Phosphorylated alpha-synuclein in Iba1-positive macrophages in the skin of patients with Parkinson’s disease

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Introduction

Idiopathic Parkinson’s disease (IPD) is a common secondary neurodegenerative disorder that affects more than 1% of the population over 65 years of age.1 The routine diagnosis of IPD depends on clinical criteria mainly focused on motor symptoms.2 However, the clinical criteria are not sufficient to precisely differentiate IPD from other parkinsonisms.3,4

The histopathological features of Parkinson’s disease (PD) are the loss of dopaminergic (DA) neurons in the substantia nigra (SN) and the presence of cytoplasmic protein aggregates known as Lewy bodies (LBs).2 The major components of LBs are β-sheet fibrillar aggregates...
of alpha-synuclein (αSyn) associated with synaptic vesicles in presynaptic nerve terminals. Intracellular αSyn forms aggregates within neurons but also spreads out into extracellular spaces. Notably, Braak, Del Tredici postulated that a neurotoxic pathogen, possibly a viral toxin, may enter the brain via two routes: (1) A nasal route, with anterograde progression into the limbic system, and (2) a gastric route, after swallowing nasal secretions in saliva. The latter nasal secretions might contain a neurotoxic pathogen that, after penetrating the epithelial lining, could enter the axons of Meissner’s plexus and reach the preganglionic parasympathetic motor neurons of the vagus nerve via transsynaptic transmission. These processes are hypothesized to be followed by retrograde transport into the medulla, pons, and midbrain, and finally, the SN might be affected. Thus, stereotypical cell-to-cell spreading of αSyn is believed to contribute to disease progression.

Several lines of evidence show that microglia are associated with the removal of extracellular αSyn aggregates and the response to neurodegeneration in the early stages of IPD pathogenesis. Although a correlation between αSyn aggregates and microglia is speculated in IPD pathogenesis, the phagocytosis of αSyn aggregates by microglia has not been demonstrated in human brain tissues. In this study, we examined possible relationships among neurodegeneration, microglia, and αSyn aggregates in brain autopsy specimens from IPD patients.

The dermal macrophages in the skin as well as the microglia in the brain are phagocytic cells. Dermal macrophages are involved in peripheral inflammation in innate immunity, autoinflammation, and acquired immunity and are involved in cutaneous tissue remodeling. Furthermore, it has been suggested that αSyn aggregates activate peripheral macrophages and that peripheral macrophages ingest pathological αSyn aggregates in aged rats or PD patients. Although phosphorylated αSyn (p-αSyn) deposits have been observed in sympathetic adrenergic and cholinergic fibers in the skin of IPD patients and cholinergic fibers have been observed in the rectal submucosa of IPD patients, whether p-αSyn deposits are observed in macrophages in the skin has not been previously reported. In this report, we demonstrated that dermal macrophages in skin biopsy specimens from IPD patients contain p-αSyn deposits.

Materials and Methods

Clinical information of the patients for skin biopsy

We studied 10 patients with IPD who met the clinical diagnostic criteria of the UK PD Society Brain Bank. The ages of the patients ranged from 56 to 77 years, with an average of 67.8 years. The 10 patients included four female and six male patients (Table 1). All patients had late-onset disorders (older than 45 years), and none of them had a family history of PD. All patients were treated with L-dopa alone or with other antiparkinsonian medications and maintained good control (CN) of their motor symptoms. We examined four consecutive autopsied decedents located at the Tokyo Metropolitan Geriatric Hospital as healthy CNs. The ages of the CNs ranged from 65 to 70 years, with an average of 67.8 years. The four CNs included two female and two male decedents (Table 1).

Brain tissue from autopsied decedents

We examined eight consecutive autopsied decedents located at the National Hospital Organization Sendai Medical Center. Four of the decedents were diagnosed with IPD, and four of the decedents were used as CNs (Table 2). CNs were defined as follows: clinical symptoms were not observed, and abnormal p-αSyn, amyloid β or phosphorylated tau aggregates were not observed in brain specimens from autopsied individuals. The ages of the decedents ranged from 62 to 87 years, with an average of 74.8 years. The entire brain tissue was fixed in 20% buffered formalin (Wako, Osaka, Japan) for 5–11 days. Serial coronal sections were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin using an automated tissue processor.

