Research report

α-Synuclein-positive structures in cases with sporadic Alzheimer’s disease: morphology and its relationship to tau aggregation

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Abstract

Alzheimer’s disease (AD) and Parkinson’s disease share common clinical and pathological features. In this study, we examined the relationship between AD pathology and α-synuclein aggregation. The frequency and distribution of α-synuclein-positive structures were systematically investigated in 27 cases with sporadic AD by α-synuclein immuno-histochemistry. Thirteen (48.2\%) of 27 cases had various α-synuclein-positive structures as well as Lewy bodies. The frequency and density of senile plaques and neurofibrillary tangles were not significantly different between cases with α-synuclein structures and those without. α-Synuclein-positive structures were found most frequently in the amygdala. The α-synuclein-positive inclusions that are different from Lewy bodies were observed at the highest rate in the hippocampus. The discovery of α-synuclein as the constituent of Lewy bodies facilitated the detection of Lewy-related structures even in AD cases with widespread and numerous neurofibrillary tangles. α-Synuclein-positive inclusions except for Lewy bodies are exposed, and the distribution of them indicates that Lewy body formation may be influenced by the degree of tau aggregation. This study also supports the suggestion that cases with AD pathology can be classified into two groups according to the existence or absence of α-synuclein aggregation.

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1. Introduction

There is a significant overlapping in clinical and pathological features between Alzheimer’s disease (AD) and Parkinson’s disease (PD), indicating the existence of common pathogenesis between the two diseases. On the other hand, it was reported that there were neurodegenerative disorders characterized by dementia and widespread occurrence of cortical Lewy bodies (LBs) [10,17,24]. These disorders are generally accompanied by varying degrees of AD pathology in addition to the occurrence of LBs in the brainstem and cerebral cortex. In 1995, cases having the above histological features were considered to be a new clinicopathological entity, termed dementia with Lewy bodies (DLB) [21]. At present, DLB has been widely recognized as the second most prevalent form of neurodegenerative dementia following AD.

Cortical LBs are the main pathological marker of DLB. They are usually less eosinophilic, less well defined than brainstem-type LBs, and generally lack a halo. It is difficult to identify cortical LBs by hematoxylin and eosin (HE) staining. Immunostaining with anti-ubiquitin antibody [18] has been recommended for detecting cortical LBs accurately. Previous immuno-histochemical studies showed that LBs and Lewy neurites (LNs) were α-synuclein-positive [4,11,27,31] and neurofibrillary tangles (NFTs) are not immunoreactive [28,30], differing from
2. Materials and methods

2.1. Cases

In the present study, we examined 27 consecutive brains obtained at autopsy from demented patients, who were clinically diagnosed as having sporadic AD. This study had the following aims: (1) to investigate the relationship between AD pathology (focusing on the distribution and frequency of senile plaques (SPs) and NFTs) and α-synuclein-positive structures; and (2) to describe the morphology of α-synuclein-positive structures.

2.2. Tissue sampling and routine staining

All brains examined in the present study were obtained at autopsy within 24 h after death. The entire brain was fixed in 10% formaldehyde for about 2 weeks and then cut in coronal slices. For overall neuropathologic evaluation, tissue blocks were obtained from selected regions: middle frontal, inferior parietal and superior temporal neocortex, hippocampus/entorhinal cortex, basal ganglia, cerebellum, midbrain, pons, medulla oblongata. These tissue blocks were embedded in paraffin and 4-μm thick sections were cut and stained with HE, Klüver-Barrera and Bodian staining. Immuno-histochemical staining of 6-μm thick sections was performed using the anti-phosphorylated tau monoclonal antibody (mAb) AT8 (Innogenetics, dilution of 1:3000), anti-human beta-amyloid mAb 6F/3D (Dako, dilution of 1:100) and rabbit anti-ubiquitin antibody (DAKO, dilution of 1:100).

2.3. Evaluation of neocortical SPs and NFTs

Neocortical SPs were identified by Bodian staining, and their distribution was assessed based on CERAD pathological criteria for AD [23] as follows: none, sparse, moderate and frequent. We examined the areas in which SPs existed in maximum density. NFTs were identified by immuno-histochemistry using AT8. The distribution pattern of NFTs and neuropil threads (NTs) was evaluated based on Braak and Braak staging of neurofibrillary changes of AD [5], and was classified into six stages as follows: transentorhinal stage, stages I–II; limbic stage, stages III–IV; and isocortical stage, stages V–VI.

