The Biochemical Spreading of Tau and Amyloid η - Precursor Protein Pathologies in Aging and Sporadic Alzheimer's Disease

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Abstract

The relationship between amyloid η - precursor protein (APP) dysfunction, that generates Aη , and tau pathology, is the key element to fully understand the physiopathology of non-familial Alzheimer's disease (AD). We studied 130 patients of various ages and different cognitive status, through a prospective and multidisciplinary approach. Aggregated Aη species and tau pathology were quantified in the main neocortical areas. Our study shows that Aη 42 aggregation is an early and constant marker of AD, while insoluble Aη 40 were found, but not systematically, at the last AD stages. During the progression of the disease, Aη aggregates increase in quantity and heterogeneity, in a close parallelism to the extension of tau pathology. Together, we observe a synergetic effect of APP dysfunction on the neuron-to-neuron propagation of tau pathology in AD.

Keywords: Amyloid, Aη, tau, aging, Alzheimer's disease, immunochemistry

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the coexistence of two degenerating processes: amyloidosis and tau pathology. Amyloidosis corresponds to the extracellular aggregation of Aη peptides into amyloidal plaques[1]. Tau pathology, also named tauopathy, corresponds to the intraneuronal association of tau proteins into abnormal filaments[2,3]. Amyloidosis is closely related to the etiology[1], while tau pathology is strongly correlated to the clinical expression of the disease[4-10] but little is known on the relationship between amyloid η - precursor protein (APP) and tau pathologies, which is the missing link to fully understand AD. Indeed, while the pathway of tau pathology is very precise in the brain of AD patients[8,7], amyloidosis seems to be more heterogeneously and randomly distributed[8]. Aη peptides derive from the catabolism of a larger transmembrane glycoprotein precursor named APP (amyloid η - precursor protein). Molecular heterogeneity of APP processing that generates Aη peptides result from different types of mutations in familial autosomic dominant Alzheimer's disease (FAD), located near the beta or the gamma cleavage sites[11]. These different pathogenic mutations can be modeled in transgenic mice[12] but AD is essentially non-familial, and includes more than 99% of all patients, according to a large scale population study[13]. Furthermore, an overexpression of Aη is well demonstrated in FAD-AD, but not in non-FAD AD, whose amyloidosis is explained by a lack of Aη clearance or an increase of fibrillogenesis.

The fate of amyloid deposits in the human brain is essentially known through immunohistochemical techniques[14], because biochemical quantification is not easy, since these amyloid deposits are extremely insoluble, even in harsh detergents[15]. EIA (Enzyme Immunoassays) were also used to quantify Aη deposits accessible to this technique[16,17]. Together, all these studies tend to demonstrate that in the human brain, amyloidosis is observed first as diffuse aggregates of Aη 42 peptides, which accumulate progressively as amyloid plaques, followed by the deposition of Aη 40 peptides. A microglial cell proteolysis of Aη 42 into Aη 40 species has also been suggested[18]. The latter peptides are also observed in large quantities in the cerebral vessel walls, to constitute amyloid angiopathy, which is found in variable amounts in AD brains [14,19].

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We have developed a method to analyze at the qualitative and quantitative levels the amyloid deposits in aging and AD. Tau pathology can also be quantified simultaneously. This method, easy to perform, efficient in that it solubilized all amyloid deposits, and informative, demonstrates that there are two different and sometimes independent profiles of amyloidosis in Alzheimer patients. Quantification of Aη 42 and 40 aggregates was performed in four brain regions of 60 cases from our prospective study, and compared to the quantification of tau pathology. This biochemical approach allows a better description of the natural and molecular history of Alzheimer's disease and help to set up the strategies for diagnostic and therapeutic approaches. Here we show that there is a synergetic interaction between APP and tau pathologies, despite their different spatiotemporal distribution.

Material and methods

Patients:

The 60 non-demented and 70 demented patients were from the geriatric department of E. Roux Hospital at Limeil-Brevannes and the Lille CH&U Hospital, France, as described in[7]. They represent all patients who were hospitalized for various disorders and died at this hospital, excluding those whose families opposed autopsy, or for whom post-mortem delay was more than 24 hours. Clinical data were detailed in[7]; Cognitive status was evaluated using the Mini Mental State Examination (MMSE) and the Clinical Dementing Rating (CDR) score. Clinical criteria for dementia were DSM III-R, for Alzheimer's disease NINCDS-ADRDA, for vascular dementia NINDS-AIREN, and for mixed dementia Hachinski score. Clinical diagnosis was summarized as AD (possible, probable), vascular dementia, mixed dementia (AD with a strong vascular involvement revealed by investigations), or dementia (for patients with an uncertain clinical diagnosis). The clinical data about the patients that were analyzed here in detail for their amyloid content are summarized in Table I. Similarly, neuropathological data for each patient were reported in the previous study (amyloid plaques, amyloid angiopathy, tangles, Braak stages, vascular pathology, etc.), and summarized in Table I.

Biochemical studies

Amyloid extraction: 100 mg of brain tissue were homogenized in 1 ml formic acid. 2 µl of the brain homogenate were used for the dot-blotting, or 50 µl were dried up under nitrogen, solubilized in 50 µl SDS sample buffer (5% SDS, 20% glycerol, 2% η-mercaptoethanol, and 150 mM Tris-HCl pH 6.8) and boiled 10 min before electrophoresis. 10 µl (100 µg protein) were loaded per well.

Immunological probes: tau pathology was revealed with AD2, a monoclonal antibody (mAb) against paired helical filaments (PHF) that is directed against phosphorylated tau proteins[20]. Amyloid plaques and aggregated Aη peptides were detected using rabbit polyclonal antisera, named ADA 40 and ADA 42 generated against synthetic peptides corresponding to the 7 last carboxy-terminal amino-acids of Aη 40 and 42. The specificity of these antibodies was checked by absorption with the corresponding synthetic Aη 1-40 and 1-42 (Bachem), by the specific labeling of these commercial peptides at concentration up to 500 ng, both using dot-blot and electrophoresis. Their immunoreactivity, specificity and sensitivity were similar to the well characterized FC3542 and FC3340 antibodies[14]. Aη 42 species were also detected using the monoclonal antibody 21F12[21].

Immunoblots: tau pathology was investigated as already described[7]. Amyloid pathology was analyzed using electrophoresis adapted to the separation of small peptides[22]. The upper part of the blot membrane (MW down to 40 kDa) was reacted with the monoclonal antibody AD2. The lower part of membranes was reacted first either with 21F12 or ADA42 for the detection of Aη 42 species, and then, after stripping, was reacted with AD40, for the detection of Aη 40 species.

