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Aggregation of α -synuclein in brain samples from subjects with glucocerebrosidase mutations

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Abstract

Recent studies show an increased frequency of mutations in the glucocerebrosidase gene (*GBA1*) in patients with α -synucleinopathies including Parkinson disease. Some patients with Gaucher disease (GD) develop parkinsonism with α -synuclein-positive inclusions *post mortem*. Proteins were extracted from the cerebral cortex of subjects with synucleinopathies with and without *GBA1* mutations, controls and patients with GD. Patients with *GBA1*-associated synucleinopathies showed aggregation of oligomeric forms of α -synuclein in the SDS-soluble fraction, while only monomeric forms of α -synuclein were seen in subjects with *GBA1* mutations without parkinsonism. Thus, brains from patients with *GBA1*-associated parkinsonism show biochemical characteristics typical of Lewy body disorders.

Keywords

Gaucher disease; Glucocerebrosidase; α -synuclein; Lewy body; Synucleinopathies; Parkinson disease

1. Introduction

Mutations in the gene encoding the lysosomal enzyme glucocerebrosidase are important risk factors for the development of Parkinson disease (PD) and related disorders. This association is based upon the concurrence of parkinsonism and Gaucher disease (GD), an increased incidence of PD in Gaucher carriers, and neuropathological findings [1–3]. Furthermore, multiple independent studies indicate that patients with PD and related synucleinopathies have an increased frequency of *GBA1* mutations [4–10]. A recent multicenter collaborative study indicated that in PD, the odds ratio for carrying a *GBA1* mutation is greater than 5, rendering mutations in this gene the most common genetic risk factors for parkinsonism identified to date [11]. However, since the vast majority of patients with GD and *GBA1* mutation carriers never develop parkinsonism, mutations in this gene are clearly a risk factor for PD, rather than a causative gene.

Gaucher disease, resulting from the inherited deficiency of the lysosomal enzyme glucocerebrosidase (GCase), is a panethnic disorder with a broad spectrum of associated

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clinical presentations. Classically the disorder is divided into type 1 (non-neuronopathic), type 2 (acute neuronopathic) and type 3 (chronic neuronopathic) forms. It is primarily a disorder of the reticuloendothelial system, and unlike other lysosomal storage disorders, lacks abundant storage of lipid in the brain. The neuropathology of neuronopathic GD disease includes periadventitial accumulation of lipid-laden macrophages (Gaucher cells), occasionally coupled with neuronal loss with crumpled, shrunken-atrophic neurons [3]. Moreover, gliosis and neuronal loss are described in the hippocampal regions CA2-4 and calcarine layer 4. Several autopsy studies of patients with *GBA1*-associated synucleinopathies indicate that there is a spectrum of associated neuropathologic findings. Most patients have Lewy bodies and Lewy neurites. In subjects with GD and parkinsonism, α -synuclein positive Lewy bodies are seen, as well as Lewy body-like synuclein inclusions in hippocampal pyramidal cell neurons [2,3,5,9]. A recent immunofluorescence study, conducted on nine patients harboring *GBA1* mutations, demonstrated that glucocerebrosidase was present in between 50 and 90% of the Lewy bodies, compared to less than 10% in subjects without mutations [12].

One of the main features of α -synuclein is its tendency to aggregate into β -sheet-like oligomers. This procedure goes through several steps leading to the formation of the insoluble fibrils that form Lewy bodies. Aggregated α -synuclein is associated with cell death and neurodegeneration, and multiple systems and organelles can be affected by α -synuclein accumulation [13,14]. The nature of the neurotoxicity associated with α -synuclein, mainly thought to be associated with the oligomeric forms, is a source of considerable debate [13,15,16].

To probe whether similar biochemical changes characteristic of synucleinopathies were present in patients with GD, we evaluated levels of soluble and insoluble α -synuclein in brain samples from patients with GD with and without synucleinopathies.

2. Material and methods

2.1. Patient samples

Autopsy samples of cerebral cortex from subjects with and without *GBA1* mutations, with or without a pathologic diagnosis of PD or dementia with Lewy bodies (DLB) were studied. All samples were screened for *GBA1* mutations by sequencing, as previously described [17]. The patients with synucleinopathies included six subjects with no *GBA1* mutations, three with GD, and six *GBA1* heterozygotes. In addition to the *GBA1* alleles described in Table 1, two patients were found to carry E326K, an alteration in *GBA1* that is often considered a polymorphism [18]. In addition, samples of cortex from subjects with all three types of GD (two with type 1, three with type 2 and two with type 3) as well as seven control brain samples were evaluated.

