Modified Bielschowsky and Immunocytochemical Studies on Cerebellar Plaques in Alzheimer's Disease

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Abstract. Senile plaques (SP) in the cerebellum of 23 cases of Alzheimer's disease (AD), three with widespread amyloid angiopathy, were studied with a modified Bielschowsky stain and immunocytochemical methods using antibodies to a beta-amyloid synthetic peptide (βASP), phosphorylated neurofilament proteins, ubiquitin, tau protein, and glial fibrillary acidic protein (GFAP). The four subtypes of SP (diffuse plaques, compact plaques, perivascular plaques, and subpial fibrillary deposits) that were observed with the modified Bielschowsky stain were also stained with antibodies to βASP. Many cerebellar SP contained ubiquitin-positive granular elements resembling dystrophic neurites. In contrast to neuritic elements in cerebral SP in AD, ubiquitin-positive elements in cerebellar SP were not labeled with antibodies to phosphorylated neurofilament or tau proteins. Various degrees of glial reaction were observed in all subtypes of SP except diffuse plaques. The absence of phosphorylated neurofilament and tau epitopes in neuritic elements in cerebellar SP is not surprising since paired helical filaments have not been seen in the cerebellum. Nevertheless, our results suggest that cerebellar SP are frequently associated with dystrophic neurites.

Key Words: Alzheimer's disease; Bielschowsky stain; Cerebellum; Immunocytochemistry; Senile plaques; Tau protein; Ubiquitin.

INTRODUCTION

Senile plaques (SP) and neurofibrillary tangles (NFT) are characteristic histopathologic hallmarks of Alzheimer's disease (AD), which includes both presenile and senile forms. The number of SP has been correlated with the degree of dementia (1), although prospective studies have documented numerous cortical SP in a subset of non-demented elderly subjects (2, 3). Other studies suggest that NFT correlate better with dementia (4). Senile plaques are heterogeneous structures, composed of amyloid deposits, degenerating neurites, microglia, and astrocytes (5). Some degenerating neurites in SP contain paired helical filaments (PHF) and those without PHF contain dense bodies, degenerating mitochondria, and ovoid lamellar bodies (5, 6). Although early studies emphasized the neuritic element in SP, more recent immunohistochemical studies using antibodies to beta amyloid synthetic peptides (βASP) have demonstrated amyloid deposits (7) referred to as very primitive plaques (8, 9) or diffuse plaques (10, 11), which are lacking apparent neuritic elements. Such amyloid deposits have been postulated to be early pathological events in SP formation (12).

Cerebellar SP in AD have been considered to be quite rare and observed in atypical
cases with a family history of dementia (13, 14), early onset of dementia (15, 16), spastic paraparesis and ataxia (17), or widespread amyloid angiopathy (18, 19). Several recent studies on the cerebellum in AD, however, have frequently demonstrated cerebellar SP (20–26). Morphologically, in addition to stellate or globular amyloid deposits in the Purkinje or granular cell layers, diffuse plaques have been demonstrated in the cerebellum in AD (24–26). It is generally accepted that cerebellar SP, including diffuse plaques, have few or no degenerating neurites (13–16, 20, 21, 23, 26). Furthermore, PHF have not been seen in the cerebellum (13, 17, 27–29).

To characterize further cerebellar SP, we undertook the present immunocytochemical study using antibodies to a βASP, phosphorylated neurofilament proteins, ubiquitin, tau protein, and glial fibrillary acidic protein (GFAP). In addition, we used a modified Bielschowsky stain, which is a sensitive method for detecting SP and NFT (30, 31).

MATERIALS AND METHODS.

We retrospectively studied 23 cases of neuropathologically confirmed AD (32), three with widespread amyloid angiopathy, and 20 cases of elderly patients without primary neurological diseases (non-AD) from the autopsy file of Montefiore Medical Center. The age at death of the cases ranged from 62 to 93 in AD and from 66 to 90 in non-AD. The brains were fixed in formalin for two to four weeks and each cerebellum was cut transversely. A few brains of AD cases had been in formalin for several months. One or more sections containing the cerebellar hemisphere, deep nuclei, or vermis were embedded in paraffin. In addition, one cerebellum from a patient with AD with numerous SP was cut perpendicular to the long axis of the cerebellar folia and processed similarly to examine the orientation of the cerebellar SP. Sections were stained with hematoxylin and eosin, and a modified Bielschowsky stain (30).