Dot-blot analyses: Amyloid was detected and quantified by dot-blot, using the procedure described by[23]. The centrifugation step was removed and formic acid homogenates were dotted directly on the PVDF membrane.

Image analysis of immunoblot and dot-blot quantification: Immunoblots and dot-blots were analyzed using the ImageMaster 1D Elite software (Amersham-Pharmacia). The quantification is expressed in µg per gram of tissue, using Aη 1-40 or Aη 1-42 synthetic peptides (Bachem) as standards.

Results

Quantification of Aη aggregates

A) Human brain amyloid is extremely difficult to dissociate and solubilize

Aη species from the human brain tissue were extracted with different buffers: SDS, guanidinium chloride, formic acid (F.A), at different concentrations. EDTA or EGTA, which improve the solubilization of physiological Aη[24]3did not yield higher the extracted amounts of Aη peptides. In parallel, different anti-Aη antibodies from our laboratory or commercially available anti-Aη antibodies were compared in order to select the best antibodies for the detection of solubilized Aη#aggregates on western-blots or dot-blots. The antibodies against the carboxy-terminal part of 42 and 40 Aη peptides, named ADA 42 and ADA 40, were the most specific and sensitive to detect Aη aggregates (estimated sensitivity of less than 1 µg/g of wet tissue). We found that only pure formic acid was able to extract totally Aη aggregates, and especially Aη 42 species that were the most insoluble.

The quantities of Aη extracted with our method were very different from one brain to another brain, and between different brain areas from the same brain. The absence of detection of Aη in the brain of young patients demonstrates that our method only detects aggregated Aη species, and not physiological Aη. The global study of all non-demented and demented cases allowed us to understand the rationale of Aη aggregation in aging and AD (Figure 1).

B) Different patterns of amyloidosis in Alzheimer cases

We quantified Aη aggregates in different brain areas, randomly among the 130 cases (60 non-demented, 70 demented patients) of our prospective study[7]. Synthetic Aη peptides were used in parallel for the quantitative analysis.

Alzheimer patients presented very contrasted amyloid phenotypes and several types of heterogeneity. First, some cases were characterized by their almost pure and large amounts of insoluble Aη 42 variants (Figure 1A, case Ci.65). Other cases had moderate (Figure 1A, case Fa.85) to large amounts of insoluble Aη 40 (Figure 1A, cases Gs.86, My.74).

Second, heterogeneity was found in the cerebral distribution of aggregated Aη variants, which where sometimes in larger quantities either in the temporal or the parietal or the occipital cortex (Figure 1A). In general, the frontal pole contained less Aη aggregates, while larger quantities of Aη 40 variants were found in the occipital region.

Third, there was a mismatch between Aη 40 and 42 aggregates in some cases. For a given brain, the two aggregated Aη species mapped differently, as shown for case Fa.85, where insoluble Aη 42 was in larger quantities in the occipital cortex while aggregated Aη 40 species were found essentially in the frontal and parietal cortex (Figure 1, panel A, B).

Fourth, heterogeneity was also observed in the detailed electrophoretic pattern of Aη deposits. Interestingly, solubilized Aη 42 variants mainly consisted of dimers and multimers (Figure 1, panel A) whereas solubilized Aη 40 consisted mainly of monomers and dimers (Figure 1, panel B).

We also investigated the biochemical patterns of amyloidosis in non-demented patients. In good agreement with our previous studies[7], we found a few aged cases with no trace of Aη aggregates, despite the presence of tau pathology. Patients up to stage 7 of tau pathology had almost exclusively Aη 42 aggregates (Figure 1, panels D-F).

C) Biochemical quantification of amyloid deposits

We quantified Aη aggregates by dot-blots and western-blots, using Aη 40 and 42 synthetic peptides as standards. Results obtained with these two approaches were similar. The dot-blot technique was faster[23], while the western blot approach gave additional values on the content of Aη aggregates, such as the percentage of monomers, dimers, and multimers. Values obtained with these methods

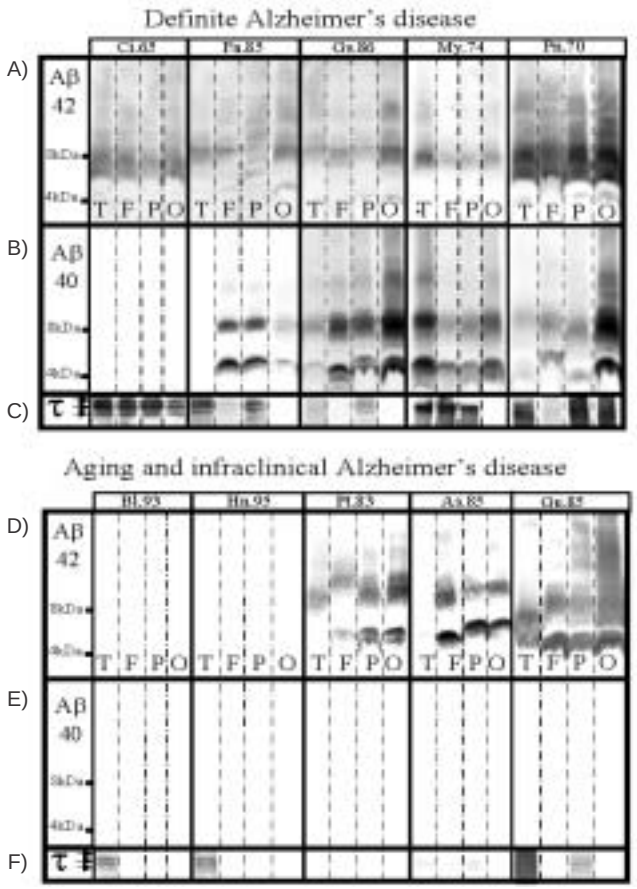


Figure 1.

Western blot analysis of Aη deposition and tau pathology in the different brains areas of Alzheimer patients (A-C), and non-demented patients with a tau pathology alone or with Aη 42 aggregates (D-F).

