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Increasing the accuracy of proteomic typing by decellularisation of amyloid tissue biopsies

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ABSTRACT

Diagnosis and treatment of systemic amyloidosis depend on accurate identification of the specific amyloid fibril protein forming the tissue deposits. Confirmation of monoclonal immunoglobulin light chain amyloidosis (AL), requiring cytotoxic chemotherapy, and avoidance of such treatment in non-AL amyloidosis, are particularly important. Proteomic analysis characterises amyloid proteins directly. It complements immunohistochemical staining of amyloid to identify fibril proteins and gene sequencing to identify mutations in the fibril precursors. However, proteomics sometimes detects more than one potentially amyloidogenic protein, especially immunoglobulins and transthyretin which are abundant plasma proteins. Ambiguous results are most challenging in the elderly as both AL and transthyretin (ATTR) amyloidosis are usually present in this group. We have lately described a procedure for tissue decellularisation which retains the structure, integrity and composition of amyloid but removes proteins that are not integrated within the deposits. Here we show that use of this procedure before proteomic analysis eliminates ambiguity and improves diagnostic accuracy.

Significance: Unequivocal identification of the protein causing amyloidosis disease is crucial for correct diagnosis and treatment. As a proof of principle, we selected a number of cardiac and fat tissue biopsies from patients with various types of amyloidosis and show that a classical procedure of decellularisation enhances the specificity of the identification of the culprit protein reducing ambiguity and the risk of misdiagnosis.

1. Introduction

One of the major therapeutic strategies of systemic amyloidosis consists in reducing the production and thus the abundance of the protein which aggregates to form extracellular amyloid deposits. Therapy is therefore dictated by amyloid fibril typing in each case [1]. Most crucially, the very toxic and expensive cytotoxic chemotherapy essential in AL amyloidosis is not beneficial in other forms of amyloidosis and can be extremely dangerous [2]. Proteomic analysis of amyloid deposits provides direct chemical characterization of the proteins present and is complementary to immunohistochemical staining for known amyloid fibril proteins and gene sequencing to identify known or potential amyloidogenic mutations. It has been reported that proteomics unambiguously identifies the major amyloid constituent in the large majority of cases [3], but in some cases more than one potentially amyloidogenic protein, even though with different score, is detected in the amyloid specimen and for these patients an accurate diagnosis can become quite challenging [4,5]. Most commonly the ambiguity can be caused by the co-presence in the specimen of both immunoglobulin light chains and transthyretin, both of which are abundant in the plasma and cause the two most common forms of systemic amyloidosis. Furthermore, the prevalence of wild-type ATTR amyloidosis, uniquely a disease of the elderly, has recently been recognised and monoclonal gammopathy with increased production of monoclonal immunoglobulin light chains, the precursor of AL amyloid fibrils, is common in this age group [6]. Indeed, up to 20% of patients with ATTR amyloidosis have an incidental monoclonal protein in serum or urine [7].

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An additional technical problem arises from the increasing and highly recommended use of fine needle fat aspiration biopsies [8] for diagnosis and typing of amyloid. Proteomic analysis of standard excision or resection biopsies involves laser capture microdissection of the amyloid deposits themselves, selectively concentrating the fibrils, but this step is omitted with fine needle fat aspiration samples. Contamination by abundant plasma proteins, not necessarily related to amyloid formation *in vivo*, is common in these specimens.

We have lately demonstrated that application of tissue decellularisation to amyloidotic organs and tissues leaves extracellular matrix proteins and amyloid deposits intact [9]. Here we show that subjecting biopsies to a rapid tissue decellularisation protocol before digestion and mass spectrometry (MS) analysis, eliminates background contamination and enables unequivocal identification of the actual amyloid fibril protein.

2. Methods

2.1. Patients

Patients attending the National Amyloidosis Centre in the Centre for Amyloidosis and Acute Phase Proteins at UCL, London, UK, all provided informed written consent in accordance with the Declaration of Helsinki. Endomyocardial biopsies were received from 3 patients. Patient 1: a 73 year old man with κ myeloma presented with periorbital bruising, macroglossia and heart failure with amyloid cardiomyopathy. ^{99m}Tc-3,3-diphosphono-1,2-propanodicarboxylic acid (^{99m}Tc-DPD) scan showed Perugini grade 3 cardiac uptake but no visceral amyloid deposits were detected by serum amyloid P component (SAP) scintigraphy. No mutations were detected on sequencing of his *TTR* and *APOA1* genes. His myeloma was treated with cyclophosphamide vincristine dexamethasone (CVD) chemotherapy. Patient 2: a 65-year-old man with heart failure and amyloid cardiomyopathy but no abnormality on Tc–DPD or SAP scintigraphy, no monoclonal protein in serum or urine and wild-type *TTR* and *APOA1* genes. Patient 3: a 67-year-old man with chronic lymphocytic leukaemia and a monoclonal κ paraprotein who presented with heart failure, amyloid cardiomyopathy and autonomic neuropathy. TC-DPD scan showed Perugini grade 2 uptake but no visceral amyloid deposits were detected by SAP scintigraphy. *TTR* gene sequencing showed that he was heterozygous for the known amyloidogenic mutation encoding T60A TTR. Fat biopsies were received from 7 other patients in which more than one potentially amyloidogenic protein was detected in the untreated tissue specimen.

