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Brief Report

Characterization of Lewy Body Pathology in 12- and 16-Year-Old Intrastriatal Mesencephalic Grafts Surviving in a Patient With Parkinson's disease

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Abstract: **We previously reported the occurrence of Lewy bodies in grafted human fetal mesencephalic neurons in two patients with Parkinson's disease. Here, we have used immunohistochemistry and electron microscopy to characterize the development of Lewy bodies in one of these cases. This patient was operated in putamen on both sides at 12 or 16 years before death, respectively. We demonstrate that 2% of the 12-year-old and 5% of the 16-year-old grafted, presumed dopaminergic neurons contained Lewy bodies immunoreactive for α-synuclein. Based on morphological analysis, two forms of α-synuclein-positive aggregates were distinguished in the grafts, the first a classical and compact Lewy body, the other a loose meshwork aggregate. Lewy bodies in the grafts stained positively for ubiquitin and thioflavin-S, and contained characteristic α-synuclein immunoreactive electron dense fibrillar structures on electron microscopy. Our data indicate that Lewy bodies develop gradually in transplanted dopaminergic neurons in a fashion similar to that in dopaminergic neurons in the host substantia nigra.** © 2010 Movement Disorder Society

Key words: neural transplantation; Lewy body; protein aggregation; α-synuclein; transmissible neurological disease

In Parkinson's disease (PD), neurodegeneration is prominent in substantia nigra dopaminergic neurons. Intraneuronal protein aggregates, rich in α-synuclein and called Lewy bodies/neurites (LBs/LNs), develop in several brain regions and become widespread with advancing disease. Patients with intrastriatal transplants of human fetal mesencephalic tissue, rich in dopaminergic neurons, have displayed long-term clinical benefits in open label trials (see Refs. 1 and 2). Brain imaging studies provide evidence that the grafted neurons survive and become functionally integrated in the host brain.[3] Postmortem studies on patients dying 18–52 months after grafting confirm that grafted dopaminergic neurons survive and innervate the host striatum, without any signs of pathology in the transplants.[4–6] Recently, we reported that large numbers of dopaminergic neurons can survive up to 16 years after implantation.[7] We and others also demonstrated that a fraction of the grafted cells developed PD pathology, i.e., LBs and LNs.[7–9] Here we report a detailed analysis of frequency, maturation and ultrastructural characteristics of LBs in two separate grafts at 12 and 16 years after implantation in the same PD patient.

MATERIALS AND METHODS

Postmortem Brain Preparation

This male patient, born in 1940, was transplanted with human fetal mesencephalic tissue in 1989 (left striatum) and 1993 (right striatum). Grafting procedure, donor tissues, neurological outcome, and imaging data have been reported previously.[10,11] He died in 2005 of acute aspiration coupled to advanced PD. The brain was fixed in 6% buffered formaldehyde solution for 2 months. Basal ganglia and left mesencephalon were cut into 10–15-mm thick blocks for frozen section preparation. Remaining brain slices, including small sections from basal ganglia and right mesencephalon,

