Kunitz Protease Inhibitor-Containing Amyloid β Protein Precursor Immunoreactivity in Alzheimer’s Disease


Abstract. The amyloid β protein (β/44) that is deposited in senile plaques and in cerebral vessels in Alzheimer’s disease (AD) is derived from a larger membrane-associated glycoprotein, the amyloid β protein precursor (APP). The gene encoding APP produces at least four major transcripts. Three of the four transcripts contain an alternatively-spliced exon encoding a Kunitz protease inhibitor domain (KPI). We now report the results of a series of experiments using novel immunohistochemical reagents to anatomically localize β/44, APP, and KPI-containing forms of APP (APP-KPI) in the hippocampal formation and temporal neocortex. A new monoclonal antibody against β/44 recognized senile plaques and vascular amyloid, but no cellular elements. Anti-APP and anti-KPI monoclonal antibodies stained neurons, including proximal axons and dendrites. The neuritic component of some plaques in patients with AD and in elderly control individuals were also immunoreactive for both APP and APP-KPI. Quantitative assessment of senile plaques in temporal neocortex showed that, on average, about one-third of β/44 immunoreactive plaques stained with either anti-APP or anti-KPI. Amyloid β protein precursor and APP-KPI immunoreactivity were also found in the white and gray matter vessels of both AD patients and control individuals. These results suggest that KPI-containing forms of APP are present in dystrophic neurites of senile plaques, and normally in neurons, neuronal processes, and in the vascular compartment in the brain. Thus, APP-KPI is in a position to be intimately associated with β/44 deposition in the neuropil, in plaques and in amyloid angiopathy.

Key Words: Alzheimer’s disease; Amyloid β protein precursor; Immunohistochemistry; Kunitz protease inhibitor; Senile plaques.

INTRODUCTION

The amyloid β protein (β/44) that accumulates in senile plaques and cerebrovascular deposits in Alzheimer’s disease (AD) is a fragment of the much larger amyloid β protein precursor (APP) (1-4). The gene encoding APP produces at least four transcripts encoding proteins that are 695, 751, 770 and 563 amino acids in length (APP695, APP751, APP770, and APP563, respectively; 2, 5-8). With the exception of APP695, these transcripts contain an alternatively-spliced exon encoding a serine protease inhibitor in the Kunitz family (KPI) (5-7). The APP message and protein have been localized to brain (9-18). While APP695 is predominantly present in brain, the KPI-containing forms of APP (APP-KPI) are expressed both in the brain and throughout the body (1, 5-7).

Interest in APP-KPI immunoreactivity in Alzheimer’s disease derives from evidence that suggests that APP-KPI molecules may contribute to the pathological alterations of AD. For example, there is a relative preservation of APP-KPI mRNA as compared to APP695 mRNA in AD brain (5, 10, 15, 19), a form of APP that contains the KPI domain but lacks the β/44 sequence is specifically increased in AD (20), and there is a linear relationship between neurons showing an increase in the percentage of APP-KPI transcripts and the density of senile plaques in the hippocampus and entorhinal cortex (11). We now report the results of a series of experiments utilizing novel monoclonal immunohistochemical reagents to localize APP and, specifically, APP-KPI in the brain. In addition to examining which populations of cells produce APP and APP-KPI, our goal was to determine the relationship between APP and APP-KPI positive cells and deposits of β/44 in plaques and the vasculature.

MATERIALS AND METHODS

Eight cases of AD, ages 58 to 99 (78.9 ± 4.6 yr, x ± SE), were studied. One of the AD cases also had Down syndrome (trisomy 21, DS). Control individuals died of non-neurologic causes and did not have neuropathological evidence of neurologic disease. Control cases included one individual age 34 and seven individuals ages 57 to 92 (75.7 ± 4.4 yr, x ± SE). Diagnosis was made by an independent neuropathologist at the Massachusetts Alzheimer Disease Research Center brain bank using Knachaturian criteria (21). Fresh tissue blocks containing the hippocampal formation and parahippocampal gyrus and adjacent temporal neocortex were immersion fixed in periodate-lysine-parafomaldehyde (PLP) for 24 hours, and then transferred to a 4°C cryoprotectant solution containing 15% glycerol in tris buffered saline (pH 7.4) for two to seven days. Fifty μm sections were cut with a slide freezing microtome.

