Progressive decrease of amyloid precursor protein carboxy terminal fragments (APP-CTFs), associated with tau pathology stages, in Alzheimer’s disease

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Abstract

Amyloid precursor protein (APP) dysfunction is a key aetiological agent in Alzheimer’s disease (AD). The processing of this transmembrane protein generates carboxy terminal fragments (CTFs) upstream of β-amyloid peptide (Aβ) production. The physiologic significance of APP-CTFs is still poorly understood, as well as the relationship that could link APP dysfunction and tau pathology in familial and non-familial AD (non-FAD). In the present study, we have investigated the quantitative and qualitative changes of APP-CTFs in different brain areas of non-demented and demented patients from a prospective and multidisciplinary study. A significant decrease of the five APP-CTFs was observed, which correlated well with the progression of tau pathology, in most cases with infraclinical AD and AD, either familial or non-FAD. Furthermore, solubility properties and the ratio between the five bands were also modified, both in the Triton-soluble and/or -insoluble fractions. Together, we show here for the first time a modification directly observed on APP-CTFs upstream of Aβ products and its relationship with tau pathology, which could reflect the basic aetiological mechanisms of AD.

Keywords: Alzheimer’s disease, amyloid precursor protein, pathological tau proteins, proteolytic processing.


Amyloidosis and neurofibrillary degeneration are the two degenerating processes that characterise Alzheimer’s disease. They correspond to dysfunctions of two basic proteins: APP (amyloid precursor protein) and microtubule-associated tau. The amyloid that accumulates in the grey matter of the cortex is composed of heterogeneous β-amyloid peptides (Aβs), which are cleavage products of APP. The enzymes that cleave the APP in the Aβ region are named secretases, with three types of activities in the α, β and γ region (Fig. 1) (De Strooper and Annaert 2000). α-Secretase generates the APPα and the α-carboxy terminal fragment (CTF). β-Secretase generates the APPβ, CTFβ and CTFβ′. Aβ peptides and CTFγ result from the cleavage of CTFβ and CTFβ′ by the γ-secretase.

The larger APP-CTFs containing the entire sequence of Aβ are neurotoxic, as demonstrated in cell or in animal models (for a review see Suh 1997). Furthermore, they could have physiologically important roles resulting from their interaction with numerous adaptor proteins such as Fe65, suggested for control of gene expression (Cao and Sudhof 2001), or signalling proteins such as X11L (Neve et al. 2000; Neve 2001).

Little is known about the fate of APP-CTFs at the different stages of AD, as well as their precise relationship with amyloid and tau pathologies. The aim of this work was essentially to determine if these fragments are modified in non-familial AD (non-FAD), and to investigate their potential link with the markers of neurodegeneration. Tools at our disposal were an important bank of frozen brain tissue from non-demented and demented Alzheimer patients, from a prospective and multidisciplinary study, and reliable immunological probes against APP-CTFs.

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Abbreviations used: AD, Alzheimer’s disease; APP, amyloid precursor protein; CTF, carboxy terminal fragment; FAD, familial AD; IP, immunoprecipitation; infra-AD, infraclinical non-FAD; IPG, immobilized pH gradient; NSE, neurone-specific enolase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
Materials and methods

Patients

We included 130 cases in the present study. Most of the samples from aged non-demented patients (n = 60) and several demented patients (n = 35) were obtained from the geriatric department of E. Roux Hospital (Limeil-Brevannes, France) and the Department of Neurology of the University Hospital Centre (Lille, France). The clinical assessments and neuropathology were already described (Delacourte et al. 1999). The post-mortem times ranged from 5 h to 61 h. The human brain biopsy sample was obtained from a tumour resection directly frozen in liquid nitrogen and kept at

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until biochemical analysis. Clinical and neuropathological data from patients are indicated in Table 1. This study included non-FAD cases (n = 14, age 75 ± 13 years), infraclinical non-FAD cases (infra-AD; n = 9, age 84 ± 10) and control subjects (Ctrl; n = 7, age 54 ± 18) (Table 1). Two patients with familial Alzheimer’s disease caused by PS1 mutation (Campion et al. 1996), FAD 1 codon 235 and FAD 2 codon 163, were included in this study.

