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The proteome of neurofilament-containing protein aggregates in blood

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ABSTRACT

Protein aggregation in biofluids is a poorly understood phenomenon. Under normal physiological conditions, fluid-borne aggregates may contain plasma or cell proteins prone to aggregation. Recent observations suggest that neurofilaments (Nf), the building blocks of neurons and a biomarker of neurodegeneration, are included in high molecular weight complexes in circulation. The composition of these Nf-containing hetero-aggregates (NCH) may change in systemic or organ-specific pathologies, providing the basis to develop novel disease biomarkers. We have tested ultracentrifugation (UC) and a commercially available protein aggregate binder, Seprion PAD-Beads (SEP), for the enrichment of NCH from plasma of healthy individuals, and then characterised the Nf content of the aggregate fractions using gel electrophoresis and their proteome by mass spectrometry (MS). Western blot analysis of fractions obtained by UC showed that among Nf isoforms, neurofilament heavy chain (NfH) was found within SDS-stable high molecular weight aggregates. Shotgun proteomics of aggregates obtained with both extraction techniques identified mostly cell structural and to a lesser extent extra-cellular matrix proteins, while functional analysis revealed pathways involved in inflammatory response, phagosome and prion-like protein behaviour. UC aggregates were specifically enriched with proteins involved in endocrine, metabolic and cell-signalling regulation. We describe the proteome of neurofilament-containing aggregates isolated from healthy individuals biofluids using different extraction methods.

1. Introduction

The formation of assemblies of proteins that have lost their soluble state is a pathological hallmark of several neurodegenerative diseases [1,2]. The confluence of proteins into aggregates may also occur physiologically as shown for the recruitment of RNA-binding proteins into stress granules [3–7]. In cells, the aggregation of misfolded proteins is usually kept in check by a quality control system, which operates through protein re-folding, autophagy and clearance by the proteasome [8,9]. Extracellularly, a range of immune mediators may contribute to the clearance of misfolded proteins and of their aggregated forms [10]. It is proposed that in biological fluids, aggregate formation reflects the propensity of proteins to assemble naturally or can be experimentally induced under conditions of stress [11–13]. Depletion of albumin from human plasma, for example, leads to a significant increase in protein aggregation, particularly when heat and shear stress are applied [14]. Recently the presence of soluble sodium dodecyl sulphate (SDS) resistant protein aggregates has been reported in plasma from older adults and in significantly lower levels in plasma from younger individuals [15]. Loss of protein homeostasis and the increased rate of intra-cellular protein aggregation seem to be important hallmarks of aging [15]. Therefore, aggregates found in circulation may originate from senescent cell that have lost their functional integrity. Equally, an age-related failure of the control of protein homeostasis (i.e., proteostasis) may condition an increase of aggregation-prone proteins in fluid state and the formation of aggregates [15].

Separation of protein aggregates from fluids for quality assessment of biopharmaceutical formulations or for diagnostic purposes can be obtained by sedimentation-density analysis using ultracentrifugation (UC) or by extraction technologies that utilize solid-state binders. The Seprion PAD-beads (SEP) technology, for example, has been applied to the capture of proteins in different states of aggregation [16]. How

Abbreviations: NCH, neurofilament-containing hetero-aggregates; Nf, neurofilaments; NfH, neurofilament heavy chain; NfL, neurofilament light chain; NfM, neurofilament medium chain; PPS, pooled plasma sample; SEP, Seprion PAD-beads; UC, ultracentrifugation

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different isolation methods compare with regards to the pool of specific aggregates found in the extraction products and their protein composition, particularly when biofluids are the source of the target particles, is not known.

Aggregates, inclusion bodies or aggregosomes described in neurodegenerative diseases contain neurofilaments (Nf), the building blocks of neurons [17,18]. Nf are type IV intermediate filaments and one of the most abundant cytoskeletal component known to stabilize axons and maintain their size and functionality. Nf isoforms include neurofilament light (NfL, 61.5 kDa), medium (NfM, 102.5 kDa) and heavy (NfH, 112.5 kDa) chains. Post-translational modifications (PTMs), including phosphorylation and glycosylation at amino acids Serine and Threonine, affect Nf properties and size which increases to 70, 170 and 200 kDa for NfL, NfM and NfH, respectively [19,20]. Nf isoforms self-assembly and interaction with other molecules depend on conformational changes and PTMs, which also condition their immunogenicity [21–23]. Nf are released in cerebrospinal fluid (CSF) and blood when neurons and axons degenerate, and changes of Nf levels are associated with the progression of several neurodegenerative disorders [22,24,25]. We have recently suggested that circulating Nf are also present in high molecular weight molecular complexes. Immunodetection of heavy chain Nf (NfH), for example, when tested in serial sample dilutions, lacks the linearity of the calibration curves seen with Nf recombinant proteins or light chain Nf (NfL), a property explained by the so-called *hook effect* [21]. This phenomenon may relate to NfH epitopes being masked due to sequestration into immunocomplexes or other molecular assemblies, which is disrupted by the dilution process [21]. These data suggest that the formation of Nf-containing hetero-aggregates (NCH) is possible in both tissues and fluids [22,24]. Understanding Nf distribution between low order oligomers and higher order hetero-aggregates in biofluids has significant implications on their utility as biomarkers. In amyotrophic lateral sclerosis (ALS), an invariably fatal neurodegenerative disorder, Nf form heterogenous protein aggregates [26] and it is assumed that these are released, essentially intact, into the blood stream following cell death. The de-novo formation of circulating heteroaggregates due to a seeding effect of proteins like Nf in the fluid phase cannot be excluded. We hypothesise that neurofilaments may be released under both normal and pathological conditions as hetero-aggregates and that the content of these formations may differ between neurologically normal and diseased individuals. Hence defining the presence and content of NCH in normal individuals is a necessary step towards developing their utility as a new source of ALS biomarkers. Establishing protocols for NCH isolation and molecular characterization is therefore mandatory for any future use of NCH as disease biomarkers.