A-B) The specific immunolabelling with Aη 42 (A) and Aη 40 (B). ADA 42 and 40 antibodies are presented. The temporal (T), occipital (O), frontal (F), and parietal (P) cortices were studied. Aη 42 aggregates were found in large quantities in all brain areas of Alzheimer patients. Patient Ci.65 had exclusively Aη 42 aggregates, since no trace or trace amounts of Aη 40 were detected in all four brain regions. Patients Gs.86 and Fa.85 had huge aggregates of Aη 40 peptides. Aη 40 was in larger quantities in the occipital area of Gs.86 while it was essentially found in the frontal and parietal cortex of patient Fa.85. Aη# 7 aggregates were essentially solubilized as Aη monomers and dimers while Aη# 9#aggregates were dissociated and solubilized as Aη dimers and multimers.

C) tau pathology revealed with AD2 antibody.
Tau detection was performed on the same membrane. Pathological tau proteins correspond to the main triplet of immunodetected band (tau 60, 64 and 69 kDa). They were abundant in all brain areas of patients Ci.65 and Cu.70. For patient Gs.86, at stage 9 and mildly affected, the temporal and parietal cortex were moderately affected by tau pathology, but not the frontal and occipital cortex. Note that the region with more Aη 40 and 42 aggregates, the occipital cortex, was not affected by tau pathology. In the same way, tau pathology and amyloid deposits did not overlap in the brain areas of patient Fa.85.

D-F) Aη 42 (D), Aη#0 (E) and tau (F) were also quantified in different brain areas from non-demented patients. We used first 21F12, because of its very high sensitivity and specificity towards Aη 42 species, and then ADA40. Some representative cases are presented here: two cases with tau pathology and no Aη aggregates (Bl.93 and Hn.95); case Pt.83, case As.85 and case Ge.85 at stages 1, 3 and 6 of tau pathology, respectively, and with various amounts of aggregated Aη 42 species, but no Aη 40 aggregates.

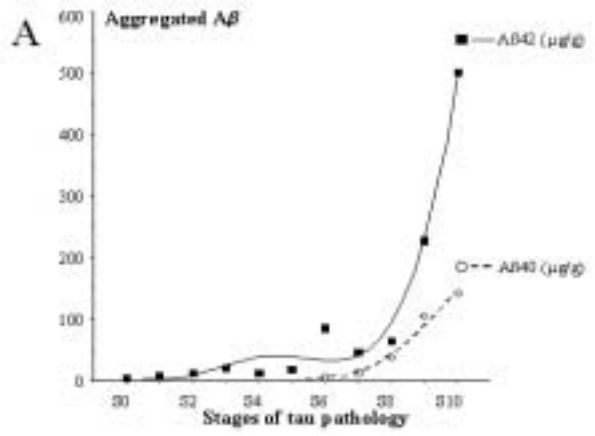
are reported in Table 1 and Figure 3. We analyzed at random at least three cases per Alzheimer stage of tau pathology, as defined in [7]. With this approach, we found insoluble Aη concentration ranging from 25 to 1250 µg per gram of human brain tissue from Alzheimer patients. Then, the quantification was performed in the 4 main brain areas of non-demented and Alzheimer patients. Our results demonstrate the strong heterogeneity of Aη aggregates, in quantity and quality, with an Aη 42/40 ratio that varied from infinite (absence of Aη 40) to 0.4. For patients with a tau pathology restricted to the hippocampal area, such as Pt.83 and Ds.59, the amyloid burden was in the range of 2 to 145 µg of insoluble Aη 42 peptide per gram of wet tissue. For these patients, Aη 40 insoluble species were never detected.

D) Spatiotemporal analysis of Ab deposits in Alzheimer's disease

In our prospective study[7], we demonstrated that the clinical features were well correlated with the extension of tau pathology in association polymodal cortical areas. For the present study, it was interesting to compare the clinical and neuropathological features of these patients with tau pathology as well as with the different biochemical phenotypes of amyloid deposition and their concentrations.

For Alzheimer demented patients, we always found severe tau pathology (stage 7 to stage 10) well correlated with a huge and widespread Aη burden. As shown in Table I, the presence of Aη 40 aggregates is a landmark of the late stages of AD. Also, the most affected AD cases, with an early onset, a rapid cognitive degradation and a huge neurodegeneration process, were cases with large amounts of Aη 42, and rare or absent Aη 40 aggregates (cases To.54, Fu.63, Ci.65, Cu.70).

When all biochemical data were collected, it was striking to observe the excellent parallelism between the increase of the disease process, tau pathology and the increase of Aη 42 aggregates (Table 1, Figure 3). However, at the level of brain areas, there was no overlap between the mappings of these two degenerating processes. It was interesting to observe brain areas such as the occipital cortex, with enormous quantities of Aη aggregates, but with absolutely no trace of tau pathology in this region (Fig. 2, case Gs.86, occipital). Conversely, brains areas with tau pathology were not those that had larger amounts of Aη aggregates (Fig. 2, Fa.85, temporal cortex). Moreover, each time we were able to detect Aη aggregates; we found tau pathology, at least in the hippocampal area. The opposite was not true since we found cases with tau pathology up to stage 6 and no trace of Aη aggregates, whatever the technique used (immunohistochemistry, western-blots, and dot-blots).



B

Polynomial regression

	n	r	p
Aß 40	58	0.984	<0.0001
Aß 42	58	0.963	<0.0001

Figure 2.

Average amounts of Aη aggregates in the brain tissue of non-demented and AD patients correlated with tau pathology staging
The amount of Aη 42 and Aη 40 aggregated species are expressed as average amounts of the four brain region analyzed (temporal, frontal, parietal and occipital region) in 58 patients distributed among the 10 stages of tau pathology (x-axis). The average amounts were calculated only for patients with Aη aggregates.
A) The average amounts are in µg/g wet tissue, the left y-axis corresponds to Aη#42 amounts and the right y-axis corresponds to Aη 40 amounts. The standard deviation is also indicated.
B) Polynomial regression between Aη aggregates and tau pathology staging. Polynomial regression of Aη 40 (round circle) and Aη 42 (black square) were both significant ($rAη40=0.984$, $rAη42=0.963$, $p<0.0001$).

