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Rapid Communication

Alzheimer's Amyloid Precursor Protein-positive Degenerative Neurites Exist Even Within Kuru Plaques Not Specific to Alzheimer's Disease

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To clarify the relationship between amyloid formation and amyloid precursor protein (APP), the brain sections from eight patients with Alzheimer's disease (AD) and four with Gerstmann–Sträussler Syndrome (GSS) were investigated immunohistochemically by the double-immunostaining method. In AD, most APP-positive senile plaques belong to classical plaques or primitive plaques, whereas in diffuse plaques, APP-positive neuritic components are rarely observed. The authors documented that anti-APP-labeled degenerative neurites surrounding kuru plaques in all four GSS patients. These kuru plaques were verified by double immunostaining using anti-prion protein and anti-APP. The APP-positive structures in kuru plaques were almost identical with those seen in the classical plaques in AD. The authors concluded that APP-positive degenerative neurites are not an early event in the amyloid formation of senile plaques. It is therefore postulated that depositions of β/A4 and prion proteins are primary events that may involve the surrounding microenvironment and result in the secondary formation of APP-positive degenerative neurites, not specific to AD. (Am J Pathol 1991, 139:1245–1250)

Amyloid deposits in the central nervous system occur both in senile plaques and kuru plaques. Senile plaques are one of the most conspicuous pathological findings of Alzheimer's disease (AD), and are also detected, to a lesser degree, in the brains of the aged individuals who do not suffer from dementia.[1] The major component of amyloid in senile plaques is a 4 kDa polypeptides, named β protein or A4 protein (β/A4 protein),[2,3] which is generated by proteolytic cleavage of a glycosylated membrane-spanning protein designated as the amyloid precursor protein (APP).[4–6] Alternatively spliced APP transcripts code for APP 695, APP 751, and APP 770.[7–9] Several investigators have hypothesized that APP accumulation precedes the formation of β/A4 protein amyloid and that proteolysis of APP to β/A4 protein occurs within the senile plaques.[10] However, the origin of the extracellular β/A4 protein found in senile plaques and the site of proteolysis of APP in AD remains to be established.

On the other hand, kuru plaques, which are seen in patients with kuru or Gerstmann–Sträussler syndrome (GSS), are composed of prion protein (PrP).[11] Although the amino-acid sequence of β/A4 protein is different from that of PrP,[4] senile plaques resemble kuru plaques morphologically, suggesting a similar morphogenesis.[12–18] Herein, we investigated the relationship between amyloid deposition (kuru or senile plaques) and APP using the double-immunolabeling method, and describe APP positive kuru plaques in the brain of patients with GSS.

Materials and Methods

Materials

We examined eight patients with AD, who fulfilled the established criteria for AD,[19] and four with GSS. All of the

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GSS patients had a missense variant of PrP with a codon 102 proline to leucine change.[20] Brain tissues were fixed in formalin, embedded in paraffin, and cut into 5 μm thick sections. The tissue sections of the cerebral cortex, hippocampus, and cerebellum from each patient were examined immunohistochemically.

Antibodies

We prepared three antibodies against human APP. Monoclonal antibody (mAb 11C22) and polyclonal antibodies to APP 695 fusion protein have been previously described.[21] The third antibody is a newly generated polyclonal antibody against APP 770-fusion protein. The plasmid designated pFd-APP 770 is based on the prokaryotic vector pFd-APP 695.[21] The plasmid pAD 770, which codes for the entire human APP 770,[21] was digested with KpnI and SacI. The resulting fragment, including Kunitz inhibitor domain, was ligated in the vector fragment of pFd-APP 695 which was digested with KpnI and SacI. The plasmid pFd-APP 770 was transformed in *E. coli* BB 1.8. The fusion protein was purified from the inclusion body fraction by preparative SDS-PAGE and electroelution. The identity of the purified protein was confirmed by Western blot analysis. A rabbit was immunized with the Fd-APP 770 fusion protein (about 250 μg) emulsified with complete Freund's adjuvant. After serial boosters (7 times), the antibody was checked for immunostain and was analyzed by Western blotting (Figure 1). The Western blotting procedures were reported previously.[22,23]