Brain samples with PD and DLB were obtained from Massachusetts General Hospital, University of Pennsylvania School of Medicine, and the National Institutes of Health.

2.2. Protein extraction and fractionation

Brain samples, about 300 mg wet weight, were minced into small fragments on ice, suspended in 10 volumes (ml/g of brain) of 1X TBS buffer (pH7.4) with 10 μ l/ml of protease inhibitor cocktail (Sigma, MO) and 50 μ l/ml of 100 mM EDTA and homogenized on ice using a mechanical homogenizer. The homogenates were spun at 1000 $\times g$ for 5 min at 4 °C. The pellet was discarded and supernatant was subsequently transferred to polycarbonate Beckman tubes and centrifuged at 100,000 $\times g$ for 1 h at 4 °C. The supernatant was labeled as the TBS-soluble fraction. The pellet from the centrifugation was washed twice with TBS buffer, resuspended in 5 volumes (ml/g of initial brain sample) of room

temperature 1X TBS/SDS buffer (pH 7.4) with 1% Triton X-100) by sonication, and centrifuged at $100,000\times g$ for 30 min at 25 °C. This supernatant is the SDS-soluble fraction. The pellet was washed twice with TBS/SDS buffer and resuspended in 5 volumes (ml/g of initial brain sample) of room temperature TBS/SDS/Urea buffer (1X TBS (pH7.4) with 8% weight/volume SDS and 8 M Urea) under sonication. This third and last fraction is the urea-soluble portion.

2.3. SDS-PAGE and western blot

The fractionated samples were separated by SDS-PAGE (4–12% NuPAGE®Novex® Bis-Tris gel, Invitrogen, CA) and transferred to PVDF membranes (iBlot PVDF, Invitrogen, CA). Blots were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (Sigma) and 5% fat-free milk for 1 h at 25 °C, incubated in blocking buffer containing primary antibody (monoclonal α -synuclein 1:1000, BD transduction, NJ) and glucocerebrosidase (1:15000, custom-made polyclonal rabbit antibody) overnight at 4 °C, followed by three 10 minute washes. The washed membrane was incubated in blocking buffer containing horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse 1:3000, KPL, MD; goat anti-rabbit 1:3000, KPL, MD) for 1 h at 25 °C. HRP immunoblots were developed using enhanced chemiluminescence (ECL Plus, GE Healthcare, NJ). β -actin antibody (Mouse monoclonal 1:2000, Abcam, MA) was used as a loading standard.

2.4. Enzyme activity

Separate tissue samples of approximately 100 mg were used to assay β -glucocerebrosidase activity. Brain samples were minced and suspended in 5 volumes (ml/g of brain) of extraction buffer (60 mM KH_2PO_4 , 0.1% Triton X-100, pH 5.9) with 10 $\mu\text{l/ml}$ of protease inhibitor cocktail (Sigma, MO) and 50 $\mu\text{l/ml}$ of 100 mM EDTA and homogenized on ice. The homogenates were centrifuged at $1000\times g$ for 10 s at 4 °C with a cell strainer (40 μm , BD Biosciences, CA). The strained homogenate was used for the enzyme assay. In a black, low-binding 96-well plate, 62 μl of assay buffer (50 mM citric acid, 0.01% Tween-20, pH 5.9 with 10 mM sodium taurocholate) was added to 4.7 μl of the homogenized sample. Conduritol B epoxide (CBE) was added to each sample as a control. For the CBE-treated samples, an additional 0.5 μl of 100 μM CBE was added to the assay buffer and the sample and incubated at 37 °C for 30 min. Substrate buffer (30 mM 4-MU beta-D-glucoside, 10 mM sodium taurocholate, 50 mM citric acid, 0.01% Tween-20, pH 5.9) was then added and incubated at 37 °C for 1 h with 750 rpm rotation in Eppendorf Thermomixer R. The enzyme reaction was halted using 100 μl of stop solution (1 M NaOH, 1 M Glycine), and fluorescence was read at 355 nm (excitation) and 470 nm (emission) on a Victor fluorescence reader (Perkin-Elmer). Serial dilutions of 4-MU standards, starting at 200 μM , were also read alongside the samples. All samples were run in triplicates.

