

Alternative Processing of γ -Secretase Substrates in Common Forms of Mild Cognitive Impairment and Alzheimer's Disease: Evidence for γ -Secretase Dysfunction

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Objective: The most common pathogenesis for familial Alzheimer's disease (FAD) involves misprocessing (or alternative processing) of the amyloid precursor protein (APP) by γ -secretase due to mutations of the presenilin 1 (PS1) gene. This misprocessing/alternative processing leads to an increase in the ratio of the level of a minor γ -secretase reaction product (A β 42) to that of the major reaction product (A β 40). Although no PS1 mutations are present, altered A β 42/40 ratios are also observed in sporadic Alzheimer's disease (SAD), and these altered ratios apparently reflect deposition of A β 42 as amyloid.

Methods: Using immunoprecipitation-mass spectrometry with quantitative accuracy, we analyzed in the cerebrospinal fluid (CSF) of various clinical populations the peptide products generated by processing of not only APP but also an unrelated protein, alcadein (Alc). Alc undergoes metabolism by the identical APP α -secretases and γ -secretases, yielding a fragment that we have named p3-Alc_x because of the parallel genesis of p3-Alc_x peptides and the p3 fragment of APP. As with A β , both major and minor p3-Alc_xs are generated. We studied the alternative processing of p3-Alc_x in various clinical populations.

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Additional Supporting Information can be found in the online version of this article.

Results: We previously reported that changes in the A β 42/40 ratio showed covariance in a linear relationship with the levels of p3-Alc $_{\alpha}$ [minor/major] ratio in media conditioned by cells expressing FAD-linked PS1 mutants. Here we studied the speciation of p3-Alc $_{\alpha}$ in the CSF from 3 groups of human subjects (n = 158): elderly nondemented control subjects; mild cognitive impairment (MCI) subjects with a clinical dementia rating (CDR) of 0.5; SAD subjects with CDR of 1.0; and other neurological disease (OND) control subjects. The CSF minor p3-Alc $_{\alpha}$ variant, p3-Alc $_{\alpha}$ 38, was elevated ($p < 0.05$) in MCI subjects or SAD subjects, depending upon whether the data were pooled and analyzed as a single cohort or analyzed individually as 3 separate cohorts.

Interpretation: These results suggest that some SAD may involve alternative processing of multiple γ -secretase substrates, raising the possibility that the molecular pathogenesis of SAD might involve γ -secretase dysfunction.

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The most common pathogenesis for familial Alzheimer's disease (FAD) involves misprocessing/alternative processing of the amyloid precursor protein (APP) by γ -secretase (for review, see Gandy¹ and Small and Gandy²). This misprocessing/alternative processing leads to a relative increase in the ratio of the level of a minor γ -secretase reaction product, amyloid- β 42 (A β 42), to that of the major reaction product, amyloid- β 40 (A β 40; Borchelt and colleagues³). Until now, little has been known about γ -secretase function in sporadic AD (SAD).

We approached this issue by studying the metabolite peptides, p3-Alc $_{\alpha}$, that are derived from the processing by α -secretases and γ -secretases of the alcadin $_{\alpha}$ (Alc $_{\alpha}$), member of the alcadin (Alc) protein family. Alc proteins colocalize with APP in healthy mouse and SAD human brain,⁴ but they are entirely distinct from APP in their polypeptide sequence. In neurons, Alc proteins are complexed to APP via X11L adaptor molecules, raising the possibility that Alcs might be sorted and processed together with APP. Experimental evidence supports this reasoning. In the absence of X11L, both Alc and APP proteins are rapidly metabolized by proteolysis.⁵ Levels of the endogenous APP metabolite, A β , are elevated in the brains of X11L-deficient mice.^{6,7} Thus, taken together with similarities in their structure and cellular distribution, APP and Alc proteins would be predicted to undergo parallel metabolic fates (for APP and X11L, see Gandy¹ and Suzuki and Nakaya⁸; for Alc, see Araki and colleagues,^{4,5,9}).