Table 1. Demographic profiles of IPD patients and CNs from whom skin biopsy specimens were collected.

| Case | Participant | Sex | Age | Duration | MMSE |
|------|-------------|-----|-----|----------|------|
| 1    | PD          | Female | 56  | 8       | 29   |
| 2    | PD          | Male  | 77  | 3       | 27   |
| 3    | PD          | Male  | 70  | 4       | 26   |
| 4    | PD          | Female | 67  | 2       | 27   |
| 5    | PD          | Female | 74  | 20      | 27   |
| 6    | PD          | Male  | 72  | 3       | 23   |
| 7    | PD          | Male  | 62  | 10      | 30   |
| 8    | PD          | Male  | 65  | 8       | 29   |
| 9    | PD          | Female | 74  | 8       | 28   |
| 10   | PD          | Male  | 61  | 2       | 29   |
| 11   | CN          | Male  | 66  | NE      |      |
| 12   | CN          | Male  | 70  | NE      |      |
| 13   | CN          | Female | 70  | NE      |      |
| 14   | CN          | Female | 65  | NE      |      |

IPD, idiopathic Parkinson’s disease; CN, control; PD, Parkinson’s disease; NE, not examined; MMSE, mini mental state examination score.
Biopsied skin tissue

According to a previous report suggesting that postmortem skin specimens from patients with Braak stage III PD demonstrated the highest incidence of dermal p-αSyn deposits, we recruited cognitively normal to mildly impaired IPD patients in the relatively early stage of IPD (Table 1). Skin specimens were collected from the cervical C8 paravertebral area, that is, close to the spinal ganglia, and from an axilla in IPD patients. Skin specimens were collected from an upper arm in CNs. Skin biopsies were performed using a 3-mm disposable punch biopsy kit under sterile conditions after topical anesthesia with lidocaine. In IPD and CNs, skin biopsy specimens were fixed in 4% paraformaldehyde for 24–48 h.

Histological immunofluorescence (IF) staining

Serial coronal 5-μm slices were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin using an automated tissue processor. Mouse anti-phosphorylated Ser129 alpha-synuclein (p-αSyn) monoclonal antibody (Immunostar, Huston, WI) was used to detect αSyn deposits in the skin or αSyn aggregates in the brain. To increase the sensitivity of detection of LB-related p-αSyn aggregates in the brain, brain sections were pretreated with formic acid. Rabbit anti-dopamine beta hydroxylase (DBH) polyclonal antibodies (Abcam, ab209487, Cambridge, MA) were used to detect adrenergic fibers in the skin. To detect microglia in the brain, rabbit anti-Iba1 polyclonal antibodies (Wako Chemicals, Richmond, VA) were used. To detect macrophages in the skin, rabbit anti-Iba1 polyclonal antibodies (Wako Chemicals, Richmond, VA) and rabbit anti-CD163 polyclonal antibodies (Abcam, ab189915, Cambridge, MA) were used. To increase the sensitivity of detection of CD163-ir positive macrophages, skin sections were pretreated with target retrieval solution pH 9 (Agilent Technologies, Santa Clara, CA). To detect cytoplasmic autophagy-associated proteins, rabbit anti-p62/SQSTM1 polyclonal antibodies (1:100, ProteinTech, Rosemont, IL) were used. The sections were double-immunostained overnight with combinations of primary antibodies: (1) Mouse anti-p-αSyn (1:1000 dilution) and rabbit anti-DBH (1:100 dilution), (2) mouse anti-p-αSyn (1:1000 dilution) and rabbit anti-Iba1 (1:100 dilution), (3) mouse anti-p-αSyn (1:1000 dilution) and rabbit anti-CD163 (1:100 dilution) or (4) mouse anti-p-αSyn (1:1000 dilution) and rabbit anti-p62/SQSTM1 (1:100 dilution). The sections were then washed and incubated with secondary antibodies for 2 h. The secondary antibodies anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 594, anti-mouse Alexa Fluor 594, and anti-rabbit Alexa Fluor 488 were obtained from Jackson ImmunoResearch (West Grove, PA) and were used at a 1:500 dilution. Then, the sections were treated with a TrueBlack™ lipofuscin autofluorescence quencher (Biotium, Hayward, CA) since brain tissue contains abundant lipofuscin. The sections were initially viewed and analyzed under a fluorescence microscope system (BZ-X700, Keyence, Osaka, Japan).

