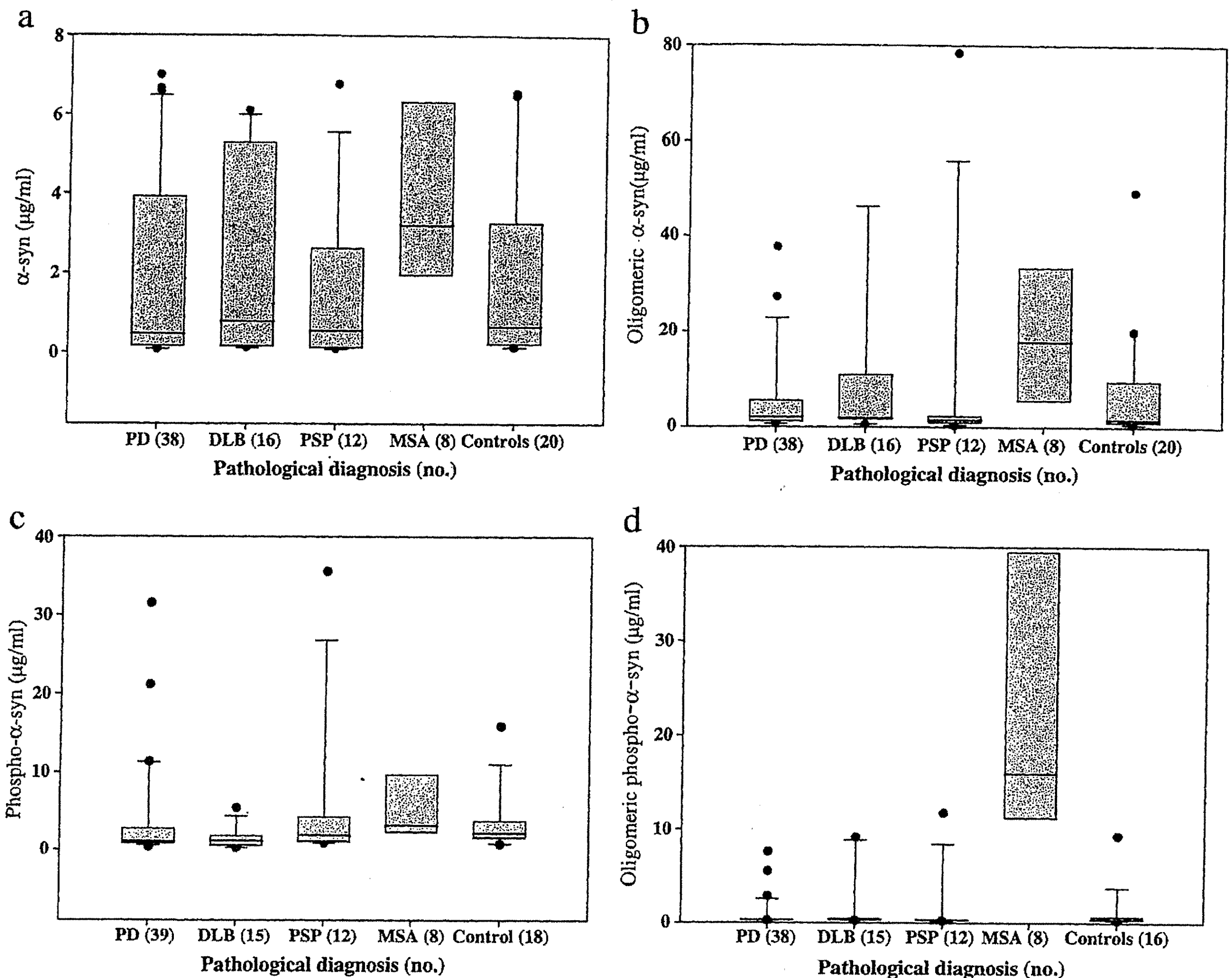

Fig. 2. Box-whisker plots for the CSF α-synuclein immunoassay results. The length of each box represents the interquartile range (75–25%) of the sample, the solid line drawn across the box the median, and outliers are denoted by dots. Immunoassay results are presented for each pathological diagnostic group for (a) total α-syn levels, (b) oligomeric α-syn levels, (c) phosphorylated α-syn levels and (d) oligomeric phosphorylated α-syn levels.

hoc Tukey test showed highly significant differences ($p<0.001$) in mean CSF levels of oligomeric phosphorylated α-syn between the MSA and all of the other diagnostic groups (Table 2). There were no significant differences in mean CSF levels of total α-syn, oligomeric α-syn, total phosphorylated α-syn or oligomeric phosphorylated α-syn between PD, PD (Cog) and PD (Dem) groups ($F_{2,37}=1.23$, $p=0.303$, $F_{2,37}=0.77$, $p=0.468$, $F_{2,38}=0.53$, $p=0.596$, $F_{2,37}=1.67$, $p=0.202$, respectively) although it is noted that the numerical levels of oligomeric phosphorylated α-syn increased progressively from PD through PD (Cog) to PD (Dem) groups (Table 2).

