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Amyloid β -Peptide and Oligomers in the Brain and CSF of Aged Canines

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Abstract

The study of Alzheimer's disease (AD) pathogenesis requires the use of animal models that develop some amount of amyloid pathology in the brain. Aged canines (beagles) naturally accumulate human-type amyloid β -peptide (A β) and develop parallel declines in cognitive function. However, the type and quantity of biochemically extracted A β in brain and cerebrospinal fluid (CSF), its link to aging and similarity to human aging, has not been examined systematically. Thirty beagles, aged 4.5–15.7 years, were studied. A β 40 and A β 42 were measured in CSF by ELISA, and from SDS and formic acid extracted prefrontal cortex. A sample of the contralateral hemisphere, used to assess immunohistochemical amyloid load, was used for comparison. In the brain, increases in A β 42 were detected at a younger age, prior to increases in A β 40, and were correlated with an increased amyloid load. In the CSF, A β 42 decreased with age while A β 40 levels remained constant. The CSF A β 42/40 ratio was also a good predictor of the amount of A β in the brain. The amount of soluble oligomers in CSF was inversely related to brain extractable A β , whereas oligomers in the brain were correlated with SDS soluble A β 42. These findings indicate that the A β in the brain of the aged canine exhibits patterns that mirror A β deposited in the human brain. These parallels support the idea that the aged canine is a useful intermediate between transgenic mice and humans for studying the development of amyloid pathology, and is a potentially useful model for the refinement of therapeutic interventions.

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Keywords

beagle; dog; cerebrospinal fluid; amyloid β -peptide; amyloid β -protein precursor; oligomer; Alzheimer's Disease

Introduction

Alzheimer's disease (AD) is a progressive and gradual decline in cognitive function accompanied by extensive neurodegeneration. It is the pattern of this neurodegeneration within the brain, the loss of specific populations of neurons and synapses, which distinguishes the clinical presentation of AD from other, less common, forms of dementia. Within the brain itself, the hallmark signs of AD include both extracellular deposition of the amyloid- β peptide (A β) and the intracellular accumulation of an abnormally phosphorylated form of the microtubule associated protein tau. Abnormal tau, found in neurofibrillary tangles, is also found in a number of other neurodegenerative diseases. These other diseases, the broader class of tauopathies, do not feature A β deposits as a primary feature of pathology, indicating that A β deposition is relatively specific to AD [1].

The A β peptide, derived from the larger amyloid- β precursor protein (APP), was first identified more than 25 years ago [2–4]. APP is sequentially cleaved by two membrane-bound endoprotease activities, β - and γ -secretase. β -secretase (primarily BACE1 in the brain [5]) first cleaves APP to release an amino terminal derivative, sAPP β . A fragment of 99 amino acids (CTF β , which begins with the N-terminal aspartyl residue of A β) remains membrane bound, and is in turn rapidly cleaved by γ -secretase to generate A β . Cleavage by γ -secretase occurs within the membrane, and is relatively unselective, resulting in the production of a population of heterogeneous peptides. The most common form of A β , 40 amino acids long (A β 40), is the most abundant (~80–90%), followed by a more hydrophobic 42 amino acid form (A β 42, ~5–10%) [6].

The issue of how much A β contributes directly to neurodegeneration has been a topic of debate for some years. It is currently believed that the toxic form of the peptide may be a higher molecular weight, oligomeric form that can readily diffuse within the brain and either directly damage neurons or interfere with their normal function [7,8]. Oligomers form readily from the A β 42 peptide, less well so from the more abundant A β 40 [9,10], suggesting that the C-terminus is critical for oligomer formation to occur. A β dimers isolated from AD brain and CSF disrupt synaptic function [11,12]. Just how oligomeric A β causes these effects has yet to be determined, although both receptor-mediated effects as well as direct interactions with the membrane bilayer are possibilities [13,14].