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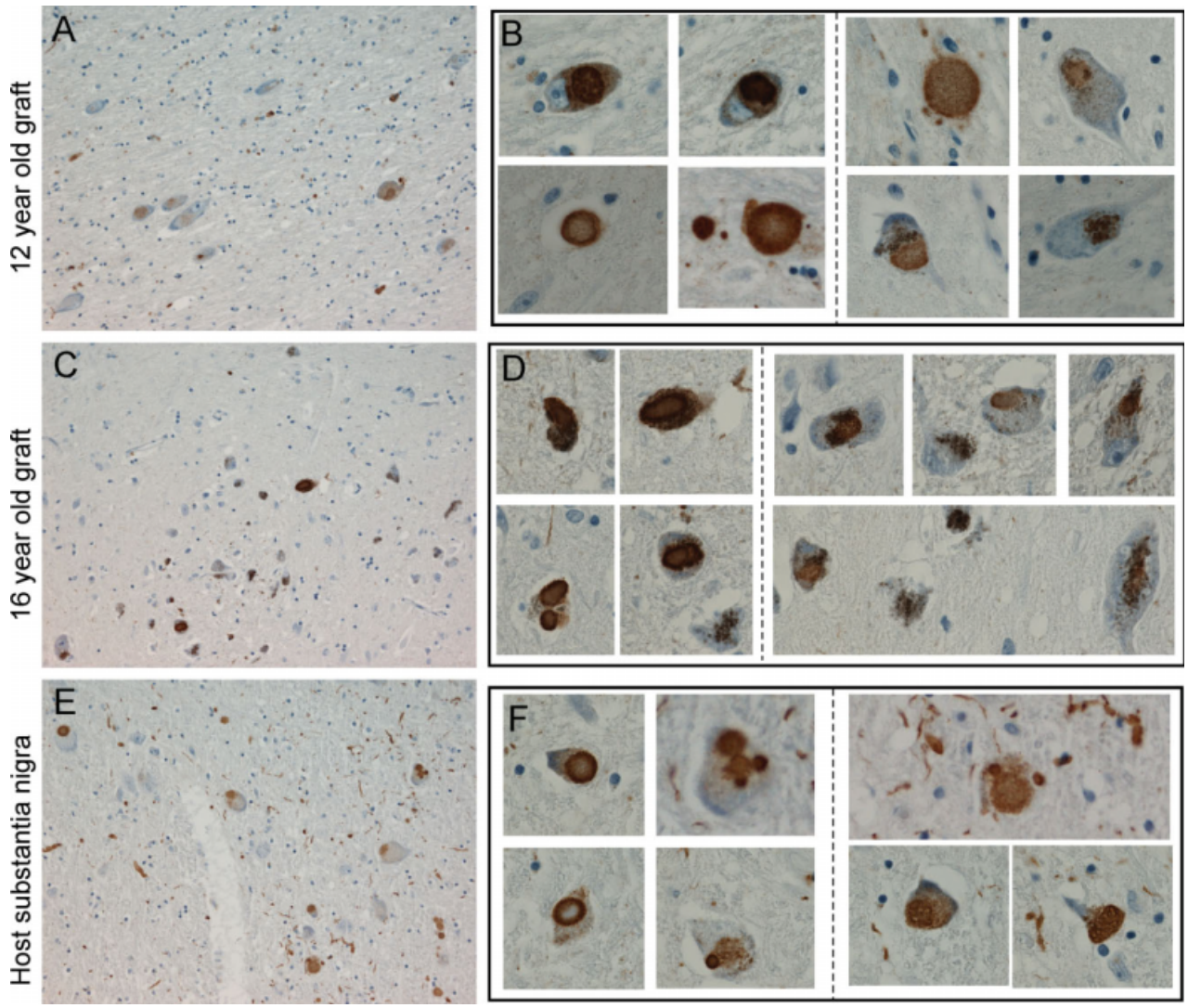

FIG. 1. Heterogeneity of LBs and LNs in the grafts. Different forms of α-synuclein positive material are present in the 12- (**A** and **B**) and 16- (**C** and **D**) year-old grafted neurons and in host substantia nigra neurons (**E** and **F**). The high power images show that some LBs and LNs exhibit densely compact α-synuclein immunoreactivity (B, D, and F, left to dashed lines), while others have a loose meshwork appearance (right to the dashed lines). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were processed for paraffin embedding and sectioning at 5 μm, followed by staining with antibodies against α-synuclein and ubiquitin.