Three monoclonal antibodies specific to different regions of APP were employed in the study. The 7H5 monoclonal antibody (anti-KPI) was derived from mice immunized with a bacterial construct containing the complete human KPI insert of APP51. The 7H5 IgG was >90% pure as judged by SDS-PAGE and

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Fig. 1. Western blot analyses using antibodies 1G5 (anti-APP) and 7H5 (anti-KPI) were carried out as described (19) on total cell extracts from kidney 293 cells transfected with APP751–lane 1, APP695–lane 2, control cells–lane 3.

contained only a single gamma-1 and only kappa light chains. Preliminary results using this antibody in lightly fixed, paraffin embedded sections have been described previously (13). A bacterial construct of APP residues 444-590 (according to the APP695 sequence [2]) was employed as the immunogen for the antibody 1G5 (anti-APP). Monoclonal antibody 10D5 (anti-B/A4) was raised against a synthetic peptide containing residues 1-38 of B/A4 (Bachem, Torrence, CA). Immunoblot analyses were performed by analyzing 20 mg of APP from transfected kidney 293 cell lysates (22) by SDS-PAGE followed by transfer to Immobilon membrane and incubation with the anti-APP and anti-KPI antibodies (22). Bound antibody was visualized with an anti-mouse IgG alkaline phosphatase conjugated antibody.

Immunocytochemistry was carried out on free-floating sections (23). Primary antibodies (3-5 mg/ml) were incubated overnight at 4°C and immunoreactivity was visualized using peroxidase-linked secondary antibodies (Jackson Labs, West Grove, PA). Preliminary experiments suggested that extended periods of fixation (an additional 24 hours in PLP or 10% buffered formalin for 24 hours) resulted in markedly reduced anti-APP and anti-KPI immunostaining. Pretreatment with 80% formic acid for 10 minutes did not appear to enhance APP or B/A4 staining. Preabsorption of each of the three antibodies with excess amounts of the initial antigen resulted in the complete loss of immunoreactivity. Immunohistochemical controls using irrelevant primary antibody or in which primary antibody was deleted were unstained. Quantitation of senile plaques was carried out using video imaging (Dage MTI CCD 72 camera) and a Bioquant image analysis system using the operator-interactive semiautomated MEG-X software. Identification of each slide was masked during analysis so that the operator was blind to case number and antibody. A strip of temporal neocortical area 20 approximately 1 mm in width by the depth of the cortex was analyzed at 100X magnification.

RESULTS
Antibody Characterization by Western Blot Analysis

Western blot analysis was performed to confirm the specificities of the three monoclonal antibodies directed against separate domains of APP. Anti-APP (1G5), directed against a region common to the three largest forms of APP, recognized APP695 and APP751 on a Western blot containing cell extracts from kidney epithelial cells transfected either with APP695 or APP751 (Fig. 1). Anti-KPI (7H5) recognized only APP751 in the 293 cells transfected with APP751 and no protein in the APP695-transfected 293 cells (Fig. 1). Neither antibody recognized material from the control cells at the dilutions used.
Immunochemistry of anti-β/A4 (Fig. 2) showed that it reacts with both synthetic β/A4 (1-38) and with secreted APP751. Preincubation with synthetic β/A4 abolished immunoreactivity in both cases. The secreted form of APP751 ends at residue 15 of β/A4. This suggests that the epitope for antibody 10D5 is located within the first 15 amino acids of β/A4. Further experiments using synthetic peptides showed that the epitope for 10D5 resides in the first 12 amino acids of β/A4.