Quantitative analysis of immunostaining

Quantitative analysis of IF staining

To examine the number of macrophages and macrophages containing p-αSyn deposits in skin sections, we measured the number of p-αSyn-immunoreactive (ir) positive deposits, Iba1-ir positive macrophages, and Iba1-ir and p-αSyn double-positive macrophages under 20-fold magnified fields in the dermis in the region of interest of one section. To examine the numbers of adrenergic fibers and adrenergic fibers containing p-αSyn deposits in skin sections, we measured the numbers of p-αSyn-ir positive deposits, DBH-ir adrenergic fibers, and DBH-ir and p-αSyn double-positive adrenergic fibers under 40-fold magnified fields in the dermis in the region of interest of one section. To examine microglial cells, p-αSyn aggregates and microglial cells containing p-αSyn aggregates in brain autopsy specimens, we identified Iba1-ir positive microglial cells, αSyn-ir positive aggregates, and p-αSyn-ir and Iba1-ir double-positive microglial cells under 20-fold magnified fields in the region of interest. For the analysis of the entorhinal cortex (EC) and amygdala, the gray matter area in the same region was defined as the region of interest.

Quantitative analysis of neuronal loss with hematoxylin and eosin staining

To evaluate neuronal loss in brain autopsy specimens, we counted the number of nuclei in neurons by hematoxylin

| Case | Participant | Age | Sex | Duration | CNS LB stage |
|------|-------------|-----|-----|----------|--------------|
| 1    | PD          | 75  | Male| Unknown  | IV           |
| 2    | PD          | 68  | Female| 10       | IV           |
| 3    | PD          | 87  | Female| 9        | V            |
| 4    | PD          | 75  | Female| 17       | V            |
| 5    | CN          | 62  | Male |          |              |
| 6    | CN          | 79  | Male |          |              |
| 7    | CN          | 83  | Female|          |              |
| 8    | CN          | 69  | Male |          |              |
and eosin (H&E) staining under 20-fold magnified fields. The nuclei of neurons were identified morphologically by hematoxylin staining.

**Statistical evaluation**

All values are expressed as the median (interquartile range). Significant differences between groups were identified using Wilcoxon rank sum test. Spearman’s rank correlation coefficient was used to correlate the number of macrophages and the number macrophages with of p-αSyn deposits. The data were analyzed with a JMP13 computer software system (SAS Institute, Tokyo, Japan). A p < 0.05 denoted a statistically significant difference.

**Results**

**Microglia with p-αSyn aggregates in brain autopsy specimens**

To identify microglia with LB-related αSyn aggregates, a double-labeling IF study was performed by incubating sections of brain autopsy specimens with anti-p-αSyn mouse monoclonal antibody and anti-Iba1 rabbit polyclonal antibodies. Iba1 is a 17-kDa EF-hand protein that is specifically expressed in microglia and macrophages. Iba1 is a 17-kDa EF-hand protein that is specifically expressed in microglia and macrophages and upregulated during immune activation.21 In IPD patients, EC (the number of neurons, 4 IPD vs. 4 CNs, 37.5 [36.25–43.25] vs. 37.5 [32.5–40.75]; [p = 0.665]) and amygdala (AM) (the number of neurons, 4 PD vs. 4 CNs, 33 [28.75–35] vs. 40.5 [33.25–44]; [p = 0.108]) were classified as the mild neuronal loss regions (MNR) because there was no neuronal loss in IPD patients compared to CNs in these regions (Fig. 1D). In IPD patients, SN (the number of neurons, 4 IPD vs. 4 CNs, 5.5 [1.25–6.75] vs. 25 [19–31]; [p < 0.05]) and dorsal motor nucleus of the vagus nerve (the number of neurons, 4 IPD vs. 4 CNs, 0 [0–0.75] vs. 11.5 [9.5–14.25]; [p < 0.05]) were classified as the severe neuronal loss regions (SNR) because of the reduced number of neurons in IPD patients compared to CNs in these regions (Fig. 1E).

