Endothelial Degeneration of Parkinson’s Disease is Related to Alpha-Synuclein Aggregation

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Abstract

Objective: We previously reported that the ability of vascular remodelling is impaired in human Parkinson’s disease, leading to endothelial degeneration and vascular dysfunction. Aggregation of α-synuclein is a hallmark of neurodegeneration in Parkinson’s disease and inflammation and autophagy may contribute to secondary neuronal degeneration. The current study examined the association between these characteristic pathologies and endothelial cell degeneration in Parkinson’s disease.

Methods: The study used the post-mortem grey matter from middle frontal gyrus (MFG) of human Parkinson’s disease and age-matched control cases. Immunohistochemical staining of phosphorylated α-synuclein, p62 for autophagy, Human Leukocyte Antigen-antigen D Related (HLA-DR) for activated microglia, Factor VIII for endothelial cells and Neuronal Nuclei for neurons were performed using either tissue microarray or free-floating sections. The expression of these factors was quantified by analysing the images of the stained sections and compared between the Parkinson’s disease and the age-matched control groups.

Results: Compared to the control cases the expression of phosphorylated α-synuclein and p62 was increased in Parkinson’s disease, whereas both neurons and endothelial cells were significantly reduced, with no changes in the number of microglial cells. The density of phosphorylated α-synuclein was negatively correlated with the total length of endothelial cell associated blood vessels when compared across normal and Parkinson’s disease cases combined. However, using double label immunohistochemistry we found that the degree of endothelial cell degeneration in Parkinson’s disease was not directly related to the degree of neuronal degeneration and accumulation of phosphorylated α-synuclein.

Conclusion: α-synuclein and autophagy are associated with endothelial degeneration in Parkinson’s disease. The degree of endothelial degeneration was not related to the extent of neuronal degeneration, both of which were co-pathological changes in PD brains. Alpha-synuclein-associated endothelial degeneration was also age-related pathology.

Keywords: Alpha-synuclein; Endothelial degeneration; Autophagy; Parkinson’s diseases; Human; Immunohistochemistry

Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder characterised by motor dysfunction [1]. Cognitive decline and dementia may appear in some cases as the disease progresses [2]. Using post-mortem human brain tissue from PD cases with no evidence of Alzheimer’s disease (AD) pathology, our recent research has reported vascular degeneration in PD, where endothelial degeneration has been suggested to be the cause of the loss of capillary networks and functions [3]. The endothelial degeneration in PD has been described in the substantia nigra (SN), caudate nucleus (CN) and the grey matter of middle frontal gyrus (MFG) which show clear neuronal degeneration [3,4]. The normal physiological turnover of endothelial cells involves the balanced loss and regeneration of endothelium. Our recent study has demonstrated impairment of vascular remodelling by showing reduction of pericytes, vascular cell proliferation and the loss of growth factors, for example VEGF, PDGF and increases in IGFBP-2 in the grey matter MFG of human PD brains [5]. The unbalanced endothelial turnover may be the result of an impaired remodelling process or accelerated loss of endothelium.

The retained basement membrane (BM) in PD leads to increased formation of string vessels which may cause secondary neuronal degeneration due to hypoperfusion, but prevent the leakage of the Blood-Brain Barrier (BBB) in a majority of brain lesions [4]. The changes in the BM, thus the BBB leakage may vary in brain regions, for example while there is no BBB leakage in the SN and MFG we found an increase in fibrinogen in the CN with increase of astrocytosis [4].

Neurodegeneration in PD involves increased α-synuclein aggregation [6], inflammation [7], altered autophagy [8,9] and

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possibly vascular dysfunction, for example the high string vessels related hypoperfusion. Lewy bodies (LbS), the hallmark of PD are mainly composed of aggregates of misfolded α-synuclein, most of which is phosphorylated at the Serine 129 site [10]. Autophagy is responsible for removal of cytoplasmic proteins and organelles where the polyubiquitin-binding protein p62 plays a key role in directing autophagy targets to the autophagosome for destruction [11]. Because p62 itself is also a substrate of autophagy, impairment of autophagy leads to p62 accumulation [12,13]. LbS were recently reported to be associated with p62 that accumulates in cell culture, implying that the α-synuclein aggregates resist degradation and impair autophagy [8]. The roles for inflammation in AD, a small vessel disease have been largely reported [14,15], whereas the involvement of inflammation in neuronal degeneration of PD is controversial [4,7]. The activation of microglia is a commonly used marker for the inflammatory response in the brain [16].