Table 2
Mean (±SD) CSF levels of α-syn (µg/ml) as determined by each immunoassay in patients with PD (non-demented (nonD), cognitively impaired (Cog) and demented (Dem), DLB, PSP, MSA and normal control individuals.

	Total α-syn (µg/ml)	Oligo α-syn (µg/ml)	pS α-syn (µg/ml)	Oligo pS α-syn (µg/ml)
PD (n=39)	1.93±2.49	7.04±1.64	3.43±6.18	0.77±1.51
PD (nonD) (n=13)	1.34±2.16	11.11±2.58	4.41±8.68	0.26±0.03
PD (Cog) (n=10)	1.47±2.10	2.35±2.02	1.76±1.02	0.68±0.78
PD (Dem) (n=16)	2.67±2.83	6.37±9.91	3.67±5.73	1.28±2.27
DLB (n=16)	2.31±2.51	9.47±2.09	1.63±1.42	1.60±3.02
PSP (n=12)	1.45±1.97	7.91±2.21	5.14±9.73	1.25±3.32
MSA (n=8)	3.80±2.40	22.49±.19	7.14±9.19	19.56±1.66*
Control (n=20)	1.87±2.29	6.78±1.14	3.58±3.85	1.05±2.23

*Indicates significantly different (P<0.001) from assay value for patients with PD (overall, and nonD, Cog and Dem subgroups), DLB, PSP and normal control individuals.

There were no significant correlations between CSF levels of total α-syn, oligomeric α-syn, phosphorylated α-syn or oligomeric phosphorylated α-syn, and pathology scores, expressed either as total pathology score or as severity scores for each individual area, either across all 71 patients or within the PD and DLB patients, separately or combined. Similarly, there were no significant correlations between CSF levels of total α-syn, oligomeric α-syn, phosphorylated α-syn or oligomeric phosphorylated α-syn; with age at onset of disease or duration of illness within PD and DLB patients, either as single or combined groups.

Immunoblotting

Immunoblots of α-syn within CSF, of PD, DLB, MSA, PSP and control cases with relatively high and low absorption α-syn values are shown in Fig. 3. Immunoblots using the polyclonal anti-α/β/γ-synuclein antibody FL-140 (Fig. 3a), showed in most/all samples irrespective of diagnostic status, an immunoreactive band at ~46–48 kDa, which was strongly present in all samples with high α-syn CSF levels (lanes C, E, G and I) but was less strongly present in those with low CSF α-syn levels (lanes B, D, F and H). In two samples with high CSF α-syn levels there was an additional α-syn species at 16 kDa which represents the monomeric protein (lanes C and G) and was not present in those with low CSF α-syn levels. Using the phosphorylated anti-α-synuclein pS129 antibody (Fig. 3b), only the 46–48 kDa species was detected, again this being strongly present in

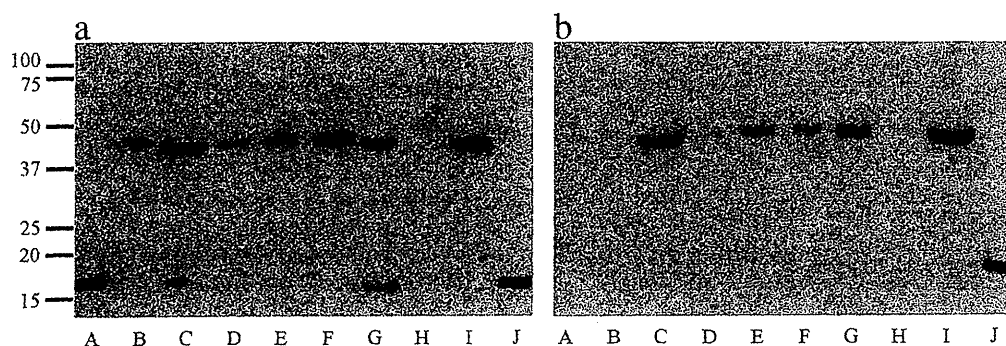

Fig. 3. Immunoblots of α-syn (a) and pS-α-syn (b) within CSF of PD, DLB, MSA, PSP and control cases with relatively high and low α-syn immunoassay levels (lanes C, E, G, I and B, D, F and H, respectively) are shown, along with recombinant non-phosphorylated standard (lane A) and recombinant oligomerised, phosphorylated standard (lane J). The immunoblot using polyclonal anti-α/β/γ-synuclein antibody FL-140 (Santa Cruz) (Figure a), shows in most/all samples irrespective of diagnostic status, an α-syn species at ~46–48 kDa, which is strongly present in all samples with high CSF α-syn levels (lanes C, E, G and I) but is less strongly present in those with low CSF α-syn levels (lanes B, D, F and H). In two samples with high CSF α-syn levels there is an additional α-syn species at 16 kDa (lanes C and G) which is not present in those with low CSF α-syn levels, but has the same molecular mass as the recombinant protein (lane A). Using the phosphorylated anti-α-synuclein pS129 antibody (Figure b), only the 46–48 kDa species is detected, again this being more strongly present in all samples with high CSF α-syn levels (lanes C, E, G and I) but less strongly present in those with low CSF α-syn levels (lanes). Recombinant α-syn (lane A) is not detected by the phosphorylation specific α-syn antibody (lane A).