Discussion

Our objective was to describe the natural and molecular history of AD as well as to develop a molecular basis for the definite diagnosis of AD. Indeed, at the present time, the definite diagnosis of AD, and the distinction from other neurological disorders, are still difficult, even working directly on the brain tissue: this is demonstrated by the fact that tau pathology has been recently reintroduced in the criteria for the definite diagnosis of AD[25], after a long absence, to fit now with Alois Alzheimer's first and sound description. This is also demonstrated by the fact that the 1997 consensus report recommends a probabilistic analysis. This is also obvious when we see that the molecular markers of neurodegenerative disorders, namely amyloid pathology, tau pathology, synuclein, are overlapping markers of many different diseases. For example, tau pathology is observed in more than 24 neurodegenerative disorders[3]. Also, Aη pathology is frequently observed in Lewy body dementia[26,27], a disease with a synucleopathy[28]. Aη deposits are also a basic component of amyloid angiopathy, a pathological entity distinct from AD[19]. Therefore, a reliable quantification of brain lesions is necessary, and only biochemical analyses can ful-

fill the work rapidly, precisely, and with good inter-laboratory reproducibility. The usefulness of such a procedure is that it could bring a quantitative and qualitative aspect to the diagnosis, as required by the consensus criteria[25], that are difficult to obtain with an immunohistochemical approach. Brain banks could use it to type precisely the extent of the degenerating processes in the frozen tissue that will be used for the search of markers or for the description of the pathological process. A molecular basis for the diagnosis at the CNS level should also open the gates of a biological diagnosis in the blood or the cerebrospinal fluid. A biochemical approach should also provide a possibility for a definite *ante mortem* diagnosis, based on the quantification of pathological tau and Aη in a stereotaxic biopsy. This controversial approach is not yet an issue these days, but could be one if an efficient anti-Alzheimer drug is discovered.

Aη quantification was performed on *post-mortem* brain tissue of 60 patients from our prospective study. Western-blot or dot-blot techniques gave similar values. The dot-blot technique is more efficient than EIA since it enables to quantify whole Aη aggregated species (from the most soluble to the most insoluble), but the most significant difference is that dot blots allow a direct loading of the antigen dissolved in pure formic acid on the PVDF membrane. This methodology avoids the possible pitfalls due to Aη reaggregation that would inevitably happen if the formic acid-treated tissue is then neutralized or treated in a less denaturant buffer. This strong tendency to reaggregation is demonstrated by the presence of smears of insoluble material on western-blots, when formic acid-treated material is removed and dissolved in a SDS buffer. These smears that are often in large quantities cannot be processed by ELISA. However, the dot blot technique necessitates working with anti-Aη antibodies that do not react with other APP catabolic products, a property which is rarely found, explaining why we choose to work with western blots.

Therefore, with our western blot methodology, it has been possible to develop a large scale study for the quantitative and qualitative analyses of Aη aggregation in the gradient of pathology of our series of 130 cases, from normal aging to severe AD, via infra-clinical stages of AD. Furthermore, it has been possible to study the temporal distribution of Aη aggregates in four brain areas (temporal, frontal, parietal, occipital), that summarizes the precise and sequential degenerating process revealed by tau pathology. Also, all our biochemical data were compared to clinical and neuropathological data.

Different biochemical phenotypes of amyloidosis in non-familial Alzheimer cases

The detection and quantification of amyloid deposits show the extensive heterogeneity and the different patterns of Aη aggregation in the brain of Alzheimer patients. For some cases, the Aη burden was almost exclusively composed of insoluble Aη 42 species. These cases had generally an early onset and a rapid evolution, as indicated in the clinical data. Aη 42 deposits were also exclusively found in cases that are at the infraclinical stage of AD. ADA42 and ADA40 had similar sensitivities, suggesting that the early and specific detection of Aη 42 aggregation in non-demented patients is revealing the first event of amyloidosis. Conversely, some cases at the last stages of tau pathology were strongly affected by Aη 40 aggregates. The profile of Aη 40 consisted of well resolved monomers and dimers, partially soluble in SDS extracts, in contrast to highly SDS-insoluble Aη 42 aggregates that consisted of dimers and multimers. Cases with Aη 40 had frequently amyloid angiopathy, but this was not always the case, showing that the production of Aη#40 can be partially independent from amyloid angiopathy. In general we found that Aη 42 burden was mainly found in temporal and parietal cortices, while Aη 40 deposits were mostly in the occipital areas. There is no clear explanation for the heterogeneous Aη 40 phenotypes. Our hypothesis is that Aη#40 species derive from a carboxy-terminal proteolytic activity on Aη 42 species, which is lately and heterogeneously activated in brain areas and among individuals. For example, this activity could be triggered by microglial cells that are known to phagocyte amyloid debris, as already suggested[18].

Aη 42 aggregates were exclusively found at the infraclinical stages of AD, showing that they are the exclusive biochemical phenotype associated with the early development of Alzheimer amyloidosis. Aη 42 aggregates were found alone or with mild amounts of Aη 40 aggregates in the most severe Alzheimer cases, showing the close relationship between Aη 42 the development of Alzheimer pathology and its severity.

The relationship between amyloidosis and tau pathology

The correlation between amyloid deposits and tau pathology is weak at the level of their spatial distribution in brain areas, but strong if we compare the stages of tau pathology versus the average amount of Aη 42 aggregates in the neocortical areas. Indeed, we demonstrated with our biochemical approach and in excellent agreement with numerous neuropathological studies [4-10], that the pathway of tau pathology is precise, sequential, predictable, stereotypical and hierarchical. Ten stages of tau pathology were defined, according to ten brain areas that are successively affected[7]. Here, our precise typing and quantification of amyloid deposits shows that the Aη burden is diffuse, widespread and extremely heterogeneous, also in good agreement with neuropathological studies[8].

Tau pathology, an aged-related disease of the human hippocampal formation

From our quantification of Aη and tau aggregation in the different brain areas of non-demented and demented people, it is interesting to note the following points:

First, each time we detected Aη 42 cortical aggregates, we found tau pathology, at least in the transentorhinal cortex. We found consistent Aη 42 deposits, up to 50 µg per gram of tissue (but not in all brain areas) of patients at stage 1 of tau pathology. We know that tau pathology spreads in brain areas, with a unique scenario (transentorhinal cortex, entorhinal, hippocampus, anterior temporal cortex, etc). When tau pathology, even at stage 1, is found with simultaneous Aη 42 aggregates in some neocortical areas, it is reasonable to think that these patients are at the earliest possible form of Alzheimer's disease pathology. This assumption is based upon the fact that insoluble Aη is considered as a marker of AD and tau aggregates as a marker of neurodegeneration.