Alzheimer's disease is specific to humans – even our most closely related primate relatives do not develop anything that can be considered to be *bona fide* AD. Hence, much of our understanding of how A β pathology develops has been driven by studies in animal models. Although there is no model that captures all the hallmarks of AD, we are able to routinely model A β deposition. The study of AD pathogenesis in animal models is primarily focused on transgenic mice over expressing human mutant APP at high levels, typically under the control of a heterologous promoter [15], and insights from these models have been indispensable for our understanding of amyloid deposition *in vivo* [16–18]. Even though over expression of APP is not an absolute requirement for the development of amyloid pathology [19,20], the introduction of some combination of familial AD mutations into APP or presenilin 1 (or both) does appear essential for the development of robust A β deposition. Although nonhuman primates (NHPs) have near identical APP sequence to humans, and are our closest evolutionary cousins, they develop surprisingly little AD-like neuropathology with age. While older NHPs

typically show small amounts of amyloid deposition, this is quite modest compared to cases of AD [21–23], and is far less than that of the aged canine. True neurofibrillary tangle pathology is not a typical feature of pathology in NHPs [22], although chimpanzees [24] and baboons [25] may be an exception.

Aged canines also develop substantial amyloid deposition with age. Unlike aged NHPs, which may require several decades, canines develop pathology at a much younger age [26]. Aged canines (beagles) naturally accumulate human-type A β [27,28] with age [29,30] and this parallels a decline in cognitive function [31,32]. A β 42 accumulation within diffuse plaques in the brain correlates with more severe learning and memory deficits in aged canines [31–33]. The quantity of deposited A β is often comparable to AD cases, and a substantial proportion is highly insoluble [34]. This suggests that the amyloid in the aged canine quantitatively overlaps with the amount of amyloid in human disease. Thus, canines may represent a useful intermediate between genetically modified mouse models and AD. In this regard it is worth noting that immunization with fibrillar A β in aged canines may be a better parallel to human trials with this therapeutic approach than preclinical mouse models [34,35].

In spite of significant progress in understanding the development of amyloid pathology in the brain of the aged dog, we still know very little about the interplay between the amount of A β in the brain and the levels of soluble oligomers in both the brain and CSF, and their link to age. This is an interesting question, since CSF A β 42 continues to undergo extensive evaluation as a potential biomarker of AD and the development of amyloid pathology in the brain [36]. From the perspective of a useful preclinical model, determining whether or not CSF A β levels predict brain levels would establish a biomarker for the selection of “at risk” animals for use in the development of novel therapeutics.

Materials and Methods

Animals

Thirty beagles, ranging in age from 4.5 – 15.7 years, were included in the study. All animals were from the colony at the Lovelace Respiratory Research Institute (Albuquerque, NM), and were housed either singly or in pairs in kennel buildings with indoor/outdoor runs measuring 91 cm $\times$ 600 cm. Animals were fed Wayne Mini Lab Dog Diet 8759 dog food once daily (Teklad Pioneer Lab Diets, Madison, WI) and water was available at all times. All dogs received regular veterinary care.

A β Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (Nembutal) in accordance with approved IACUC protocols. The brains were removed and alternating 0.5 cm coronal sections were frozen at -80°C or fixed in 4% paraformaldehyde at 4°C for 72 hours. The fixed tissue was then transferred to phosphate buffered saline (pH 7.4, with 0.02% sodium azide) and stored at 4°C . A β was detected using a rabbit polyclonal antibody, designated anti-A β 42, raised against a synthetic A β peptide containing amino acids 1 through 42 [32]. Fifty μm vibratome sections were taken from the dorsolateral prefrontal cortex and pretreated for 4 minutes with 90% formic acid (FA) prior to overnight incubation at room temperature in anti-A β 42 (1:500) in Tris-saline with 2.0% bovine serum albumin and 0.1% Triton X-100. Bound antibody was detected using a biotinylated anti-rabbit ABC peroxidase kit and visualized with DAB substrate (Vector Labs, Burlingame, CA).

To determine A β load, five semi-random fields from each section (2 sections in total from each animal) were digitized and the cross-sectional area occupied by A β quantified using gray scale thresholding as described previously [32]. Briefly, a random region within a random 10X field

with A β deposition was centered and digitized at 20X. Additional fields were semi-randomly selected by moving laterally by one field. A Sony high-resolution CCD video camera (XC-77) and the built-in video capture capabilities of a Macintosh 8100/80AV were used to digitize images. All sections from a given region were captured sequentially during one session and were analyzed blind with respect to age. Files were saved as gray scale (256 level) images that were subsequently binarized with a cutoff value of 110. This eliminates the background along with some faint A β immunoreactivity, and is hence a conservative estimate of the actual amount [29]. Public domain image analysis software (NIH Image 1.55) was used to calculate the percentage area occupied by A β .