all samples with high CSF α-syn levels (lanes C, E, G and I) but less strongly present in those with low CSF α-syn levels (lanes B, D, F and H). Recombinant α-syn at 16 kDa (lane A) was only detected by the FL-140 antibody, and not the phosphorylation dependent α-syn antibody pS129 (see Fig. 3a, lane A). Inspection of the immunoblots (Fig. 3) suggests, therefore, that this 46–48 kDa α-syn species might represent an oligomerised and phosphorylated form of α-syn (the estimated molecular mass would suggest a trimer). Minor quantities of non-phosphorylated (monomeric) α-syn were also present, but these were only detectable in those cases with high CSF α-syn levels.

Confounding factors

It has been suggested from previous studies (Mollenhauer et al., 2008) that levels of total α-syn within CSF may progressively increase with increasing post mortem delay time. However, we found no correlation between levels of α-syn within CSF and post mortem delay time for any of the 4 immunoassays, either when all 96 patients were considered as a group, or separately according to diagnosis (data not shown). Moreover, mean post mortem delay time did not differ significantly between any of the diagnostic groups ($F_{4,89}=1.90$, $p=0.118$).

Furthermore, because, some of the CSF samples were obviously contaminated with blood, and because previous studies have suggested that such contamination of CSF samples might contribute to α-syn measures through lysed red cells (Hong et al., 2010), we assayed the CSF samples for haemoglobin. Mean haemoglobin levels within CSF samples did not differ significantly between the various diagnostic groups (PD $=4.6\pm2.3$ μg/ml; DLB $=5.1\pm2.6$ μg/ml; PSP $=5.8\pm2.2$ μg/ml; MSA $=4.4\pm2.0$ μg/ml; Controls $=6.1\pm3.2$ μg/ml; $F_{4,73}=1.01$, $p=0.408$), nor was there any correlation between CSF haemoglobin level and any one of the 4 measures of α-syn (total α-syn $r=0.168$, $p=0.148$; oligomeric α-syn $r=0.156$, $p=0.178$, phosphorylated α-syn $r=0.094$ $p=0.418$, oligomeric phosphorylated α-syn $r=0.027$ $p=0.818$).

Discussion

In the present study, we have shown by immunoblotting that both phosphorylated and non-phosphorylated forms of α-syn can be detected in CSF of patients with PD, DLB, PSP, MSA, and also in control individuals, and that the levels of these can be measured by immunoassay. Consequently, we have not only employed conventional immunoassays to measure total levels of α-syn, as many other groups have done previously, but we have developed new assays based on the detection of phosphorylated and/or oligomerised forms of α-syn, since these may have more relevance in targeting and indexing the pathological species of α-syn that is accumulated in the brains of patients with these disorders (Fujiwara et al., 2002; Anderson et al., 2006; Obi et al., 2008).

The main finding to emerge from this study is the observation that measurement of oligomeric phosphorylated forms of α-syn in CSF can differentiate patients with MSA from all of the other diagnostic groups. Although this finding is based on a relatively small number of MSA cases, and may therefore be considered preliminary, the distinction between MSA and other patient groups was robust, and higher α-syn levels were consistently seen across all 4 assays in MSA compared to PD/DLB and other non-synucleinopathies. Recent findings by Hirohata et al. (2011) showing that an unidentified factor in CSF promotes the *in vitro* aggregation of α-syn, and that CSF from patients with MSA was more effective in this respect than CSF samples from patients with PD, would be consistent with our findings.

There have been a few other recent studies looking at biomarkers in MSA. Aerts et al. (in press) compared total α-syn in lumbar CSF from 47 patients with MSA with those from 58 patients with PD, 3 with DLB, 10 with PSP and others with CBS and vascular parkinsonism, but detected no significant differences in mean level between any of the diagnostic groups. Similarly, Shi et al. (2011) did not find any differences in mean α-syn levels between 32 patients with MSA and 126 with PD, though in both instances such levels were significantly lower than 137 control subjects and 50 patients with Alzheimer's Disease (AD). Mollenhauer et al. (2011) also reported α-syn levels to be decreased in patients with MSA (as well as in those with PD and DLB) compared to patients with AD, and ones with other neurological disorders. As seen in these other studies, we also found that measurements of total α-syn did not clearly distinguish patients with MSA from those with PD (and other parkinsonian disorders).

Our present data therefore suggest that raised levels of phosphorylated forms of α-syn, rather than total α-syn, might provide a test for not only distinguishing MSA from normal individuals, but perhaps more importantly from other synucleinopathies. However, it is accepted that these are very preliminary data and will need verification in larger sample cohorts, especially in samples taken from living patients earlier in the course of their illness before it is possible to categorically state the value of this as a diagnostic test.

There are several potential confounders in our study which we have taken into consideration, leading us to believe that our present findings are genuine.