Iba1-positive microglial cells with p-αSyn aggregates in brain autopsy specimens from IPD patients and CNs were analyzed. Interestingly, Iba1-positive microglia with p-αSyn aggregates were observed (Fig. 1A and B) in the EC of brain autopsy specimens from IPD patients but not CNs (Fig. 1C).

Next, LB-related Iba1-positive microglial cells and p-αSyn aggregates in brain autopsy specimens from four IPD patients were analyzed in the SNR and MNR. LB-related p-αSyn-positive cells were 155 observed in both the SNR and MNR (the number of p-αSyn-positive cells, 8.5 [8–12.25] vs. 9.5 [8–12.5]; [p = 0.668]) of brain autopsy specimens from four IPD patients (Fig. 1F). Iba1-positive activated microglial cells (amoeboid form) were more abundant in the MNR compared to SNR (the number of Iba1-positive activated microglial cells, 15 [10.75–19.5] vs. 1 [0–1]; [p < 0.001]) of brain autopsy specimens from four IPD patients (Fig. 1G). In addition, Iba1-positive microglial cells with p-αSyn aggregates were observed in the MNR but not SNR (the number of Iba1-positive microglial cells with p-αSyn aggregates, 6.5 [5–8.75] vs. 0; [p < 0.001]) of brain autopsy specimens from four IPD patients (Fig. 1H). Thus, in the brains of patients with IPD, the results suggest that Iba1-positive microglia containing LB-related αSyn aggregates are mainly localized in brain regions where neuronal cell death is not prominent. Our observation is in accordance with a previous report suggesting that αSyn aggregate-related synaptic dysfunction may trigger later neurodegeneration in IPD.22

**Figure 1.** p-αSyn aggregate-positive microglia and neurons in brain autopsy specimens from PD patients. IF staining of Iba-ir microglia (red) and p-αSyn-ir aggregates (green) in the EC classified as the mild neuronal loss region of IPD patients and CNs. Low (A) and high (B) magnification images are shown in the EC of IPD patients. High (C) magnification images are shown in the EC of CNs. Quantitative analysis of the numbers of neurons in the EC and AM of four IPD patients and four CNs (D). Quantitative analysis of the numbers of neurons in the SN and DNV of four IPD patients and 4 CNs (E). Quantitative analysis of the numbers of p-αSyn-positive neurons (F). Quantitative analysis of the numbers of Iba1-positive activated microglial cells (G), and Iba1-positive microglial cells with p-αSyn aggregates in the SNR and MNR of four IPD patients (H). IF images show Iba1-positive activated microglial cells with p-αSyn aggregates (A and B) in the EC of IPD patients but not CNs (C). In IPD patients, EC (p = 0.665) and AM (p = 0.108) were classified as the MNR because there was no neuronal loss compared to CNs (D). In IPD patients, SN (p < 0.05) and DNV (p < 0.05) were classified as the SNR because of the reduced number of neurons compared to CNs (E). LB-related p-αSyn aggregate-positive cells were observed in both the SNR and MNR of IPD patients (p = 0.668) (F). Iba1-positive activated microglial cells (amoeboid form) were more frequently observed in the MNR compared to SNR of IPD patients (p < 0.001) (G). Moreover, Iba1-positive microglial cells with p-αSyn aggregates were observed in the SNR but not SN of brain specimens from IPD patients (p < 0.001) (H). ***p < 0.001, *p < 0.05. Scale bars: 50 μm (A–C). Circles indicate the data points between the lower and upper whiskers, and x indicates the average marker in a box/whisker diagram. p-αSyn, phosphorylated alpha-synuclein; IF, immunofluorescence; EC, entorhinal cortex; IPD, idiopathic Parkinson’s disease; CNs, controls; SN, substantia nigra; SNR, severe neuronal loss regions; MNR, mild neuronal loss regions; AM, amygdala; LB, Lewy body; DNV, dorsal motor nucleus of the vagus nerve.
p-αSyn in IPD Skin Macrophages