Alc proteins exist in mammalian neurons as 4 isoforms⁴: Alc $_{\alpha 1}$, Alc $_{\alpha 2}$, Alc $_{\beta}$, and Alc $_{\gamma}$. Alc $_{\alpha}$, Alc $_{\beta}$, and Alc $_{\gamma}$ are encoded by independent genes, while Alc $_{\alpha 1}$ and Alc $_{\alpha 2}$ are splice variants derived from the Alc $_{\alpha}$ gene. All 3 members of the Alc family (Alc $_{\alpha}$, Alc $_{\beta}$, and Alc $_{\gamma}$) are cleaved by ADAM 10 and ADAM 17, which have been identified as the α -secretases for APP.^{10–13} Subsequent cleavage of the remaining Alc C-terminal fragments involves predominantly the presenilin 1-(PS1)-dependent γ -secretase, and this reaction liberates a short peptide that we have designated p3-Alc $_{\alpha}$ into cell-conditioned media and into cerebrospinal fluid (CSF). The amino acid sequences of the various alternatively cleaved p3-Alc $_{\alpha}$ peptides in human CSF are shown with those of APP-p3 and A β (Fig 1). The current study is based on the hypothesis that examination of AD-related processing

of p3-Alc $_{\alpha}$ might reveal evidence for γ -secretase dysfunction in SAD. In so doing, we seek to confirm and extend the report of Yanagida and colleagues,¹⁴ who described similar alternative processing of another γ -secretase substrate, APLP. Taken together with the data from Yanagida and colleagues,¹⁴ we suggest that multiple γ -secretase substrates are subjected to altered processing in SAD and that this potentially implicates an “acquired” γ -secretase dysfunction that might contribute the pathogenesis of SAD.

Materials and Methods

CSF Sample Collection

Standard protocols for CSF collection varied slightly according to site. Complete details of collections protocols are provided in the Supporting Information.

Aggregated characteristics and data for each cohort are shown in the Table. Detailed descriptions of all 158 subjects including clinical backgrounds from the 3 cohorts and the raw values for A β 42/40 and p3-Alc $_{\alpha}$ 38/total p3-Alc $_{\alpha}$ are shown in Supporting Information Table 1. The total p3-Alc $_{\alpha}$ used for the calculation of p3-Alc $_{\alpha}$ 38/total p3-Alc $_{\alpha}$ was the sum of all recognized p3-Alc $_{\alpha}$ species; ie, p3-Alc $_{\alpha}$ 34, p3-Alc $_{\alpha}$ 35, p3-Alc $_{\alpha}$ 36, p3-Alc $_{\alpha}$ 37, and p3-Alc $_{\alpha}$ 38. It is worth noting that for US cohort 1, but not for US cohort 2 or the Japanese cohort, CDR 0 subjects were verified as “true” controls by positron emission tomography (PET) scan analysis using [¹¹C]Pittsburgh compound B (PiB).¹⁵

Matrix-Assisted Laser Desorption/Ionization–Time of Flight/Mass Spectrometry and Matrix-Assisted Laser Desorption/Ionization–Tandem Mass Spectrometry Analysis of p3-Alc $_{\alpha}$ Secreted into Human CSF

In the initial pilot study (Fig 2), pooled CSF (300 μ l) from 5 individuals (70–90 years old) was subjected to immunoprecipitation with anti-Alc $_{\alpha}$ UT135 and anti-A β 82E1 (Immuno-Biological Laboratories/IBL, Fujiloka, Japan) antibodies. In the extended studies (Fig 3A–D), aliquots (300 μ l) of CSF from well-characterized individual subjects were derived from 2 U.S. cohorts (designated US cohort 1 and US cohort 2) and 1 Japanese cohort (see Table). Samples were subjected to immunoprecipitation individually with anti-Alc $_{\alpha}$ UT135 antibody and Protein G Sepharose.¹⁰

After washing the beads, samples were eluted with trifluoroacetic acid/acetonitrile/water (1:20:20) saturated with sinapinic acid. The dissolved samples were dried on a target plate, and matrix-assisted laser desorption/ionization–time of



FIGURE 1: Amino acid sequences and cleavage sites of p3-Alc α and A β in human CSF. The amino acid sequences of p3-Alc α (black-underline) along with the sequences of p3 (gray double-underline) and A β 40 (black double-underline) of APP. The major primary (black arrowheads) and secondary (black arrowheads) cleavage sites of Alc α 1 are indicated together with those of APP (α , the cleavage site by α -secretase or ADAM 10/17; β , the cleavage site by β -secretase or BACE). Numbers on amino acids indicate their positions. The shaded area indicates the putative transmembrane region. Alc = alcadin; APP = amyloid β protein precursor; CSF = cerebrospinal fluid.

flight/mass spectrometry (MALDI-TOF/MS) analysis was performed using an Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany). In all immunoprecipitation studies prior to MALDI-TOF/MS analysis, protease inhibitor mixture (5 μ g/ml chymostatin, 5 μ g/ml leupeptin, and 5 μ g/ml pepstatin) was added in samples to prevent nonspecific proteolysis. Molecular masses were calibrated using the peptide calibration standard (Bruker Daltonics). The quantitative accuracy of mass spectrometry analysis with immunoprecipitation was confirmed by studies with a mixture of synthetic p3-Alc α peptides as described previ-

ously.¹⁰ A β 40 and A β 42 levels were quantified with sELISA systems (Innotest; Innogenetics, Ghent, Belgium and IBL, Fujioka, Japan). All analyses were performed with operators blinded to diagnosis until data tables were generated. Diagnoses and data tables were exchanged at the time of unblinding.