Firstly, as compared to lumbar puncture where this is less of a problem, it is almost inevitable that the practicalities of taking CSF

samples at post mortem will inadvertently include contamination of some samples by red blood cells, which themselves are a potential source of α-syn (Barbour et al., 2008; Hong et al., 2010). Indeed, a recent study (Hong et al., 2010), based on the analysis of CSF samples obtained at lumbar puncture by Luminex assays, noted that levels of α-syn were significantly lower than those of controls, but only when those samples contaminated by blood (with haemoglobin concentrations greater than 200 ng/ml) were excluded from the analysis. Consequently, we assayed our CSF samples for haemoglobin to assess whether varying degrees of blood contamination might have contributed to the output from our immunoassays. Our findings of a lack of correlation between CSF haemoglobin level and any of the α-syn measures, either over all the patients and controls, or within any of the diagnostic groups, makes us confident that the assay results presented here are reliable and have not been influenced to any great extent by contamination by red blood cells. Moreover, it is known that α-syn within platelets is not phosphorylated at serine 129 (Shults et al., 2006), and therefore contamination of CSF samples by blood may not, in any case, be expected to influence the output from the immunoassays for phosphorylated forms of α-syn.

Secondly, it has been suggested from previous studies (Mollenhauer et al., 2008) that levels of total α-syn within CSF may progressively increase with increasing post mortem delay time and hence, the variations in α-syn measured in this study might simply reflect group differences in post mortem delay times. However, we found no correlation between levels of α-syn within CSF and post mortem delay time for any of the 4 immunoassays, either when all 96 patients were considered as a group, or separately according to diagnosis (data not shown). Moreover, mean post mortem delay time did not differ significantly between any of the diagnostic groups. Therefore, we have no reason to believe that the findings presented here are not reliable. To our knowledge, there have been no other studies except that of Mollenhauer et al. (2008) indicating a rise in levels of α-syn within CSF at post mortem, and so it is still uncertain as to whether these levels do indeed change after death.

There have been a number of previous studies investigating whether measurement of total α-syn can also be employed as a biomarker for PD, though these have not always provided consistent results. For example, an earlier study by Tokuda et al. (2006) found levels of total α-syn on average to be lower in samples of CSF taken from a group of patients with PD compared with a group of normal or neurological controls. However, in a later study (Tokuda et al., 2010) no such clear differences were seen. Elsewhere, Mollenhauer et al. (2008, 2011) have similarly reported a lowering of total α-syn levels between PD patients and controls, though this finding has not been replicated by others (Ohrfelt et al., 2009; Spies et al., 2009; Reesink et al., 2010; Aerts et al., in press). In the present study, using a similar immunoassay protocol to Tokuda et al. (2006, 2010), we also found no significant differences in total α-syn levels between PD patients and controls, or between DLB patients and controls. Hence, it remains unclear whether measuring total α-syn levels in CSF has any utility in discriminating between patients with LBD (i.e. PD and DLB) and control subjects.

Because recent studies have suggested that oligomeric forms of α-syn may be the toxic species that induce neuronal cell death, it has been suggested that measurement of these particular forms of α-syn might be better biomarker for PD than total α-syn (Tokuda et al., 2010). Indeed, it has been shown that soluble oligomeric forms of α-syn are elevated in brain homogenates of patients with PD and DLB (Sharon et al., 2003, Paleologou et al., 2009), supporting such an argument. In keeping with this hypothesis, El-Agnaf and colleagues have found elevated levels of oligomeric α-syn in plasma (El-Agnaf et al., 2006) and CSF (Tokuda et al., 2010) of PD patients compared to controls. However, again using similar methodologies, we were unable to replicate these findings with our results showing no clear distinctions between PD or DLB patients and controls with respect to oligomeric forms of α-syn.

It is not clear from a methodological standpoint why we have obtained results dissimilar to those of Tokuda et al. (2010), though it is notable that these latter authors employed CSF samples obtained at lumbar puncture from living patients most of whom were sampled within 5 years of onset of illness. In the present study we employed post mortem samples from end-stage PD and DLB patients dying 11–44 years on average after onset of illness. Hence, increases in oligomeric forms of α-syn early in the course of the disease could diminish with time. This same kind of situation has been seen in Motor Neurone Disease where CSF levels of TDP-43 protein were found to be increased within the first 11 months of illness, but after this time fell and became not significantly different from control subjects (Kasai et al., 2009).

In summary therefore, we present some new methods of assessing α-syn levels in CSF from patients with parkinsonian disorders, and show that those assays based on the detection of phosphorylated oligomeric forms of α-syn may have utility in differentiating patients with MSA from other parkinsonian disorders in which the underlying pathology is also α-syn based (i.e. PD and DLB) or is tau-based (i.e. PSP).

Supplementary materials related to this article can be found online at doi:10.1016/j.nbd.2011.08.003.

