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Simple Protocol for Amyloid Extraction from FFPE Sections using Organic Solvent

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Abstract

Background: Recently, we have established a simple method of amyloid extraction from Formalin-Fixed, Paraffin-Embedded (FFPE) sections using organic solvent. This method provides an amyloid-rich extract that can be analyzed by electrophoresis and mass spectrometry. This study aimed to show the utility of the established method and present a simple protocol of amyloid extraction for electrophoresis and mass spectrometry.

Methods: Amyloid deposition was confirmed pathologically in two dogs diagnosed with medullary carcinoma and plasmacytoma. Amyloid deposition was classified by immunohistochemistry. Amyloid proteins were extracted from FFPE sections of these cases of plasmacytoma and medullary carcinoma using Dimethyl Sulfoxide (DMSO). Amyloid extracts were analyzed by electrophoresis and mass spectrometry.

Results: Amyloid deposition was confirmed pathologically in both cases. Immunohistochemistry revealed that the proteins forming amyloid deposits were calcitonin in medullary carcinoma and the lambda chain of immunoglobulin in plasmacytoma. DMSO extracts showed case-specific isolation patterns of proteins in electrophoresis. Mass spectrometry identified the specific peptides of calcitonin and the lambda chain of immunoglobulin in DMSO extracts from the cases.

Conclusion: We demonstrated the utility of the amyloid extraction method using DMSO for calcitonin and AL amyloid in canine cases. This simple method might contribute to the diagnosis and study of amyloidosis.

Keywords: Amyloid extraction; Formalin-fixed; Paraffin-embedded sections; Organic solvent

Introduction

Amyloidosis is caused by the extracellular deposition of amyloid fibrils formed by amyloid precursor proteins [1,2]. In total, 36 and 10 amyloid precursor proteins have been identified in tissue deposits in humans and animals, respectively [1]. To diagnose amyloidosis, it is essential to identify the precursor protein of amyloid deposited in tissue. The gold standard for identifying this precursor protein is immunohistochemistry against Formalin-Fixed, Paraffin-Embedded (FFPE) sections using specific antibodies. However, in various amyloidosis cases, there is no specific functioning antibody.

Several studies have demonstrated the utility of Mass Spectrometry (MS)-based amyloid identification in combination with Laser Microdissection (LMD) for the diagnosis and accurate typing of amyloidosis [1,2]. This approach is expected to provide the next gold standard of amyloid identification for highly sensitive and accurate protein identification. In this MS-based approach, LMD is necessary to specifically isolate the area of amyloid deposition from tissue sections for accurate identification of the amyloid protein. Recently, we have established an amyloid-specific extraction method without LMD [3]. Here, we would like to introduce our simple protocol of amyloid protein extraction from FFPE sections using organic solvents instead of using LMD.

Materials and Methods

Specimens

Two dogs affected by plasmacytoma and medullary carcinoma in which amyloid deposition had been confirmed were studied. Tumor tissues from biopsy specimens were evaluated in this study. For pathological examination, FFPE sections were stained with hematoxylin-eosin and Congo red. For immunohistochemistry, sections were incubated with antibodies against dog lambda chain (Kyowa Medex Co., Ltd., Tokyo, Japan) or human calcitonin (DAKO, Tokyo, Japan) at room temperature for 1 h. Peroxidase-conjugated anti-IgG [Histofine Simple Stain MAX-PO (Multi); Nichirei, Tokyo, Japan] was used as the secondary antibody. Immunoreactions were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB Tablet; Wako, Tokyo, Japan).

Procedure of amyloid extraction

FFPE section preparation

1. Fix the tissues in 10% buffered formalin. The fixation time should be under 1 week. Embed the fixed tissues in paraffin.

2. Move one 10 µm thick section into a low-adsorption treated 1.5 ml tube.

3. For dewaxing, add hexane until the section is immersed (200-500 µl) and incubate for 10 min at room temperature. Remove the solution from the tube. Repeat this step twice.

