Methods for identification and diagnosis of amyloidosis

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ABSTRACT

The amyloidoses are biochemically heterogeneous diseases with pathophysiologic deposits of various proteins. Amyloid deposits can occur either localized to one organ or tissue or as part of a systemic disease with deposits in many different tissue. The clinical course, prognosis and therapy are different for each type of amyloidosis and therefore a type specific diagnosis is demanded as early as possible. We describe a method for typing of the most common systemic amyloidoses based on Western blot analysis combined with specific in-house antibodies, using subcutaneous fat biopsies. We found that the method is reliable and easy to perform and the tissue sample needed is obtained by minor surgery.

In the aortic intima amyloid deposits are often associated with atherosclerosis plaques. In our study we also investigated the prevalence of intimal amyloid from 10 patients age 58-94, amyloid deposits were present in 50% of the cases.

Key words: subcutaneous fat tissue, systemic amyloidosis, Western blot analysis, immunohistochemistry, intimal amyloid
SAMMANFATTNING

INTRODUCTION

Protein misfolding and aggregation have been linked to several human diseases, including Alzheimer’s disease, type2 diabetes, systemic and local amyloidosis, by mechanisms that are not yet completely understood. The hallmark of amyloidosis is the formation of highly ordered and β-sheet-rich aggregates referred to as amyloid fibrils [1].

Amyloidosis is not a single disease entity but rather a diverse group of disease processes characterized by the abnormal extracellular deposition of insoluble fibrillar protein, generically termed amyloid, in one or many organs [2]. Amyloid deposits contain the fibrillar protein that defines the type of amyloidosis and several common components such as, the serum amyloid P and glycosaminoglycans. Glycosaminoglycans stain blue with iodine and in 1854 Virchow was the first to use iodine stain to study cerebral amylosea under the microscope. He described its appearance as that of starch or cellulose, thus giving the disease its name, amyloid is Greek for starch. All of the components have been claimed to be involved in the amyloid fibril stability as well as acceleration of amyloid formation. Amyloid fibrils are arranged in an antipararell conformation with a β-sheet structure [2,3,5]. The classification of amyloidosis is based upon the tissue distribution of amyloid deposits (local or systemic amyloidosis), the absence or presence of preexisting disease (primary or secondary amyloidosis) and the chemical type of amyloid protein fibril [2].

Table 1 presents the classification of representative systemic amyloidosis and local amyloidosis [4].

<table>
<thead>
<tr>
<th>Type of amyloid</th>
<th>Fibril protein</th>
<th>Protein precursor</th>
<th>Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL (primary)</td>
<td>Monoclonal immunoglobulin light chain</td>
<td>λ or κ light chain (S,L)</td>
<td>Primary systemic amyloidosis</td>
</tr>
<tr>
<td>ATTR (familial)</td>
<td>Transthyretin</td>
<td>Variant forms of transthyretin (S)</td>
<td>FAP, Senile systemic amyloidosis</td>
</tr>
<tr>
<td>AA</td>
<td>Amyloid A protein</td>
<td>Serum amyloid A protein (SAA) (S)</td>
<td>Secondary amyloidosis</td>
</tr>
<tr>
<td>AMed</td>
<td>Lactadherin</td>
<td>Medin (L)</td>
<td>Aortic (media)</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ precursor protein</td>
<td>Amyloid β peptid (L)</td>
<td>Alzheimer’s disease</td>
</tr>
</tbody>
</table>

S: systemic; L: localized
AL systemic amyloidosis is caused by deposition of amyloid fibrils, derived from the
immunoglobulin light chain, most often the N-terminal part but also the full-length protein
and parts of the C-terminal have been demonstrated in the deposits. This abnormal protein is
produced by plasma cells in the bone marrow [6]. The free immunoglobulin light chains can
be found in the urine as Bence-Jones protein. AL amyloidosis includes primary amyloidosis,
multiple myeloma and other plasma cell dyscrasias such as β-cell lymphoma and
Waldenström macroglobulinemia. Immunoglobulin light chain can also rise to an uncommon
form of localized amyloid described for many different tissues including skin, breast, brain
and respiratory tract. In this form of AL-amyloidosis, the monoclonal protein is most likely
produced at the site of deposition by a clone of plasma cells, present locally in the tissue.

The organs most commonly involved are the heart and the kidney, either individually
or together with progressive amyloid deposition, inevitably leading to organ failure [5,7].

Familial or hereditary amyloidosis is less common than primary amyloidosis. It is caused by
an autosomal dominant mutation in the transthyretin (TTR) gene [4,5]. Most patients with
hereditary amyloidosis are characterized by the presence of peripheral somatic and autonomic
neuropathy and thus this type of amyloidosis have been named familial amyloid
polyneuropathy (FAP). FAP was long regarded as an endemic disease. Four well-known
endemic foci of this disease in the world are: Oponto in Portugal, the northern part of Sweden,
Arao and Ogawa in Japan. However, a number of FAP families have been found in non-
endemic areas, during the past 20 years [4,5].