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Molecular Dissection of TDP-43 Proteinopathies

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Abstract TDP-43 has been identified as a major component of ubiquitin-positive tau-negative cytoplasmic inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and in amyotrophic lateral sclerosis (ALS). We raised antibodies to phosphopeptides representing 36 out of 64 candidate phosphorylation sites of human TDP-43 and showed that the antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 labeled the inclusions, but not the nuclei. Immunoblot analyses demonstrated that the antibodies recognized TDP-43 at ~45 kDa, smearing substances and 18–26 kDa C-terminal fragments. Furthermore, the band patterns of the C-terminal fragments differed between neuropathological subtypes, but were indistinguishable between brain regions and spinal cord in each individual patient. Protease treatment of Sarkosyl-insoluble TDP-43 suggests that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between the diseases. These results suggest that molecular species of abnormal TDP-43 are different between the diseases and that they propagate from affected cells to other cells during disease progression and determine the clinicopathological phenotypes of the diseases.

Keywords Propagation · Phosphorylation · Tau · α-Synuclein · Prion · Cancer

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Introduction

TAR DNA-binding protein of M_r=43 kDa (TDP-43) is a nuclear factor that functions in regulating transcription and splicing. It is structurally characterized by two RNA recognition motifs and the C-terminal tail containing a glycine-rich region, and resembles a heterogeneous ribonucleoprotein (hnRNP) (Ayala et al. 2005). It has been shown to interact with several nuclear ribonucleoproteins (RNP), including hnRNP A and B and survival motor neuron protein, inhibiting alternative splicing (Buratti et al. 2005; Bose et al. 2008). In 2006, TDP-43 was identified as a major component of ubiquitin-positive inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) (Arai et al. 2006; Neumann et al. 2006). Subsequent immunohistochemical examination demonstrated abnormal accumulation of TDP-43 in neurodegenerative disorders other than FTLD-U and ALS, including ALS/parkinsonism–

dementia complex of Guam (Geser et al. 2007; Hasegawa et al. 2007), Alzheimer's disease (AD) (Amador-Ortiz et al. 2007; Higashi et al. 2007; Arai et al. 2009), dementia with Lewy bodies (DLB) (Higashi et al. 2007; Nakashima-Yasuda et al. 2007; Arai et al. 2009), Pick's disease (Arai et al. 2006; Freeman et al. 2008; Lin and Dickson 2008), argyrophilic grain disease (Fujishiro et al. 2009) and corticobasal degeneration (Uryu et al. 2008). These diseases with TDP-43 pathologies are now referred to as TDP-43 proteinopathies. In 2008, mutations in the TDP-43 gene (*TARDBP*) were discovered in familial and sporadic cases of ALS (Yokoseki et al. 2008; Gitcho et al. 2008; Sreedharan et al. 2008; Kabashi et al. 2008; Van Deerlin et al. 2008; Barmada and Finkbeiner 2010; Pesiridis et al. 2009), FTD-MND (Benajiba et al. 2009) and FTD (Borroni et al. 2009), clearly indicating that abnormality of TDP-43 protein causes neurodegeneration.

Identification of Abnormal Phosphorylation Sites of TDP-43

Biochemical analyses of the detergent-insoluble fraction extracted from brains of patients afflicted with FTLD-TDP and ALS show that TDP-43 accumulated in these pathological structures is phosphorylated and cleaved (Arai et al. 2006; Neumann et al. 2006). By producing antibodies against synthetic phosphopeptides containing 36 different phosphorylation sites from among the 56 serine/threonine residues of TDP-43, five abnormal phosphorylation sites were identified at serine residues in the C-terminal region (Hasegawa et al. 2008). The antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD-U, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulation in neuropathological examinations (Hasegawa et al. 2008). Immunoblotting of the Sarkosyl-insoluble fraction from control, FTLD-U and ALS cases using these phospho-specific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 at ~45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD-U and ALS (Hasegawa et al. 2008).