We also used anti-human PrP, and anti-β/A4 protein, which were also previously described.[22,23]

Immunohistochemistry

The sequential double immunostaining method has been described elsewhere.[24] To enhance the immunoreactivity, formic-acid pretreatment[23] and hydrated autoclaving[25] were used. After pretreatment, the sections were incubated with anti-Fd-APP 770 (1:2000) at 4°C overnight. The following steps were performed with the biotin-streptavidin method (ICN Immunochemicals) according to the manufacturer's instructions using diaminobenzidine (DAB) as a chromogen. After developing the DAB reaction, the anti-Fd-APP 770 was eluted with glycine

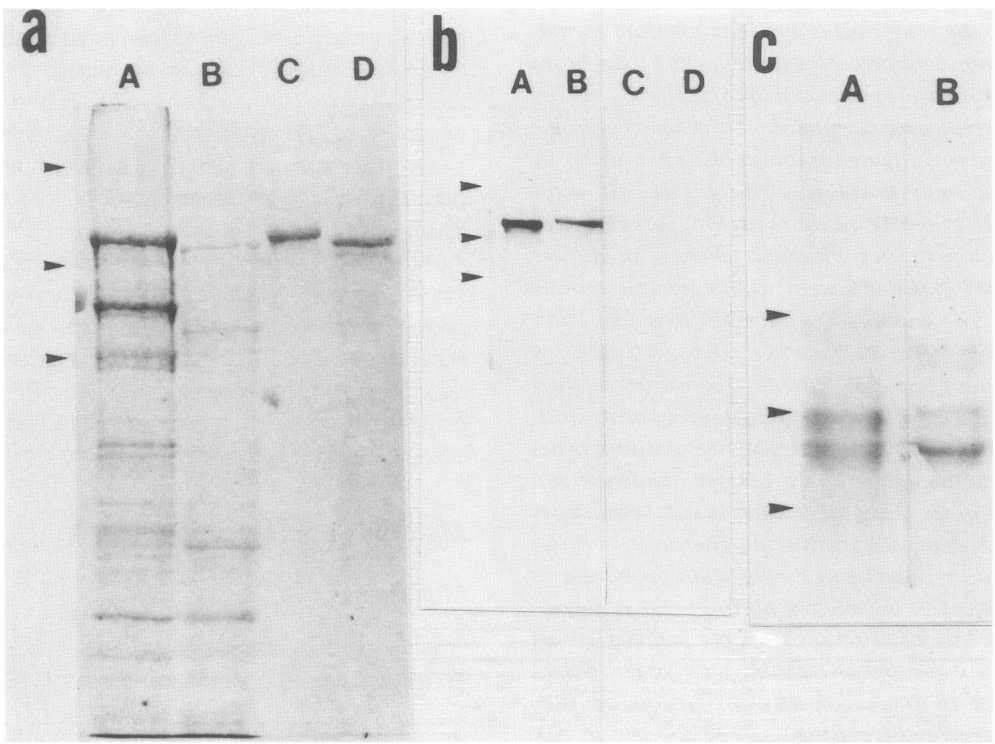


Figure 1. *Characterization of APP fusion protein and APP antibodies.* **a:** *SDS-polyacrylamide gel (8%) electrophoresis. Lane A, inclusion body fraction of Fd-APP 770. Lane B, inclusion body fraction of Fd-APP 695. Lane C, purified Fd-APP 770. Lane D, purified Fd-APP 695. Gel was stained with Coomassie brilliant blue. Arrows show molecular marker (180, 116, and 84 KD).* **b:** *Western blotting with anti-Fd-APP 695 (Lane A and B) and without the primary antibody (Lane C and D). Lane A and C, Purified Fd-APP 695, Lane B and D, Purified FD-APP 770. Electrophoresis was performed with 8% gel. Arrows show molecular marker (180, 116 and 84 KD).* **c:** *Western blotting using human brain homogenate. Electrophoresis was performed with 6% gel. Lane A was stained with monoclonal antibody (mAb 11c22). Lane B was stained with anti-Fd-APP 770. Arrows show molecular marker (180, 116 and 84 KD).*

buffer (100 mM Glycine-HCl pH 2.2). Then, the sections were incubated overnight with a second primary antibody, anti-human PrP (1:5000) or anti-β/A4 protein (1:2000) overnight at 4°C. The biotin-streptavidin method was used again in the same manner. In the final step, the color was developed with DAB and cobalt chloride.[24,26]

For single immunostaining, we used DAB as a chromogen.

