, DAKO) was performed for 1h at room temperature (RT), followed by incubation with biotinylated anti-mouse IgG for 30min at RT and avidin-biotin complex for additional 30min. Colour development was performed with di-aminobenzidine/H_2O_2, as described previously [46].

Frozen tissue preparation

Fresh frozen tissue (~90-110mg pieces, consisting of both grey and white matter) from occipital lobe was homogenized in 500µL tris(hydroxymethyl)aminomethane (Tris) buffered saline (TBS), pH 7.6, containing complete protease inhibitor per 100mg tissue in a TissueLyser (Qiagen) for 4min at 30Hz. The TBS-soluble fraction (~550mL) was discarded and the homogenate was centrifuged at 31,000×g for 1h at +4°C and the pellet was resuspended in 1ml of 70% FA, followed by further homogenization in the TissueLyser for 2min at 30Hz and subsequent sonication for 30s. The homogenate was centrifuged again and the supernatant (FA-soluble fraction) was dried down in a vacuum centrifuge. The TBS-soluble fraction was not included for practical reasons; however, we have previously analysed its Aβ content and found it to be minor compared to that of the FA-soluble fraction analysed.

Immunoprecipitation

Dried FA-soluble fractions were reconstituted in 20µL 70% FA, shaken for 30min at RT and centrifuged again at 31,000g for 1h at +4°C. The supernatant was removed and neutralized with 0.5M Tris before IP.

IP was performed with a KingFisher magnetic particle processor as previously described with some modifications [47]. Briefly, 4µg of the Aβ-specific antibodies 6E10 and 4G8 were separately added to 25µL of Dynabeads M-280 sheep anti-mouse suspension, according to the manufacturer's product description. The washed antibody-bead complexes were combined (50µL in total) and added to the neutralized FA fraction together with 20% (v/v) Triton X-100 to a final concentration of 0.2% (v/v) and incubated over night at +4°C. The beads/FA fraction was transferred to the KingFisher for automatic washing (in 0.2% Triton X-100, phosphate buffered

saline (PBS), pH 7.6, and 50mM ammoniumbiocarbonate) and elution in 0.5% FA. The eluate was dried down in a vacuum centrifuge pending MS analysis. From recovery experiments we estimated the efficiency of the IP to be ~80%.

Mass spectrometry

Prior to MS analysis, samples were reconstituted in 5µL 0.1% FA in 20% acetonitrile. MS analysis was performed using a Bruker Daltonics UltraFleXtreme matrix-assisted-laser-desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) instrument. MALDI samples were prepared using the seed layer method as previously described [48]. An average of 10,000 shots were acquired for each spectrum (2,000 at a time using a random walk mode). Individual peak areas were normalized to the sum of the four generally most abundant Aβ peak areas (1-40, 1-42, 4-42, and 5-42) before further analysis. At different stages in the sample extraction and preparation, quality control experiments were performed, by IP of both CSF and brain control samples, to ensure that the protein extraction and the IP performed normally.

For a more detailed analysis, we performed nanoflow liquid chromatography (LC) coupled to electrospray ionization (ESI) hybrid quadrupole–orbitrap tandem MS (Dionex Ultimate 3000 system and Q Exactive, both Thermo Fisher Scientific) in a similar way as described previously [49]. Samples were reconstituted in 7µL 8% FA/8% acetonitrile in water (v/v/v). An Acclaim PepMap 100 C18 trap column (length: 20mm; inner diameter: 7 µm; particle size: 3µm; pore size: 100Å) was used for online desalting, and a reversed-phase Acclaim PepMap RSLC column (length: 150mm, inner diameter: 75µm; particle size: 2µm; pore size: 100Å) was used for separation (both Thermo Fisher Scientific). Mobile phases were 0.1% FA in water (v/v) (A) and 0.1% FA/84% acetonitrile in water (v/v/v) (B). The separation was performed at a flow rate of 300nL/min by applying a linear gradient of 3% to 40% B for 50min at 60°C. The mass spectrometer was operated in positive ion mode and set to acquire spectra between 350 and 1,800 mass-to-charge (m/z) units. Both MS and MS/MS acquisitions were obtained at a resolution setting of 70,000 using 1 microscan. MS/MS acquisitions were obtained using higher-energy collisional dissociation fragmentation (HCD) using a normalized collision energy (NCE) setting of 25, exclusion of singly charged ions and ions with unassigned charge, target values of 10^6, and maximum injection time of 250ms. Database search (including isotope and charge deconvolution) was performed with PEAKS Studio v8.5 (Bioinformatics Solutions Inc.) against a custom made APP database. All suggested fragment mass spectra were then evaluated manually.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v7.02. Since the CAA+ group only consisted of two cases, this group was combined with the AD/CAA+ group and this combined group (CAA positive) was compared to AD/CAA- (CAA negative) for the statistical analysis. Since

the groups were not normally distributed the Mann-Whitney U-test was used to test the statistical significance.