Immunohistochemistry

Paraffin sections were mounted on capillary glass slides and treated in a microwave oven in citrate buffer at pH 6.0 for 15 minutes at 800 W. The sections were washed three times with 0.1 M PB. Thereafter, we incubated sections with a primary antibody against α-synuclein (Clone, LB509, 1:600, Zymed) and ubiquitin (1:200, Dako) overnight at room temperature. After rinsing, we incubated with the secondary antibodies (Biotinylated horse-anti-mouse or goat-anti-rabbit, 1:200, Jackson Lab, West Grove, PA), ABC-solution (Vector Lab) and finally DAB. Immunoreactivity was assessed using a Nikon microscope and images were processed with Adobe Photoshop software. Grafts were easily distinguished from the host brain. Since we observed decreased TH immunoreactivity in grafted neurons, especially in 16-year-old transplant, we used pigment granule-containing neurons to estimate the proportion of dopaminergic neurons within the grafts that contained LBs.

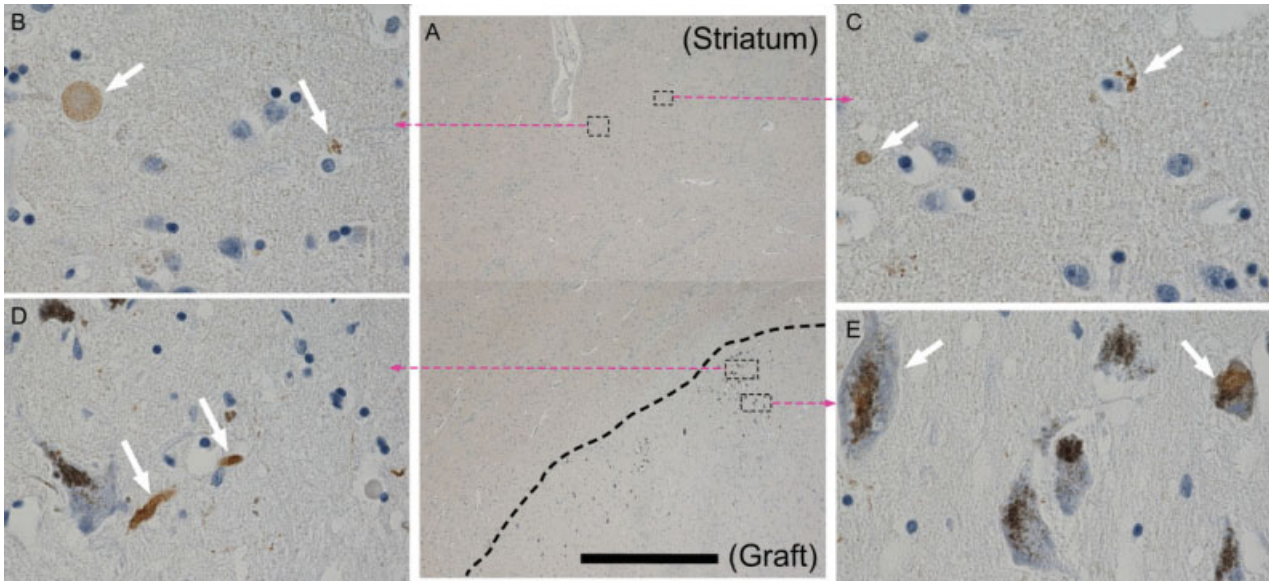

FIG. 2. Photomicrographs showing LBs/LNs in grafted neurons and surrounding host tissue. Dashed line (**A**) depicts the border between graft and host striatum. High power images demonstrate many LNs around the graft (arrows, **B** and **C**), LBs and LNs are present in the grafted neurons (arrows, **D** and **E**). Scale bar is 1 mm (A). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Thioflavin-S Staining

Sections through grafts were mounted on glass microscope slides, air-dried, treated in mixture of chloroform and absolute ethanol (1:1) for 2 hours, and hydrated through graded ethanol to distilled water. Sections were then placed in 0.1% thioflavin-S (Sigma) for 10 minutes in the dark and developed in 80% ethanol for about 15 seconds. After rinsing in distilled water, cover slip was placed over the sections, and examined in a Nikon fluorescence microscope and a Leica confocal microscope.