3. Results

Brain homogenates from the cerebral cortex of cases with *GBA1*-associated synucleinopathies were compared to cases with synucleinopathies without *GBA1* mutations, as well as other subjects with GD, and controls. The immunoblots showed that most patients with *GBA1* mutations and synucleinopathies exhibited oligomeric forms of α -synuclein in the SDS-soluble fraction (Fig. 1B; Subjects #14–21), while controls and patients with GD without synucleinopathies had only the monomeric form of α -synuclein in the same fraction (Figs. 1B, C; Subjects #24–30). Insoluble α -synuclein oligomers, appearing as a ladder in the SDS- and urea-soluble fractions, were seen in most patients with synucleinopathies with or without *GBA1* mutations (Figs. 1B,C; Subjects #8–21). Compared to the normal controls (Fig. 1, Subjects #1–7), cases with a more extensive Lewy body burden tended to have

distinct bands of the oligomeric forms of α -synuclein. None of the patients with Gaucher disease without synucleinopathies, including brains from older Gaucher probands with type 1 GD (Fig. 1, Subjects 24–25), as well as infants with type 2 GD manifesting with extensive neurologic involvement (Fig. 1, Subjects 26, 29–30), demonstrated aggregated forms of α -synuclein by this technique. One faint band, found at approximately 51 kDa in samples 22, 23, 24, and 25, was considered to be non-specific, for it was also seen in a normal control (Subject 1).

We also probed blots with an antibody to glucocerebrosidase. While levels of glucocerebrosidase were greatly diminished in samples from patients with neuronopathic Gaucher disease (Fig. 1D; Subjects # 17, 26–30), we were not able to establish whether glucocerebrosidase was present in the aggregates, in part because the molecular weight of glucocerebrosidase was right where the highest band of aggregated α -synuclein occurs. However, we did not see higher molecular weight bands above 60 kDa. Enzyme activity assay confirmed the diagnosis in patients with GD, and documented the range of glucocerebrosidase activity among the samples (Table 1).

4. Discussion

Neurodegeneration in PD is accompanied by the formation of Lewy bodies and Lewy neurites, intracellular inclusion bodies containing aggregated fibrillar proteins including α -synuclein [14]. The presence of glucocerebrosidase in α -synuclein positive intracellular inclusions in cases with GD and parkinsonism prompted this current study to characterize oligomeric aggregation intermediates in patients with GD, looking for new insights into the mechanism of α -synuclein aggregation.

Previous studies of brain samples from subjects with *GBA1*-associated synucleinopathies demonstrated neuropathological findings typical of Lewy body disorders, and showed that glucocerebrosidase is a component of α -synuclein positive intraneuronal inclusions [12]. This current evaluation similarly shows that such patients also have biochemical findings characteristic of other synucleinopathies. Here we show that in brain homogenates from patients with synucleinopathies with *GBA1* mutations, oligomeric forms of α -synuclein are present in the insoluble SDS- and urea-soluble fractions. However, no oligomeric forms are seen in subjects with GD without parkinsonian manifestations.

Although in this study the tools used to characterize intracellular aggregation were limited, and the initial preservation and length of storage of the autopsy samples varied, the absence of increased aggregated insoluble α -synuclein in patients with GD without parkinsonism suggests that neither glucocerebrosidase deficiency nor the presence of *GBA1* mutations alone result in α -synuclein aggregation. Unraveling the role of mutant glucocerebrosidase in the development of this pathology should further expand our understanding of pathways contributing to the aggregation and/or clearance of α -synuclein.

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Fig. 1. Solubility and aggregation of α -synuclein in brain (A, B and C). Brain samples from neurologically normal control subjects (#1–7), patients with synucleinopathies (#8–13), patients with GD and carriers of *GBA1* mutations exhibiting synucleinopathies (#14–23), and patients with GD (#24–30) were fractionated by solubility. Tris-buffered saline (TBS)-soluble (panel A), sodium dodecyl sulfate (SDS)-soluble (panel B), and urea-soluble (panel C) fractions were blotted for α -synuclein using monoclonal Syn-1. Arrow indicates the major band of monomeric α -synuclein (17 kDa); asterisk indicates oligomeric α -synuclein. The SDS-soluble fraction was additionally blotted with anti-glucocerebrosidase antibody (R386). GCase is shown as a single band at 60 kDa. (panel D). Blots were reprobed with β -actin to assess loading (panels below each figure).