2.2. Laser capture microdissection and proteomic mass spectrometry analysis

Sections (6 μm) of formalin-fixed paraffin-embedded tissue on Director™ slides, stained with alkaline alcoholic Congo red (AMRESCO, Solon, OH, USA) [10] and haematoxylin (Pioneer Research Chemicals, Colchester, UK), were viewed under intense cross polarized light. Areas showing the pathognomonic apple green birefringence of amyloid were excised by laser capture microdissection and trapped on adhesive caps of microcentrifuge tubes. Following the method of Rodriguez et al. [11], proteins were extracted from each sample into 10 mM Tris/1 mM EDTA/0.002% Zwittergent buffer solution (35 μl) by heating (99 °C, 1.5 h) followed by sonication (1 h) and then digested with trypsin (1.5 mg *w*/*v*) overnight (~18 h) at 37 °C. Each digested sample was reduced with dithiothreitol (50 μg) at 99 °C for 5 min, freeze dried, reconstituted in 0.1% v/v trifluoroacetic acid in HPLC grade water (20 μl) and analysed by HPLC-MSMS.

Tryptic digests were applied to a trap column (180 μm ID × 20 mm bed, 5 μm Symmetry C18 packing; Waters Corporation, Massachusetts, USA) and separated on a reverse phase column (100 μm ID × 150 mm bed, 5 μm C18 packing; Nikkyo Technos Company Ltd., Tokyo, Japan) using a linear gradient from 1% to 60% acetonitrile/water over 44 min at 400 nl/min. Ten partial system washes (injector and trap) using injection boluses containing formic acid, ammonia, methanol and acetonitrile followed by a full solvent blank were run after each sample. Nanoflow liquid chromatography-electrospray tandem MS was performed using a Waters nanoACQUITY™ UPLC system (Waters Ltd., Elstree, Hertfordshire, UK) coupled to a Thermo Scientific Orbitrap Velos Mass Spectrometer (Thermo Electron, Bremen, Germany) operated in the positive ion mode. Each tryptic digest was analysed in three technical replicates unless otherwise stated (Supplementary Table1).

Instrument control and data acquisition used Thermo Scientific Xcalibur Version 2.1. MS data files were analysed using MASCOT software (Matrix Science, London, UK) [12] to search the SwissProt database. Searches were conducted with trypsin as the digestion enzyme (with 2 missed cleavages) and oxidation of methionine set as a variable modification; mass tolerances were 10 ppm for precursor ions, 0.60 Da for fragment ions with possible charge states of +2, +3 and +4. Protein identities were expressed in terms of MASCOT probability-based scores with a significance value set at $p < 0.05$ (http://www.matrixscience.com/help/scoring_help.html).

In addition Mascot output data files were also analysed and validated by the use of Scaffold 4.6.1 (Proteome Software, Inc., Portland, OR). This tool uses a Local False Discovery Rate (LFDR)-based scoring system for peptide validation based on a Bayesian approach to confirm peptide probabilities. The likelihood of peptides is calculated on parent ion mass accuracy and parent ion delta masses [13,14]. Filtering parameters for protein identification by Scaffold were set at a protein threshold confidence level > 99%, a minimum of 2 assigned peptides with a probability >95%.

In addition, to increase confidence in protein identification and peptide validation, Scaffold software was run using Mascot and X!Tandem search engines.

A semi-quantitative analysis was performed on the group of fat aspirates by considering the total number of spectra matched to a single protein group (TS) [15]; TS values in the three replicates per sample were averaged. Ratios of the resulting TS between non-amyloid and amyloid protein in both untreated and decellularised samples were calculated and plotted. In addition, we carried out a label free quantification (LFQ) of proteins using MaxQuant software (version 1.5.8.3), which is based on the ion intensities of the extracted ion chromatogram [16].

For the first and the main search, peptide mass tolerances were 20 ppm and 4.5 ppm respectively, whereas for MS/MS the threshold was 20 ppm. For the protein identification, a minimum of 2 peptides and at least 1 unique peptide were set. The search was performed in revert decoy mode with peptide spectra matches false discovery rate (PSM FDR), protein FDR and site decoy fraction set at 0.01. An average of LFQ of 3 replicates for each experiment was determined and the ratio between LFQ of non-amyloid and amyloid protein in untreated and decellularised samples was calculated and plotted.

Statistical analysis for the group of 7 fat biopsies was performed by using the Mann Whitney test on GraphPad Prism 5 software (GraphPad Prism Inc., San Diego, CA). Statistically significant differences between the untreated and decellularised TS (or LFQ) ratios are indicated by $p < 0.05$.

2.3. Decellularisation protocol

Fresh, unfixed, snap frozen cardiac or adipose tissue biopsies were decellularised by sequential washing with 500 mM NaCl containing 2 mM calcium chloride; 4% w/v sodium deoxycholate; and Tris-HCl containing 140 mM NaCl, 2 mM calcium chloride, pH 8.0 (all reagents from Sigma-Aldrich, St. Louis, MO, USA). All the steps were carried out at room temperature using a minimal volume of the respective buffer in Eppendorf safe-lock tubes placed in a tissuelyser (Qiagen, Hilden, Germany) at 25 Hz per 2 min. Typically two set of washings were repeated for the tissue to become translucent. Decellularised cardiac biopsy

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