Results

The recent production of an anti-Alc α antibody raised against the Alc α extracellular juxtamembrane sequences

TABLE: Summary of Subject Information

	CDR 0	CDR 0.5	CDR 1	OND
JP Cohort				
Age, yr (mean \pm SD)	79.4 \pm 0.802	75.3 \pm 2.79	76.9 \pm 1.55	
Gender (F%)	80.0	55.6	45.5	
MMSE (mean \pm SD)	—	24.7 \pm 2.37	20.3 \pm 3.57	
HDS-R (mean \pm SD)	—	23.5 \pm 1.95	17.6 \pm 2.40	
Duration of disease, yr (mean \pm SD)	—	0.78 \pm 0.171	1.85 \pm 0.700	
US cohort 1				
Age, yr (mean \pm SD)	68.8 \pm 2.85	75.5 \pm 1.26	76.2 \pm 1.73	65.6 \pm 3.18
Gender (F%)	50.0	55.0	53.8	18.8
MMSE (mean \pm SD)	28.8 \pm 0.244	26.4 \pm 0.514	23.1 \pm 1.03	
Duration of disease, yr (mean \pm SD)		2.70 \pm 0.334	4.25 \pm 0.700	
US cohort 2				
Age, yr (mean \pm SD)	71.2 \pm 1.13	74.5 \pm 2.57	70.4 \pm 2.41	73.3 \pm 1.18
Gender (F%)	53.3	30.8	45.5	23.1
MMSE (mean \pm SD)	28.5 \pm 0.325	27.0 \pm 0.361	22.4 \pm 0.875	

Details of individual subjects are shown in the Supporting Information Table.
 CDR = clinical dementia rating; F = female; HDS-R = Hasegawa's dementia scale; JP = Japanese; MMSE = Mini-Mental State Examination; OND = other neuronal and neurodegenerative diseases except for AD; SD = standard deviation; US = United States.

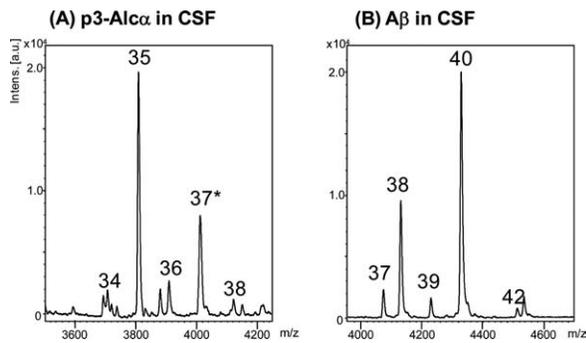


FIGURE 2: Representative MS spectra of p3-Alc_x peptides and A β peptides in human CSF. (A) p3-Alc_x in CSF and (B) A β in CSF. The CSF (300 μ l) were subjected to immunoprecipitation with (A) UT135 or (B) 82E1 antibodies, respectively. The precipitates were analyzed for molecular mass with MALDI-TOF/MS. (A) "34", p3-Alc_x34; "35", p3-Alc_x35; "36", p3-Alc_x36; "37*", a mixture of p3-Alc_x37 and p3-Alc_x2N+35 (see Hata and colleagues¹⁰ for p3-Alc_x2N+35); "38", p3-Alc_x38. (B) "37", A β 37; "38", A β 38; "39", A β 39; "40", A β 40; "42", A β 42. Alc = alcadin; CSF = cerebrospinal fluid; MALDI-TOF/MS = matrix-assisted laser desorption ionization–time-of-flight/mass spectrometry.

enabled us to recover p3-Alc_x secreted into conditioned media and CSF.¹⁰ The amino acid sequences of the major and minor p3-Alc_x were determined by matrix-assisted laser desorption/ionization–tandem mass spectrometry (MALDI-MS/MS) analysis and shown in Figure 1. The p3-Alc_x species with Thr851 as the C-terminal residue is the major species in human CSF (p3-Alc_x35), as described in detail elsewhere.¹⁰