Fifteen cases of AD, three with widespread amyloid angiopathy, and six cases of non-AD were immunostained using the avidin–biotin–peroxidase complex (ABC) method. The following antibodies were used: affinity purified rabbit antibodies raised to a synthetic peptide derived from the N-terminal 28 amino acid sequence (33) of the amyloid beta protein (βASP, diluted 1:200) (34), a mouse monoclonal antibody binding to phosphorylated high molecular weight (200 and 160 kDa) neurofilament proteins (NP14, diluted 1:2) (35), and affinity purified rabbit antibodies to ubiquitin (UBQ, diluted 1:500) (36). In addition, four cases of AD were also stained with another monoclonal mouse antibody which recognizes a phosphorylated neurofilament epitope (NP18, undiluted) (35, 37). Six cases of AD, three with widespread amyloid angiopathy, and two cases of non-AD were stained with two mouse monoclonal antibodies to tau protein (Tau-1, diluted 1:1,000, or Tau-2, diluted 1:2) (38, 39), and a monoclonal mouse antibody to glial fibrillary acidic protein (GFAP, Labsystems Oy, Helsinki, Finland, diluted 1:1,000). In several cases, serial 6 μm thick sections were stained with βASP, NP14, or UBQ. Sections were incubated with the primary antibody for 18 hours at 4°C. Before incubation with βASP, sections were immersed in formic acid (88%) for 60 minutes at room temperature as reported by Kitamoto et al (40). In a few cases of AD, βASP immunostaining was carried out without formic acid pretreatment. Before incubation with Tau-1, sections were digested with 20 IU/ml of E. coli alkaline phosphatase (Sigma, type III-S) in 0.1 M Tris-HCl, pH 8.0, and 2 mM phenylmethylsulfonyl fluoride for 18 hours at room temperature. Bound antibody was detected using an ABC kit (Vector Laboratories, Burlingame, CA) and visualized with diaminobenzidine and hydrogen peroxide. All sections except those stained with UBQ were counterstained with hematoxylin. In control incubations, normal rabbit serum or phosphate buffered saline was substituted for the primary antibody.

We classified cerebellar SP stained with the modified Bielschowsky method into four subtypes (20, 26) as follows:

1. Diffuse plaques: ill-defined areas in the molecular layer, consisting of fine fibrillar material, apparently aligned with parallel fibers.
TABLE 1
Incidence of Cerebellar Senile Plaque Subtypes with Respect to Pathological Diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Total number of cases</th>
<th>Diffuse plaques</th>
<th>Compact plaques</th>
<th>Perivascular plaques</th>
<th>Subpial fibrillar deposits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alzheimer's disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Without widespread amyloid angiopathy</td>
<td>20</td>
<td>14/20</td>
<td>13/20</td>
<td>4/20</td>
<td>15/20</td>
</tr>
<tr>
<td>b. With widespread amyloid angiopathy</td>
<td>3</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td><strong>Non-AD</strong></td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2. Compact plaques: stellate or globular structures with or without radiating margins, usually seen in the Purkinje cell or granular cell layer.

3. Perivascular plaques: fibrillar or globular structures in the parenchyma closely associated with small blood vessels.

4. Subpial fibrillar deposits: fine or coarse fibrillar deposits just beneath the pial membrane with occasional core-like structures, including diffuse type of SP in the subpial regions.

RESULTS

Cerebellar SP were observed in all AD cases examined, but not in non-AD cases. The number of cerebellar SP varied from case to case. The incidence of various subtypes of cerebellar SP with respect to pathologic diagnosis is summarized in Table 1. Diffuse plaques were noted particularly in AD without widespread amyloid angiopathy, whereas perivascular plaques were present in all cases of AD with widespread amyloid angiopathy. Compact plaques and subpial fibrillar deposits were frequently seen in AD with or without widespread amyloid angiopathy.

With the modified Bielschowsky method, diffuse plaques were observed in the cerebellar hemisphere and vermis. In the transversely cut sections, they were visualized as fine fibrillar material running horizontally in the molecular layer (Fig. 1A). Some diffuse plaques were confluent, measuring up to 1,000 μm in greatest diameter. Apparently normal nerve fibers and occasional capillaries traversed some diffuse plaques. There were a few diffuse plaques that contained stellate or globular structures. In the sections cut at a right angle to the long axis of the cerebellar folia, diffuse plaques were characterized by well defined areas that were arranged vertically in the molecular layer (Fig. 1B), and composed of closely packed dots and ovoids.