In addition, vascular degeneration and dysfunction is pathogenic and leads to neuronal degeneration in AD [17,18]. Although the potential hypoperfusion may contribute to secondary neuronal degeneration in PD [4], this study investigated whether the endothelial degeneration [3] is the cause or the result of neuronal degeneration by assessing the co-pathology of neuronal and vascular degeneration in the areas with a different extent of neuronal degeneration and PD pathology within the grey matter of the MGF, where there is no PD-related BBB leakage [4]. Due to limited supply of post-mortem tissues from brain regions with degeneration, we only evaluated the grey matter of MGF in this study which has clear neuronal [4] and endothelial degeneration [3]. More importantly the region is larger than other regions with degeneration and the degree of degeneration throughout the region of interest can be largely variable which allows us to compare the degree of endothelial degeneration with extent of neuronal degenerations. The current study evaluated changes of phosphorylated α-synuclein (pα-synuclein), autophagy, microglia and their activation, and their relationship to endothelial degeneration in human PD cases. The relationships of vessel changes to α-synuclein aggregation and neuronal survival were also compared based on the degrees of neuronal degeneration and α-synuclein pathology.

Methods

This study has received approval from the University of Auckland Human Participants Ethics Committee (Reference number 2008/279).

Tissue preparation and case selections

The tissue preparation procedures for both the tissue microarray (TMA) [5,19] and free-floating sections [3,4,19] have been described previously. Briefly, the brains were provided by the Neurological Foundation of New Zealand Douglas Human Brain Bank. The Formalin fixed tissue came from brains that were perfused first with 1% sodium nitrite and then with 15% formalin in 0.1 M phosphate buffer solution. Brain regions were dissected into blocks. The blocks used for pathological examination and TMA production were embedded in paraffin. The blocks used for making free-floating tissue sections were post-fixed and cryoprotected by transferring the blocks sequentially into 20% and 30% sucrose solution before they were stored at -80°C. The TMA block was sectioned at a thickness of 7 μm. The free-floating blocks were sectioned at 50 μm and stored at 4°C.

The criteria for case selection have also been previously described [4,5]. Briefly, only pathologically diagnosed idiopathic PD cases without dementia and age-matched control cases with significant pathological changes were selected. All the brains from the selected cases have a PM delay below 48 h. Lists including age, sex, PM delay and cause of death are provided (Tables 1 and 2). Information such as duration and clinical diagnosis are listed for PD cases (Table 1). This study used paraffin embedded tissue microarray (TMA) sections

Table 1: Clinical information of the PD cases.

| Case | Age | Sex | PM Delay (h) | Cause of Death |
|------|-----|-----|-------------|----------------|
| H127 | 59  | F   | 21          | Pulmonary embolus |
| H137 | 77  | F   | 12          | Coronary atherosclerosis |
| H139 | 73  | M   | 5.5         | IHD |
| H144 | 76  | M   | 18.5        | Ruptured aortic aneurysm |
| H150 | 78  | M   | 11          | Ruptured myocardial infarction |
| H152 | 79  | M   | 18          | Congestive heart failure |
| H153 | 76  | M   | 8           | IHD |
| H154 | 71  | M   | 23          | IHD |
| H156 | 89  | M   | 15          | Atherosclerosis |
| H169 | 81  | M   | 24          | CO poisoning |
| H180 | 73  | M   | 33          | IHD |
| H181 | 78  | F   | 20          | Aortic aneurysm |
| H190 | 72  | F   | 19          | Ruptured myocardial infarction |
| H191 | 77  | M   | 20          | IHD |
| H193 | 71  | M   | 23          | IHD |
| H196 | 85  | M   | 15          | Metastatic adenocarcinoma - colon |
| H198 | 67  | F   | 27          | IHD |
| H202 | 83  | M   | 14          | Ruptured abdominal aortic aneurysm |
| H204 | 66  | M   | 9           | IHD |
| H215 | 67  | F   | 23.5        | IHD |

NA: Not Available; IHD: Ischemic Heart Disease

Table 2: Clinical information of the age-matched controls.
Containing grey matter of the MFG from 17 PD and 17 age-matched control cases and free-floating tissue sections of the MFG from 9 PD and 6 age-matched control cases.