Second, we observed that a few cases had not a trace of amyloid, as tested with our immunochemical technique as well as at the immunohistochemical level, but tau pathology up to stage 6. The absence of detection of Aη deposits in the brain of a few aged non-demented patients could not result from a lack of sensitivity of our technique, since less than 1 µg of Aη aggregates per gram of wet brain tissue could be detected. This sensitivity was equal to that described by ELISA[29]. That sensitivity was less than 1000 times lower that the amounts of Aη detected in some Alzheimer brains. Furthermore, similar results were obtained with the monoclonal anti- Aη#42 antibody 21F12, which, in our hands, was the most sensitive and specific tool for the detection of insoluble Aη#species (sensitivity below 0.1 µg per gram of tissue). Thise results demonstrate clearly that tau pathology is independent from amyloid production, since it can develop in a rather large part of the brain (the hippocampal formation and the half of the temporal cortex), without the detection of Aη 42 aggregates.

From this observation, two conclusions can be drawn: the first one is that tau pathology is an aged-related degenerating process, since all (100%) patients older than 75 years have tau pathology at least in the transentorhinal cortex. The second one is that tau pathology is not caused by aging, since we were able to observe the brain of centenarians that had very mild tau pathology (Table 1). They show that the intensity of tau pathology is not directly linked to aging. In fact, age is a window that allows detecting a vulnerability of the human hippocampal formation to a degenerating process that develops slowly, namely tau pathology. Tau pathology can be considered as a disease rather than an aging process, because we know that tau pathology has different molecular substrates (mutation on tau gene that generates loss of function or abnormal tau splicing; indirect abnormal splicing; polymorphisms; decreased synthesis; abnormal phosphorylation,etc.)[30].

Third, our study shows that tau pathology is prone to spread in polymodal association brain areas, at revealed by the neuropathological Braak stages or our biochemical stages of tau pathology. However, we note that this pathological tau spreading in association areas is exclusively found in the presence of Aη 42 aggregates, demonstrating that the progression of tau pathology in human brain areas is fueled by events associated with Aη 42 production.

Together, our data presented here still reinforce the idea that non-familial autosomic AD is an amyloid (neurotoxic) or APP (loss of trophic activities) burden that will extend tau pathology from the temporal cortex to the association brain areas[31], as represented in Figure 3. The data are in favor of a synergy of tau and APP pathologies. They show that tau pathology can be initiated before and likely independently of amyloidosis or APP dysfunctions, up to stage 6 of tau pathology. But our data also show that both neurodegenerative processes evolve systematically in parallel after stage 6, and that APP pathology amplifies tau pathology. Indeed, the progression of tau pathology in the association cortical areas is only observed when there is a simultaneous amyloid burden. Therefore, our study demonstrates that tau and APP pathologies work together to produce non-familial AD, and that tau pathology cannot be considered as a secondary event of non-familial AD pathology, as frequently suggested. In that respect, the recent mice models proposed[32,33] fit well with the human physiopathology[31] and as described here, because they demonstrate a synergy between APP and tau pathology. Furthermore, it is interesting to note that in one model, the amyloid burden is acting through neuronal connections and not by a direct contact to nerve cells, as observed here in human brains[32].

Amyloid patterns and CEBDAD

We found a good correlation between clinical data, amyloidosis and tau pathology, if a notion of threshold is added. Indeed, brains lesions at low levels, observed at the beginning of the pathology, do not impede cognitive functions. Neuroplasticity is certainly a significant factor that will modify the threshold of clinical manifestations, almost independently of the physiopathological process[7,34].

Our study shows that all clinically defined "probable AD" cases had huge amounts of amyloid deposits and widespread tau pathology, in good agreement with the recent neuropathological criteria[25]. As mentioned previously, the polymodal association areas are the most informative brain areas for an accurate definite diagnosis of AD. The hippocampal area, early and frequently affected by tau pathology and amyloidosis in non-demented patients, is only informative to stage the infraclinical stages of AD. In agreement with our previous reports, this technique of amyloid quantification is able to define the Criteria to Establish a Biochemical Diagnosis of Alzheimer's disease (CEBDAD) and shows that it is possible, with only biochemical means to distinguish between normal aging and infraclinical stages of AD on one hand and between infraclinical and clinical AD on the other hand.

Clinical AD: All of our clinically and then neuropathologically diagnosed cases with Alzheimer's disease had tau pathology in the frontal pole and the parietal cortex (Stage 7 to 10) and the presence of Aη 42 aggregates above 50 µg /gram of wet tissue in these cortical areas. Therefore,