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Dermal macrophages with p-αSyn deposits are markedly increased in skin specimens from IPD compared with those from CNs

To investigate the distribution of abnormal αSyn deposits in skin specimens from IPD patients, a single-label IF study was performed by incubating skin sections from IPD patients with anti-p-αSyn (phospho-Ser129, mouse monoclonal). p-αSyn deposits were observed in both the posterior cervical C8 paravertebral and axillary skin of all IPD patients.

We identified Iba1-ir microglial cells with p-αSyn aggregates in the central nervous system (CNS) of PD patients. This suggests that microglial cells are directly involved in the degradation of αSyn aggregates. Thus, we hypothesized that the presence of p-αSyn deposits in dermal Iba1-positive macrophages, which are analogous to microglia, could be a biomarker based on immunological mechanisms in IPD. Based on this hypothesis, we examined whether macrophages in the axillary skin from IPD patients contained p-αSyn deposits. The double-labeling IF study with anti-p-αSyn mouse monoclonal antibody and anti-Iba1 rabbit polyclonal antibodies revealed macrophages with p-αSyn deposits in the dermis of IPD patients but not CNs (Fig. 2A). Iba1-ir-positive macrophages with p-αSyn deposits were found diffusely in the dermis of IPD patients but not CNs (Fig. 2C). The double-labeling IF study with anti-p-αSyn mouse monoclonal antibody and anti-CD163, another marker of macrophages, rabbit polyclonal antibodies also revealed macrophages with p-αSyn deposits in the dermis of IPD patients but not CNs (Fig. 2D). p-αSyn deposits were colocalized with p62/SQSTM1, a cytoplasmic protein associated with autophagy, in the dermis of IPD patients but not CNs (Fig. 2E).

The number of p-αSyn-ir and Iba1-ir double-positive macrophages in the dermis was observed in 10 IPD patients but not four CNs (the number of p-αSyn-ir and Iba1-ir double-positive cells, 10 [5.75–20] vs. 0; [p < 0.01]) (Fig. 3A). There was no difference in macrophages with p-αSyn deposits staining pattern between the cervical C8 paravertebral and axillary skin in all IPD patients.

Adrenergic nerve fibers with p-αSyn deposits in skin specimens

In the skin, adrenergic DBH-ir fibers mainly exist around arterioles. It is reported that p-αSyn deposits in adrenergic fibers in the cervical C8 paravertebral skin from IPD patients. Thus, in this study double-labeling IF studies were performed by incubating sections with anti-p-αSyn mouse monoclonal antibody and anti-DBH rabbit polyclonal antibodies in the cervical C8 paravertebral skin sections obtained from IPD patients and CNs. The double-labeling IF studies revealed the localization of p-αSyn deposits in DBH-ir adrenergic vasomotor fibers in the dermis of IPD patients but not CNs (Fig. 2B). p-αSyn deposits in DBH-ir adrenergic vasomotor fibers were mainly observed around blood vessels in the dermis of IPD patients. The % p-αSyn-ir and DBH-ir double-positive adrenergic fibers among DBH-ir adrenergic fibers were observed in the dermis of 10 IPD patients but not four CNs (the % p-αSyn-ir and DBH-ir double-positive adrenergic fibers among DBH-ir adrenergic fibers, 73.2 [57.5–80] vs. 0; [p < 0.01]) (Fig. 3B).