**Immunohistochemistry**

**TMA sections:** TMA sections were used for both peroxidase-DAB immunohistochemistry and double-labelled fluorescent immunohistochemistry. DAB-peroxidase immunohistochemistry was performed using antibodies directed against alpha-synuclein (phospho S129) (1:500, Abcam, Melbourne, Australia) [20] and p62 (1:100, BD Biosciences, CA, USA) for macrophagy [21]. The double-labelled fluorescent immunohistochemistry was performed using antibodies directed against lba1 (1:100; Abcam, Melbourne, Australia) for microglia [22] and Human Leukocyte Antigen - antigen D Related (HLA-DR, 1:200, Dako Corporation, Carpinteria, CA, USA) for activated microglia [23]. The experimental procedures have been described previously [3,19]. All washes, unless noted specifically, were carried out three times for five minutes each in phosphate buffer solution (PBS). All antibodies were diluted with 1% donkey serum (v/v) in PBST (0.2% Triton X-100 (v/v) in PBS).

The TMA sections were incubated at 60°C for one hour and de-paraffinized in two changes of xylene, ten minutes each. The sections were then rehydrated through a graded alcohol series and three changes of water. Antigen retrieval was performed by heating the sections in 10 mM Tris-EDTA buffer with pH9 using a 2100 Retriever (Prestige Medical). For DAB-peroxidase immunohistochemistry, endogenous peroxidase was deactivated by incubating the sections in a solution containing 50% methanol (v/v) and 1% hydrogen peroxide (v/v) for twenty minutes. The sections were then washed and incubated in the primary antibodies for 48 h at 4°C followed by washes and incubation with biotinylated secondary donkey anti-mouse or donkey anti-rabbit antibodies (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for twenty four hours at 4°C. The sections were washed and incubated with ExtrAvidin-HRP (1:1000; Sigma-Aldrich Corporation, Saint Louis, MO, USA) antibodies for twenty four hours at 4°C. The sections were then incubated with ExtrAvidin-HRP (1:1000, Sigma-Aldrich Corporation, Saint Louis, MO, USA) antibodies for twenty four hours at room temperature. Black NeuN and pα-synuclein staining was visualized by incubating the sections in DAB-nickel solution containing 0.05% Ammonium Nickel sulphate (w/v). After the DAB-nickel reaction, the experiment was repeated from the endogenous peroxidase deactivation step, following by incubation in the Factor VIII antibody, secondary antibody and ExtrAvidin-HRP as described above. The staining of Factor VIII was visualized with DAB solution to generate brown stain. Finally, the sections were mounted, air-dried, dehydrated through a graded alcohol series and xylene and cover-slipped.

**Image processing and analysis**

**Fluorescently labelled TMA sections:** For the fluorescently stained TMA sections, the automated ViSlide scanner (MetaSystems) was used to obtain 16-bit grayscale images for each colour (Alexa 594 and Alexa 488) with a 20x objective lens. Ten random sampled images were selected from each core for image analysis. A macro was developed in MetaMorph™ software (Molecular Devices) based on the method described in previous publications to analyse the images [24,25]. Briefly, the images were directly processed by the "Neurite Outgrowth" algorithm which segmented the cell bodies and processes of the microglia. The "Neurite Outgrowth Summary Log" was configured to count the number of microglia.

**Peroxidase-DAB stained sections:** Methods for image analysis have been described previously [4,5]. Briefly, the peroxidase-DAB images were captured using the Nikon E800 microscope equipped with a digital camera (MBF Bioscience) as described previously [4]. Briefly, images of DAB single or double labelling were obtained from each section by automatic randomized sampling. For TMA single labelled sections, ten randomly images were selected from each section for image analysis. For double labelled sections, nine images (3 images x 3 sections) per case were selected randomly for image analysis.

The number and average size of the pa-synuclein and p62 profiles from the TMA images were analysed using macros developed in ImageJ software (V1.50) which enables automatic high throughput image processing. The images were first converted into 8-bit format and background was subtracted. The “Threshold” tool was used to select the targets of interest and create binary images. The number and average size of both pa-synuclein and p62 profiles were obtained using the “Analyse Particles” tool.