Electron Microscopic Analysis

Sections through the grafts (40 μm) were stained with the α-synuclein antibody and fixed with 2.5% glutaraldehyde in PBS for 1 hour and 1% OsO_4 in 0.1 PBS for 30 minutes. After rinsing in PBS, sections were dehydrated in ethanol and propylene oxide and flat-embedded in Epon 812 between two transparent film leaves. After polymerization, sections were examined in a bright field microscope, and we identified and cut small pieces (about 1 mm^2) containing α-synuclein immunoreactive grafted cells. These pieces were mounted on plastic blocks for semithin and ultra-thin sectioning. After contrasting the ultra-thin sections in 4% uranyl acetate, images were acquired in a Philips CM-10 electron microscope.

RESULTS

Frequency of Lewy Bodies in Grafted Cells

In mesencephalic brain, 95% of dopaminergic neurons in substantia nigra contain neuromelamin, the amount of pigment progressively increasing with age.[12] We found LBs in both the 12- and 16-year-old grafts as evidenced by α-synuclein, ubiquitin, and thioflavin-S staining (Figs. 1 and 3). In the 12-year-old grafts, α-synuclein positive LBs were detected in 1.9% of the neuromelanin-containing, presumed dopaminergic neurons (1,267 cells sampled). The frequency of α-synuclein positive LBs had increased to 5.0% (1,196 cells sampled) in the 16-year-old grafts (Fig. 1). We observed that 2.1% of the pigmented neurons were ubiquitin-immunopositive in the 12-year-old graft (905 cells sampled), with only a slight change to 2.6% in the 16 year-old graft (966 cells counted). Interestingly, α-synuclein positive LNs were sparsely distributed throughout host striatum around the grafts (Fig. 2). In sections stained with thioflavin-S, recognizing the beta sheet structure of α-synuclein, we observed several positive cells and neurite-like structures in the grafts (Fig. 3).

Different Forms of Lewy Bodies in Grafts

The α-synuclein positive aggregates in the grafts exhibited different morphologies. Some of them were classical compact LBs and LNs, whereas others were

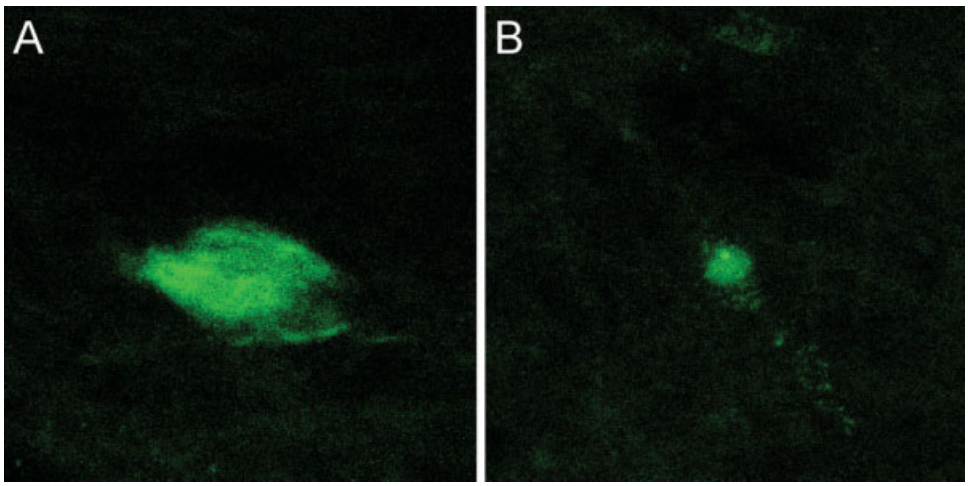

FIG. 3. Photomicrographs showing protein aggregates in grafted cells. The cell body (**A**) and neurite (**B**) are stained by thioflavin-S. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

fine granular aggregates, sometimes forming a loose meshwork of α-synuclein positive material (Fig. 1). The compact and granular forms of α-synuclein aggregates were detected in both the 12- (Fig. 1A and B) and 16- (Fig. 1C and D) year-old grafts, as well as in the host substantia nigra (Fig. 1E and F). We also frequently found LN structures in the transplants, presumably belonging to grafted neurons (Fig. 1A–D).