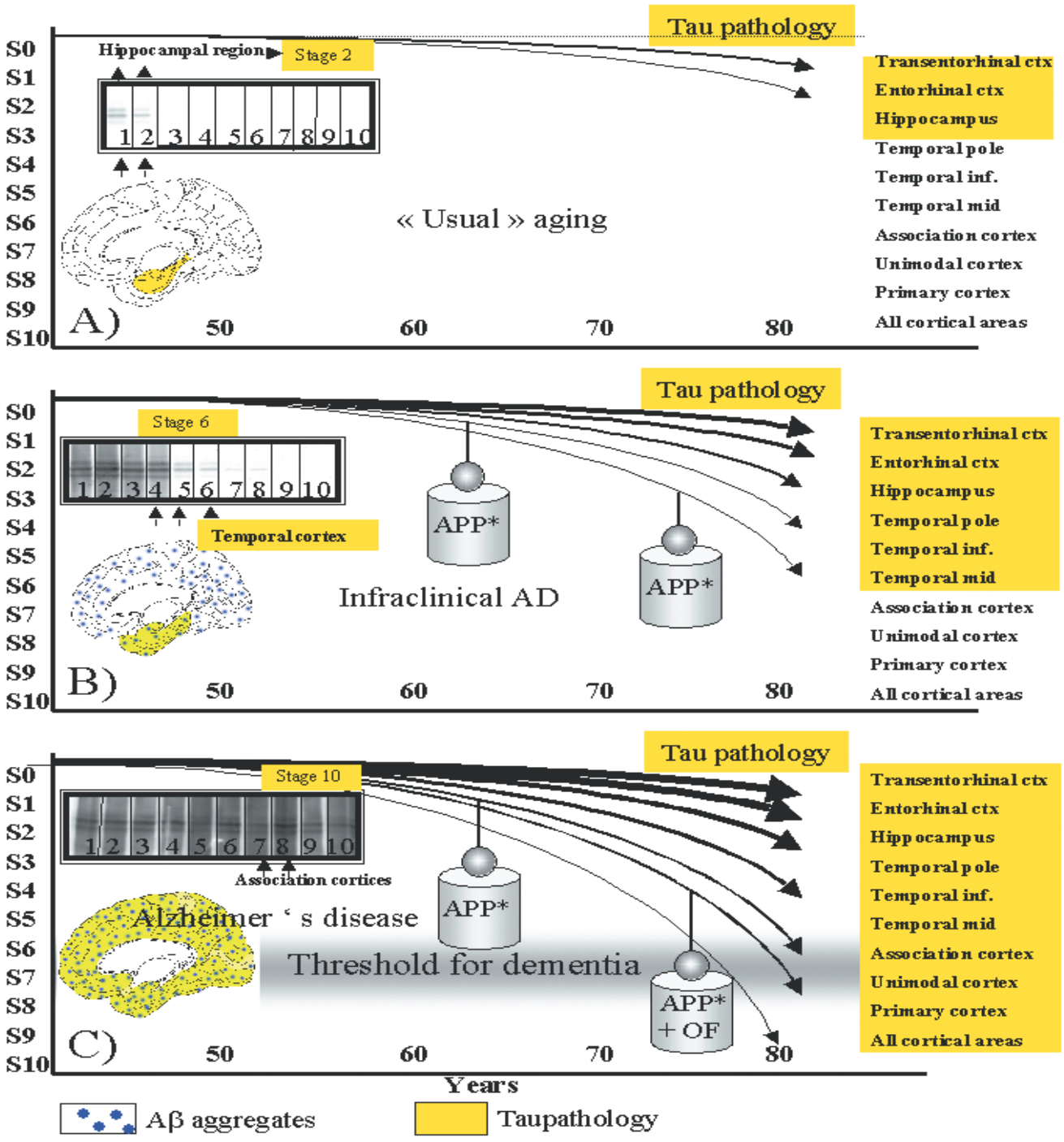


Figure 3

The pathway of tau pathology and amyloidosis in aging and Alzheimer's disease

Facts: tau pathology is always detected, but in various amounts, in the hippocampal region of non-demented patients over 75 years old. In Alzheimer's disease (AD), tau pathology always extends in other brain areas, along a precise pathway, which is sequential and hierarchical. The 10 brains that are successively affected define 10 stages of tau pathology (S1 to S10). Tau pathology can be observed without Aη aggregates, while the opposite is not true. However, extension of tau pathology in association areas is always observed with Aη deposits.

Hypotheses: There is a threshold of NFD extension near stage 6 that separates infraclinical AD from clinical AD. Clinical manifestations are always observed at stage 7 or above, when association brain areas are affected. In AD, APP dysmetabolism (loss of function of APP or neurotoxicity of Aη peptide) is likely to affect the most vulnerable brain areas and to extend tau pathology. The process of NFD that occurs systematically in aging is dramatically intensified, because of the weight of APP dysfunctions. The progression of NFD in other brain areas is also likely boosted by numerous other factors (OF), such as the lack of trophic factors (TrF) that are no longer supplied by affected neuronal networks, apoptosis that could be programmed following the lack of trophic factor and inflammation due to brain lesions and activation of microglial cells. The progression of NFD that will occur along a precise pathway of cortico-cortical connections could have its own dynamic, relatively independent from the etiology. Slowing down this dynamic will delay the progression of AD.