Cellular Models of TDP-43

To establish cellular models of TDP-43 proteinopathies, several deletion mutants of human TDP-43 in SH-SY5Y cells were expressed and the accumulation of TDP-43 was analyzed by use of the phospho-TDP-43 antibodies and ubiquitin. Wild-type (WT) full-length TDP-43 was localized to nuclei and no inclusions were observed, whereas in cells transfected with C-terminal fragments as GFP fusions, round cytoplasmic inclusions with intense GFP fluorescence were formed (Nonaka et al. 2009b). In addition, a deletion mutant lacking the nuclear localizing signal (NLS) and six amino acids similar to the NLS also formed aggregates in cells without any treatment (Nonaka et al. 2009a). These inclusions are strongly positive for antibodies to phosphorylated TDP-43 and ubiquitin. Using these cellular models, the effect of pathogenic mutations of the TDP-43 gene was analyzed. Of 14 mutants examined, seven mutants showed a significantly higher number of aggregates than the WT C-terminal fragment, strongly suggesting that these mutations of TDP-43 accelerate aggregation of the C-terminal fragments (Nonaka et al. 2009b). In addition, when GFP-tagged C-terminal fragments were co-expressed with DsRed-tagged full-length TDP-43, cytoplasmic inclusions with both GFP and DsRed signals were formed, suggesting that exogenous full-length TDP-43 is trapped in cytoplasmic inclusions formed by C-terminal fragments. This may explain why normal nuclear staining of TDP-43 is lost in neuronal cells with inclusions in diseased brains (Nonaka et al. 2009b). Furthermore, we identified two cleavage sites of TDP-43 deposited in FTLD-U by mass spectrometric analysis, and confirmed that expression of these fragments as GFP fusions also afforded cytoplasmic inclusions positive for ubiquitin and phosphorylated TDP-43 (Nonaka et al. 2009b). The cleavage sites identified in the 23-kDa C-terminal fragment of FTLD were different from that of caspase-3, suggesting that caspase is not the enzyme responsible for generating the 23-kDa fragment (Nonaka et al. 2009b). These cellular models recapitulate many of the features of TDP-43 in patients, and therefore, should be useful for screening small molecules for activity to inhibit TDP-43 aggregate formation. We tested whether or not methylene blue and dimebon have the ability to suppress formation of pathological TDP-43 inclusions. Compared to controls, a 50% reduction in the number of inclusions with 0.05 μM methylene blue, a 45% reduction with 5 μM dimebon and an 80% reduction with the combination of 0.05 μM methylene blue and 5 μM dimebon were observed (Yamashita et al. 2009). The effects were statistically significant and the results were also confirmed by Western blotting. These results suggest that these two compounds may be effective in the therapy of ALS, FTLD-U and other TDP-43 proteinopathies.

TDP-43 C-Terminal Fragments

Based on neuropathological studies, TDP-43 proteinopathies have been classified into 4 subtypes (Cairns et al.

2007). Type 1 is characterized by dystrophic neurites (DNs) with few neuronal cytoplasmic inclusions (NCIs) and no neuronal intranuclear inclusions (NIIs), Type 2 has numerous NCIs with few DNs and no NIIs, Type 3 has numerous NCIs and DNs and occasional NIIs and Type 4 has numerous NIIs and DNs with few NCIs, a pattern which is specific for familial FTLD-U with mutations of VCP gene. There appears to be a strong relationship between other subtypes of TDP-43 pathology and clinical phenotype. Type 1 is associated with semantic dementia, type 2 with FTLD with motor neuron disease (MND), ALS or clinical signs of MND, and type 3 with progressive nonfluent aphasia or FTD with mutation in the progranulin gene. Recent studies of ALS have clarified the wide distribution of neuronal and glial TDP-43 pathology in multiple areas of the central nervous systems (Geser et al. 2008; Nishihira et al. 2009), suggesting that ALS does not selectively affect only the motor system, but rather is a multisystem neurodegenerative TDP-43 proteinopathy affecting both neurons and glial cells.

By immunoblot analyses of the Sarkosyl-insoluble fractions from FTLD-U and ALS patients, we found that the band patterns of the C-terminal fragments of phosphorylated TDP-43 corresponded to the neuropathological subtypes. Type 1 FTLD-U showed two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa, while type 2 ALS showed three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa. Type 3 FTD with mutation in the progranulin gene showed an intermediate pattern between those two. These results clearly indicate that TDP-43 proteinopathies subclassified by neuropathological differences can also be distinguished biochemically. This strong association between the neuropathology and the biochemistry is critical for understanding the molecular pathogenesis of TDP-43 proteinopathies.

Biochemical Analysis of TDP-43 in FTLD-U and ALS

The biochemical differences of TDP-43, as shown in the different band patterns of TDP-43 C-terminal fragments, are closely linked to the morphologies of inclusions. The properties of the abnormal TDP-43 may determine the neuropathological and clinical phenotypes of TDP-43 proteinopathies. Similar biochemical and neuropathological differences have been reported in tau between PSP and CBD. Both PSP and CBD are tauopathies with deposition of 4-repeat tau isoforms; however, distinct types of C-terminal fragments are detected, i.e., a 33-kDa band in PSP and ~3-kDa bands in CBD (Arai et al. 2004).

So, what do the different band patterns mean? It is clear that the fragments are produced by cleavage at multiple sites of TDP-43. The band patterns also suggest that the cleavage sites are slightly altered between the diseases. Based on these observations, it is likely that the event may occur after the assembly or aggregation of abnormal TDP-43, and represent relatively protease-resistant domains of TDP-43, which form beta-sheet structure. That is, the different band patterns in TDP-43 proteinopathies represent different conformations of abnormal TDP-43 in the diseases.

To test this idea, we performed protease treatment of the abnormal TDP-43 recovered in the Sarkosyl-insoluble pellets, and analyzed the protease-resistant bands. Proteins can be easily cleaved by proteases if they are denatured or unstructured, but domains that have rigid structures such as beta-sheet structure, or that are structurally buried or interacting with other molecules, are highly resistant to proteases. Figure 1 shows the result of immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment. Before treatment, hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. Upon trypsin or chymotrypsin treatment, the full-length 45-kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16–26 kDa (Fig. 1). As expected, the protease-resistant band pattern of ALS is different and clearly distinguishable from that of FTLD-U. Doublet bands at ~16 kDa and a band at 25 kDa were detected in ALS, but only a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment (Fig. 1). Similarly, multiple protease-resistant bands were detected at 16–25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U (Fig. 1). These results strongly support the idea that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between ALS and FTLD-U.