Results

Demographics and immunohistochemical characterization of CAA and plaque pathology

Demographic data for the 12 cases included in the study are shown in Table 1. The severity of both amyloid plaque load and amyloid cerebrovascular load was determined on the basis of the extent of immunohistochemical staining. Patients were assigned into three different groups, where the neuropathological diagnosis was AD with no CAA (AD/CAA- , n=5), AD with CAA (AD/CAA+, n=5) and severe CAA (CAA+, n=2). Representative images of the immunohistochemical staining of each group are shown in Figure 1.

Distinct Aβ peptide patterns in patients with and without CAA pathology

We compared the relative levels of different Aβ peptides, using MALDI-TOF/TOF MS, across different groups. Most of the Aβ peptides were present in all groups including Aβ1-40, Aβ1-42, Aβ2-40, Aβ2-42, Aβ4-40, Aβ4-42, Aβ5-42, as well as the pyroglutamate-modified pGLu Aβ3-40, pGlu Aβ3-42, and pGlu Aβ11-42 forms. On average, Aβ4-42, Aβ1-42, Aβ5-42, pGLu Aβ3-42, and Aβ1-40 were, in order, the most abundant peptides in cases with AD/CAA- (Figure 2A and D). Contrary to this, the most abundant peptides for the combined CAA positive group (AD/CAA+ and CAA+) were, in order, Aβ1-40, Aβ4-42, Aβ4-40, Aβ4-42, and pGLu Aβ3-40 (Figure 2B, C and D). Two of the AD/CAA+ patients (8 and 9) exhibited an Aβ pattern similar to AD/CAA-; *i.e.*, Aβ4-42 was the most abundant in these samples. In addition, two of the cases in the combined CAA positive group (patients 10 and 11) also had abundant Aβ 1-37/38/39 peaks (Figure 2D). The relative levels of Aβx-42 was higher in AD/CAA-, while Aβx-40 was higher for AD/CAA+ and CAA+ cases (Figure 2D and 3). Significant differences between the AD/CAA- and the combined CAA positive group were observed for a number of the more abundant peptides including Aβ1-42 (P=0.0101), Aβ2-42 (P=0.0051), pGlu Aβ3-42 (P=0.048), and pGlu Aβ11-42 (P=0.0088) which were higher in AD/CAA- group compared to the combined CAA positive group, while Aβ1-40 (P=0.048) and Aβ2-40 (P=0.048) was higher in the combined CAA positive group (Figure 3).

High resolution MS identification of Aβ peptides in AD/CAA-, AD/CAA+ and CAA+ cases

Analysis with LC-MS allowed a more in depth identification of low abundant Aβ peptides that MALDI measurement were unable to detect. In total, 126 endogenous Aβ peptides (including oxidized and pyroglutamate forms) were identified in the sample set. Sixty of the peptides were not detected in the AD/CAA- group; only in the combined CAA positive group. The two cases (10, 11) that had abundant 1-37/38/39 peaks in the MALDI-TOF/TOF analysis were confirmed with the LC-MS analysis. Case 11 from the CAA+ group also exhibited many shorter Aβ peptides, truncated either at the N- or the C-terminus, although the signal of these peptides were low

compared to those ending at amino acids 37-42. A summary of all the Aβ peptides identified is shown in Suppl. Table 1.

Discussion

Using IP-MS of brain tissue extracts from patients with and without CAA, we tested the hypothesis that the brain Aβ peptide profile in AD-type Aβ pathology is different from the Aβ pathology of CAA. In AD/CAA- cases, there was a variety of Aβ peptides of different lengths of which Aβ1-42 and Aβ4-42 were among the most abundant whereas Aβ1-40 was found more abundantly in the AD/CAA+ and CAA+ cases. By using high resolution LC-MS, we confirmed the Aβ peptides identified with MALDI and in addition identified more than 100 endogenous Aβ peptides in all groups.