Here we studied circulating highly stable Nf-containing aggregates in plasma of neurologically healthy individuals using gel-based separation and described their protein composition. For the isolation of these aggregates from biological fluids, we have tested different conditions of ultracentrifugation including detergents and high salt concentrations and compared this approach to extraction obtained using aggregate capture binders (Seprion Ligand, SEP [27]). Liquid chromatography tandem mass spectrometry (LC-MS/MS) was then used to characterize the protein content of the complexes isolated by UC and SEP.

2. Methods

2.1. Plasma samples

Plasma samples from 6 healthy individuals with no known neurological disorders were pooled to be used in the enrichment methods. The selected individuals were aged between 51.2 and 62.9 years at the time of blood sampling. Neurofilament heavy chain (NfH) concentration ranged from 7.0 and 42.9 ng/ml (NfH levels were determined using immunodetection by sandwich ELISA as previously described by Lu et al. [22]). The pooled plasma sample (PPS) was divided in 1.1 ml aliquots and stored at − 80 °C. Ethical approval for recruitment, sampling and for the experimental procedures was obtained by the East London and The City Research Committee (09/H0703/27).

2.2. Total protein quantification

Total protein quantification was carried out using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) or Bio-Rad Protein Assay Kit (Bio-Rad) according to compatibility with reagents used in the protocol.

2.3. Western blotting

HiMark™ pre-stained Protein Marker (Fisher Scientific UK Ltd) and samples were loaded into 3–8% Tris-Acetate gels (Fisher Scientific UK Ltd) and, after electrophoresis, proteins and marker were transferred onto a nitrocellulose or polyvinylidene fluoride (PVDF) membrane (Fisher Scientific UK Ltd). The membrane was blocked with 5% skimmed milk in TBS 0.1% Tween-20 buffer at room temperature for 1 h. Overnight incubation was performed with primary antibody at 4 °C followed by incubation with secondary antibody for 1 h at room temperature, with membrane washes between steps using TBS 0.1% Tween-20. The membrane was then incubated with enhanced chemiluminescence substrate (ECL) and visualised using a BioRad Chemi-Doc system. For serial probing of the same membrane with different antibodies, stripping with ReBlot Plus Mild Antibody Stripping Solution (Millipore) for 15 min was performed.

2.4. Nf expression in pooled plasma samples

To first evaluate the presence of neurofilaments (Nf)-containing high molecular weight protein aggregates in pooled plasma samples (PPS) by western blot (WB), aliquots were first filtered twice with Amicon filters 100 K (Millipore). In this step, NfL isoform (~70 kDa) was likely to be retained by the filter. Different conditions known to solubilise plasma aggregates were also employed as previously reported and detailed hereafter [21]. Pooled plasma samples aliquots prepared for NfH, NfM and NfL analysis were divided into three fractions and processed as follows: 1) pre-treatment with 0.5 M urea and $Barb_2EDTA$ buffer for 1 h at RT, 2) dilution 1:1 with $Barb_2EDTA$ Buffer for 1 h at RT and 3) left untreated at + 4 °C.

2.5. Antibodies

The following antibodies and relative dilutions (in brackets) were used in this study: anti-Neurofilaments light (NfL; 1:1000) (clone EP675Y, Rabbit, Millipore), anti-Neurofilament Medium (NfM; 1:1000) (AB1987, Rabbit, Millipore), anti-Neurofilament heavy (NfH; 1:1000) (N4142, Rabbit, Sigma-Aldrich), Swine Anti-Rabbit Immunoglobulins (1:50,000) (P021702-2, DAKO).

2.6. Protein aggregate enrichment methods

2.6.1. Ultracentrifugation

To identify the best conditions for enrichment of NCH using ultracentrifugation (UC), we tested different detergents (SDS, Triton X-100, Sarkosyl) at either 0.5% or 2% and different NaCl concentrations (0.5 M, 1 M, 1.5 M) for salting in (Supplementary Table 1). The best conditions were selected based on Nf detection, both in native and within high MW forms. Total protein concentration was measured to evaluate sample protein enrichment (Supplementary Fig. 1). 111 µl of 20% Triton X-100 was added to 1 ml of PPS (final concentration 2% Triton X-100) and incubated in agitation for 10 min at RT. Centrifugation at 21,000*g* for 15 min was performed at RT and supernatant was collected for ultracentrifugation (UC). At this stage, 800 µl Sucrose Cushion (1 M sucrose, 50 mM Tris-HCl, 1 mM EDTA and 2% Triton X-