Antibodies

Two New Zealand rabbits were immunized with a synthetic peptide corresponding to the last 17 amino acids of the human APP sequence. The serum was directly used or purified by affinity chromatography with the peptide covalently coupled to NHS-Sepharose. This antibody is named APPCter-C17. The B10 antiserum (a kind gift from Bart De Strooper, Leuven, Belgium) is raised against the C-terminal region of APP (De Strooper et al. 1995). A lab-made specific monoclonal antibody against the neurone-specific enolase (NSE), named 1C1, was used as neuronal marker. Briefly, 1C1 detects a single band at 47 kDa on brain tissue homogenates. Using two-dimensional gel electrophoresis followed by western blotting, it was shown to react with a single spot at 47 kDa, the isoelectric point of 4.9. This spot was isolated and analysed by mass spectrometry and it matched with the c-enolase or NSE. WO2 monoclonal antibody (Abeta, GmbH, Heidelberg, Germany) is raised against the amino-terminal region of Aβ and reacts strongly with APP fragments that contain the sequence 4–10 of Aβ (Fassbender et al. 2001). Anti-mouse or anti-rabbit antibodies coupled with horseradish peroxidase were purchased at SIGMA Immunochemicals (Saint Quentin Fallavier, France).

Brain tissue samples preparations and immunoprecipitation

Tissues were homogenized using a Teflon potter in 10 volumes of Laemmli sample buffer containing 5% (w/v) sodium dodecyl sulfate (SDS) and boiled for 10 min. For fractionation experiments, the cortical brain samples were homogenized in 10 volumes of 10 mM Tris-HCl pH 6.8 buffer and centrifuged at 100 000 g for 1 h. The supernatants (fractions F1) were collected, the pellets were re-suspended in Tris buffer containing 1% (v/v) of Triton X-100

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(1) Patients (n = 32) are ranked as a function of tau pathology stages. (2) The extent of tau pathology in the different brain areas is mentioned and the brain areas affected are indicated ■. (3) The presence (+) or absence (0) of an amyloid burden, detected at the neuropathological and biochemical level is indicated. (4) The main clinical data are summarized. (5) The final diagnosis was based upon the biochemical amounts of $\alpha$ and tau in the different brain areas (CEBDAD; Delacourte et al. 1999). (6) The main APP-CTFs changes in total brain tissue homogenates, F2 (Triton-soluble) and F3 (Triton-insoluble) brain extracts are indicated. ↓ Decreased, → not modified, ↑ increased, APP-CTFs; AB and DE specifies the bands that are specifically modified. 0, APP-CTFs were totally absent.
and centrifuged at 100,000 g for 1 h. The supernatants (fractions F2) were collected and the pellets (fractions F3) were homogenized in Laemmli sample buffer.

For immunoprecipitation (IP) experiments, 100 µL of the F2 fraction were diluted in three volumes of IP buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40 and EDTA-free protease cocktail inhibitors (Roche Molecular Biochemicals, Meylan, France) and pre-cleared with 10 µL of Immunopure® protein A-agarose (Pierce, Bezons, France) for 1 h at 4°C. Following centrifugation at 7000 g, the supernatant was added with 10 µL of APP-CterC17 antiserum and incubated overnight at 4°C. Immunopure® protein A-agarose (40 µL) was added and incubated for 1 h at 4°C. Agarose beads were washed three times with the IP buffer and 50 µL of non-reducing Laemmli sample buffer was added and incubated at 60°C for 5 min prior to centrifugation at 7000 g. The supernatant was collected for western blot analyses.

Treatment of immunoprecipitated APP-CTFs with calf intestine alkaline phosphatase (EC 3.1.3.1; Molecular Roche Biochemicals) was performed according to the manufacturer’s instructions. Briefly, following IP, agarose beads were washed three times with IP buffer, 50 µL of calf alkaline phosphatase buffer and 400 U/mL of enzyme was added and incubated at 37°C overnight. The reaction mixture was centrifuged at 7000 g, the supernatant discarded and 50 µL of non-reducing Laemmli sample buffer was added, as was done for the IP experiments. In a control experiment, immunoprecipitated APP-CTFs were processed without the addition of the enzyme.