A β Immunoassay

Frozen tissue samples were directly homogenized in 70% formic acid (FA; 1.0 ml / 150 mg of tissue), with complete protease inhibitor cocktail (PIC; Roche). Samples were centrifuged at $100,000 \times g$ for 1 hour, and the aqueous layer collected, aliquoted and frozen at -80°C . For the assay [20,37,38], samples were initially neutralized by a 1:20 dilution in TP buffer (1 M Tris base, 0.5 M Na 2 HPO 4), followed by a further dilution as needed (but at least 1:1) in capture buffer [0.02 M sodium phosphate buffer (pH = 7), 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace (Serotec), 0.2% bovine serum albumin, 0.05% CHAPS, and 0.05% sodium azide]. CSF samples were diluted in capture buffer, as necessary. Standard curves of either synthetic A β 40 or A β 42 were prepared in the same buffer as the samples. All samples and standards were run at least in duplicate on Ab3160 coated ELISA plates, followed by detection with either HRP conjugated BA27 (for A β 40) or BCO5 (for A β 42). A β values were determined by comparison to the appropriate standard curve. An additional subset of samples (N=14) across a range of ages were extracted first in 2% SDS (+PIC), centrifuged for 30 minutes at $20,000 \times g$, and the supernatant collected and frozen. The resulting pellet was then extracted in 70% FA, as described above.

Oligomer Blots

PBS soluble fractions were tested for total protein concentration by the BCA protein assay and diluted with double-distilled water to a standardized concentration. 7.6 μg of total protein was spotted onto a nitrocellulose membrane (Protran BA83, 0.2 μm pore size). Samples were dried and then blocked (10% Non-Fat Milk in Low-Tween TBS, pH 7.6 (20 mM Tris, 137 mM NaCl, 0.001% Tween 20) for 1 hour at room temperature. After blocking, the blot was washed in Low-Tween TBS, pH 7.6 (20 mM Tris, 137 mM NaCl, 0.001% Tween 20) three times for 5 minutes each. The blot was then incubated in anti-oligomer antibody (A11-1:2000 [8]) in 5% Milk- Low Tween TBS overnight at 4°C . After washing the blot 3 times for 5 minutes each, the blot was transferred into goat-anti-rabbit IgG (Jackson Immuno Research), diluted 1:12000 in Low-Tween TBS. Secondary antibody incubation was for 1 hour at room temperature. After the final 3 washes (5 min each), the blot was exposed to ECL reagent (Amersham) for 1 minute, exposed to film and the film developed. The film was scanned and pixel density was determined using Scion Imaging Software (Version: Beta 4.0.2). Experiments were repeated twice and the average densities used in data analyses.

For additional comparison, SDS extracts were run on 10–20% SDS-PAGE gels, transferred to nitrocellulose, and blotted with 1 $\mu\text{g}/\text{ml}$ of 6E10 (which will detect fragments of the amyloid- β precursor protein that contain A β , as well as A β oligomers). Analyses were performed on the A11 signal since this represents “total oligomers”, and since it cannot be determined which bands should be summed for a comparable measure with 6E10.

Data Analysis

SPSSR® for Windows was used to test for effects of age, and to determine the significance of all correlations and regression analyses.

Results

In the brain, both A β 40 ($p<0.001$) and A β 42 ($p<0.001$) increased exponentially with age (Fig. 1A), as did the A β 42 load ($p<0.001$) as determined by immunohistochemical analysis (Fig. 1B). Immunohistochemical A β 42 load and total A β 42 determined by ELISA were significantly correlated with each other ($R^2=0.23$, $p<0.01$). A β 42 deposition began at a slightly earlier age (7.6 years) as compared to A β 40 (8.1 years), based on examination of the x-intercept data. The extractable A β in younger animals was primarily A β 42, with A β 40 appearing only in the older subjects, similar to what has been observed in humans. To examine this point further, animals were grouped into three age groups of approximately equal width (aged 4 to 8 years, $N = 8$; aged 8 to 12 years, $N = 9$; aged 12 to 16 years, $N = 13$); this illustrates that A β 42 is much more prominent in middle aged (ranging from 8–12 years of age) canines, with A β 40 appearing in substantial quantities only in later years (Fig. 1C). This is also reflected in the ratio of A β 42/A β 40, which tended to decrease with age in the brain ($p<0.04$; Fig. 1D). As with a previous report in canine brain, there are a small number of individual aged dogs (>10 years) that show low levels of A β [39].