Senile systemic amyloidosis (SSA) is a disorder affecting the elderly and is also
caused by transthyretin but an unmutated protein. SSA is associated with aging and affects
approximately 25% of patients over the age of 80. This type of amyloidosis mainly involves
the atria and less often is isolated in the aorta or involves the entire heart. SSA is not always a
benign condition and can result in heart failure, atrial fibrillation and other conduction
disturbances [8].

Secondary amyloidosis is characterized by amyloid fibrils, composed of the amino acid
terminus of the acute phase protein serum amyloid A (SAA). This form of amyloidosis occurs
mainly as a complication of chronic inflammatory disease such as rheumatoid arthritis,
tuberculosis, leprosy, chronic lung disease and chronic osteomyelitis. Typical organs involved
include the spleen, kidney and liver, before it finally presents with generalized vascular and
interstitial deposits. SAA is an apolipoprotein, one may speculate that amyloid occurs initially
in the spleen, liver and kidney because these are major sites of lipid and cholesterol metabolism. Approximately 45% of all systemic amyloidoses are secondary amyloidosis [4,5,9].

The systemic amyloidoses are life-threatening diseases, but early diagnosis and more specific treatment have given promising results in many cases. Treatment of an underlying metabolic abnormality demands an exact knowledge of the type of amyloidosis. The prognosis for patients with AL amyloidosis is poor. The median survival rate is 13 months without treatment and can be extended to 17 months with cyclic oral melphalan and prednisone therapy. Only 5% of patients survival longer than 10 years and at the time of definitive diagnosis 15 to 20% suffered from cardiac amyloidosis with heart failure. FAP is commonly treated with liver transplantation. FAP was long considered to be incurable, but liver transplantation is now a very promising therapy, because the liver produces most of the TTR in serum. Secondary amyloidosis is often treated with anti-inflammatory drugs [4,5,10].

Localized amyloidosis affects only one organ or tissue in the body in contrast to systemic amyloidosis. Age related localized amyloid is common in vascular tissue and the human aorta may be the most common site for this type of amyloid deposition. Two biochemically different forms of localized amyloid have been identified. The most common form of localized amyloid is aortic medial amyloidosis and it occurs in most people older than 60 years. The amyloid fibril protein is called medin (AMed). Medin is a 50 amino acid fragment of its precursor lactadherin, a glycoprotein expressed by mammary epithelium. Lactadherin is a 378 amino acid protein and its normal function is unknown [11,12]. Intimal amyloid is less common and is seen in association with severe arteriosclerosis. Based on a study of one patient with aortic intimal amyloid, the fibrilar protein was claimed to consist of a 69 amino acid residues long N-terminal fragment of apolipoprotein A1. However, trials to confirm this result have not been successful. Whether or not amyloid is involved in the pathogenesis of arteriosclerosis is not known [13, 14].

Alzheimer’s disease (AD) is also a form of localized amyloidosis characterized by cerebral cortical amyloid plaques. AD is the most common progressive neurodegenerative disorder in elderly people and is characterized by neuronal loss with the accumulation of senile plaques. The amyloid precursor is amyloid-β peptid (Aβ), a cleavage product from the larger amyloid-β precursor protein (AβPP). The APP gene is expressed in all major tissues but predominantly in the brain [15].
Diagnosis of amyloidosis of any type is achieved by examination of biopsy material from involved organ and tissue, where the amyloid substance stained with Congo red gives a characteristic green birefringence viewed in polarized light microscope. Following diagnosis, amyloid is classified either immunohistochemically or biochemically by extracting the fibril proteins from native or formalin-fixed amyloidotic tissue and submitting the protein to amino acid sequencing. Amyloid proteins from biopsy tissue can been typed by ELISA (enzyme linked immunosorbent assay), Western blot or immunohistochemistry. The most commonly used biopsy material for diagnosis of systemic amyloidosis is taken from subcutaneous fat, rectum, kidney or liver [4,5,9].

The aim of this study is to investigate the prevalence of amyloid in intima using immunohistochemistry and diagnose systemic amyloidosis by Sodium Dodecyl Sulfate Polyacrylamid Gel Electrophoresis (SDS-PAGE) based on Western blot analysis combined with specific amyloid fibril protein antibodies.

**MATERIALS AND METHODS**

**Electrophoresis and Western blot analysis**

*Material*

Subcutaneous fat biopsies were used for the typing of systemic amyloidosis.

*Antisera*

The antisera used were raised against amyloid fibril proteins or against synthetic peptides derived from amyloidogenic proteins [10]. The antibodies were diluted in TBS and a positive control for each antibody was used.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Amyloid type</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>A 126</td>
<td>AA</td>
<td>1:2000</td>
</tr>
<tr>
<td>A 1898</td>
<td>ATTR</td>
<td>1:5000</td>
</tr>
<tr>
<td>A 147</td>
<td>AL κ</td>
<td>1:5000</td>
</tr>
<tr>
<td>A 132</td>
<td>AL λ</td>
<td>1:50</td>
</tr>
</tbody>
</table>
**Electrophoresis and Western blot analysis**

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique for separating denatured proteins on the basis of charge (which is proportional to molecular weight) in an electrical field.