Compact plaques were more frequently found in the vermis than in the cerebellar hemisphere, and commonly seen in the Purkinje or granular cell layer, occasionally in the molecular layer, and rarely in the white matter (Fig. 1A).

Perivascular plaques were far less frequently observed than other subtypes of SP, but were one of the major findings in AD with widespread amyloid angiopathy.

In AD without widespread amyloid angiopathy, subpial fibrillar deposits consisted of fine fibrillar material and could not be differentiated from diffuse plaques in the subpial regions (Fig. 1A), while in AD with widespread amyloid angiopathy, most of them were coarsely fibrillar and contained occasional core-like structures. These subpial coarse fibrillar deposits were sometimes found in confronting folia.

The immunoreactivity of cerebellar SP subtypes with various antibodies is summarized in Table 2.
Diffuse plaques were demonstrated with βASP immunostaining only when sections were pretreated with formic acid (Fig. 2A). On the other hand, cores of compact plaques and perivascular plaques were labeled with βASP without formic acid pretreatment. Subpial coarse fibrillar deposits in AD with widespread amyloid angiopathy also reacted with βASP without pretreatment. Radiating margins of compact plaques were better visualized following formic acid pretreatment. Compact plaques in the granular cell layer were clearly recognized with βASP immunostaining (Fig. 3A). Perivascular plaques were observed as clusters of small granular deposits or amorphous masses in the parenchyma around blood vessels, which also contained amyloid deposits (Fig. 3C). Similar to their appearances on the Bielschowsky stain, βASP revealed subpial fibrillar deposits composed of fine fibrillar material in AD without widespread amyloid angiopathy and coarse fibrillar or small granular deposits with occasional core-like structures in AD with widespread amyloid angiopathy (Fig. 3E). Amyloid angiopathy in the cerebellum was detected with βASP immunostaining in nine cases of AD, including all three cases of AD with widespread amyloid angiopathy, and one of six non-AD cases (72 years old). Amyloid angiopathy in the cerebellum was more marked in AD with widespread amyloid angiopathy than either AD without widespread amyloid angiopathy or the one non-AD case.

NP14 and NP18 immunostained axons in the white matter and basket cell fibers in the deep molecular layer in both AD and non-AD. In several cases of AD, the axons of the Purkinje cells in the granular cell layer showed swellings, consistent with "torpedoes." Examination of serial sections in several cases revealed no neuritic labeling with NP14 or NP18 in diffuse plaques, compact plaques, perivascular plaques,
or subpial fibrillar deposits. Occasional thick neuronal processes in the molecular layer, which were not directly associated with SP, were labeled. Nuclear staining of neurons and glia was sometimes observed with NP14 (35).

The staining with UBQ revealed many granular elements resembling dystrophic neurites in the cerebellar cortices in AD. Examination of serial sections showed that ubiquitin-positive elements were present in many diffuse plaques, in some compact plaques, and in most perivascular plaques and subpial coarse fibrillar deposits (Figs. 2B, 3B, D, F). In some diffuse plaques, ubiquitin-positive elements had the same orientation as the amyloid deposits. In compact plaques, perivascular plaques, and subpial coarse fibrillar deposits, ubiquitin-positive elements were usually located in the periphery of the plaques. Corresponding structures could not be definitely identified in sections examined with hematoxylin and eosin, or the modified Bielschowsky silver stain. Many smaller ubiquitin-positive “dot-like” bodies were demonstrated in the white matter in both AD and non-AD cases as previously reported (41, 42). Immunoreactivity with UBQ was not detected in the cerebellar cortices of non-AD cases except in the nuclei of some cells.

Tau-1 or Tau-2 did not recognize any definite structure in cerebellar SP, although Tau-2 stained scattered astrocytic processes in the cerebellar cortices, dentate nuclei, and white matter in AD as reported previously (43). Both Tau-1 and Tau-2 labeled neurofibrillary tangles, neuritic elements in SP, and abnormal neuropil neurites in the hippocampus in AD cases (not shown).

Gliad fibrillar acidic protein (GFAP) immunostaining was not remarkable in diffuse plaques, but showed variable reaction in many of the compact plaques and some of the perivascular plaques and subpial fibrillar deposits. The ubiquitin-positive elements seen in cerebellar SP were not labeled with GFAP. In non-AD, GFAP immunostaining was usually confined to the subpial and perivascular regions, white matter, or fibrils of Bergmann’s glia in the molecular layer.