Correlation between p-αSyn deposits and macrophages in skin specimens from IPD patients

Spearman’s rank correlation coefficient was used to correlate the number of Iba1-ir macrophages and the number of Iba1-ir macrophages with p-αSyn deposits in the dermis of IPD patients. Interestingly, the number of Iba1-ir macrophages was significantly positively correlated with the number of Iba1-ir macrophages with p-αSyn deposits in the dermis of IPD patients. (ρ = 0.9693, p < 0.0001) (Fig. 3C). These results suggest that the increase in αSyn deposition in dermal macrophages of IPD patients may be directly related to an increase in macrophages.

Figure 2. p-αSyn deposits in macrophages and adrenergic sympathetic fibers in skin specimens from IPD patients. IF images of dermal p-αSyn (red) and Iba1 (green)-positive macrophages in the dermis of IPD patients and CNs (A and C). IF images of dermal p-αSyn (red) and DBH-ir positive vasomotor fibers (green) around blood vessels in the dermis of IPD patients and CNs (B). IF images of dermal p-αSyn (red) and dermal CD163, another marker of macrophages, (green)-positive macrophages in the dermis of IPD patients and CNs (D). IF images of dermal p-αSyn (red) and p62/SQSTM1, cytoplasmic proteins associated with autophagy, (green)-positive deposits in the dermis of IPD patients and CNs (E). IF images (high magnification) show Iba1-ir positive macrophages with p-αSyn deposits (A) and DBH-ir adrenergic nerve fibers with p-αSyn deposits around blood vessels (B) in the dermis of IPD patients but not CNs. IF images (low magnification) show that Iba1-ir positive macrophages with p-αSyn deposits were found diffusely in the dermis of IPD patients but not CNs (C). IF images (high magnification) show CD163-ir positive macrophages with p-αSyn deposits in the dermis of IPD patients but not CNs (D). IF images (high magnification) show p-αSyn-ir positive deposits were colocalized with p62/SQSTM1 in the dermis of IPD patients but not CNs (E). Scale bars: 20 μm (A–E). IPD, idiopathic Parkinson’s disease; IF, immunofluorescence; CNs, controls; DBH, dopamine beta hydroxylase; p-αSyn, phosphorylated alpha-synuclein.
### A

| PD | CN |
|----|----|
| p-αSyn | lba1 |
| p-αSyn/lba1 | p-αSyn/lba1 |

### B

| PD | CN |
|----|----|
| p-αSyn | DBH |
| p-αSyn/DBH | p-αSyn/DBH |

### C

| PD | CN |
|----|----|
| p-αSyn | lba1 |
| p-αSyn/lba1 | p-αSyn/lba1 |

### D

| PD | CN |
|----|----|
| p-αSyn | CD163 |
| p-αSyn/CD163 | p-αSyn/CD163 |

### E

| PD | CN |
|----|----|
| p-αSyn | p62 |
| p-αSyn/p62 | p-αSyn/p62 |
and the $\alpha$-Syn-ir and DBH-ir double-positive adrenergic fibers among $\alpha$-Syn-ir positive deposits to compare the frequency of macrophages and adrenergic nerve fibers with $\alpha$-Syn deposits in the dermis of IPD patients. There was no difference between the frequency of macrophages and adrenergic nerve fibers with $\alpha$-Syn deposits in the dermis of 10 IPD patients (the % macrophages or adrenergic nerve fibers among $\alpha$-Syn deposits, 38.4 [39.6–49.1] vs. 35.9 [27.1–43.3]; $p = 0.426$) (Fig. 3D). These results suggest that $\alpha$-Syn deposits were observed not only in adrenergic fibers but also in macrophages in the dermis.