Secondary cleavage sites, determined by MALDI-MS/MS analysis,¹⁰ are also shown in Figure 1 (black arrowheads), together with the major secondary γ -secretase-dependent cleavage sites of APP that generate A β 40 and A β 42. We have shown that HEK293 cells expressing FAD-linked PS1 mutants generated qualitatively altered p3-Alc_x, and the ratios of p3-Alc_x 38/35 [minor/major] were strongly correlated with the ratio of A β 42/40 [minor/major] ($R^2 > 0.5$).¹⁰ This suggested that altered processing of Alcs can reflect intrinsic (ie, genetic) γ -secretase dysfunction. Thus, we undertook a study of CSF from various clinical populations including sporadic AD.

In initial experiments, p3-Alc_x species were recovered from pooled human CSF samples by immunoprecipitation with anti-p3-Alc_x and anti-A β antibodies. The major and minor species of p3-Alc_x and A β were compared by analyses with MALDI-TOF/MS (see Fig 2). This preliminary study demonstrated that the levels of p3-Alc_x35 [major] and p3-Alc_x38 [minor] are correlated with those of A β 40 [major] and A β 42 [minor], supporting the usefulness of our immunoprecipitation–MS analysis in estimating the relative amounts of p3-Alc_x species and obtaining p3-Alc_x38/total p3-Alc_x ratios with quantitative accuracy.¹⁰

We first studied CSF p3-Alc_x38 in a small Japanese (JP) population (see Fig 3A). CSF p3-Alc_x38/total p3-Alc_x tended to be elevated in both CDR 0.5 and CDR 1 but only CSF p3-Alc_x38/total p3-Alc_x in the CDR 1 group reached statistical significance when compared against age-matched nondemented elderly ($p < 0.05$). In the US1 cohort (see Fig 3B), a nonsignificant trend toward elevation of CSF p3-Alc_x38/total p3-Alc_x was observed. In the US2 cohort (see Fig 3C) as in the Japanese cohort (see Fig 3A), CSF p3-Alc_x38/total p3-Alc_x tended to be elevated in both CDR 0.5 and CDR 1 but only CSF p3-Alc_x38/total p3-Alc_x in the CDR 1 group reached statistical significance ($p < 0.05$). Thus, elevated CSF p3-Alc_x38/total p3-Alc_x was associated with CDR 1 SAD in 2 of the 3 cohorts tested. Whenever all 3 cohorts were pooled and analyzed as a single group (see Fig 3D), both CDR 0.5 and CDR 1 were elevated and reached statistical significance, depending upon whether CDR 0 control or OND control was used as the reference group.

We also sought to determine whether correlations existed between the ratios of p3-Alc_x38/total p3-Alc_x and Mini-Mental State Examination (MMSE) scores (N.B., for some Japanese subjects, revised Hasegawa's dementia scale [HDS-R] scores were also examined). The results are shown in Supporting Information Figure 1. The ratios of p3-Alc_x38/total p3-Alc_x and MMSE scores were negatively correlated in all 3 cohorts. We also sought to determine whether there existed any correlation between the ratio of p3-Alc_x38/total p3-Alc_x and disease duration in US cohort 1. A weakly positive correlation was detected. Disease duration was not available for other cohorts. There was no correlation between age at onset and ratios of p3-Alc_x38/total p3-Alc_x.

Discussion

Our preferred interpretation of the foregoing data is that a non-APP γ -secretase substrate undergoes alternative γ -secretase processing in SAD. Because CSF A β 42 levels fall during MCI and SAD, presumably due to deposition as amyloid,¹⁵ we also considered the possibility that the altered levels of p3-Alc_x38 were caused not by γ -secretase dysfunction but by aggregation of A β and/or p3-Alc_x peptides. Although we cannot completely exclude this possibility, we would note that Alc immunoreactivity is apparently confined to intraneuronal vesicles and dystrophic neurites in AD brains,⁴ mimicking the distribution of APP. Neither non-A β holoAPP epitopes nor p3-Alc_x epitopes are detectable in amyloid deposits.⁴ Further, in unpublished experiments, we have determined that synthetic p3-Alc_x peptides undergo little or no detectable spontaneous aggregation. For this reason, too, we do not favor the idea that the altered CSF p3-Alc_x38 levels are generated by differential deposition.