Results

APP Accumulations in Senile Plaques in AD

Anti-Fd-APP 770, anti-Fd-APP 695 and mAb 11c22 gave essentially similar results. Negative control studies including preimmune serum and omission of primary antibodies did not show any positive reactions. In AD, these antibodies identified neurons and structures surrounding senile plaques. APP-positive structures in senile plaques resembled degenerative neurites in agreement with the data of other groups.[10,27–33] The number of APP-positive senile plaques was small, compared with the number of β/A4 protein-positive plaques. To examine the population of β/A4 and APP-positive plaques, double immunostainings with anti-β/A4 protein and anti-Fd-APP 770 were performed. Most of the APP-positive senile plaques had a well-defined amyloid core, designated as classical or typical plaques (Figure 2A). However, some primitive plaques without an amyloid core were also immunolabeled with anti-APP. While in diffuse plaques, APP-positive structures were rarely seen (Figure 2A). Diffuse plaques are a recently described plaque type, which do not have neuritic component. To differentiate diffuse plaques from primitive plaques, tissue sections with silver staining need to be examined. It is difficult to perform double immunostaining and silver staining simultaneously on the same section. Since it is well known that many AD patients have an early stage of β/A4 deposit in the cerebellum,[34,35] we examined the cerebellar tissue sections from patients with AD. Among eight AD patients, five had only diffuse plaques in the cerebellum. In these five patients, anti-Fd-APP 770 did not immunolabel the β/A4 deposits in the cerebellum (Figure 2B). In the other three AD patients, who had classical or typical plaques in the cerebellum, anti-APP immunolabeled the degenerative neurites in senile plaques even in the cerebellum (Figure 2C).

APP Accumulations in Kuru Plaques in GSS

Anti-Fd-APP 770, anti-Fd-APP 695 and mAb 11c22 gave essentially similar results. To examine APP accumulations in kuru plaques, we used cerebellar sections from patients with GSS. Our previous study showed that elderly GSS patients have senile plaques in the cerebral cortex, but not in the cerebellum.[36] APP positive structure in kuru plaques corresponded to the degenerative neurites as previously reported[37] (Figure 3D). These APP-positive structures appeared almost identical with those observed in senile plaques in AD. Double immunolabeling using anti-human PrP and anti-Fd-APP 770 confirmed APP accumulations in kuru plaques (Figure 3E). These plaques in the cerebellum were not immunolabeled with anti-β/A4 protein (Figure 3C).

In the cerebral cortex, two patients with GSS had both senile and kuru plaques, whereas two other patients had only kuru plaques. In GSS patients without senile plaques, anti-Fd-APP 770 recognized the degenerative neurites in large kuru plaques (Figure 3A,B) which were not immunolabeled with anti-β/A4 protein. The proportion of APP-positive kuru plaques to the total kuru plaques was about 50 to 90% in hippocampus, and about 10 to 50% in neocortex. With GSS patients with senile plaques, we performed double immunostainings with anti-PrP and anti-Fd-APP 770, and with atni-β/A4 protein and anti-Fd-APP 770 to confirm the amyloid typing. These patients had not only APP-positive senile plaques, but also APP-positive kuru plaques.

Discussion

The predicted amino-acid sequence of APP is consistent with a membrane-associated glycoprotein resembling a cell surface-receptor protein.[4–6] The proteolytic cleavage of APP remains to be established, but is a necessary process for the release of β/A4 fragment. One of the key questions is where APP is processed to the β/A4 protein. Previous immunohistochemical studies have showed that APP is located in the degenerative neurites of senile plaques.[10,29–33] Based on this result, it can be concluded that the senile plaques are one of the sites of APP processing.[10] To examine the possibility of APP proteolysis in senile plaques, we investigated the APP-positive neurites in various types of senile plaques. APP depositions were rarely seen in diffuse plaques that were believed to be an initial stage of amyloid depositions.[37] We observed APP-positive neurites within many typical or classical plaques. Therefore, we can postulate that APP-positive neurites are not associated with initial amyloid genesis, but may still be associated with amyloid formation in the advanced stage of senile plaques.