For analysing DAB double labelled images the changes of Factor VIII positive endothelium with either NeuN positive neurons or pa-synuclein positive profiles were analysed in the same microscopic images. The number of NeuN positive neurons and pa-synuclein were analysed using macros developed in ImageJ (V1.50). The "Colour Space Converter" plugin in ImageJ was used to convert the original RGB images to grayscale HSb images to segment the black objects. These images were converted into 8-bit format and thresholded to
select the targets of interest and create binary images. The numbers of both neurons and pα-synuclein were obtained by using the “Analyse
Particles” tool.

To measure the total length of Factor VIII positive blood vessels, the “line” tool in ImageJ was used to manually trace and measure the blood
vessels. The total length of the blood vessels was calculated by adding
up the length of all the tracing lines in that image. The average length of
vessels was calculated by dividing the total length of the vessels by the
number of vessels in that image.

Statistical analysis: The difference between the age-matched
control and PD cases was analysed by using two-tailed unpaired t-test.
Two-way ANOVA was used to analyse the difference between the age-
matched control and PD cases at locations with different densities of pa-
synuclein and neurons, where Bonferroni multiple comparison test was
performed to compare the differences between the groups and between
severities of degeneration. Correlation between vascular density and pa-synuclein density was analysed by using Pearson correlation test,
where the r value represents the Pearson correlation coefficient. P
values less than 0.05 were considered as statistically significant.

Results

Neuronal and endothelial degeneration, phosphorylated α-synuclein and p62

Unpaired t-tests showed that the density (number/mm²) of neurons
(p<0.01, Figure 1A) and the total length of endothelial cell associated
blood vessels (p<0.0001, Figure 1C) were significantly reduced in the PD
cases compared to the age-matched controls while the density
(number/mm²) of pα-synuclein profiles were significantly increased in PD (p<0.001, Figure 1B). Furthermore, a Pearson correlation test
showed that the total length of endothelial cell associated blood vessels
is negatively correlated with the density of pa-synuclein profiles in the brain
(r=-0.6384, p<0.05, Figure 1D). When the measurements only included LBs larger than 7 µm, unpaired t-test showed that the density
(p<0.0001, Figure 2A) and average size (p<0.01, Figure 2B) of the
LBs were significantly increased in PD compared to the age-matched
controls. Similar increase in the density (p<0.0001, Figure 2C) and
average size (p<0.0001, Figure 2D) of the pα-synuclein profiles smaller
than 7 µm² were also observed in PD.

Immunofluorescent labelling of pa-synuclein was observed in large
round shaped inclusions and fibrous structures resembling cellular
processes in the PD brain (Figures 3B and 3E). The big round shaped
pa-synuclein positive inclusions were located inside neuron-like cells
and were morphologically identified as LBs (Figure 3B) and the fibrous
structures were identified as Lewy neurites (Figures 3B and 3E). The
fluorescent labelling of p62 was observed in inclusions with different
sizes known as p62 bodies (Figures 3A and 3D). The study also showed
LB-like p62 aggregates, a few of which showed co-localisation with pa-
synuclein (Figure 3C) and the others did not (Figure 3F).

Although no difference in the density (Figure 4A) of p62 staining
was found between the two groups, the average size of p62 bodies
larger than 7 µm² was significantly reduced in the PD cases compared to
the control cases (p<0.05, Figure 4B). When only the p62 bodies larger than 7 µm² were included, there was no difference in the density
(Figure 4C) of the large p62 bodies between the two groups, but the average size of the large p62 bodies
was increased in the PD cases (p<0.05, Figure 4D). When only the p62
bodies smaller than 7 µm² were included, no difference was found in
the density (Figure 4E) or average size (Figure 4F) of the small p62
bodies between the two groups.

![Figure 1](image1.png) (A) Densities (number/mm²) of neurons and (B) pa-synuclein profiles and (C) total length of endothelial cell associated blood vessels in the PD and age-
matched control cases, and (D) the correlation between the total blood vessel length and the pa-synuclein density. Compared to the age-matched controls, density
of neurons (A. **p<0.01) and total length of endothelial cell associated blood vessels (C. ***p<0.0001) were significantly reduced in the PD cases and density of pa-synuclein profiles was significantly increased in PD (B. ***p<0.001). The total length of endothelial cell associated blood vessels was negatively correlated with the density of pa-synuclein profiles in the PD and control brains (D, r=-0.6384, *p<0.05).
Co-pathologies of endothelium with α-synuclein and neurons