Since the CAA+ group only consisted of two individuals, these two subjects were merged with the rest of the AD/CAA+ cases for statistical analysis. As previously reported [41, 50, 51], we observed a difference in the abundance of Aβ1-40 and Aβ1-42 between CAA and AD. In the present study, we have expanded the number of Aβ peptides investigated and shown that the differences between these two groups is not limited to the full length Aβ1-40 and Aβ1-42, but also peptides such as Aβ4-40 and Aβ4-42. Significant differences between the CAA negative and combined CAA positive group were observed for six out of the nine selected high abundant peptides visible in all samples using MALDI-TOF/TOF MS. Four peptides belonging to the Aβx-42 group (1-42, Aβ2-42, pGlu Aβ3-42, and pGlu Aβ11-42), had higher relative levels in the CAA negative group compared to the combined CAA positive group, while for the Aβx-40 group (Aβ1-40 and Aβ2-40) were significantly higher in the combined CAA positive group. However, the three investigated peptides that did not differ significantly also followed the same general pattern, with Aβx-40 being higher in CAA positives and Aβx-42 being higher in CAA negative only. In line with this, immunohistochemistry has shown that CAA is characterized by high deposition of Aβx-40, contrary to Aβx-42 in plaque-only AD. What should be noted is that CAA pathology is present in more than 80% of AD cases [17, 52-56] and that CAA can also be present in patients without AD diagnosis, although more advanced CAA pathology is generally observed in AD cases compared to controls [3].

There may also be differences in Aβ peptide profiles between different CAA subtypes. This could not be formally examined in the current study due to the low number of cases. The MALDI-TOF/TOF data on two of the CAA positive samples showed the presence of Aβ1-37/38/39 peptides (Figure 2D and Suppl. Figure 1). A more detailed investigation using LC-MS/MS confirmed this finding and these peptides were also observed in other AD/CAA+ samples at a lower intensity. Moreover, particularly in one of the patients with CAA+ pathology an extensive series of shorter Aβ peptides was found (Suppl. Table 1). Their abundance was low relative to the

longer Aβ peptides ending at amino acids 37-42. This might indicate a blood contribution since many of these peptides have been previously identified in plasma [57]. One possible explanation for the variation observed in the CAA+ group may be the precise location of the Aβ deposits in the different types of CAA. Moreover, two neuropathologically diagnosed CAA subjects had Aβ patterns that were more similar to plaque-only AD. A possible explanation for this may be that these patients have an Aβ plaque pathology dominating over an Aβ CAA pathology, making the latter difficult to observe.

There are multiple potential reasons for the observed differences in the Aβ fragment profiles between AD and CAA. Firstly, the extracellular matrix of the brain parenchyma may favour aggregation of Aβ42 over Aβ40 and the opposite may be true for the vessel wall. Local production of Aβ40 and Aβ42 may also be different in the two matrices, resulting in different local concentrations, which could lead to differences in the Aβ peptide composition of the aggregates. There might also be differences in how Aβ peptides drain from the brain between different cases. In the presence of Aβ plaque pathology, newly formed Aβ42 might stick to plaques and never reach the vessels. Aβ40, on the other hand, may be soluble enough to diffuse into the perivascular spaces and there occasionally aggregate. However, this would not explain the Aβ peptide patterns observed in CAA-only cases. The differential association of *APOE* ε2 and ε4 with CAA may also provide important clues; although no such observation was made in the current study, further research on the topic is needed.

The main limitation of the study is the small cohort size, which is due to the limited availability of CAA positive brains. Other limitations include the non-gender matched groups, and the post-mortem time range of 36-134 h. Furthermore, only the relative abundances of the Aβ peptides were investigated. In addition, while the combined epitopes of 6E10 and 4G8 covered a large variety of Aβ peptides, including the major variants, a number of shorter Aβ peptides may not be included in the analysis [58]. Therefore, interpretation should be made with caution. However, the results clearly show the biochemical difference between the AD and CAA pathology in terms of Aβ peptide composition, which is an important starting point for further studies and the development of disease-specific biomarkers. To summarize, the present study should be considered a pilot and the findings need to be verified in additional, larger, cohorts.

Conclusions

CAA has an Aβ peptide pattern that is distinct from plaque-only AD, with Aβ1-40 and Aβ4-40 being more abundant in patients with CAA pathology, while Aβ1-42 and Aβ4-42 are more abundant in AD patients without the pathology. There is a clear, general pattern differentiating AD with CAA from plaque-only AD, where the relative abundance of Aβx-40 is higher in CAA and

Aβx-42 higher in AD only. These results might pave the way for the development of disease-specific Aβ biomarkers.

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Author's contribution

EG, EP, GB, HZ, and TL drafted the study design. EG carried out sample processing, MS data collection, analysis and interpretation. EP and GB assisted with MS data analysis and interpretation. CET and TL carried out the immunohistochemistry experiments and analysis, and selected patient samples for MS analysis. HZ and KB provided support and expertise. All authors participated in writing the manuscript.

Conflicts of interest

HZ has served at scientific advisory boards for Eli Lilly, Roche Diagnostics, Wave, Samumed and CogRx, has received travel support from Teva and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg.

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