**Discussion**

### Microglia with $\alpha$-Syn aggregates may indicate an early stage of neurodegeneration in IPD patients

The microglial cells containing $\alpha$-Syn aggregates were mainly localized in brain regions where neuronal cell death was not remarkable. Activated microglia within the CNS are detectable using macrophage markers, including major histocompatibility complex II and Iba1. Depending on the inflammatory environment, microglia exhibit both phenotypic and functional changes from resting to activating states. Activated microglia are amoeboid (larger cell bodies, shorter, and thicker processes) and are morphologically similar to the macrophages in peripheral tissues. A correlation between microglial activation and DA terminal loss in the affected nigrostriatal pathway in a PD mouse model also supports the hypothesis that neuroinflammatory responses induced by activated microglia contribute to the early neurodegeneration process. Other previous reports also suggest that microglial activation is associated with the early stage of neurodegeneration in PD models. In addition, microglia are capable of clearing extracellular $\alpha$-Syn aggregates by internalization and degradation in PD models. Although microglial activation in PD models has been suggested, the presence of $\alpha$-Syn aggregates in the microglia of human PD brains has not been reported. In this study, $\alpha$-Syn-positive round structures within Iba1-positive cells were observed in the MNR of PD patients but not CNs. Thus, for the first time, we demonstrated that LB-related $\alpha$-Syn aggregates were observed in Iba1-ir microglial cells of autopsy brain specimens from IPD patients. In addition, $\alpha$-Syn-positive round structures within Iba1-negative cells were more observed in the SNR compared to MNR of IPD patients. On the other hand, $\alpha$-Syn-positive round structures within Iba1-positive microglial cells were observed in the MNR but not SNR of IPD patients. Thus, microglia with $\alpha$-Syn aggregates...
may indicate an early stage of neurodegeneration in IPD patients. We also examined whether amyloid β or phosphorylated tau aggregates were observed in the MNR of IPD patients by using immunohistochemical analysis. We could not observe abnormal amyloid β or phosphorylated tau aggregates in the MNR of PD patients (data not shown). Thus, in this study, we suggested that there was no influence of concomitant amyloid β and tau pathologies on microglial activation in the MNR. In summary, our results suggest that the phagocytosis of p-αSyn aggregates by microglia is a good marker for early-stage synucleinopathies.

**p-αSyn deposits as a potential useful biomarker for IPD**

In this study, Iba1-ir microglial cells with p-αSyn aggregates in the CNS of IPD patients suggests that microglia are directly involved in the degradation of αSyn aggregates. Thus, we hypothesized that the presence of p-αSyn deposits in dermal Iba1-positive macrophages, which are analogous to microglia, could be a biomarker-based on immunological mechanisms in IPD. Although αSyn accumulation in human induced pluripotent stem cell macrophages from PD patients has been observed,15 macrophages with p-αSyn deposits in human skin tissues have not been reported. Therefore, we examined whether macrophages in the dermis of IPD contain p-αSyn deposits. In this report, we first demonstrated dermal macrophages with p-αSyn deposits in IPD. Moreover, we propose that not only dermal nerve terminals containing p-αSyn deposits but also dermal macrophages with p-αSyn deposits are good markers to differentiate IPD patients from CNs.

We also demonstrated that the number of Iba1-ir macrophages significantly correlated with the number of Iba1-ir macrophages with p-αSyn deposits in the dermis in IPD patients. Increasing evidence suggests that the spread of αSyn is the underlying mechanism for LB-related neurodegeneration.5,6 In addition, it has been suggested that microglia in the CNS, which are analogous to macrophages, may clean extracellular αSyn and prevent the spread of αSyn-related pathology.26 Phillips et al. showed that macrophages adjacent to dystrophic nerve terminals were swollen and contained insoluble αSyn in the myenteric plexus of aged rats.14 Haenseler et al. studied the removal role in αSyn using human induced pluripotent stem cell macrophages from PD patients. In this study, macrophages have the ability to remove fibrillary αSyn by phagocytosis, but high levels of exogenous or endogenous αSyn may reduce this ability and cause PD.15 These results suggest that macrophages play an active removal role in αSyn. Thus, our results indicated that Iba1-ir peripheral macrophages were directly related to p-αSyn deposits in the dermis in IPD patients in the present study.

In the present study, we demonstrated that p-αSyn deposits in biopsied skin tissues of IPD patients were coexpressed with the cytoplasmic autophagy-related protein p62. αSyn aggregates are also preferred targets for p62-dependent autophagy in IPD.27,28 Recently, αSyn aggregates with p62 accumulation were shown to be induced by autophagy dysfunction in DA neuron-specific autophagy-deficient mice.20 Thus, our results suggested that p-αSyn deposits with p62 accumulation in biopsied skin tissues of IPD patients might be degraded by the autophagy pathway.