2.4. Immuno-histochemical analysis using anti-α-synuclein antibodies

We examined immunoreactivities of α-synuclein against two antibodies. One is a monoclonal antibody (LB509), raised against purified LBs specifically from DLB brains [4], and recognizes amino acids 115–122 [14], the other is a polyclonal antibody (#17) [7], raised against a synthetic peptide corresponding to amino acids 1–10 of human α-synuclein. For immuno-histochemistry using anti-α-synuclein antibodies, 6-μm thick paraffin sections were obtained from four selected regions of the brain of each case: the hippocampus/entorhinal cortex, amygdala, midbrain, and cingulate gyrus. The sections were deparaffinized and pretreated with hydrolytic autoclaving in citrate buffer for 15 min at 121°C to enhance the immunoreactivity. Thereafter, they were incubated in 0.01% H2O2 in methanol at room temperature for 30 min to block endogenous peroxidase activity and pretreated to block nonspecific immunoreactivity prior to the addition of LB509 (dilution of 1:50) or #17 (dilution of 1:1000) with normal rabbit serum, or normal goat serum respectively. This was followed by one-overnight incubation at 4°C. Staining was performed using the avidin–biotin complex technique with 3,3′-diaminobenzidine (DAB) as the chromogen and followed by counterstaining with hematoxylin. Sections of the midbrain of PD patients, in which typical brainstem-type LBs were detected on HE staining, were used as positive controls.

All four sections were immunolabeled with anti-α-synuclein antibodies, and were then examined for the presence of α-synuclein-positive structures in each case. In cases in which α-synuclein-positive structures could be found in at least one region, five additional regions were selected. These additional regions were the frontal, parietal, temporal neocortex, pons and medulla oblongata, which were recommended in the guidelines for brain sampling by the consortium on DLB international workshop [21], and were immunolabeled in the same manner.

2.5. Quantiﬁcation of α-synuclein-positive structures

Initially, each section was scanned at a magnification of ×100 to check whether or not there is any α-synuclein-positive structures and identify the regions in which the most abundant α-synuclein-positive structures were observed. α-Synuclein-positive structures were divided into
α-synuclein-positive intra-cytoplasmic inclusions and α-synuclein-positive neurites. To semiquantitatively evaluate them, a score ranging from 0 to 3 was assigned according to the number of intra-cytoplasmic inclusions and the density of neurites in an area of maximum density for each region. Intra-cytoplasmic inclusions were defined as α-synuclein-positive structures including the nucleus in neurons in the plane of sections. Their number was counted in three nonoverlapping fields at a magnification of ×200 for each section, and the final score was the average of the three fields as follows: (score 0, 0; score 1, 1–5; score 2, 6–10; score 3, >10). Similarly, the density of α-synuclein-positive neurites was scored as follows: (score 0, none; score 1, a few neurites; score 2, some neurites; score 3, many neurites).

Additionally, α-synuclein-positive intra-cytoplasmic inclusions were morphologically subdivided into two groups according to whether or not they were of the LBs-type, which are revealed as spherical, ellipsoidal, angular and reniform by staining. The regional rate of the α-synuclein-positive intra-cytoplasmic inclusions, which were impossible to identify as LBs morphologically at a magnification of ×400 (=non-LB type), was expressed as the percentage relative to the total α-synuclein-positive intra-cytoplasmic inclusions. The quantitation was performed for all cases and sections by the same examiner.

2.6. Confocal microscopy

To assure that α-synuclein-positive intra-cytoplasmic inclusions were colocalized with NFTs, sections were double-labeled with the anti-α-synuclein (#17) antibody and anti-tau (AT8) antibody. Then, #17 and AT8 were fluorescence-labeled with Alexa 488 (Anti-Rabbit IgG (H+L)), detected as green, and with Alexa 594 (Anti-Mouse IgG (H+L)), detected as red, respectively. Sections were observed under a confocal microscopy system (TCS-SP, Leica, Heidelberg, Germany).

3. Results

3.1. Summary of 27 cases (Table 1)

In α-synuclein immuno-histochemistry, 13 (48.2%) out of 27 cases had α-synuclein-positive structures in at least one region of the four selected regions, and were defined as the positive group. Nine cases in the positive group had some α-synuclein-positive structures in all four regions, and the four cases in the positive groups had α-synuclein-positive structures in only two or three regions. The remaining 14 cases did not have α-synuclein-positive structures in all four regions, and were defined as the negative group. We compared the positive group with the negative group with respect to age of onset, age of death, duration of illness, and brain weight. There was a tendency that cases of the positive group were slightly older at the time of death, and had shorter duration of illness, and lighter brain weight than those of the negative group. The two groups were not found to be significantly different by the Mann–Whitney U-test.