	PHF-tau	Hippocampal reg.			Temporal ctx				Cing.	Par.	Frontal ctx				Occ. ctx		Amyloid Ab 42				Amyloid Ab 40				Braak Stg		Neuropath	MMSE	CDR	Clinical
Cases	Scores	A35	Ent	CA1	A38	A20	A21	A22	A23	A39	A10	A44	A4		A18	A17	Tp	Fr	Pa	Oc	Tp	Fr	Pa	Oc	NFT	Am.	other	duratn		diagnosis
De.26	S0, 1	0	0	0	0	0	0			0	0	0	0	0			0	0			0	0		0	0	0		30	0	Control
Re.42	2	0	0	0	0	0	0			0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0			0	Control
Cu.43	3	0	0	0	0	0			0		0	0	0	0			0	0	0		0	0	0		0	0		30	0	Control
Le.64	4	0	0	0	0	0			0		0	0	0	0			0	0	0	0	0	0	0	0	0	0		30	0	Control
Pt.83	S1, 1	0.1	0	0	0	0			0	0	0	0	0	0			6	15	12	33	0	0	0	3	1	A		*	0	Control
Ru.78	2	0.3	0	0	0	0	0			0	0	0	0	0			0	0	0	0	0	0	0	0	1	0		30	0	Control
Mg.95	S2, 1	0.1	trace	0	0	0	0			0	0	0	0	0			35	37	40	23	0	0	0	0	1	A			0	Control
Co.72	2	0.6	0.3	0	0	0	0			0	0	0	0	0			0	0	0	0			0		1	0			0	Control
Bn.89	3		2.5	0			0		0		0	0	0	0			0	0	0	0	0	0	0	0	II	0		30	0	Control
Ds.59	S3, 1	trace	trace	trace		0	0	0		0	0	0	0	0	0			13		16			0		1	A	Nigra cell loss	20	0	Control
As.85	2	0.2	0.75	0.25		0	0		0		0	0	0	0			5	7		10	5	0	0	0	1	A		*	0	Control
Cc.91	3	0.2	0.8	0.1	0	0	0	0		0	0	0	0	0	0		53			145	0			0	I	A	+ vascular	15	3	Poss Vasc Dem
Gr.80	4	trace	0.4	1	0	0	0	0		0	0	0	0	0				6					0		1	0	+ vascular		3	Poss Vasc Dem
Bu.88	5		0.5	0.8		0	0		0		0	0	0					0					0		II	0		*	0	Control
Vr.84	6	1.2	1.2	1.1	0	0	0	0		0	0	0	0	0	0			27					0		II	A		28	0	Control
Ry.72	7		4.75	1.85	0	0	0	0		0	0	0	0	0	0			3					0		II	0	+ vascular + AA		0.5	Control
Bn.90	8	1.4	2.7	1.6	0	0	0	0			0	0	0	0	0		6	2	0	0					III	A	+ vascular	*	1	Poss Vasc Dem
Lc.92	S4, 1	0.5	0.3	0.25	0.1		0		0		0	0	0	0	0		1	3		23	7	0	0	0	III	A		*	0.5	Memory imp.
Mc.88	2	0.2	0.2	0.1	0.05	0	0	0		0	0	0	0	0	0		6	0	0	0	0	0	0	0	III	0		*	0.5	Memory imp.
Dl.98b	3	1.1	0.1	0.6	0.1	0	0	0			0	0	0	0	0			11					0		III	A		*	0	Control
Bl.93	4	0.4	0.3	0.4	0.5	0	0	0		0	0	0	0	0	0		0	0	0	0			0		III	0		26		Control
Hn.95	5	1.6	0.7	0.6	0.2	0	0	0			0	0	0	0	0		0	0	0	0			0		III	0			0	Control
Cu.81	6	3.7	1.8	0.1	0.01	0	0	0		0	0	0	0	0	0		53	24		145	0	0		0	III	A	nigra cell loss	*	0	Control
Le.69	7		4	3.7	2.5	0			0		0	0	0			0	0	0	0		0	0	0		III	0		30	0	Control
Ml.88	S5, 1	0.2	3	1	0.1	trace	0	0	0		0	0	0				0	0	20	0	0	0	0		III	0		*	1	Memory imp.
Eu.82	2	0.1	0.5	0.5	0.5	0.75	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	III	0		28	0	Control
Bo.92	3	3.3	1.5	0.4	0.25	0.6	0	0	0	0	0	0	0				13	78			0	0			III	0	+ vascular		1	Poss Vasc Dem
Fu.83	4	0.5	3.5	1	1.5	1.75	0	0	0	0	0	0	0	0				36	31			0			III	A		30	0	Control
Rs.93	5	0.5	5	2.75	0.25	0.25	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	III	0		20	0.5	Memory imp.
Ti.85	6		5	9		2.5	0	0		0	0	0					51		20	0	16		0	0	III	A		27	0.5	Memory imp.
Py.93	S6, 1	0.7	0.6	0.6	0.5	0.1	0.1	0	0	0	0	0	0	0			213	66	112	7	0	4	0				11	III	A		27	0.5	Memory imp.
Lm.92	2	0.1	1.9	1.3	0.1	trace	1	0	0	0	0	0	0	0			39	61				0	2					III	A		*	0.5	Memory imp.
Am.86	3	0.9	0.8	1	1.1	0.8	0.7	0	0	0	0	0	0	0			201	127	263	189					IV	B	nigra cell loss		0	Control
Bu.90	4	2	2	2	1.5	1	0.3	0		0	0	0	0	0			0	0			0	0			III	0		12	2	Dementia
Ge.85	5		2	2.5	1.75	1	0.1	0	0	0	0	0	0	0			81	166	300	263	0	0	0	0	III	B		*	0.5	Memory imp.
Ti.86	6	9	3	2	1.5	0.5	trace	0		0	0	0					85	55	73	46	63	17	52	32	III	B		20	0.5	Memory imp.
Ce.92	7	10	5	2	3.25	2.25	0.15	0	0	0	0	0	0	0			36	8	0	0	14	2	0	0	III	A		22	0.5	Memory imp.
Fs.71	8	10	10	1.25	1	1	1.8	0	0	0		0	0	0	0			67				0			IV	B		20	0	Control
Vn.88	S7, 1	1.45	1.45	3.2	1.4	1.5	trace	0.3	0	0	0	0	0	0			15	0	0	0	0	0			V	A		24	0.5	Memory imp.
So.84	2	2.2	2.4	1.3	3.5	3	0.3	0.2	0.2	0.1	0.1	0	0	0			56	18	8	17	0	0	0	0	IV	B		20	0.5	Probable AD
Al.87	3	7.3	3	8.1	9.2	7.6	4.2	5.4		6.1	1.6	0	0	0	0		172			50	0			0	V	B	+ vascular		3	Poss Vasc Dem
Tf.77	S8, 1			5	2.4	0.8		1		1	1.2	0.75	0	0			87	2	80	14	5	0	2	1	IV	C		8	3	Probable AD
Vr.91	2	15	13	11	2	2	3.1	0.25		1.25	1.25	0.75	0	0	0		6	3		22	29	0	0	0	0	IV	B		27/1	1	Possible AD
Vr.89	3	2	3.3	2.6	0.9	2.4	0.1	0	0.1	0.8	1.2	0.6	0	0	0		88	111			2	2			IV	C	+ vascular	1	2	Poss Vasc Dem
Mi.91	4		9	2	9	9	1			11	6	12	9	0	0		93	200	76	31	132	173	145	150	IV	C	+ vascular	1/5	3	Probable AD
Gs.86	S9, 1	3	4	2	2	1.2	1.8	0.5		0.3	1	0.4	0.6	0	0		190	162	106	532	59	292	273	645	IV	C	+ AA	7/13	2	Probable AD
Lu.91	2	7.5	2.5	1	5.7	4.5	3.2	4.6	0.7	2.8	0.5	0.2	0.5	0	0		96	89			0	0			IV	B			2	Probable AD
My.74	3		10	7		10	11			9.5	10	8.5	11	0	0		382	123	62	230	318	114	134	216	IV	C	+ vascular + AA	16	3	Possible AD
Ks.82	4	5	4	6	5	5	5			1.8		5	5	1.4	0.4	2		130	25	4	408	20	4	0	16	V	C		5/5	3	Probable AD
Ci.65	5	13	7.8	1.4	7.25	8.5			9.5		13	5	1.5	0	4.7	2	570	354	642	602	9	0	0	0	V	C		0/6	3	Probable AD
Ta.85	S10,1				10	15					4	0.1		10		3	332	368	550	582	10	376	341	162	VI	C	+ vascular + AA			atypical DLB
Mr.88	2	17	6	4.5	6.25	5	5	6.7	1	9.2	6.5	1.5	1.4	3.2	1		366	221	168	466	5	0	0	10	VI	C		12	1	Probable AD
Du.63	3		9	6.5				9.4	6.2	9.5	8.7		6.5	10	2.5		435	165	290	413	12	4	6	7	VI	C	+ vascular	7/13	3	Probable AD
Cu.70	4		12	12				13	10		15		6.5		4		692	52	218	322	108	8	21	55	VI	C	+ AA		3	Probable AD
Ct.66	5		8	9	10	10	10	10	10	10	10	12	10	10	10					200				450	VI	C		7/10	3	Aphasia, FTD
Nv.78	6	9	9	9	11	11	11	11	10	10	10	10	10	10	10					300				0	VI	C		7/17	3	Atypical FTD
Pn.70	7				8		10			10	10		9		10		836	588	1260	1250	56	99	108	625	VI	C	rare AA	8/12	3	Probable AD
Fa.64	8		10	11	10	10		15		13	12	12	15	10	9.5		30	466		742	50	135		688	VI	C	+AA	9/8	3	Probable AD
To.54	9		9.8	8	10	12	10	9	9	18	18	9.3	6.7	3	0.7		1044	1774	1034	1000	24	102	71	29	VI	C		6/12	3	Probable AD

Table 1.