In CSF, A β 40 remained remarkably stable with age while A β 42 decreased slightly (Fig. 2A). We expressed the A β 42 data as a percentage of the total, a common method of examining A β in biological fluids that better controls for individual variability. The percentage (or ratio) of A β 42 undergoes a linear decrease with increasing age ($p<0.001$; Fig. 2B). Lower percentages of CSF A β 42 were correlated with larger amounts of formic acid extractable A β 42 in the brain ($p<0.001$), as well as brain A β 40 ($p<0.005$; Fig. 2C). Similarly, higher prefrontal immunohistochemical A β 42 loads were correlated with lower CSF A β 42 percentages ($p<0.05$; Fig. 2D).

Finally, we performed an examination of A11 positive oligomers in the brain and CSF within a subset of the subjects (Fig. 3A). As expected, 6E10 immunoblots run for comparison purposes displayed bands corresponding to full length APP, possible fragments of APP, and various oligomeric species of A β . Brain A11 immunoreactivity did not correlate with the total amount of either A β 40 or A β 42 (data not shown). However, although the number of subjects that overlapped on this measure was small, A11 immunoreactivity against soluble oligomers did correlate with SDS extractable A β 42 ($R^2=0.68$, $p<0.02$) (Fig. 3B). In the CSF, A11 immunoreactivity was inversely correlated with both A β 40 ($R^2=0.24$, $p<0.05$) and A β 42 ($R^2=0.26$, $p<0.04$) in the prefrontal cortex, indicating that as the total amount of brain A β increases, the amount of soluble oligomers in the CSF decreases (Fig. 3C).

Discussion

Formic acid extractable A β 40 and A β 42 increases in the brain with age in canines, and parallels similar changes in immunohistochemically detectable A β . In contrast, in the CSF, A β 42 decreased with age while A β 40 showed little change. Decreasing A β 42 in the CSF is consistent with the concept of a “peripheral pool effect”: as A β is deposited in brain, soluble CSF pools decrease [40]. The association between brain and CSF A β is similar to that observed for both AD patients [41–44] and transgenic mice [38]. Brain extractable A β from younger animals was almost exclusively A β 42, with A β 40 appearing later. This extends earlier immunohistochemical studies in the canine showing that A β 40 accumulation occurs at later ages than A β 42, paralleling observations in human brain [33,45].

As the proportion of A β 42 in the CSF decreased, brain A β accumulation increased. The percentage of A β 42 in the CSF was not only able to predict the amount of formic acid soluble A β 42 in the brain, but also formic acid extractable A β 40, as determined by ELISA. Also, the amount of A β 42 measured as a percentage of occupied cortical area, determined by

immunohistochemical techniques using a different antibody, was strongly related to the percentage of A β 42 in the CSF. It is not surprising that the percentage of A β 42 in the CSF is able to predict not only A β 42 in the brain, but also A β 40, since there is a high degree of correlation between the amounts of each peptide in the brain. Although CSF A β has yet to fulfill its potential as a biomarker of AD, these data suggest another possible use: measures of A β derived from CSF samples may be useful for predicting the levels of A β in brain as well as the extent of plaque formation in animal models of amyloid deposition. This measure could be particularly useful for selecting animals with extensive amounts of pre-existing A β pathology to use in targeted intervention studies aimed at reducing A β levels (*e.g.*, immunization, statin therapy, nonsteroidal anti-inflammatories, *etc.*) and to potentially monitor the effects of ongoing treatments. This is particularly important in animal model research, since *in vivo* imaging of A β is expensive and usually impractical for most studies and investigators. Also, at least in the case of the most widely studied amyloid imaging agent, Pittsburgh Compound B, amyloid deposited in the brain of species other than humans does not image nearly as well, likely due to differences in the interaction between the dye and the proportion of high affinity binding sites on the amyloid fibrils *in vivo* [46–48]. Since there is a current lack of good *in vivo* imaging agents that can assess the amount of A β in the brain from non-humans, CSF A β 42 may prove a useful tool that behaves similarly across all species.

We have previously shown that A β in diffuse deposits is correlated with cognitive test error scores [32,33]. For example, visual reversal learning scores, a measure of frontal function correlates with the prefrontal cortex A β load [32]. Cognitive impairment in humans is associated with a decline in the level of A β 42 in the CSF [43,49], with an increase in the amount of brain extractable A β 40 and A β 42 [44], and with some aspects of plaque pathology [50]. A similar relationship is seen between FA extractable A β and water maze performance in Tg2576 mice [51]. It would thus seem that extracted A β is a functionally relevant measure that reflects soluble species of A β in addition to the accumulation of insoluble A β detected by immunohistochemistry. It will be interesting to see whether or not CSF A β 42 is able to predict aspects of cognitive function in the aging beagle.