3.2. The frequency of SPs and NFTs in two groups

The SP density determined based on CERAD neuropathological criteria for AD [23] did not differ significantly among the neocortical three regions between the two groups. The average SP density was the highest in the temporal cortex, followed by the parietal cortex and by the frontal cortex (Fig. 1). The distribution pattern of NFTs and NTs based on Braak and Braak staging [5] showed that the majority of examined cases were classified into stages V–VI except for three cases of the positive group, which were classified into stage IV (Table 2). In the positive group, all 13 cases were categorized as having definite AD based on CERAD diagnostic criteria [23], and diagnostic criteria for the neuropathology of AD by the

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Table 1
Comparison of characteristics of two groups of study patients

<table>
<thead>
<tr>
<th></th>
<th>Number (Sex; M/F)</th>
<th>Age of onset (years) average (range)</th>
<th>Age of death (years) average (range)</th>
<th>Duration (y) average (range)</th>
<th>Brain weight (g) average (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-synuclein-positive group</td>
<td>13 (4/9)</td>
<td>73.5 (61–89)</td>
<td>83.2 (72–93)</td>
<td>9.7 (2–19)</td>
<td>998.3 (840–1220)</td>
</tr>
<tr>
<td>α-synuclein-negative group</td>
<td>14 (3/11)</td>
<td>72.8 (55–89)</td>
<td>80.9 (60–94)</td>
<td>11.0 (4–22)</td>
<td>1048.2 (720–1270)</td>
</tr>
</tbody>
</table>

*The patients analyzed in this study were separated in two groups with respect to whether they had α-synuclein-positive structures in all four selected regions or not. The two groups did not have statistical differences between each other for all heading of the Mann–Whitney U-test.
Table 2
Comparison of stages of neurofibrillary changes between two groups

<table>
<thead>
<tr>
<th>Stage</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive group</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Negative group</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>

*The extent of neurofibrillary changes was evaluated by immuno-histochemistry of tau (AT8) in all cases of each group according to classification of Braak and Braak. Most of the cases in two groups were assigned to an isocortical stage.

National Institute on Aging, and Regan Institute Working Group [1], ten cases as high likelihood and three cases as intermediate likelihood. In the negative group, 13 cases categorized as having definite AD or high likelihood, and the remaining one case as having probable AD or intermediate likelihood.

3.3. The frequency and distribution of α-synuclein-positive structures

In this study, the difference in immunostaining between LB509 and #17 was hardly recognized, and the immunostaining for LB509 was evaluated.

3.3.1. α-Synuclein-positive intra-cytoplasmic inclusions

Fig. 2 shows the distribution and frequency of α-synuclein-positive intra-cytoplasmic inclusions in brains of the positive group. In 12 (92.3%) out of 13 cases, α-synuclein-positive inclusions were most frequently observed in the amygdala and temporal cortex among all regions examined. The next frequent occurrence of α-synuclein-positive inclusions was observed in the parahippocampal gyrus, in which 11 (84.6%) out of 13 cases had α-synuclein-positive inclusions. The parietal cortex, in which nine cases (69.2%) had α-synuclein-positive inclusions, was the region in which the occurrence of these inclusions was the most infrequent among the three neocortices. This frequency was the same as that observed in the cingulate gyrus, which is considered as one of the preferred regions [17]. Moreover, the amygdala showed the highest density of α-synuclein-positive inclusions, followed by the parahippocampal gyrus > the cingulate gyrus, CA2/3 and CA4 regions of hippocampus formation.

3.3.2. α-Synuclein-positive neurites

The distribution and frequency of α-synuclein-positive neurites among the positive group are described in Fig. 3. α-Synuclein-positive neurites were present not only in CA2/3 region of hippocampus formation [8], but also in the other regions. Twelve cases (92.3%) had α-synuclein-positive neurites in the amygdala, and ten cases (78.5%) had them in CA2/3 region of hippocampus formation, parahippocampal gyrus, temporal cortex, substantia nigra and locus ceruleus. Five cases (38.5%) had α-synuclein-positive neurites in the frontal cortex, and six cases (46.2%) had them in the parietal cortex. In the frontal and parietal cortices, the α-synuclein-positive neurites were much less dense than in the other regions. The region with the highest density of positive neurites was the amygdala,
followed by CA2/3 region of hippocampus and parahippocampal gyrus.