Figure 5 shows the distributions and morphologies of endothelium co-labelled with α-synuclein (Figures 5A-5D) and neurons (Figures 5E-5H) in both age-matched control (left panel) and PD cases (right panel). Similar to our previous report [3], the endothelial cell associated blood vessels in the PD cases were more fragmented (Figures 5B and 5G) with endothelial clusters (Figure 5, insert H) compared to the age-matched control cases (Figures 5A and 5E). The α-synuclein positive aggregates, with various sizes and shapes, were widely distributed throughout the PD cases (Figure 5B), but were much less evident in the control cases (Figure 5A). The aggregates were observed as large (general size ≥ 7 µm²) round shaped Lewy bodies (Figure 5B, insert C), dots and fibrous structures (Figure 5B, insert D). In the age-matched control cases, the NeuN positive neurons were evenly distributed with normal neuronal morphology of both cytoplasm and processes (Figure 5E, insert F). Compared to the control cases, there were fewer NeuN positive neurons that were more faintly stained with a patchy distribution indicating neuronal loss in the PD cases (Figure 5G, insert H).

To evaluate the relationship of vascular degeneration with neuronal pathology, the co-pathologies of endothelium with α-synuclein and neurons were assessed in the same areas with DAB double labelling.
Figure 4: (A) Density and (B) average size of p62 bodies in the PD and age-matched control cases. In the PD cases, there was a significant increase in the average size of p62 bodies (B, *p<0.05) but not in density (A) of the p62 staining, compared with the age-matched control cases. There was no difference in the density (C) of p62 bodies larger than 7 µm² between the two groups but the average size (D, *p<0.05) of the large p62 bodies increased in the PD cases. There was no difference in the density (E) or average size (F) of p62 bodies smaller than 7 µm² between the two groups.

Figure 5: Double colour peroxidase-DAB immunohistochemical staining of endothelium using Factor VIII (brown) co-labelled with pα-synuclein (A-D, black) and neurons labelled with NeuN (E-H, black) respectively in both age-matched control (left panel) and PD (right panel) cases. Compared to the age-matched control case (A, E and insert F), the blood vessels in the PD case were more fragmented (B and G) with clustered endothelial morphology (insert H). Aggregates of pα-synuclein were very rare in the control case (A), but widely distributed in the PD case (B), where the aggregates were observed in forms of Lewy bodies (arrow and insert C), fine punctate staining and fibres (arrowheads and insert D). The NeuN positive neurons in the age-matched control case were evenly distributed with normal cytoplasm and process morphologies (E and insert F). In the PD case, the NeuN positive neurons were fewer; less evenly distributed and faintly stained (G and insert H). Scale bars: 100 µm and 20 µm (inserts)
The sections with more than 1000 neurons/mm$^2$ and less than 230 pa-syn profiles/mm$^2$ were specified as ‘moderate’ degeneration whereas locations with less than 1000 neurons/mm$^2$ and more than 230 pa-synuclein profiles/mm$^2$ were specified as ‘severe’ degeneration. Compared to the age-matched controls the total vessel length was reduced in the PD cases in both areas with ‘moderate’ (<230 pa-synuclein profiles/mm$^2$, p<0.01, Figure 6A) and ‘severe’ (>230 pa-synuclein profiles/mm$^2$, p<0.001, Figure 6A) degeneration specified by pa-synuclein number. Two-way ANOVA analysis suggested a significant difference between PD and age-matched controls (p<0.0001, F (1,21)=46.06, Figure 6A), but there was no difference between moderate and severe degeneration, when the degree of degeneration was specified by number of existing neurons, and no interactions between the groups and severity of the degeneration. Similarly, the vessel length was reduced (p<0.0001, F (1,18)=25.4, Figure 6B) in PD in both areas with moderate (>1000 neurons/mm$^2$, p<0.01, Figure 6B) and severe (<1000 neurons/mm$^2$, p<0.05, Figure 6B) degeneration. There was no difference in vessel length between the areas with moderate and severe degeneration and there was no interaction between the groups and severity of the degeneration. To investigate the relative changes of endothelial cells with pa-synuclein aggregation and neuronal loss, this study further analysed the ratios of total vessel length to pa-synuclein profile number and total vessel length to neuronal number. Two-way ANOVA suggested a significant reduction in the ratio of the vessel length to pa-synuclein number between the groups (p=0.003, F (1,21)=11.16, Figure 6C), and significant reduction between the areas with moderate and severe degeneration (p<0.0001, F (1,21)=28.61, Figure 6C) specified by pa-synuclein number. However, post-hoc test showed a significant decrease in the vessel length to pa-synuclein number ratio in areas with severe degeneration in both PD (p<0.05, Figure 6C) and control (p<0.01, Figure 6C) groups. When the degree of degeneration was specified by neuronal number the study showed a significant increase in the vessel length to neuronal number ratio in areas with severe degeneration compared to those with moderate degeneration (p=0.005, F (1,18)=9.98, Figure 6D). There was no difference in the vessel length to neuronal number ratio between the two groups. Post-hoc test suggested an increase in the vessel length to neuronal number ratio in areas with severe degeneration in the PD cases (p<0.05, Figure 6D), but not in the control cases.