### Western blot analyses

All protein samples were processed in Laemmli sample buffer and the same quantity of total brain proteins (100 µg/lane) was loaded on a 16.5% polyacrylamide gel. Tris-Tricine SDS–polyacrylamide gel electrophoresis (PAGE) was performed following the procedure of Schägger and von Jagow (1987) with a Protean II Xi Cell (Bio-Rad Laboratories, Hercules, CA, USA). Following electrophoresis, proteins were transferred to nitrocellulose membrane at 2.5 mA/cm² per gel using the semidry Novablot transfer system (Amersham Pharmacia Biotech). Following electrophoresis, the IPGs were equilibrated three times for 10 min in Laemmli sample buffer and was disposed over a 16.5% Tris-Tricine SDS–polyacrylamide gel. Tris-Tricine SDS–polyacrylamide gel electrophoresis (PAGE) was performed following the procedure of Laemmli (1970) applying a total of 75 kV/h, according to the manufacturer’s instructions (Westermeier et al., 1983; Gorg 1993).

The IPG-strip was equilibrated three times for 10 min in Laemmli sample buffer and was disposed over a 16.5% Tris-Tricine SDS–polyacrylamide gel. Tris-Tricine SDS–polyacrylamide gel electrophoresis (PAGE) was performed following the procedure of Laemmli (1970) applying a total of 75 kV/h, according to the manufacturer’s instructions (Westermeier et al., 1983; Gorg 1993).

For two-dimensional gel electrophoresis, 400 µg of protein was loaded on immobilized pH gradient (IPG) strip 3–10 (Bio-Rad), isoelectrofocalization was performed with the Protean IEF Cell System (Bio-Rad) applying a total of 75 kV/h, according to the manufacturer’s instructions (Westermeier et al., 1983; Gorg 1993). The IPG-strip was equilibrated three times for 10 min in Laemmli sample buffer and was disposed over a 16.5% Tris-Tricine SDS–PAGE.

### Quantification and statistical analysis of data

Western blots films were digitized using an Umax scanner calibrated for optical densities (Amersham Pharmacia Biotech). The IMAGE-MASTER 1D ELITE software (Amersham Pharmacia Biotech) was used to quantify the signal, and data were collected using EXCEL software (Microsoft, LesUlis, France). Statistical analyses were performed with StatView software (StatView SE+Graphics™, Abacus Concept Inc., Meylan, France).

### Results

#### Carboxy terminal proteolytic products of APP in human brain tissue

The specificity of APPCter-C17 antiserum was compared with the purified antiserum B10 (De Strooper et al. 1995) (Fig. 2a, lane 1). The same electrophoretic profile was observed, consisting of five individual bands ranging from 8 to 15 kDa (Fig. 2a, lane 2) as previously described (Estus et al., 1992; Golde et al., 1992; Haass et al., 1992; LeBlanc 1994; Russo et al. 2001). These APP-CTFs bands were detected by the purified antiserum APPCter-C17, whereas the absorbed serum collected after affinity chromatography showed no immunoreactivity (Fig. 2a, lane 3). Thus, the five bands A–E correspond to human APP-CTFs. Moreover, in biopsy derived human brain tissue, the five APP-CTFs were detected with the same electrophoretic profile as that observed in autopsy brain tissue homogenates, demonstrating that none of the APP-CTFs are generated during postmortem delays (Fig. 2b).

A quantitative analysis of APP-CTFs was performed in control, infra-AD, AD and FAD (PS1 mutation) total brain tissue homogenates (Figs 2b and c). Infra-AD patients correspond to non-demented patients, with an important amyloidosis and tau pathology (Table 1), as defined in Delacourte et al. (1999). The five APP-CTFs bands were detected with a similar pattern in control, infra-AD, AD and FAD brain tissue homogenates (Figs 2b and c). However, a reduced signal of APP-CTFs was observed in the infra-AD and AD cases, as well as in FAD when compared with the control cases. In contrast, APP holoprotein was observed with similar expression profile even in the brain tissue with important loss of APP-CTFs (Fig. 2b). Statistical analysis showed that the decrease in the quantity of APP-CTFs was 1.5-fold (p < 0.03) for the infra-AD and 1.7-fold (p < 0.002) for AD (Fig. 3a).