4. To dry up the section, open the tube cap and incubate for 10 min at room temperature.

Amyloid extraction

5. Add organic solvent [we usually use Dimethyl Sulfoxide (DMSO) or Kamyloid kit (Cosmo Bio, Tokyo, Japan); if you use Kamyloid kit, the dewaxing step can be omitted) until the section is immersed (100-300 µl). Incubate it for 16 h at 37°C.

6. After incubation, spin down the section by centrifugation at 15,000 × g for 15 min. Collect the supernatant in a new tube. This supernatant is the amyloid extract.

7. Dry up the solvent completely using a vacuum dryer with heating at 60°C. The drying time should be under 30 min. (A long duration of this step induces aggregation of the extracted amyloid.).

For Mass Spectrometry analysis

8. To dissolve the dried-up extract, add 50 µl of a buffer containing 1.2 M urea and 100 mM Tris-HCl, pH 8.0, to the tube. Incubate for 30 min with agitation at room temperature.

9. Then, digest the extract in 0.1 µg of TPCK-treated trypsin (Trypsin Gold; Promega, USA) overnight at 37°C.

10. Desalt the tryptic peptides using a C18 spin column (spin trap C18; GL Science, Tokyo, Japan). Dry the eluates from the C18 column and dissolve them in 20 µl of 0.3% formic acid. Analyze 5 µl of each sample by MS.

For electrophoresis

11. To dissolve the dried-up extract, add 10 µl of Laemmli's sample buffer. After incubation for 15 min at 37°C, electrophoretically separate the protein components. The Tris-tricine system is recommended for the electrophoresis of amyloid proteins.

Mass spectrometry for identification of amyloid precursor proteins

Tryptic peptides of amyloid extracts were injected into a nano-flow-LC system (Thermo Fisher Scientific) coupled with an MS system (Q-Exactive, Thermo Fisher Scientific). MS spectra were acquired in Data-Independent Acquisition (DIA) analysis. The specific peptide signals derived from amyloid precursor proteins from the DIA dataset were detected using Skyline software, which set transitions for peptides derived from amyloid proteins in the database.

SDS-PAGE

Protein components in extracts were electrophoretically separated through a 5%-20% gradient polyacrylamide gel using a Tris-tricine system (ET-15S e-Pagel; ATTO, Japan). The proteins on the gel were visualized by silver staining (Silver Quest kit; Invitrogen, USA).

Results

Both cases contained amorphous materials that were Congo-red-positive amyloid deposits in tumor tissues **(Figure 1)**. The amorphous materials were identified as calcitonin and immunoglobulin light chain respectively by immunohistochemistry **(Figure 1)**. SDS-PAGE analysis demonstrated that DMSO extracts showed case-specific isolation patterns of proteins **(Figure 2a)**. The extract from medullary carcinoma showed single band at almost 6 kDa that was like previous report [4]. The extract from plasmacytoma contained two proteins of different size. 15 kDa protein was much the immunoglobulin light chain in molecular weight. The lower protein had a molecular weight close to 6 kDa might be proteolytic digest of the immunoglobulin light chain. DIA analysis detected specific peptides of CSLNSTCVLGTYSK derived from canine calcitonin in the medullary carcinoma and YAASSYLSLTPDK derived from canine immunoglobulin lambda light chain in the plasmacytoma **(Figures 2b and Figure 2c)**.