Ultrastructural Appearance of Lewy Bodies in Grafted Neurons

Electron microscopy showed electron dense material in the soma and proximal dendrites of several pigmented neurons (Fig. 4A, asterisks). Some of them contained fibrillar structures that were immunoreactive for α-synuclein and formed presumed LBs (Fig. 4B) and LNs (Fig. 4C).

DISCUSSION

Here we provide further evidence that the α-synuclein-containing aggregates detected in neural grafts in PD patients are LBs and LNs, and that α-synuclein is present in a fibrillar form. Our data suggest that LBs in grafted neurons exhibit different stages of maturation, and that their formation is a slow, gradual process.

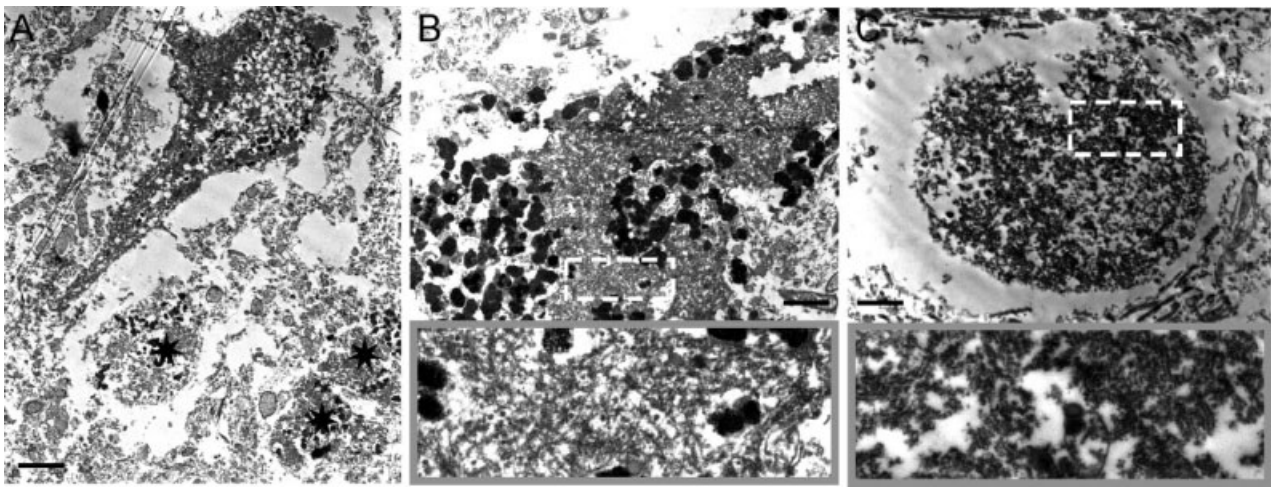

FIG. 4. Electron microscopic photomicrographs showing protein aggregates in grafted cells. Tissue sections were stained with an antibody against human α-synuclein. (**A**) In the graft, three pigment-granule containing neurons (asterisks) did not exhibit any α-synuclein immunoreactive material, while one neuron was intensely positive for α-synuclein immunoreactivity. (**B** and **C**). The high power images (dashed squares) show details of LB (B) and LN (C). Fibril forms of α-synuclein positive structures are seen on zoomed up images (Insets). Scale bars = 2 μm.