Precise IPD diagnosis is important for predicting prognosis, selecting therapeutic options, and conducting appropriate clinical studies. To improve the accuracy of IPD diagnosis, several ongoing studies aim to identify biomarkers. Possible biomarkers include olfactory function, cerebral spinal fluid tests, and cardiac scintigraphy.29-34 Since specific biomarkers have not yet been identified to distinguish IPD from other neurodegenerative parkinsonisms, a novel biomarker with high specificity and sensitivity is required. In the present study, dermal p-αSyn deposits were observed in 10 (100%) of 10 IPD patients in the posterior cervical paravertebral skin. Several studies demonstrated that the incidence of dermal p-αSyn staining in distal skin18,35 was less than that in proximal skin in IPD patients.17,23,36,37 Interestingly, dermal p-αSyn deposits were observed in 80–100% of IPD patients when proximal cervical skin was used as the skin biopsy site.17,23,36,37 Our results are in good accordance with previous studies demonstrating that cervical skin might be a promising biopsy site. We also observed dermal p-αSyn deposits in the auxiliary skin of 10 (100%) of 10 IPD patients. Thus, we suggest that p-αSyn deposits in axillary skin and posterior cervical skin might be a good target biomarker for IPD, and the former might be a more cosmetically suitable site for skin biopsy.

Here, we identified p-αSyn deposits in the DBH-ir adrenergic nerve vasomotor fibers of IPD patients. Our results were in accordance with several studies that observed dermal p-αSyn deposits in postganglionic adrenergic fibers16,23,35 in IPD patients. Thus, the detection of p-αSyn deposits in the skin autonomic fiber terminal may be a useful biomarker for IPD diagnosis.

**Limitations of this research**

There are several limitations of this research. First, the number of samples was relatively small. Second, cross-sectional longitudinal studies need to be performed.
Third, and possibly the major limitation of this study, the IPD patients from whom skin biopsy specimens were taken were not genetically and pathologically diagnosed. Fourth, autopsy skin specimens of autopsy confirmed PD cases were not available, so IF study of these specimens could not be performed. Fifth, the skin biopsy samples are from different areas between IPD patients (C8 and axilla) and CNs (upper arm). We chose C8 and axilla to obtain highest incidence of dermal p-αSyn deposits in IPD. Collaborators have reported that 149 of 1241 consecutive autopsy cases in the elderly were incidental LB.38 We considered that skin specimens with autopsy confirmed CNs were necessary for this study. Therefore, although skin biopsy samples are from different areas, the autopsy confirmed CNs obtained from the collaborator was included in this analysis. Sixth, this study was analyzed by IF staining only. Electron microscopic analysis was not performed. Thus, electron microscopy should be performed in future studies.

Summary of the results
The main results of our study are as follows: (1) p-αSyn deposits were detected in DBH-ir adrenergic nerve vaso-motor fibers and in Iba1-positive macrophages in skin specimens; and (2) the number of macrophages was significantly positively correlated with the number of macrophages with p-αSyn deposits in the dermis of IPD patients. These results suggest that p-αSyn deposits in dermal macrophages, as well as adrenergic fibers are useful biomarkers to differentiate IPD patients from CNs and that peripheral macrophages are directly related to p-αSyn deposits in the dermis in IPD. These results provide new insights into our understanding of pathophysiological processes in synucleinopathies.

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Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions
Hideki Oizumi, Kensi Yamasaki, Hiroyoshi Suzuki, Yuko Saito, Shigeo Murayama, Takaumi Hasegawa, Kohji Fukunaga and Atsushi Takeda designed the study; Hideki Oizumi, Kensi Yamasaki, Hiroyoshi Suzuki, and Yuko Saito performed the experiments; Hideki Oizumi, Kenshi Yamasaki, and Hiroyoshi Suzuki analyzed the data; and Hideki Oizumi, Kensi Yamasaki, Hiroyoshi Suzuki and Atsushi Takeda wrote the manuscript.

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