Amyloid and pathological tau content in the different brain areas from non-demented and demented patients, ranked as a function of tau pathology stages.

Each patient is represented by a coded reference + the age. Patients were ranked as a function of the extent of PHF-tau in the different Brodmann areas: A35 = transentorhinal region, Ent: entorhinal region (A28), CA1: Hippocampus (A34), A38: anterior temporal cortex, A20: inferior temporal cortex, A21: mid temporal cortex, A22: superior temporal cortex, Par. A39: inferior parietal cortex, A10: frontal pole, A44: Broca area, A4: motor cortex, A18: secondary occipital cortex, A17: occipital pole. Patients were ranked in ten stages S1 to S10, according to ten brain areas that are successively affected. Values of pathological tau are indicated, as in[7]. The main clinical and neuropathological features are also presented. Some features of the cognitive status are presented. MMSE (mini mental state examination) and duration (duratn) of the dementing process are represented. * is for MMSE which were incomplete but was presented in [7]. CDR (clinical dementia rating) score is also presented.

The mean of the values of immunodetected Aη 42 and 40 are indicated. Note the sequential progression of NFD in the different brain areas. All patients with two association brain areas affected by tau pathology were demented. Note also the early and progressive increase of Aη 42 aggregates and the late appearance of Aη multimers.

these biochemical criteria are necessary and sufficient to demonstrate that an Alzheimer process is present, which could explain the cognitive deficit.

Infraclinical AD: All non-demented patients, or with a mild cognitive impairment, with insoluble Aη 42 at a concentration of 10 µg / gram of tissue in a polymodal association area, such as the frontal pole or the parietal cortex, and a tau pathology in the hippocampal area can be reasonably considered as patients at the infraclinical stage of Alzheimer's disease. The choice of 10 µg is arbitrary and corresponds to a sizeable detection of insoluble Aη, 10 times higher than the threshold of sensitivity of the technique with most antibodies against Aη At this concentration, there is no doubt that the amyloidogenic process of Alzheimer's disease has started. Staging of tau pathology is important to precise the extent of the neurodegenerating process. Tau pathology can be asymptomatic up to stage 6, and rarely but possibly up to stages 7 or even 8.

Normal aging: All non-demented patients can be considered as pure "controls", as far as tau and APP pathologies are concerned, if tau pathology is absent in all cortical areas, including the hippocampal area and if there is no trace of Aη 42 aggregates in neocortical areas. If the patients are older that 75 years, tau pathology, very discrete or moderate, is likely to be found in the hippocampal area (stages 1 to 3), due to an aging or a pathological process that remains to be determined. But these aged non-demented patients can be considered as controls as they have no detectable Aη 42 aggregates.

Here we show that Aη 42 aggregates are the main and sufficient variable to demonstrate and quantify amyloidosis. Our CEBDAD criteria can be used first for the staging of Alzheimer disease, in complement to the neuropathological one. Our method gives a rapid and reliable quantification of amyloid deposition and tau pathology. This method can be used to further characterize Alzheimer pathology of the frozen tissue from brain banks or from animal models. This approach is likely to be extremely valuable, since the brain tissue is the source for the search of biochemical (proteomic) or molecular markers (differential display, DNA chips). The CEBDAD should also be useful for the search of markers for the biological diagnosis, in the context of a prospective study and a parallel collect of biological fluids.

Amyloid patterns and the biological diagnosis of AD

The data presented here show the great variety of insoluble Aη patterns in AD patients, from Aη#42 aggregates found alone or associated with huge Aη 40 peptide accumulations. The ratio Aη 42/40 is varying from infinite to 0.4 according to the cases studied. This heterogeneity is likely to be mirrored by the Aη peptides that are released in the CSF or the blood. It could explain the relative lack of specificity observed for the biological tests based upon the ratio Aη 40/42[35]. Indeed, at the early stages of AD, we demonstrate here that all amyloid deposits are in the Aη 42 form. Biological parameters dealing with Aη 40 could bring a lack of specificity, due to the extreme heterogeneity of Aη 40 deposits as well as to the late appearance of insoluble Aη 40 in the course of the disease. Therefore, we recommend testing exclusively Aη 42 for the early diagnosis of AD.

Amyloid patterns and the etiology of AD

The quantification of Aη in the different brain areas also demonstrates that the pathway of tau pathology remains constant, even in a brain containing extremely large quantities of Aη aggregates in all brain areas. We conclude that if there is a toxicity of amyloid, this one is acting through neuron to neuron connections, and not by a direct neurotoxicity of Aη towards surrounding neuronal cell bodies[31] In that perspective, our results suggest more a loss of APP function as a neurotrophic factor than an amyloid toxicity as the cause of AD, Aη#; 9 being clearly the earliest marker of Alzheimer pathology, but not necessarily the etiologic factor.

Amyloid quantification methods

Surprisingly, we found up to 2 mg/g of Aη aggregates in AD brain tissues, in contrast with the 20 µg/g and 80 µg/g of Aη per gram of tissue measured with other immunochemical approaches[17,24] This is likely to be due to a lack of solubilization of Aη deposits, since these authors did not use pure formic acid for their extraction. This is also due to the fact that western blots are more adapted to insoluble material quantification than EAI, as reported by [17]. Conversely, EAI methods are more sensitive to detect Aη peptides soluble in non denaturant buffers, and especially adapted for the detection of physiological Aη that was not detected with our western blot method.

In conclusion, the values of Aη 42 aggregates observed in the human brain could be used for cellular and animal models of Aη 42 toxicity. Our quantitative approach was also validated by comparison with the neuropathological one, and led us to refine the criteria to establish a biochemical diagnosis of Alzheimer's disease (CEBDAD). This method was used to analyze the spatiotemporal development of tau and amyloid processes in aging and AD. The main result is the evidence that these two processes cooperate and evolve in parallel in AD. Their biochemical description presented here should facilitate the approach of Alzheimer's disease at all levels where therapy, diagnosis, search of markers and modeling are concerned.

Acknowledgments

We are deeply grateful to Innogenetics (E. VanMechelen) for the gift of 21F12 monoclonal antibody.

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