TDP-43 in Different Brain Regions

Similar protease-resistant bands and differences in the band patterns have been reported in prion diseases, CJD and BSE (Collinge et al. 1996). Protease-resistant prion from new-variant CJD cases showed a different characteristic pattern from that in sporadic CJD cases, and the band pattern is indistinguishable from that of mice infected with BSE prion. This is biochemical evidence that the BSE agent has been transmitted from bovine to human.

Applying this to TDP-43 in TDP-43 proteinopathies, it is possible to determine whether there is any difference between the abnormal TDP-43 accumulated in cortex and that in spinal cord by analyzing the band patterns of the C-

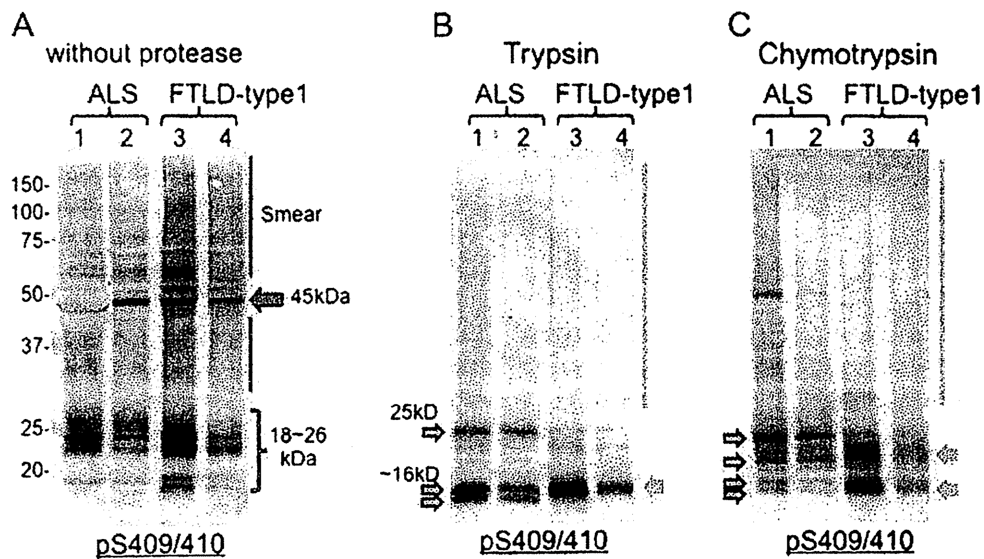

Fig. 1 Immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment with a phosphorylation dependent anti-TDP-43 rabbit polyclonal antibody (pS409/410). **a** Hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410 before treatment. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. **b** Upon trypsin treatment, the full-length 45 kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16~26 kDa. Doublet bands at ~16 kDa and a band at 25 kDa were seen in ALS, but a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment. **c** Multiple protease-resistant bands were detected at 16~25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U

terminal fragments of TDP-43. Thus, we have prepared Sarkosyl-insoluble fractions from cortex and spinal cords of three ALS cases, immunoblotted them with pS409/410 and compared the results. In all three cases, type 2 C-terminal fragments of TDP-43 were detected, and there was no significant difference between the band pattern in cortex and that in spinal cord (data not shown). This strongly suggests that the same form of abnormal TDP-43 molecule is deposited in different brain regions. Similar results were also obtained from the analysis of the C-terminal band pattern of TDP-43 in FTLD-U. It seems highly unlikely that the same conformational change would occur synchronously in different brain regions. Instead, it seems more likely that abnormal protein produced in cells is transferred to different regions and propagated. These biochemical data obtained from the brains of patients provide biochemical evidence that abnormal species of TDP-43 are transmitted from cell to cell and propagated in vivo.

Discussion

Amyloid-like protein deposition is a common neuropathological feature of many neurodegenerative diseases. Hyperphosphorylated tau in Alzheimer's disease and related tauopathies, hyperphosphorylated alpha-synuclein in Parkinson's disease and other alpha-synucleinopathies, and expanded polyglutamines in polyglutamine diseases have been identified. Importantly, the extent of the abnormal protein pathologies is closely correlated with the disease progression (Braak and Braak 1991; Braak et al. 2003; Saito et al. 2003). The proteins or protein fibrils deposited in cells in these diseases have been shown to have a common structural feature. Cross-beta structure, which is the same as in abnormal prion protein, has been demonstrated in filaments or fibrils composed of tau (Berriman et al. 2003), alpha-synuclein (Serpell et al. 2000) or expanded polyglutamines (Perutz 1999). It has not been demonstrated in TDP-43 yet, but we have shown by electron microscopy that phosphorylated TDP-43 in motor neurons of ALS patients has a fibrous structure (Hasegawa et al. 2008), suggesting that TDP-43 is also an amyloid-like protein.