3.4. Morphological features of α-synuclein-positive structures by light microscopy

3.4.1. α-Synuclein-positive intra-cyttoplasmic inclusions

In immuno-cytochemistry using an anti-α-synuclein antibody, brainstem-type LBs (Fig. 4A) exhibited spherical immunoreactivity with a halo. This pattern of staining was very similar to that of brainstem-type LBs immunostained with an anti-ubiquitin antibody. On the other hand, in cerebral cortices, α-synuclein-positive inclusions were recognized as various shapes. For example, the staining patterns of cortical α-synuclein-positive inclusions were spot-shaped (Fig. 4C), flame-shaped (Fig. 4D), filamentous in neuronal perikarya (Fig. 4E,F), besides the typical shape of cortical LBs (Fig. 4B). The number of these inclusions that are different from LBs (= non-LB type) varied from case to case and their percentages with respect to the total number of α-synuclein-positive inclusions also varied depending on the region (Fig. 5). Namely, in the hippocampus formation (especially the CA4 region), the percentage is higher than in the other regions, and the temporal cortex has the highest percentage among the neocortices. Moreover, in the cingulate gyrus and brainstem, the percentage was much lower than in the other regions.

3.4.2. α-Synuclein-positive neurites

In the superficial layer of temporal cortex in particular, α-synuclein-positive fine neurites that morphologically resemble NTs, were more dense in some cases. Such cases had much more numerous α-synuclein-positive structures including LBs in the deep layer, although individual cases showed slight variability.

3.5. Double-labeling confocal microscopy of α-synuclein and tau

Laser scanning confocal microscopy of sections double-immunolabeled with antibodies against α-synuclein (#17) and tau (AT8) showed that the respective immunoreactivity colocalized in some neurons. These neurons exhibited mainly two patterns as follows: (1) intermingled with tau and α-synuclein-positive aggregation in the neuron (Fig. 6A–C); and (2) LBs and α-synuclein-positive masses surrounded by tau immunoreactivity (Fig. 6D–I). In the present study, the latter pattern was observed much more frequently than the former. Conversely, NFTs and tau-positive masses surrounded by α-synuclein immunoreactivity were not observed in the same neuron. Moreover, only a few neurites were colabeled with #17 and
Fig. 4. Photomicrographs of the immuno-histochemical staining with the anti-α-synuclein antibody (LB509). (A) Brainstem-type LBs in the substantia nigra. (B) Typical cortical LBs in the cingulate gyrus. (C) Spot-shaped α-synuclein-positive inclusions in the subiculum. (D) Flame-shaped α-synuclein-positive inclusions in the CA1 region of the hippocampus. (E) Filamentous inclusions in neuronal perikarya in addition to typical cortical LBs in the frontal cortex. (F) Filamentous inclusions in neuronal perikarya in the hippocampus. Scale bar=10 μm in each panel.

AT8. These neurites appeared to be covered with tau-positive coarse granular deposits (Fig. 6J–L).

4. Discussion

α-Synuclein, also known as the precursor of the non-Ab component of AD amyloid (NACP) [29], is a soluble protein, and it is predominantly localized to presynaptic nerve terminals [13,15] in a normal brain. Some previous studies have identified α-synuclein as the major component of LBs and LNs. In the present study, the cases with SPs and NFTs numerous enough to meet the criteria of AD could be subdivided into two groups according to whether α-synuclein aggregation was present or not. The majority of α-synuclein-positive cases have α-synuclein-positive structures in various amounts in almost all the regions examined in this study. This finding indicates that the aggregation of α-synuclein was distributed more extensively in the central nervous system than we had expected. Among all the regions examined, the amygdala contained the highest amount of α-synuclein-positive structures. This result is consistent with those of DLBD [25], familial AD [19], Down’s syndrome with AD [20] and Parkinsonism–dementia complex of Guam [33], which implies that the amygdala would be one of the easiest regions for the α-synuclein protein to aggregate. Immuno-histochemistry of α-synuclein revealed a variety of α-synuclein-positive inclusions that are different from LBs. These inclusions were considered to represent an early stage of LB formation in PD and DLB, because they were recognized more numerous on patients with shorter disease duration [30], or their ultrastructural features were similar to LBs [2]. However, in these sporadic AD cases, it is uncertain whether the so-called non-LB type α-synuclein aggregation would be that of LBs or not.