Table 1

Summary of brain samples. Brain samples from neurologically normal control subjects (#1–7), patients with synucleinopathies (#8–13), patients and carriers of *GBA1* mutations with synucleinopathies (#14–23), and patients with Gaucher disease (#24–30) were tested for their enzyme activities. All samples were run in triplicates and % enzyme activity was derived by comparing the average of the normal subjects (47.672±7.961 μmol 4MU/g protein/hour) with the patients and carriers of *GBA1* mutations.

	#	<i>GBA1</i> mutations	Sex	Age at PD diagnosis	Age at death	Pathology	GCase activity (μmol 4MU/g/hr)	% wt activity
Normal controls	1	WT/WT	F		77	Normal control	51.69 ± 0.638	
	2	WT/WT	M		84	Normal control	N/A	
	3	WT/WT	F		56	Normal control	45.46 ± 0.844	
	4	WT/WT	F		26	Normal control	45.77 ± 1.412	
	5	WT/WT	F		68	Normal control	47.42 ± 1.197	
	6	WT/WT	M		77	Normal control	45.78 ± 3.110	
	7	WT/WT	F		61	Normal control	31.95 ± 1.386	
Subjects with synucleinopathies	8	E326K/WT	M	65	70	DLB	N/A	
	9	WT/WT	M	N/A	79	DLB	N/A	
	10	WT/WT	F	N/A	69	DLB	N/A	
	11	WT/WT	M	69	81	DLB	58.49 ± 0.219	
	12	WT/WT	M	68	77	DLB	54.82 ± 1.730	
	13	WT/WT	M	58	78	PD	N/A	
	14	L444P/E326K	M	67	77	PD	N/A	N/A
Patients and carriers with <i>GBA1</i> mutations with synucleinopathies	15	84GG/WT	F	60	83	DLB	26.42 ± 0.399	55.4% ± 0.84%
	16	N370S/N370S	M	N/A	75	GD//DLB	0.90 ± 0.022	1.9% ± 0.05%
	17	D409H/L444P	F	42	52	GD//PD	3.08 ± 0.028	6.5% ± 0.06%
	18	K198T/WT	M	57	58	DLB	N/A	N/A
	19	N370S/N370S	M	44	54	GD//DLB	1.29 ± 0.006	2.7% ± 0.01%
	20	A359X/WT	M	37	58	DLB	19.30 ± 0.136	40.5% ± 0.29%
	21	T267I+E326K/WT	M	54	75	DLB	32.43 ± 0.437	68.0% ± 0.92%
	22	84GG/WT	M	80	84	DLB	26.77 ± 0.071	56.1% ± 0.15%
	23	N370S/WT	M	N/A	68	PD	23.51 ± 0.786	49.3% ± 1.65%
	24	N370S/N370S	M		86	Type 1 GD	1.46 ± 0.033	3.1% ± 0.07%

#	GBA1 mutations	Sex	Age at PD diagnosis	Age at death	Pathology	GCase activity (μmol 4MU/g/hr)	% wt activity
25	N370S/c.208delC	M		58	Type 1 GD	0.99 ± 0.012	2.1% ± 0.03%
26	Rec Ncd/L444P	F		1	Type 2 GD	2.41 ± 0.044	5.1% ± 0.09%
27	G377S/g.5245del T	M		14	Type 3 GD	2.39 ± 0.001	5.0% ± 0.00%
28	L444P/L444P	M		5	Type 3 GD	3.18 ± 0.042	6.7% ± 0.09%
29	IVS2+1/L444P	F		0.6	Type 2 GD	2.10 ± 0.004	4.4% ± 0.01%
30	IVS2+1/F251L	F		Newborn	Type 2 GD	0.82 ± 0.004	1.7% ± 0.01%

* WT = wild type, GD = Gaucher disease, DLB = dementia with Lewy bodies, N/A = not available. E326K is considered to be a polymorphism.