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ALZHEIMER'S DISEASE AMYLOID PRECURSOR PROTEIN IS PRESENT IN SENILE PLAQUES AND CEREBROSPINAL FLUID:

Immunohistochemical and Biochemical Characterization

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The amyloid fibrils deposited in cerebral vessel walls and senile plaques in Alzheimer's disease are polymeric forms of a 4 kDa fragment produced by proteolysis of a putative precursor protein (APP). Using antibodies to several fragments of the deduced precursor, we were able to demonstrate the presence of APP in senile plaques, brain extracts and cerebrospinal fluid. Membrane-associated APP is detected as a group of 105-135 kDa proteins while soluble APP is predominantly 105 kDa, does not react with an anti C-terminal antibody, and is 10 kDa shorter than the membrane-bound APP. Amino terminal sequence of the tissue 105 kDa protein indicates that APP begins at residue 18 of the cDNA sequence. These findings imply that i) two forms of APP are detected: membrane-bound and secreted, and ii) APP can be processed in situ.

Alzheimer's disease (AD) is the most common form of human amyloidosis and the major cause of dementia. The amyloid fibrils deposited in blood vessels (β-amyloid protein, AP) and plaque cores (A4) (1-3) are similar and composed of 39 and 42 residues respectively, with no sequence homology to known brain peptides or subunit proteins of other types of amyloid. AP is also the main component of amyloid fibrils deposited in a group of diseases showing different patterns of amyloid distribution and clinical manifestations: e.g. Hereditary Cerebral Hemorrhage with Amyloidosis (Dutch type) (4), cerebral amyloid angiopathy,

Abbreviations used: AD, Alzheimer's Disease; CSF, Cerebrospinal Fluid; AP, Amyloid Protein; APP, Amyloid Protein Precursor; SDS-PAGE, Sodium dodecylsulfate polyacrylamide-gel electrophoresis.

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asymptomatic age-related amyloidosis (5), and Guamanian parkinsonism-dementia (6). Oligonucleotides based on the AP sequence allowed several groups to isolate cDNAs that encode a 695 amino acids amyloid protein precursor (APP), and to localize its gene to the long arm of chromosome 21 (7-10). The amino terminal of the AP maps at position 597 of the precursor. It has recently been shown that APP is codified by discontinuous DNA (11), and 16 exons have been identified and sequenced. Additional forms of APP mRNAs, encoding proteins of 751 and 770 residues, represent alternative splicing products of the same gene (12-14).

The presence of AP deposits in AD brain can not be explained by an APP gene defect (15), or an overexpression of a particular exon (11); therefore, posttranslational changes may be implicated in the anomalous cleavage and deposition of the highly insoluble AP. Using antibodies directed to different parts of the putative APP, several groups reported immunoreactive bands of different molecular weight on immunoblots in a wide range of tissues and cultured cells (16-19); however, the amino terminal sequence of the mature protein remains unknown.

We are reporting the immunochemical characterization of APP in brain tissue and cerebrospinal fluid (CSF) from AD and control patients, by using polyclonal antibodies against different portions of the APP. The N-terminal sequence of tissue APP indicates that the protein begins at position 18 of the precursor sequence deduced from cDNA.

MATERIALS AND METHODS

Synthetic peptides and polyclonal antibodies

According to the sequence deduced from the APP cDNA (8), several peptides were synthesized in the Center for the Analysis and Synthesis of Macromolecules (SUNY, Stony Brook), using solid phase techniques (20). Among them, peptides SP18 (amino acids 45-62) and SP28 (amino acids 597-624) were used to obtain polyclonal antibodies in rabbits. Peptide SP18 was coupled to Keyhole Limpet Hemocyanin before injection while peptide SP28 was used uncoupled. The rabbits were immunized every fifteen days with 200 μg of antigen dissolved in 0.15 M NaCl mixed with complete Freund's adjuvant in a 1:1 ratio. The animals were bled according to the evolution of the antibody titer (evaluated by ELISA), and boosted every 20 days. Polyclonal antibody anti-C_1 (amino acids 676-695) was previously characterized (17).

Biological samples

The frontal lobes from three patients with histologically confirmed Alzheimer's disease (age of death: 55, 62 and 93 years) and two control subjects (age of death: 67 and 72 years) were used for this study. The tissues were obtained with a postmortem delay of 2.5, 5, 7.5, 1.5, and 4 hours, respectively, and kept frozen at -70°C.