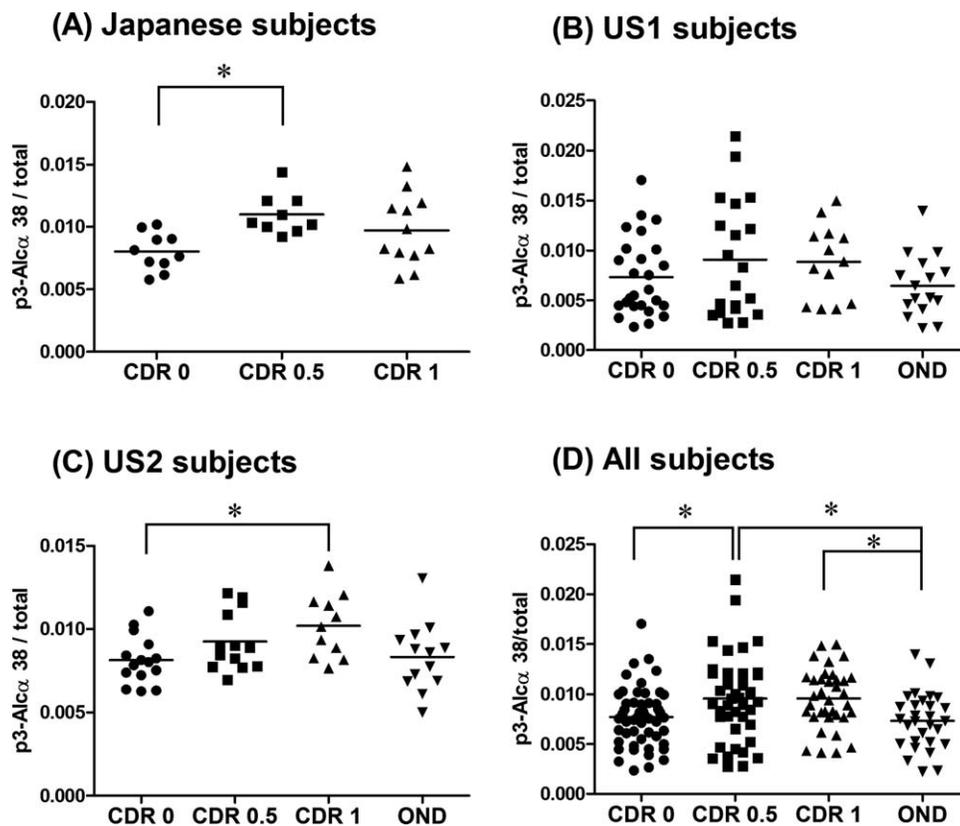


FIGURE 3: Comparison of the distribution of the ratio of p3-Alc₃₈/total p3-Alc₃₈ in CSF of elderly nondemented subjects, AD subjects and other neurological disease subjects according to cohort. (A) Japanese cohort; nondemented CDR 0 (n = 10), AD CDR 0.5 (n = 9), and AD CDR 1 (n = 12). (B) US1 cohort; nondemented CDR 0 (n = 26), AD CDR 0.5 (n = 20), AD CDR 1 (n = 13), and OND (n = 16). (C) US2 cohort; nondemented CDR 0 (n = 15), AD CDR 0.5 (n = 13), AD CDR 1 (n = 11), and OND (n = 13). (D) Combined subjects of 3 cohorts; nondemented CDR 0 (n = 51), AD CDR 0.5 (n = 42), AD CDR 1 (n = 36), and OND (n = 29). See the Table for raw data of the ratio and subject information. Statistical analysis was performed by a one-way analysis of variance followed by the Tukey Kramer's test (**p* < 0.05). AD = Alzheimer's disease; Alc = alcadin; CDR = clinical dementia rating; CSF = cerebrospinal fluid; OND = other neuronal and neurodegenerative diseases except for AD.

We have demonstrated that C-terminal speciation of p3-Alc₃₈ enables an analysis that distinguishes SAD CSF from CSF taken from other clinical populations. Yanagida and colleagues¹⁴ have recently proposed that levels of another non-APP γ -secretase reaction product, derived from APLP1 and designated APL1 β 28, may serve as a surrogate marker for A β 42. Likewise, our data imply that APP is not the only γ -secretase substrate that undergoes variant processing in association with the AD clinical phenotype. Since alternative γ -secretase processing was not observed in the OND subjects, the most parsimonious explanation is that environmental or otherwise acquired γ -secretase modulators may occur in nature, and, conceivably, that these compounds contribute specifically to the risk for SAD. Indeed, examples of such compounds (eg, fenofibrate¹⁶) have been described, and a major challenge will be to determine whether this or some other compound with similar allosteric action on γ -secretase¹⁷ can be associated with an increase in risk for SAD.

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Potential Conflicts of Interest

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