**DISCUSSION**

Amyloid beta protein immunostaining with formic acid pretreatment has been shown to detect more SP, particularly diffuse plaques, than conventional histologic
methods (7, 10–12, 26). In this study, diffuse plaques were similarly detected with both βASP immunostaining and the modified Bielschowsky stain. Other studies have also demonstrated this correspondence (10, 11, 26).

Although cerebellar SP have been frequently observed in AD (20–26), the presence of degenerating neurites in cerebellar SP have not been emphasized (13–16, 20, 21, 23, 26). Pro et al (14) showed that cerebellar SP consisted largely of periodic acid-Schiff-positive homogeneous cores with little or no microglial or neuritic element resembling “kuru plaques” found in the cerebellum in kuru and Creutzfeldt-Jacob disease. Yamaguchi et al (26), using beta protein immunostaining and silver impregnation methods, demonstrated that both cerebellar diffuse and compact plaques consisted mainly of amyloid deposits. No neuritic elements could be demonstrated with Bodian stain.

The results of our study, however, indicate that many cerebellar SP have ubiquitin-positive granular elements resembling dystrophic neurites in cerebral SP (44–46). In contrast to cerebral SP in AD, where degenerating neurites include both tau-positive and tau-negative neurites (47), the ubiquitin-positive elements in cerebellar SP were uniformly tau-negative.

Previous electron microscopic studies on cerebellar compact plaques showed that amyloid cores were sometimes surrounded by dystrophic neurites which did not contain PHF (13, 27–29). Moreover, Aikawa et al (17) demonstrated in cerebellar perivascular plaques in a case of atypical AD with spastic paresis and ataxia, many dystrophic neurites containing dense bodies and abnormal mitochondria. We spec-
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Fig. 3. A, B. Serial sections containing compact plaques, C, D. perivascular plaque, and E, F. subpial fibrillar deposits stained with antibodies to a beta amyloid synthetic peptide (βASP) (A, C, E) and ubiquitin (B, D, F). A, C and E are counterstained with hematoxylin. βASP immunostaining demonstrates several compact plaques, one in the molecular layer (arrow) in A, a perivascular plaque in C, and subpial fibrillar deposits with one core-like structure (arrow) in the confronting folia in E. Ubiquitin-positive granular elements are seen in each senile plaque subtype (B, D, F). A, B, AD without widespread amyloid angiopathy; C, D, E, F, AD with widespread amyloid angiopathy. ×185.

ulate that such dystrophic neurites may be the morphological counterparts of the ubiquitin-positive elements in our study.

One of the known functions of ubiquitin, a highly conserved 76-amino acid protein, is ATP-dependent non-lysosomal degradation of abnormal and short-lived proteins (48). Ubiquitin is synthesized in response to many stresses such as heat shock and toxic agents (49, 50). Recent studies have demonstrated that ubiquitin is associated, not only with Alzheimer's changes such as NFT and neuritic elements in SP (44, 45, 51), but also with various neuronal inclusions such as Lewy bodies and Pick bodies (41, 52, 53). The involvement of the ubiquitin system in various conditions suggests that ubiquitination may be a response to abnormally accumulated cellular
proteins, particularly intermediate filaments, which are resistant to degradation (53). Ubiquitin-positive elements, seen in every subtype of cerebellar SP, may have resulted from ubiquitination of abnormal proteins in response to cellular stresses such as amyloid accumulation in the neuropil, since even perivascular plaques, which are considered to be derived from vascular walls (54, 55) contained many of them. The precise identification of the ubiquitin-positive elements remains to be further elucidated by electron microscopic immunocytochemistry.

Recent immunocytochemical studies on cerebral SP in AD have demonstrated that tau-positive, PHF-containing degenerating neurites in SP and throughout the neuropil may be the morphological feature more closely associated with dementia rather than amyloid deposition (9, 46). Cerebellar SP, on the other hand, did not contain tau-positive neurites. Since tau is the major component of PHF (56), and since PHF have not been seen in the cerebellum, ubiquitin-positive elements almost certainly do not contain PHF. The results of this study suggest that even in the cerebellum, amyloid deposition is frequently associated with neuritic changes. The difference in the immunoreactivity of the neurites in cerebellar SP compared to cerebral SP may reflect the absence of PHF in the cerebellum.

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