Intriguingly, among the AD patients analysed an increase of APP-CTFs of 1.5-fold was observed, but this was for one AD case exclusively (Fig. 2b, AD lane 5; Fig. 3a, □). Overall, our results show a significant decrease in the quantity of APP-CTFs in non-FAD and FAD cases (Fig. 3a).

#### Relationship between APP-CTFs reduction and tau pathology stages in AD

At least one patient per stage of tau pathology was studied among the patients included in the present study. The quantity of APP-CTFs was compared with the stages of tau...
pathology that reflect well the progression of the disease (Delacourte et al., 1999). Significant correlation was observed between the level of APP-CTFs and the progression of tau pathology in the temporal ($p < 0.0001$) and in the occipital cortex ($p < 0.003$) (Fig. 3b and statistics table). No correlation was found between the APP-CTFs modifications and other known factors such as age, sex, ApoE genotype and post-mortem delay (data not shown).

Modification of the solubility distribution of APP-CTFs
To investigate further the APP-CTFs in the human brain, a solubility fractionation of APP-CTFs was performed as already described (Allinquant et al., 1994; Bouillot et al., 1996; Ikezu et al., 1998; Hayashi et al., 2000). In control brain tissue, the first Tris-buffer fraction (F1) did not contain APP-CTFs (Fig. 4a, F1). A large quantity of APP-CTFs was extracted in fraction F2, in 1% of non-ionic detergent
Triton X-100, and no additional APP-CTFs were detected after a repeated extraction in the same conditions (Fig. 4a). The third fraction (F3) was recovered in Laemmli sample buffer. The five APP-CTFs bands were detected in both fractions F2 and F3 without any differential solubility between APP-CTFs bands, and more than 50% were recovered in the F2 fraction (Fig. 4a).

The same approach was used to analyse the differential solubility distribution of APP-CTFs in control, infra-AD and AD cases (Fig. 4b). Temporal and occipital regions were analysed and showed similar results (shown for temporal region). Modifications of APP-CTFs were observed in F2 and F3 fractions in infra-AD and AD cases when compared with control cases. Indeed, the labelling was more intense for APP-CTFs bands D and E in F2 fraction in infra-AD and in AD cases (Fig. 4b and Table 1). In F3 fraction, APP-CTFs bands A and B were increased in AD patients. In infra-AD and AD cases with a decreased quantity of APP-CTFs, the decrease was stronger in the F3 fraction (Fig. 4b, lanes 6 and 8 of infra-AD and AD). The increase was observed in F2 and F3 fractions (Fig. 4b, AD lane 9) in the only AD case showing a global increase in APP-CTFs (Fig. 3a, ○). However, as for infra-AD and AD cases, in the F2 fraction APP-CTFs bands D and E were more intensively labelled, and in the F3 fraction the APP-CTFs bands A and B staining was stronger. APP-CTFs are therefore qualitatively modified in infra-AD and AD when compared with control, even at the early stages of AD (stages 1–3) where the global quantity of APP-CTFs was not significantly decreased.

We also investigated if the decrease of APP-CTFs was the result of a complete insolubilization, as observed for Aβ aggregation into totally SDS-insoluble aggregates (Delacourte et al. 1999). For that purpose, we solubilized the brain tissue with progressive concentrations of formic acid, and then we neutralized and dissolved the brain tissue homogenate in SDS buffer for SDS–PAGE analysis. Aβ peptides were released using this method but no additional quantity of APP-CTFs was recovered (data not shown).

Characterization, phosphorylation and two-dimensional analysis of APP-CTFs

To further characterize the APP-CTFs bands, an immunostaining was performed with WO2 antibody that recognizes the amino-terminal region of Aβ as well as APP fragments including this sequence (Ida et al. 1996; Fassbender et al. 2001). Thus, the use of WO2 would enable us to distinguish between β-stub and β′-, α- and γ-stubs. Following IP with APPCter-C17, APP-CTFs bands A and B were detected with WO2 showing that they both correspond to β-stubs fragments (Fig. 4c). Consequently, APP-CTFs bands C, D and E might correspond to β′-, α- and γ-stubs. Alkaline phosphatase treatment of IP APP-CTFs reduced the number of bands from five to three (Fig. 4d). APP-CTFs bands A and C disappeared, whereas a stronger signal was observed for bands B and E. These results suggest that band B and band E correspond to the unphosphorylated β-stub and α-stub, respectively. Conversely, band A corresponds to phosphorylated β-stub. The dephosphorylated band D could