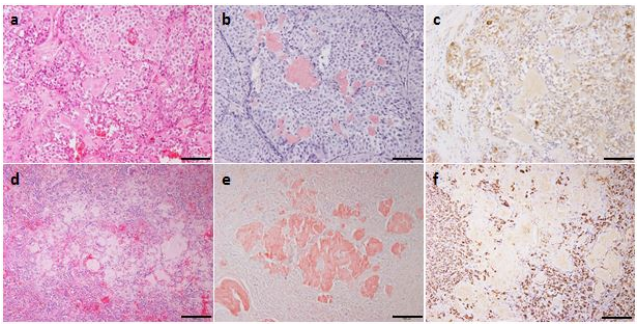

Figure 1: Histopathological findings of the kidney of a dog with medullary carcinoma (a-c) and plasmacytoma (d-f). (a) HE staining shows amorphous material deposition in interstitial tissues of tumour. (b) Amorphous materials were stained by Congo red. (c) Amorphous materials were immunohistochemically positive against calcitonin (scale bar on a-c: 100 µm). (d) Amorphous material deposited in interstitial tissue and macrophage infiltrated in tumour. (e) Amorphous materials were stained by Congo red. (f) Amorphous materials were immunohistochemically positive against lambda chain. (scale bar on d-f: 200 µm).

Discussion

We have developed a simple method of extracting amyloid from FFPE sections using organic solvent. This approach successfully extracted amyloid including SAA from human kidney, amyloid β from brain of a 5XFAD mouse, which is an animal model of Alzheimer's disease [3], as well as the immunoglobulin lambda light chain and calcitonin from tumor tissues (in this study). The mechanism of amyloid extraction with organic solvents depends on the conversion of amyloid protein structure from β-sheet to α-helix. β-sheet is a common structure

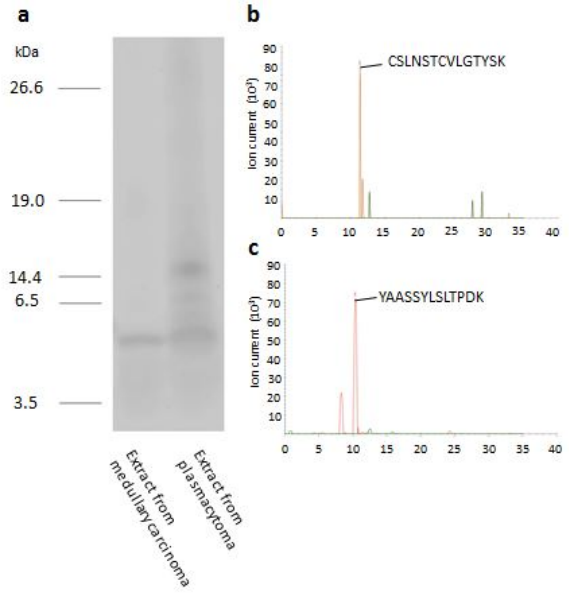

Figure 2: Extraction of amyloid from dog tumour tissues using DMSO. (a) SDS-PAGE (silver stain) of extracts from FFPE tissues of dog medullary carcinoma and plasmacytoma with DMSO. (b) MS chromatograms of the doubly charged ion with specific mass-to-charge ratio 738.35 for CSLNSTCVLGTYSK derived from canine calcitonin. (c) MS chromatograms of the doubly charged ion with specific mass-to-charge ratio 708.36 for YAASSYLSLTPDK derived from canine immunoglobulin lambda light chain.

of amyloid proteins forming amyloid fibrils [3]. Therefore, it is suggested that organic solvents could extract all amyloid proteins from FFPE specimens.

Electrophoresis of the extracts showed that the extracts obtained with DMSO from FFPE specimens were amyloid-rich. We explain this amyloid selectivity of organic solvent extraction as follows: Proteins fixed with formaldehyde *via* chemical cross-linking are difficult to dissolve in any solution [5]. Exceptionally, the proteins forming amyloid fibrils can resist chemical cross-linking due to formaldehyde fixation [6-9]. This resistance to fixation is believed to be related to the amyloid fibril structure, consisting of packed aggregates of β-sheets. Therefore, it is conceivable that organic solvents can extract amyloids selectively from FFPE tissues by converting their secondary structure to α-helix.

Conclusion

In summary, we demonstrated the selective amyloid extraction from FFPE specimens using organic solvents. The obtained extracts can be applied to protein analysis including electrophoresis and MS. We hope that this simple method contributes to the diagnosis and study of amyloidosis.

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