**Iba1 positive microglia and HLADR positive activated microglia**

Figure 7 shows the immunofluorescent labelling of Iba1, a marker of microglia (Figure 7A) and HLADR, a marker of microglial activation (Figure 7B). The Iba1 expression was observed in cell bodies and processes of microglia. HLADR was co-localised with Iba1 in cell bodies and processes in a subgroup of the activated microglia (Figure 7C).
No significant difference was found in the densities (number/mm$^2$) of either Iba1 positive microglia or HLADR positive microglia between PD and age matched controls (Figure 8).

**Discussion**

The results have demonstrated that the endothelial degeneration in the MFG of the PD brains was associated with α-synuclein aggregation and autophagy impairment as measured by p62. The pa-synuclein-associated endothelial degeneration is age related and may, therefore, not be specific to PD. Endothelial degeneration is more likely to be a co-pathology of neuronal degeneration in PD. No changes were seen in microglial numbers or activation state indicating that inflammation may not be a contributing factor to neuronal and vascular degeneration in the MFG of pathologically diagnosed idiopathic PD.

The main feature of vascular degeneration in PD appears to be the degeneration of endothelial cells [3] possibly due to the impairment of vascular remodelling [5]. In this study we attempted to examine the relationships of endothelial cell degeneration with neuronal degeneration and pathology of human PD. We found increased α-synuclein and p62 aggregation with profound neuronal and endothelial cell degeneration in the grey matter of the MFG. The degree of endothelial degeneration was significantly correlated with the extent of pa-synuclein accumulation. It has been well demonstrated that α-synuclein [26] and its associated autophagy alterations [9] and inflammation [7,27,28] contribute to the pathogenesis of neuronal degeneration of PD. Regardless of the size range of the aggregates, the pa-synuclein profiles were increased in number and size in PD brains. The findings agree with the previous reports regarding the aggregation of misfolded α-synuclein in PD [6]. Aggregation of p62 was observed in the LBs by showing co-localisation with pa-synuclein but not in the Lewy neurites. The co-expression of autophagosomes and α-synuclein has been previously reported in an *in vitro* study [8]. Some large LB-like p62 aggregates were not associated with the LBs (Figures 3F). The pathophysiological role of these large aggregates could be related to the degradation of other large intracellular materials given the broader function of p62 in autophagy [11]. The increased expression of p62 in PD group was mainly caused by large aggregates as when the analyses excluded the large LB-like p62 aggregates; neither the number nor the average size of the small p62 aggregates was different between the two groups. This implies that the accumulation of p62 or impairment of autophagy in PD could be related to the formation of LBs. A recent *in vitro* study reported that α-synuclein overexpression lead to increased level of p62 as a result of autophagy impairment [9]. Another *in vitro* study showing co-localisation of LB-like α-synuclein aggregates and p62 suggests that the LB-like α-synuclein aggregates resist degradation and impair autophagy [8]. P62 is digested together with the autophagy substrates by autophagosomes [12,13]. Our study provided the first evidence showing in human brain tissues that impaired autophagy could be associated with α-synuclein aggregation and LB formation in PD.

Using human tissues for investigating the timing of events, for example whether endothelial degeneration happens before neuronal degeneration, is limited if not impossible. Thus to investigate the
relationship of endothelial degeneration with α-synuclein pathology and neuronal loss, we assessed the co-changes of endothelial cells with pα-synuclein (Figures 5A-5D) and neurons (Figures 5E-5H), respectively. The co-pathology between the PD and age-matched control groups were compared using DAB double labelled sections. The age matched control cases used also had mild neuronal and endothelial degeneration, as well as age-related pathologies, for example α-synuclein and p62 expression. The PD cases however showed more severe endothelial degeneration, neuronal degeneration and increased pα-synuclein. The increase of pα-synuclein was strongly correlated to the endothelial degeneration in both PD and age-matched controls (Figure 1D). The α-synuclein pathology and its association with neuronal degeneration are characteristic of PD. This study provided evidence of the association of α-synuclein with endothelial cell degeneration in PD.