Fig. 3 APP-CTFs and tau pathology. (a) APP-CTFs levels in the temporal cortex from four groups: Control cases (Ctrl), intractinal AD cases (infra-AD), AD cases (AD) and familial AD cases (FAD). A significant decrease was observed between infra-AD and AD vs. Ctrl (IAD, p < 0.007; AD, p < 0.001), using the non-parametric statistic test of Mann–Whitney. Average decrease of APP-CTFs was of 1.5-fold for infra-AD and of 1.7-fold for AD vs. controls. Patient S9,c (○) had a 1.5-fold increase of APP-CTFs. The decrease of APP-CTFs in the infra-AD and AD groups was not significantly different. (b) Linear regression between APP-CTFs levels and tau pathology in human temporal cortex of patients at different stages of tau pathology. A significant relationship was observed between APP-CTFs levels in the temporal cortex and in the occipital cortex of each patient studied and the different stages of tau pathology (statistics table).
correspond either to unphosphorylated β'-stub or to incompletely dephosphorylated α-stub. Lower molecular weight APP-CTFs were not detected suggesting that the γ-stub (expected at 6.5 kDa) is not detected in our conditions.

Dephosphorylation of APP-CTFs was performed on brain tissue fraction F2 of the temporal cortex of control (S0,c) and the temporal cortex of an AD patient (S9,c). A lower level of band B and a higher level of band E was observed in AD (Fig. 4d). This result corroborated that obtained directly on fractions F2 (Fig. 4b, lanes 5, 7 and 9) and it suggested in addition a difference in the phosphorylation state of APP-CTFs in AD.
Post-translational modifications of APP-CTFs were investigated using two-dimensional gel electrophoresis coupled with western blotting. Analysis of APP-CTFs was performed in the F2 fraction of one control (S0,a), one infra-AD (S1,a) and one AD (S9,b) sample. Sixteen isovariants of APP-CTFs were detected in the control, whereas 23 isovariants were detected in AD (Fig. 5, Ctrl and AD panels). This figure shows that nine isovariants of APP-CTFs spots are common to control, infra-AD and AD, six isovariants are only detected in control and 13 additional isovariants of APP-CTFs are detected in AD. These additional acidic isovariants corresponded mainly to APP-CTFs bands D and E, and they are consistent with the stronger staining of these bands on Tris-Tricine SDS–PAGE (Fig. 4b, case S9,b).

**Discussion**

APP dysfunction is the aetiologic factor of AD, as demonstrated by the pathological mutations on APP, PS1 and PS2 genes. These mutations provoke a degenerating process, an increased ratio of Aβ42 : Aβ40 peptides (except for the Swedish mutation) and amyloid plaques. Well correlated to dementia, tau pathology is also an inescapable and constant
event of FAD and non-FAD (Delacourte et al. 1999). Apart from these facts, we do not yet know precisely the origin of neurodegeneration. Aβ toxicity is frequently suggested, by indirect means, but either a loss of function of APP or a role of other metabolic products such as APP-CTFs can also be suggested (Neve 2001). In particular, it has been recently demonstrated that APP-CTFs bind to Fe65 to form an active transcriptional complex with Tip60 (Cao and Sudhof 2001).

In the present study, our aim was to investigate whether APP-CTFs were significantly modified in AD. For that purpose, we analysed the qualitative and quantitative changes of APP-CTFs in the temporal and occipital brain areas of numerous patients at different stages of AD, from normal controls to severely affected patients. Our results demonstrate dramatic changes of APP-CTFs as a function of the progression of the disease, illustrated by the progressive extent of tau pathology in neocortical areas.