Samples of CSF from AD and control patients were collected in the presence of proteinase inhibitors and stored at -70°C.

Immunohistochemistry

Immunohistochemical studies were carried out on 8 μm thick cryostat sections of unfixed frontal cortex and paraplast-embedded serial sections of frontal blocks fixed either in 10% phosphate buffered formalin or Bouin's solution. The tissue was incubated with anti-SP28, anti-SP18 and anti-C_1 anti-sera (1:50, 1:200 and 1:150 dilution, respectively), at 4°C overnight. In order to improve the staining with anti-SP28, relevant sections were pretreated with 98% formic acid, as described (21,22). The immunodetection was carried out using an avidin-biotin-peroxidase system (Amersham). Peroxidase substrate was 3-3'diaminobenzidine (Sigma) containing 0.015% H_2O_2. Negative control sections were incubated with rabbit preimmune sera as a first antibody, at the same dilution as that the indicated above. Specificity of immunoreactions was confirmed by peptide absorption. The antibodies were incubated with 10 μM of the corresponding SP for 1 hour at 37°C and overnight at 4°C. After centrifugation at 10,000 g the supernatants were used as primary antibodies.

Purification procedure

Tissue: Gray matter was dissected from the frontal cortex and aliquots (2 - 3 g wet weight) were homogenized in 2 volumes of buffer A (50 mM Tris - 150 mM NaCl - 5 mM EDTA - 5mM EGTA - 5 μg/ml soyabean trypsin inhibitor - 10 μg/ml aprotinin - 0.1 μg/ml pepstatin - 1 μg/ml TLCK (7 amino-1 cloro-3 tosylamido-2 heptanone) - 2 mM PMSF (phenylmethylsulphonyl fluoride), pH 7.6) followed by centrifugation at 100,000 g for 60 minutes. The material yielded a supernatant and a pellet; the latter was further extracted by homogenization with 2 volumes of buffer B (idem buffer A plus 2% Triton X-100) followed by sonication for 45 seconds (Heat Systems Sonicator). After centrifugation in the same conditions as above, a second supernatant and a pellet were obtained. The latter was rehomogenized and sonicated in buffer C (idem buffer B, without NaCl, containing 0.6 M KCl). After centrifugation, a third supernatant and a pellet were obtained; all the samples were stored at -20°C until further studies.

CSF: Samples of CSF were dialyzed 24 h against distilled water containing 2 mM PMSF, pooled, and lyophilized.

Immunodetection of APP

The search for APP was carried out via immunoblot analysis. Fractions from tissue extraction and samples of CSF were subjected to SDS-PAGE (23) in 7.5% minigels under reducing conditions. After electrophoresis, the samples were transferred onto PVDF membranes (Immobilon, Millipore Corp.) using 3-cycloexylamino-1-propanesulfonic acid (CAPS) buffer, pH 11. The membranes were blocked with 0.2 % gelatin in TBS (20 mM Tris - 150 mM NaCl, pH 7.5) and incubated overnight with anti-SP antibody in appropriate dilution (anti-SP18, 1:1000; anti-C_1, 1:200). Alkaline phosphatase labeled goat anti-rabbit IgG (Bio Rad) 1:2000 was used as a second antibody. Immunoblots were developed using 5-bromo-4-chloro-3-indolyl-phosphate and Nitroblue tetrazolium (Kirkegaard & Perry Lab. Inc.).

Amino acid sequence analysis

Sequence analysis was carried out on a 477A protein sequencer, and the PTH amino acids identified using an on-line 120A PTH analyzer (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Several antisera raised against fragments of the predicted precursor were used to detect the APP in tissue and CSF. In histological sections, senile plaques detected either by thioflavine S or anti-SP28, were also immunostained by anti-SP18 (Fig. 1). Each antibody labeled different structures: anti-SP28 (24-26) recognized the plaque cores and the amyloid bundles intermingled with degenerating neurites (Fig. 1a) while anti-SP18 immuno-stained small round or larger rod-shaped structures at the plaque periphery. These structures varied in size from 1 to 15 μm and, on some occasions, resembled dystrophic neurites (Fig.1 b-c). Preimmune sera and preabsorbed antisera did not label any structure, indicating the specificity of the reaction. Since different staining patterns were found, we concluded that AP and its precursor coexist in the same plaque, with the amyloid fibrils surrounded by APP. A pattern similar to anti-SP18 was obtained with anti-C_1 antiserum (not shown).