**Immunocytochemical Observations**

*Control Brains:* Both anti-KPI and anti-APP displayed strong immunoreactivity in neurons of control brain sections. The pattern of staining of the two antibodies was very similar (Fig. 3). Granule cells of the dentate gyrus and large neurons of the hilus were strongly immunoreactive with both antibodies, as were large neurons in the CA3, CA1, and subicular areas. Smaller neurons within the hilus and in the presubiculum were stained less strongly. In the entorhinal cortex (anterior parahippocampal gyrus) all neuronal layers were immunoreactive, with strongest staining in layers II, III and IV. Immunoreactivity was seen in the neuronal soma, apical and proximal basal dendrites, and in fine axonal processes that occasionally could be followed for several hundred microns (Fig. 4). In the temporal neocortex (Brodmann's area 20) and posterior parahippocampal gyrus, the large neurons of layers II, III and V were most immunoreactive and the smaller neurons of layer IV and in layer VI near the grey-white junction were less immunostained. The APP immunoreactivity within neurons was punctate and associated with intracytoplasmic aggregates. Distinction from possible artefact due to lipofuscin was made by the autofluorescence of lipofuscin in fluorescence microscopy. Astrocytes appeared to be very weakly stained by both anti-APP and anti-KPI in some brains, although in some brains astrocytic staining could not be discerned.
The three most elderly control cases (ages 83, 83, and 92) had senile plaques. Dystrophic neurites associated with some plaques were visualized with both anti-APP and anti-KPI. Neither anti-APP nor anti-KPI recognized plaque cores.

Both anti-APP and anti-KPI immunoreactivity were evident in association with cerebral vessels in white matter, and somewhat less so in the cortical grey matter (Fig. 3). The staining was not age-dependent, and even the youngest control (age 34) contained definite immunostaining of vessels. The endothelium of capillary walls and the intima and adventitia of small and medium sized vessels were stained. The tunica media, by contrast, did not appear to contain either APP or KPI immunoreactivity.

The anti-β/A4 antibody showed intense immunoreactivity with senile plaque cores in the most elderly individuals. No cellular staining occurred with anti-β/A4. Amyloid angiopathy was not found in these control individuals.

Alzheimer’s Disease: The same populations of neurons that were APP and KPI immunoreactive in the control individuals were also stained in the AD cases. Neuronal immunoreactivity in the AD cases and the DS individual did not differ in degree or pattern from that of control cases, except in that areas severely affected by pathological changes such as the CA1/subiculum field appeared patchy or cell-poor in both Nissl and immunostains. Neuronal somas, proximal basal and apical dendrites, and fine axonal processes were all immunoreactive. In addition, numerous dystrophic neuritic processes of plaques were immunostained with both anti-KPI and anti-APP (Fig. 5). Comparison of the sections stained with anti-KPI or anti-APP and those stained with anti-β/A4 revealed that not all amyloid senile plaques contained immunoreactivity for the amyloid precursor proteins (see “Quantitation of Senile Plaques,” below).

Dystrophic neurites of neuritic plaques were most strongly immunoreactive in plaques within the hilus of the dentate gyrus, the molecular layer of the dentate gyrus (perforant pathway terminal zone), and in layers III and V of the temporal neocortex (Fig. 5). Astrocytes were lightly stained with both anti-APP and anti-KPI in many of the cases. There was no apparent increase in astrocyte staining in the vicinity of senile plaques.

The anti-APP and anti-KPI antibodies did not recognize β/A4 present in mature plaque cores or diffuse β/A4 deposits. For example, lamina principalis externa of the presubiculum contained a cloud of diffuse β/A4 deposition in the AD cases, in agreement with earlier observations (24, 25). No APP or KPI positive dystrophic neurites were seen overlapping these β/A4 deposits. However, the neurons that make up the islands in lamina principalis externa were both APP and KPI immunoreactive.