Finally, the amount of soluble oligomers in CSF was inversely related to brain extractable A β , whereas oligomers in the brain were correlated with SDS soluble A β 42. It is now widely believed that soluble oligomeric forms of A β are responsible for the biological, and neurotoxic, effects of the peptide [7,8]. Oligomeric A β species, which can exist at concentrations in the pM range in the CSF or brain interstitial fluid [52], are orders of magnitude more neurotoxic than either monomeric or fibrillar A β [53]. It is also likely that soluble oligomeric A β pools exist in equilibrium with other forms of A β in the brain; for example, neuritic plaques and other amyloid deposits may serve as stable reservoirs [54]. It is possible that the decrease in CSF soluble A11 positive oligomers in canine CSF relates to the exponential increase in the amount of insoluble A β deposited in the brain; this is a similar argument to that proposed above to account for the decreasing proportion of A β 42 in the CSF. We also observed in a small subset of animals that A11 immunoreactivity in the brain is related to the amount of SDS soluble A β 42. SDS-stable A β oligomers with size corresponding to a dimer have been isolated from AD brain and CSF, and have been put forward as likely candidates for the major form of toxic A β [11,12]. However, A11 positive prefibrillar oligomers did not increase with age or with AD whereas fibrillar oligomers were higher in AD tissue [55] suggesting that future studies to measure the latter pool of oligomers would be interesting in the canine model.

The canine has proven to be a useful model for preclinical drug development due to similarities with humans in terms of absorption, toxicity and other pharmacokinetics. This contrasts to that of drugs developed in rodents and subsequently translated to the human [56]. Several recent potential AD therapeutic agents, with a heavy reliance on preclinical data developed in transgenic mouse models of A β deposition, have ultimately failed in clinical trials. In light of

this lack of success, it may be justified to include a complementary animal model in addition to mice in order to bolster the justification for proceeding into human trials. The present findings combined with previous data suggest that the aged canine provides a useful intermediate between transgenic mice and humans for the development of AD therapeutic interventions, particularly those aimed at reducing brain A β .

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Figure 1. Age related changes in Aβ from the Canine Prefrontal Cortex

(A) Formic acid extractable Aβ₄₀ and Aβ₄₂, as measured by ELISA, each increase exponentially with age (note the logarithmic scale); (B) amyloid load (using rabbit anti-Aβ₄₂; see text), as determined immunohistochemically, also increases exponentially with age in brain, but the correlation is not as strong as Aβ ELISA measurements; (C) Aβ₄₂ can be seen as the prominent peptide species deposited in middle age when animals are grouped into approximately equal age categories (young, middle-aged, and old), whereas Aβ₄₀ appears in significant quantities only in older animals; (D) Aβ₄₂, as a percentage of total Aβ (Aβ₄₀ + Aβ₄₂) in the brain, decreases with age (note the increasing individual variability after the age of 12 years).

Figure 2. Age related changes in Aβ in Canine CSF

(A) Aβ₄₀ remains relatively constant with age while Aβ₄₂ decreases slightly; (B) Aβ₄₂, expressed as a percentage of total Aβ, decreases linearly with age in the CSF; (C) The percentage of CSF Aβ₄₂ is inversely correlated to the amount of formic acid extractable Aβ in the brain; (D) Although the relationship is weaker, CSF Aβ₄₂ percentage is also inversely correlated with prefrontal immunohistochemical Aβ₄₂ load.

Figure 3. Relationship Between A β and Oligomers in the Brain and CSF

(A) Dot (top; A11) and Western (bottom; 6E10) blots illustrating oligomers in the canine brain (animal numbers are indicated adjacent to each dot). Species quantified from dot blots represent a population of A β oligomers from the brain that can vary over a wide range of sizes. For instance, the intense set of bands at ~110 kDa is mostly full length APP, and 6E10 positive bands between 50 and 60 kDa could represent either fragments of APP or oligomeric A β . Hence, analyses are focused on A11 positive material only. (B) Soluble, A11 positive oligomers in brain are correlated with the amount of SDS extractable A β 42, but with nothing else. (C) Soluble, A11 positive oligomers in CSF are inversely correlated to the amount of A β 40 and A β 42 in the brain.