Lewy Body Formation in Grafts Resembles That Seen in the Substantia Nigra

We and others previously reported that α-synuclein accumulates in grafted dopaminergic neurons, and that in some cases it misfolds and forms LBs beyond one decade after transplantation.[7–9] Other earlier studies showed no LBs in patients dying 18–52 months after surgery.[4–6] Here, we demonstrate that once LBs have started to form, their frequency increases at least over a 4-year period. In the 12-year-old grafts, 2% of pigmented, presumed dopaminergic neurons contained LBs, compared to 5% in the 16-year-old graft in the same patient. Recently, LBs were reported to be present in around 3.6% of nigral dopaminergic neurons in PD,[13] regardless of disease stage after onset of motor symptoms. The authors suggested that cells with LBs die after 6 months and that the generation of new LBs and death of neurons reach a steady-state. Whether the grafted neurons die or are dysfunctional after LBs have been formed is not known. Kordower et al. have suggested that grafted neurons, albeit not only those containing LBs, become functionally impaired a decade after surgery as reflected, e.g., by downregulation of the dopamine transporter.[8,9] Arguing for a detrimental effect, LBs are only rarely found in the brains of cognitively normal aged individuals and typically never seen in the young normal brain. On the other hand, LBs and LNs have also been suggested to be neuroprotective.[14] Thus, while their presence may signify an ongoing disease process in the grafts, their role remains elusive.

We examined some of the LBs and LNs using thioflavin-S staining and electron microscopy and found them to contain typical beta sheet/fibrillar structures. Interestingly, we observed different morphological types of α-synuclein immunoreactive LBs and LNs in the grafts. These types possibly represent different degrees of maturity, which is consistent with our findings in cases of Lewy body dementia of different duration (E. Englund, unpublished). Recently, the BrainNet Europe Consortium proposed a staging protocol of LBs in α-synucleinopathy,[15] noting that aggregates can appear in four forms, i.e., grain cytoplasmic-like, intracytoplasmic LB-like inclusions, extracellular LB-like inclusions, and α-synuclein LNs.[15]

Mechanisms Underlying Lewy Body Formation in Grafted Neurons

Alpha-synuclein is located to synapses and not found in the neuronal cell body in young rodent,[16] monkey and human brain.[17] In humans, α-synuclein becomes detectable in some cell bodies of nigral neurons from 21 years of age, and cytoplasmic protein level then increases with time.[17] We previously reported that the majority (80%) of the 16-year-old grafted neurons, but only 40% of the cells in the 12-year-old graft in the present patient exhibit high cytoplasmic levels of α-synuclein.[7] In contrast, we find here that only a minority of the grafted neurons contains LBs. These observations suggest that the formation of LBs in grafted neurons occurs in two steps, starting by increased cytoplasmic levels of soluble α-synuclein followed by initiation of the aggregation process. The mechanisms leading to increased cytoplasmic levels of α-synuclein, followed by the generation of LBs and LNs in grafted neurons are not known. We have speculated that neuroinflammation, oxidative stress, excitotoxicity or loss of trophic support could contribute.[18] We also suggested that α-synuclein transfer from host neurons to grafted cells may act as a seed for α-synuclein aggregation, in a prion-like manner.[7,18] In line with this idea, we found LBs and LNs in the cortex[7] and in the host striatum around the grafts in the present patient. This hypothesis is further supported by a recent study demonstrating that α-synuclein can move from one cell to another in vitro and promote formation of aggregates in the new cells.[19] Moreover, α-synuclein from host brain was found to enter neural precursors grafted into transgenic mice overexpressing α-synuclein.[19] Transfer of aggregation-prone proteins between cells is now suggested to play a role in many proteinopathies.[20] Protein containing expanded polyglutamine proteins and aggregates of mutant tau can penetrate the outer membrane of cultured cells, act as a seed and promote nucleation of endogenous proteins expressing homologous sequences.[21,22] Furthermore, mutant human tau injected into mouse brains causes aggregation of wild-type tau, and pathology can spread from injection site to neighboring regions.[23]

Concluding Remarks

The findings of LBs in the grafted neurons provide new insights into PD pathogenesis and may help to explain how the pathology spreads within the patient's brain.[18] Our observations are in line with the Braak hypothesis[24] concerning the propagation of Lewy pathology according to stereotypic neuroanatomical patterns, and suggest that transfer of misfolded protein can be a key step of the process. The protracted development of Lewy pathology indicates that there is a large time window within which future therapies targeting the transfer of proteins between cells can act.

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