In this study, the ratio of the number of α-synuclein-positive inclusions other than LBs to the total number of inclusions was less in the brainstem and the cingulate gyrus, where these are generally considered to be the preferential regions for LB formation. Meanwhile the hippocampus, in which few LBs were usually found [9], had higher ratio than in any other regions. These findings indicate that there are two kinds of regions in the brain: one is susceptible to LB formation, and the other to non-LB-type of α-synuclein aggregation. The amount of NACP/α-synuclein was not equal across the brain, and α-synuclein was relatively more abundant in the hippocampus and less in the brainstem in rats [12]. It is uncertain whether the hippocampus is abundant with α-synuclein in humans as well. However, it was indicated that the process of α-synuclein aggregation was dependent
Fig. 5. Percentage of non-LB-type α-synuclein-positive inclusions with respect to the total number of α-synuclein-positive ones in each region. α-Synuclein-positive inclusions were morphologically subdivided into LBs-type and non-LB-type. In all cases of the positive group, the numbers of non-LB-type in each region was the percentage of the total α-synuclein-positive inclusions. The highest percentage was observed in the hippocampus. On the other hand, the brainstem and cingulate gyrus had lower percentages than the other regions. F, middle frontal gyrus; P, inferior parietal lobule; Cin., cingulate gyrus; T, middle temporal gyrus; CA4, CA2/3, CA1, CA4, CA2/3 and CA1 regions of the hippocampus, respectively; sub., subiculum; para., parahippocampal gyrus; Amy., amygdala; SN, substantia nigra; LC, locus ceruleus; vagus, dorsal motor nucleus of vagus.

Acknowledgements

We thank Professor Takeshi Iwatsubo and Ms Minami Baba of the Graduate School of Pharmacological Science, on its concentration and nucleation in vitro [32]. Taking these reports into consideration together with our observations, it could be supposed that in the hippocampus, the aggregation of α-synuclein could not always lead to LB formation and non-LB-type α-synuclein aggregation could secondarily occur under the influence of tau aggregation. Alternatively, a large number of NFTs could inhibit LB-type α-synuclein aggregation because tau aggregates and α-synuclein could interact with each other. In the brainstem, tau aggregation was not prominent and α-synuclein could aggregate to form typical LBs. Although the cellular mechanism underlying the aggregation of α-synuclein and LB formation is unknown, there are some reports indicating the relevance of tau and α-synuclein aggregation. It was reported that α-synuclein binds to tau and stimulates the protein kinase A-catalyzed tau phosphorylation in vitro [16]. Moreover, in some neuropathological reports, it was indicated that neocortical LBs were significantly more frequent in cases having both AD and PD lesions than in cases with either PD or AD lesion alone [6]. It was also reported that in the limbic area [12], particularly in the amygdala [26], LBs and NFTs were colocalized in the same neurons.

A recent immunoelectron microscopical study demonstrated that the tau-epitope was occasionally incorporated even into brainstem-type LBs [3]. The above studies assumed that tau and α-synuclein could interact with each other both in vitro and in vivo. There could be two possibilities with respect to non-LB-type α-synuclein aggregation: one was that these non-LB-type inclusions represent the early stage of LB formation, and the other is that these non-LB-type α-synuclein inclusions are formed secondary to tau aggregation and never lead to LB formation. We could not determine which possibility applies. However, we could not usually observe typical LBs in the hippocampus, and it would be difficult to conclude that all of these α-synuclein-positive inclusions signified the early stage of LB formation.

In summary, the discovery of α-synuclein as a marker of LBs facilitated the detection of LB-related structures even in AD cases with widespread and numerous NFTs. Moreover, α-synuclein-positive inclusions other than LBs were observed, and their distribution indicates that LB formation may be influenced by the degree of tau aggregation. The cases with AD might be subdivided into two groups according to the existence or absence of α-synuclein aggregation.
Fig. 6. Confocal microscopy images of sections double-stained for α-synuclein (#17) and tau (AT8) immunoreactivity. #17 was labeled with Alexa 488 and appeared green in the left column (A, D, G, J). AT8 was labeled with Alexa 594 and appeared red in the middle column (B, E, H, K). Merging of two signals is shown in the right column (C, F, I, L). (C) shows the intermingling of tau and α-synuclein aggregation. (F, I) show that α-synuclein aggregations are surrounded by tau substrates. (L) shows that α-synuclein-positive neurites are covered with tau-positive coarse granular deposits.
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References