Endothelial degeneration leads to an increase in the formation of string vessels which are unable to function in blood circulation [4], thus a possible role for vascular dysfunction in contributing to secondary neuronal loss in PD was proposed. It is a difficult task to evaluate the timing of events during the disease process and whether vascular degeneration would be a contributor or the outcome of neuronal degeneration or simply the co-pathology in PD. To answer these questions the study would require human brain tissues at different stages of the disease development. But with post-mortem human tissue, it is often difficult to have enough subjects available at each stage of the disease, especially when there is not a clear criterion for the early stages of PD. However, the degree of neuronal degeneration and a-synuclein aggregation in PD is not uniform in an individual brain or a large brain region. Thus we hypothesised that the endothelial degeneration may be differentially related to neuronal degeneration and a-synuclein aggregation in the areas with severe or moderate degenerative pathology. The grey matter of MFG is a large region where the degree of degeneration varied considerably. We classified the severity of the pathology according to the densities of α-synuclein aggregates (<230 or >230 pα-synuclein profiles/mm²) and neurons (>1000 or <1000 neurons/mm²) in specified areas with double labelling of either endothelium/neuron or endothelium/pα-synuclein in both PD and age-matched controls. The loss of endothelial cells that was measured as total length of vessels, in PD cases did not correspond to the severity of either α-synuclein aggregation (Figure 6A) or neuronal loss (Figure 6B). We also analysed the ratios of endothelium to either pα-synuclein or neuronal density in the same images from double labelled sections. The analysis revealed more significant loss of vessel length in the areas with severe pα-synuclein aggregation than the areas with moderate aggregation, surprisingly of both PD and age-matched control groups. The data suggested the relative changes of endothelium to pα-synuclein aggregation were not a PD specific, rather an age-related pathology given PD is an age related condition.

Although the α-synuclein pathology is a defining marker in PD, it also appeared in some of the age-matched control cases, even though the level of the α-synuclein deposition was lower (Figure 2) [29]. Without a young control group to compare, the evaluation of the relative changes of α-synuclein to endothelial cells may help to reveal the change with age.

The relative changes of vascular length to neuronal number were increased in the tissue with more severe neuronal degeneration particularly in the PD cases (Figure 6D). The results may suggest that the blood vessel degeneration is slower than the neuronal loss in the brain or is a compensatory response through improved vascular remodelling. Therefore, the evidence suggests that during the pathological progression of PD, the neuronal loss is likely to precede the vascular degeneration.

The relationships among α-synuclein aggregation, neuronal loss and blood vessel changes may be dynamic. Observing the ratios in the areas with different degrees of pathology may provide an initial insight into the relationship between these pathological features through disease progression. Our current observation may suggest that the increased α-synuclein aggregation may not be a cause or the result, rather the co-pathology of vascular degeneration due to advanced age. However, the significant change in relationship of endothelial cells to neuronal degeneration was observed only in PD brains.

Accumulating evidence suggests the role for inflammation in neuronal degeneration of PD, particularly in the SN of the PD brain, where activation of microglia has been suggested to contribute to the death of dopaminergic neurons [7]. However we did not see higher levels of microglial activation in the MFG of the PD cases (Figure 8). This may suggest that there was no inflammation in the MFG of the PD cases. We have previously reported that the BBB function is maintained in the MFG of these PD cases due to the retained basement membrane [4]. It is likely that the retained BBB [4] has played a key role in preventing the inflammation in PD brains [30].

The current study analysed the MFG region where the BBB is not leaking and shows no astrocytosis [4]. Thus the relationship of neuronal, vascular and pathological changes, for example α-synuclein aggregation and inflammation may be different in the brain regions with BBB leakage and activation of glia, such as the CN [4], which needs to be evaluated in a separated study.

Conclusion

In conclusion, α-synuclein aggregation and autophagy impairment, but not inflammation, were related to endothelial degeneration in PD. The α-synuclein-associated endothelial degeneration is not specific to PD itself, but is rather an age-related pathology, which nevertheless is more severe in PD compared with the aged controls.

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