Anti-SP18 was able to recognize several bands on immunoblots of human cortical homogenates. When the tissue was extracted with different solutions, immunoreactive material of 105 kDa was released in isotonic conditions. The detergent containing buffer yielded a fraction enriched in proteins of 105-115, 125 and 135

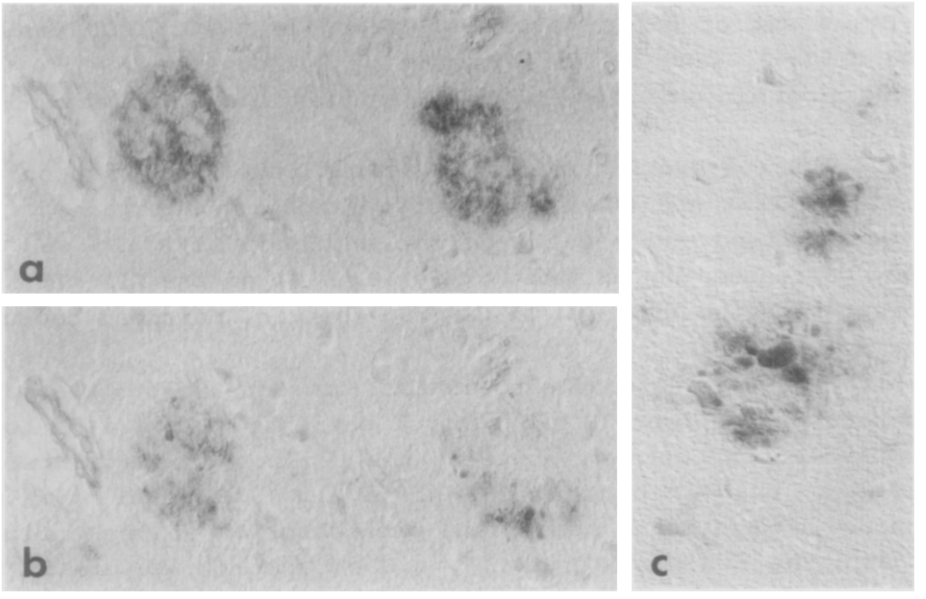

Figure 1 Positive immunohistochemical reaction of APP and AP to the anti-SP18 and anti-SP28 antibodies. Magnification x 360. (a) and (b) are adjacent sections. a) anti-SP28. b and c) anti-SP18.

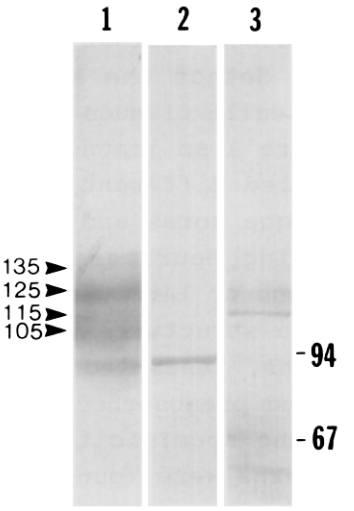

Figure 2 Identification of APP proteins in tissue extracts. Western Blot analysis in 7.5% SDS-PAGE; all lanes loaded with 200 μg of protein. Molecular weight markers: 94 -: Phosphorylase B, (94 kDa); 67 - : Bovine serum albumin, (67 kDa). Membrane-bound APP recognized by anti-SP18 (Lane 1) and anti-C_1 (Lane 3). The reaction is negative after absorption of the anti-SP18 (Lane 2).

kDa, indicating their membrane-associated nature (Fig 2, lane 1). The reaction was negative with preimmune serum, and preabsorption of the antibody abolished the staining (Fig. 2, lane 2). Using anti-C_1, a set of bands migrating from 115 to 135 kDa was detected (Fig. 2, lane 3) as described by Selkoe et al. (17). The 105 kDa protein was not labeled, suggesting a truncated C-terminal.

The 105 kDa protein extracted from tissue was fractionated on 7.5% SDS-PAGE and transferred onto Immobilon membranes. The band was excised and sequenced; the sequence LEVP()DG was obtained, corresponding to amino acids 18 to 24 of the APP cDNA, in agreement with the findings in CSF (S. Younkin, personal communication).