A significant decrease of APP-CTFs in AD

Using a western blot approach, we have been able to detect precisely and quantitatively the five (A–E) main bands that correspond to APP-CTFs in the human brain. A significant decrease was observed in non-FAD and FAD cases. Indeed, to explain the increase of Aβ production demonstrated well in FAD, an increase of APP-CTFs would have been expected. This is not the case, with the exception of one AD patient. Interestingly, the decrease was also observed at the infraclinical stages of AD. This general decrease was found evenly in two different neocortical brain areas, the temporal and occipital cortices.

It was also unexpected to have a global decrease of APP-CTFs in non-FAD patients as well as in some FAD cases, showing similar patterns of APP-CTFs. These data are in favour of a general dysmetabolism of APP-CTFs in all subtypes of AD. Intriguingly, only one case had a pattern opposite to all others, with huge quantities of APP-CTFs, but in agreement with the well known fact that this disease is extremely heterogeneous on many points, even those directly involved in the aetiology, as demonstrated by the numerous different mutations on APP and presenilin genes.

Characterization of APP-CTFs and modifications of their solubility distribution in AD

β-Stubs were selectively identified from β⁺-, α- or γ-stubs of APP-CTFs using WO2 antibody and we show that bands A, C and D correspond to phosphorylated variants of β⁺, β⁻ or α-stubs, because dephosphorylation of APP-CTFs result in the disappearance of corresponding bands. Our characterization is in agreement with that described by Russo et al. (2001) and also suggests that the recently described γ-stub or AICD (amyloid precursor protein intracellular domain) (Cupers et al. 2001; Kimberly et al. 2001; Sastre et al. 2001; Yu et al. 2001) is not a part of the five APP-CTFs bands described in human brain tissue.

Using a differential solubility assay, we show that in addition to reduced expression of APP-CTFs, α- and/or β-stubs are present in higher levels in the Triton-soluble fraction, whereas β-stubs are present in higher levels in the SDS-insoluble fraction in AD. Moreover, dephosphorylation and two-dimensional analysis also suggest that α-stubs are phosphorylated more and, hence, it could account for their higher solubility. Conversely, the reduced solubility of β-stubs could be related to a reduced phosphorylation. Thus, our data suggest a relationship between the change of specific solubility, the progressive disappearance and post-translational modifications such as phosphorylation. This is illustrated by the presence of APP-CTFs isovariants that were only detected in the tissue affected by Alzheimerization, thus demonstrating a specific dysmetabolism of APP-CTFs in AD.

Conclusion

This is the first report demonstrating modifications of APP-CTFs in the human brain affected by AD. This demonstration was possible because we have a large bank of brain tissue that was carefully characterized at the clinical, neuropathological and biochemical levels. In particular with our biochemical approach we were able to differentiate clearly aging from the infraclinical stages of AD, using a quantification of tau and Aβ pathologies in numerous brain areas. The staging of tau pathology was particularly helpful for grading the extent of Alzheimer pathology (Delacourte et al. 1999).

However, despite the complete characterization of the brain tissue used for our experiments, the APP-CTFs changes observed were not found in a precise and linear relationship with the other parameters of Alzheimer pathology. This is not surprising because AD is extremely heterogeneous, and is likely to be a disease resulting from a combination of numerous factors, each of them weighing more or less on the development of the pathology (Delacourte 2000). The statistical significance of APP-CTFs changes in AD demonstrated here is, however, very strong.

Two different hypotheses can explain our observations. First, loss of APP-CTFs could result from a modification of secretase activities, and especially from an activation of γ-secretases, and therefore a transformation of APP-CTFs into γ-CTFs, Aβ peptides and smaller fragments (see Fig. 1). The second hypothesis is that the targeting of APP in subcellular compartments is dys-regulated. The differential decrease of APP-CTFs in our differential solubility assay, and the different ratio between the five bands is in favour of such a hypothesis. The challenge to verify these hypotheses in relevant models remains.

Together, we demonstrate a dysfunction of APP that could be deleterious for neurones, and that could trigger tau pathology (Mesulam 1999; Delacourte 2000). One additional benefit of our study is that the precise modifications of
isovariants found in the nervous tissue in AD could be observed also in the peripheral fluids, as APP is an ubiquitous protein. If so, the decrease or the post-translational modification of APP-CTFs could be used as an early marker for the biological diagnosis of AD.

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