Capillary walls and the intima and adventitia of small and medium sized arterioles were immunoreactive with both anti-APP and anti-KPI. The immunostaining was
relatively uniform, and there did not appear to be any “skip” regions. Examination of leptomeningeal arteries that contained substantial amyloid angiopathy also showed anti-APP and anti-KPI staining in the intima and adventitia, whereas anti-β/A4 staining was localized primarily in the tunica media and, to a lesser extent, the intima. The APP and KPI immunostaining were not different in vessels that contained β/A4 deposits as compared to vessels that were apparently spared.

Some meningeal, grey and white matter blood vessels in the AD cases were stained with the anti-β/A4 antibody (Fig. 5). In addition, both senile plaque cores and “diffuse” neuropil deposits were β/A4 immunoreactive. The pattern of senile plaque staining was identical to that seen using thioflavine S, although the antibody revealed a substantially larger number and morphological variety of total lesions. Occasional “tombstone” neurofibrillary tangles were visualized by anti-β/A4, as had been noted previously using polyclonal anti-β/A4 antisera (26). No neuronal or astrocytic staining was seen with anti-β/A4.

Quantitation of Senile Plaques

Adjacent sections were immunostained with anti-β/A4, anti-KPI, and anti-APP and the number of immunostained plaques in area 20 neocortex was assessed. A strip of approximately 1 mm in width by the depth of the cortex was analyzed using the interactive Bioquant image analysis software (Fig. 6). Anti-β/A4 consistently revealed a substantially greater number of plaques than identified with either APP antibody. The percentage of plaques seen with anti-β/A4 that were APP or KPI positive varied from 17 to 50% in AD individuals. In the control cases, the percentage varied from 0 (in those cases with no plaques) to 47% (in an 83 year old). Within an individual case, an essentially identical number of total plaques were positive using either anti-KPI or anti-APP (Fig. 6).

DISCUSSION

Our results show strong APP and APP-KPI immunoreactivity in normal neurons as well as in association with a subset of the senile plaques observed in aging, AD, and DS. The pattern of immunostaining observed with anti-APP and anti-KPI antibodies was similar. These results suggest that KPI-containing forms of APP are normally expressed in neurons and, in AD, are associated with dystrophic neurites in a subset of β/A4 plaques.

Anti-APP is directed against a domain common to all forms of APP, and so would be expected to visualize both KPI and non-KPI forms of APP. Because a reagent directed uniquely against APP695 (lacking the KPI domain) is not available, we cannot directly estimate the degree to which APP-KPI contributes to the immunoreactivity seen with anti-APP. Anti-APP and anti-KPI do not differentiate between secreted and full-length forms of the precursor. Nonetheless, quantitative data suggest that, at
the least, KPI-containing APP is present in the vast majority of APP positive plaques.

The immunocytochemical results showing neuronal staining with anti-KPI in control, AD and DS brains confirm earlier in situ hybridization studies suggesting that neurons express APP-KPI transcripts (11). Both neurons that are prone to neurofibrillary degeneration (entorhinal cortex-layer II, subiculum) and neurons that are relatively resistant to neurofibrillary tangle formation (dentate gyrus granule cells, 24, 27) are strongly immunoreactive for APP-KPI. Moreover, both normal axons and proximal dendrites are immunostained, suggesting that KPI-containing APP molecules are widely dispersed within the neuron. These findings are consistent with constitutive expression of APP-KPI in neurons.

Amyloid β protein precursor immunoreactivity is present in dystrophic neurites that are associated with a subset of senile plaques (Fig. 5; 12-14, 28-35), and it is possible that this APP (and APP-KPI) thereby contributes to the β/A4 in the plaque core. However, if APP within dystrophic neurites is a major source of β/A4 deposition, why are only 17-50% of amyloid plaques associated with APP and KPI immunoreactivity (see also 13, 14)? Several hypotheses could explain this observation. For example, APP positive dystrophic neurites might be present only transiently during the life history of a senile plaque. If this were the case, a single observation at the time of autopsy might show both β/A4 deposits currently interacting with APP positive dystrophic neurites as well as β/A4 deposits that had previously been associated with APP positive neurites. However, this hypothesis predicts that β/A4 deposits would be present only in brain regions that are vulnerable to APP immunoreactive dystrophic neurite formation. The observation that β/A4 deposits occur in areas such as the presubiculum and cerebellum, where APP immunoreactive dystrophic neurites are rare, argues against this possibility.