A striking finding is that APP-positive neurites exist even within PrP (kuru) plaques. Two GSS patients did not have any β/A4 protein depositions, but had many APP-positive PrP (kuru) plaques, whereas the other two GSS

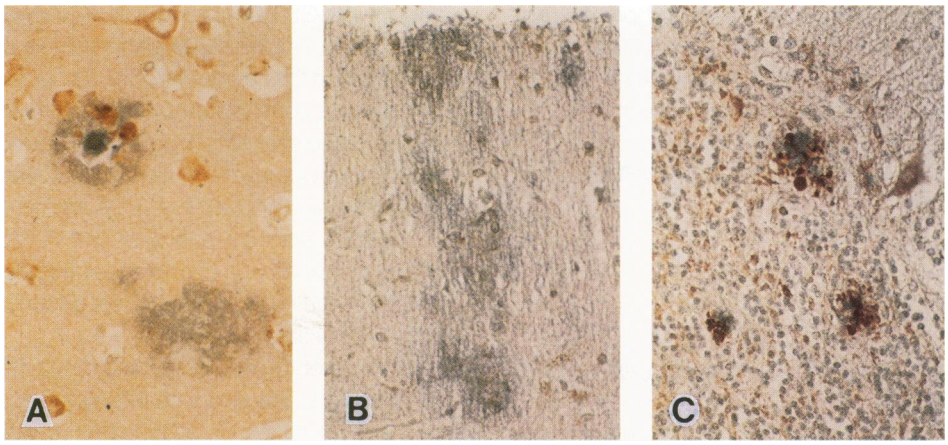
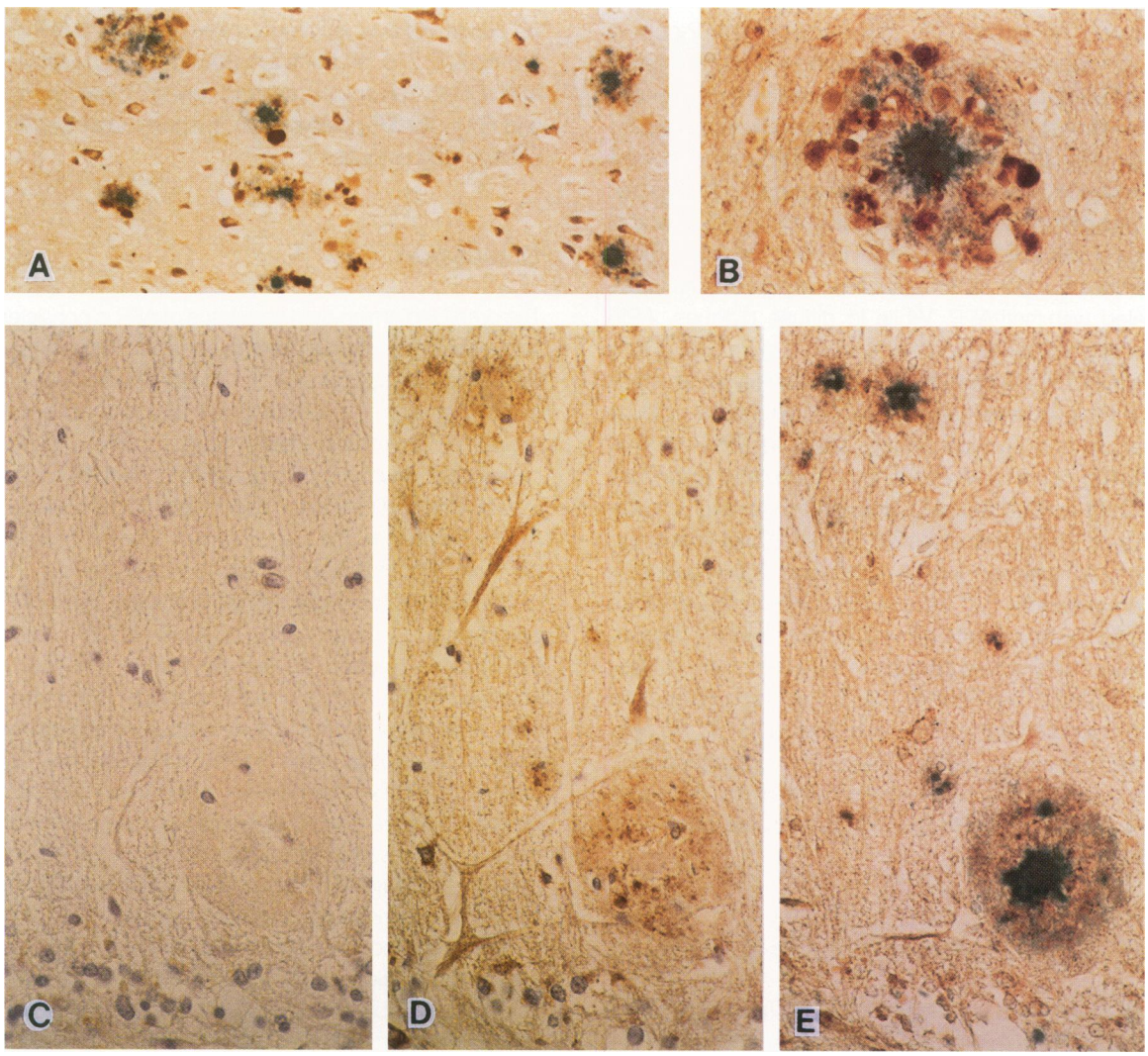