Anti-SP18 was also able to detect similar 105-115 kDa bands in samples of CSF from AD and control patients (Fig. 3a). Loading equivalent amounts of protein, the reaction was stronger in AD (lane 1) than in control CSF (lane 2). The bands were not recognized by preimmune serum, and preabsorption of the antibody abolished the staining (lane 3). When anti-C_1 was used, the reaction with CSF was negative (Fig 3b lane 1). These findings indicate that soluble forms of APP detected in the CSF and tissue are shorter (approximately 10 kDa) than the membrane-bound form; the difference is located at the C-terminal end, as indi-

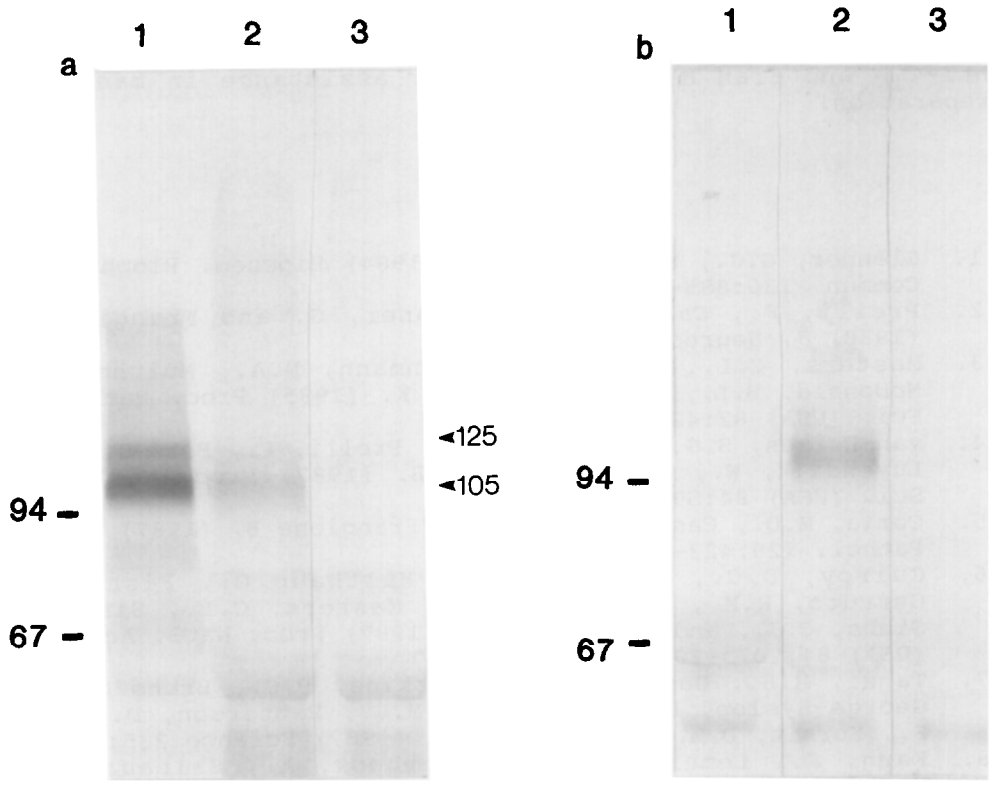

Figure 3 Identification of APP proteins in CSF by anti-SP18.
a) Soluble APP is recognized by anti-SP18 in AD CSF (Lane 1) and control CSF (Lane 2). Absorption of anti-SP18 abolishes the staining (Lane 3).
b) Soluble APP is not recognized by anti-C_1 in AD CSF (Lane 1), while anti-SP18 is able to stain the 105-115 kDa bands (Lane 2) and absorption of the antibody abolishes the staining (Lane 3).

cated by the amino terminal sequence of the 105 kDa protein and the lack of reactivity with anti C-terminal antibody.

In summary, our data demonstrate that APP is present in the periphery of neuritic plaques and in CSF, and two main forms of precursor proteins are detected: membrane (APPm) and secreted (APPs). The presence of APP and amyloid fibrils in neuritic plaques suggests that the processing of the precursor takes place in situ, although it is not known from which form of APP the amyloid fibril is derived. Preliminary observations indicate that the APP is also present in vessel walls. Further studies are necessary to determine the cell/tissue origin of the APP involved in the pathogenesis of amyloid formation and deposition, as well as its relationship with the process of aging of the brain (5).

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