For the assembly of amyloid fibrils, nucleation-dependent protein polymerization has been proposed. This comprises nucleation and elongation phases, and nucleation is the rate-limiting step. It takes a long time to form the first aggregated seed from the monomer, but once the seed is formed, the elongation step proceeds relatively quickly. More importantly, by addition of amyloid-seed, proteins are often converted to the same conformation as that of the seed. For example, WT monomeric alpha-synuclein is converted to A30P-type amyloid fibrils when it is incubated with a small amount of fibril-seeds formed with A30P mutant alpha-synuclein (Yonetani et al. 2009). Differences in the conformations of the amyloid fibrils are detected based on the differences in the protease-resistant band

patterns, as in the typing of prion proteins. There is another example of nucleation-dependent amyloid fibril formation in cultured cells. We developed a novel method for introducing amyloid seeds into cultured cells using lipofectoamine, and presented experimental evidence of seed-dependent polymerization of alpha-synuclein, leading to the formation of filamentous protein deposits and cell death (Nonaka et al. 2010). Overexpression of alpha-synuclein itself in cells does not generate abnormal inclusions, but if fibril seeds formed with alpha-synuclein are introduced into cells, abundant filamentous alpha-synuclein aggregates positive for PSer129 and ubiquitin are developed, and cells with inclusions undergo cell death. This was also clearly demonstrated in cells expressing different tau isoforms by introducing the corresponding tau fibril seeds (Nonaka et al. 2010).

The above results obtained from biochemical analyses of abnormal proteins in patients strongly suggest that intracellular amyloid-like proteins, including TDP-43, propagate from cell to cell and this propagation is the cause of disease progression, analogously to metastasis of cancer cells to multiple different tissues in cancer progression. From this point of view, we have proposed as a hypothesis that neurodegenerative diseases with amyloid-like proteins can be regarded as "protein cancers." The term prion, coined in 1982 by Stanley B. Prusiner, describes an agent transmissible among humans and a variety of mammals. On the other hand, the term "protein cancers" describes diseases that involve the spreading or propagation of abnormal proteins in tissues or individuals, even though the mechanism of propagation is basically the same as that of prions. Amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation, and the amplified amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions (Fig. 2). As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. Therefore, it is important to regulate the propagation of abnormal proteins for clinical therapy.

Conclusions

1. In ALS, FTLD-U and other TDP-43 proteinopathies, abnormally phosphorylated, ubiquitinated, and truncated TDP-43 is accumulated in a filamentous form.
2. We established cellular models which recapitulate many of the features of the abnormal TDP-43 in FTLD-U and ALS
3. ALS-related pathogenic mutations of the TDP-43 gene accelerate aggregate formation by the C-terminal fragments.
4. The band pattern of the TDP-43 C-terminal fragments is different between diseases with different clinicopathological phenotypes, and it represents different conformations of the abnormal TDP-43 between the diseases.
5. The C-terminal band patterns in several brain areas and spinal cord in each individual case of sporadic ALS are indistinguishable.
6. These and other results suggest that abnormal TDP-43, tau and alpha-synuclein are transmitted and propagated from cell to cell in different regions during disease progression. It is important to find drugs that can block the propagation of abnormal proteins for clinical therapy.

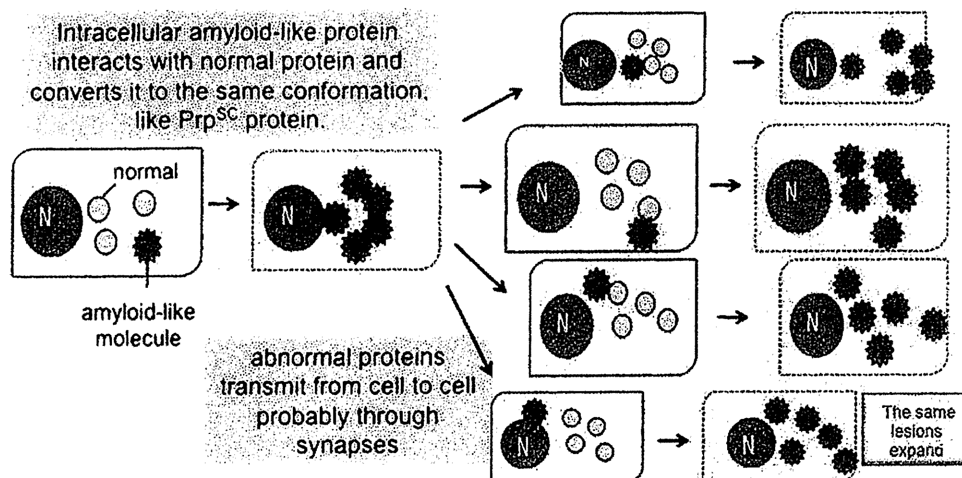

Fig. 2 Schematic representation of prion-like conversion of normal protein into amyloid-like protein and its propagation in neurodegenerative diseases. Intracellular amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation. Amplified abnormal amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions. As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. From this point of view, neurodegenerative diseases with amyloid-like proteins can be regarded as 'protein cancers"

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