Figure 2. *Double labeling with the anti-β/A4 protein (dark blue) and anti-Fd-APP 770 (brown) in the AD brain sections.* **A:** *Hippocampus. Anti-APP labeled degenerative neurites (brown) of the senile plaque with amyloid core (dark blue), whereas APP immunoreactive structures were not observed in the diffuse plaques (blue).* **B:** *Cerebellum. Anti-APP did not immunolabel the β/A4 depositions (diffuse plaque).* **C:** *Cerebellum. Anti-APP labeled the degenerative neurites (brown) in typical or compact plaque (dark blue).* **A,** *×320,* **B** *and* **C,** *×200.*



patients had both β/A4 protein plaques and PrP plaques in the cerebral cortex. In the cerebral cortex, we sometimes observed the coexistence of β/A4 protein and PrP in the same plaques (Miyazono et al., unpublished data). We therefore performed double immunostainings using anti-PrP or anti-β/A4 protein to verify the amyloid typing. In addition, tissue sections of the cerebellum were examined because these patients did not have β/A4 deposition in the cerebellum.[36] APP-positive neuritic components in kuru plaques were confirmed in all four GSS patients. In view of this finding, it is doubtful whether proteolysis of APP to β/A4 protein occurs primarily in the degenerative neurites of senile plaques. We therefore conclude that the presence of APP in degenerative neurites is probably an event that follows amyloid formation within the plaques.

Koo et al. reported that APP molecules are transported by fast axonal flow.[38] We also found APP accumulations in axonal swelling (spheroid) around areas of old cerebral infarction (Ohgami et al., unpublished data). We postulate that the deposition of β/A4 or prion protein in the neuropil is a primary event that secondarily alters the microenvironment of surrounding tissues and later results in the formation of APP-positive degenerative neurites.

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Figure 3. *Double labeling with anti-human PrP (dark blue) and anti-Fd-APP 770 (brown) in the GSS brain sections.* **A, B:** *Hippocampus.* **A:** *Anti-APP recognized degenerative neurites (brown) within the kuru plaques (dark blue).* **B:** *Observation of APP positive kuru plaques under higher magnification. In this cerebral cortex of this patient, there were no β/A4 deposition.* **C, D, E:** *are adjacent the section of the cerebellum;* **C:** *Anti-β/A4 protein. There was no β/A4 deposition.* **D:** *Anti-APP labeled degenerative neurites (brown) surrounding kuru plaques.* **E:** *Double immunolabeling with anti-APP and anti-human PrP showed APP (brown) in kuru plaques (dark blue).* **A,** ×*132;* **B, C, D, E,** ×*320.*

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