We suggest that APP immunoreactive dystrophic neurites are not the primary source of the APP that is deposited as β/A4 in the neuropil and in plaque cores. Instead, the neuropil, with its abundant dendrites and axons, may act as a reservoir of APP that is synthesized in neurons and ultimately deposited as β/A4. For example, the strong APP immunostaining of neurons in layer II of entorhinal cortex and the granule cells of the dentate gyrus suggests that axons and dendrites of these cells, located in the molecular layer of the dentate gyrus, could contribute precursor molecules to the senile plaques that frequently occur in the dentate molecular layer (24, 30).

Cerebrovascular β/A4 angiopathy involves the deposition of β/A4 in cerebral vessels as well as in the abnormal vessels of central nervous system vascular malformations (16, 17, 31, 36). β/A4 deposits have also been observed in vessels in the skin and intestines of patients with AD and DS (37). Recently, APP has been shown to be secreted by activated platelets and to inhibit the clotting factor Xlla (38, 39). Amyloid β protein precursor immunoreactivity is seen in vessels in the control cases (Fig. 3) and both β/A4 and APP immunoreactivity are present in vessel walls in AD (Fig. 5; reference 17). Our data are consistent with either a circulating or tissue source of this APP and β/A4.

Other recent studies have examined central nervous system APP immunoreactivity with a variety of anti-APP antibodies and have provided slightly differing results (12-14, 28-35). In accord with the present data, there is agreement that APP immunoreactive neurites are present only in a subset of senile plaques. Our quantitative results show that the exact percentage of APP positive plaques varies substantially between cases, and that there is overlap between AD and some control individuals in terms of APP neurite immunostaining. Arai et al (14) suggest that controls have much less APP immunoreactivity; this difference is likely due to the individual variability in the cases studied. Martin et al (32) examined APP immunostaining in aged, nonhuman primates using unfixed, frozen brain sections and showed APP immunoreactivity in neuronal cell bodies, proximal dendrites, and axons of cortical neurons as well as in dystrophic neurites surrounding plaques. This result is analogous to our observations in lightly fixed human material (Fig. 3; see also references 33-35). Immunostaining in neurites is consistent with the observation that neurons contain mRNA for APP (9, 11, 30). In contrast, other observations suggest
weak or occasional neuronal immunostaining for APP in human tissue (13, 14). Additionally, our results and some previous studies report APP immunoreactivity in association with vessels (16, 17, 31), while others do not (13, 14, 32). The differences among these studies may be due to differences in fixation and tissue processing, and possible pre- or post-mortem influences. Arai et al (14) and Cras et al (34) have shown marked effects on APP immunoreactivity as a result of different fixation procedures. Our experience suggests diminution of APP staining with longer PLP or formalin fixation. For example, vessel immunoreactivity and the majority of neuronal staining is not apparent in paraffin sections even with antibody 7H5 (13).

In summary, using novel monoclonal antibodies directed against APP and the KPI domain of APP, we find APP and APP-KPI-like immunoreactivity associated with neurons, cerebral vessels, and a subset of senile plaques. The presence of the KPI domain in APP-KPI may confer important biological function. The secreted form of KPI-containing APP is identical to PN-II which is able to bind to serine proteases including the kallikreins, nerve growth factor-gamma subunit, and epidermal growth factor binding protein (40, 41). Another protease inhibitor, alpha-1-antichymotrypsin, has been identified as a component of plaque cores (42). Regardless of the molecular mechanisms of B/A4 deposition in the vessel and plaque amyloid, a common denominator may be a contribution from KPI-containing forms of APP.

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References


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