The biopsy sample was dissolved of sample buffer containing 20 mM dithiothreitol (DTT), and separated by a 16.5% gradient SDS-PAGE.

For Western blot analysis the proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia). The membrane was blocked with 3% dry milk in 0.1% TBS-Tween and incubated over night. The membrane was incubated with the primary antibodies in different dilutions, see table 2, for 1-2 h in room temperature. After washing, the membrane was incubated for 1 h with swine anti-rabbit HPR (DakoCytomation) diluted 1:10000 in TBS. Labeling was visualized by enhanced chemiluminescence (ECL) system (Amersham Pharmacia) according to the manufacture’s instructions.

**Immunohistochemistry**

**Material**

Aortic tissue was obtained at autopsy from 10 individuals, aged 58-94 years (8 males and 2 females). The material was formalin fixed and paraffin-embedded. 5 µm sections were prepared.

**Antisera**

Rabbit antisera A 179 was raised against synthetic peptides corresponding to amino acid 245-256 (within medin) of lactadherin [13].

**Immunohistochemistry**

Immunohistochemistry is a method for detection of antigens in tissue using antibodies. Immunohistochemistry was performed on deparaffinized sections. For antigenic retrieval the sections were incubated for 45 min with boiling 0,02 M sodium citrate buffer, pH 6.0. The sections were incubated with antiserum A 179:7 diluted 1:4000 in TBS in room temperature over night. For controls, the antiserum was replaced by null serum in the same concentration. After incubation with primary antibody, biotinylated goat anti-rabbit antibody (DakoCytomation) diluted 1:200 in TBS was used and incubated for 30 min. Then the sections were incubated with streptavidine-HPR (DakoCytomation) diluted 1:500 for 30 min. The reaction was visualized with diaminobenzidine (DAB) and stained with Mayers
hematoxylin for 30 sec. The sections were also stained with Congo red B solution diluted 1:10 for 20 min and then mounted.

**Light Microscopy**
The sections were stained in Congo red B solution, diluted 1:10 with Congo red A solution, for 20 min and was studied in polarized light for deposition of amyloid.

**RESULTS**

**Light Microscopy**
The presence of amyloid was demonstrated by the appearance of green birefringence from Congo red under polarized light.

![Figure 1. Amyloid in fat biopsy visualized by polarized light.](image)

**Typing of systemic amyloidosis**
Analysis was performed on Congo positive fat biopsies that were sent to the Rudbeck laboratory. Western blot analysis for clinical assessment has been used for the last three years in this laboratory. In figure 2 the Western blot analysis with an SAA antiserum has been performed and the results show a band in the lanes 1 and 2. Such bands were regarded as full length SAA and ignored in the interpretation of the immunoblotting results, which were only based on low molecular protein bands (< 20 kDa) since the amyloid protein consist of N-terminal fragment of SAA. Figure 3 show the Western blot analysis with a TTR antiserum and as seen the result, it reacts with patient materials in lane 1, which shows a strong immunoreaction and that means the patient has a TTR amyloidosis. Figure 4 and 5 show the Western blot analysis with antiserum against amyloid protein derived from κ type and λ type of immunoglobulin light chain. In figure 4, lane 2, a band was detected but the band probably was a contamination because immunoglobulins are common contents of several tissues.
Figure 5, lane 1, show a strong immunoreaction that is represented λ type of immunoglobulin light chain.

Figure 2. Lanes 1 and 2 contain extracts of fat biopsies from patients with unknown type of systemic amyloidosis. Lane 3 contains negative control and Lane 4 contains control material with SAA-derived amyloid.

Figure 3. Lane 1 contains extract of biopsy from patient with unknown type of systemic amyloidosis. Lane 2 contains negative control and lane 3 contains control material with transthyretin-derived amyloid.

Figure 4. Lane 1 contains control material with amyloid of AL-κ type. Lane 2 contains extracts of fat biopsy from patient with unknown type of systemic amyloidosis. Lane 3 contains negative control.
Prevalence of amyloid in intima

Microscope analysis of the immunohistochemistry revealed that intimal amyloid deposits were present in 5 cases (50%) of the specimens.

DISCUSSION

The systemic amyloidoses are usually life-threatening diseases due to the involvement of the heart and kidneys. Early diagnosis and more specific treatment have given promising results in many cases. Treatment of an underlying metabolic abnormality demands an exact knowledge of the type of amyloidosis [10]. The diagnosis of systemic amyloidosis is still based on tissue biopsy followed by staining of sections with Congo red and examination under polarized light. However, establishing the diagnosis on clinical impression alone is difficult due to the wide variety and often vague presenting signs and symptoms. In the early 1970s, Westermark and Stenkvist noted that skin biopsies of amyloidosis patients demonstrated amyloid deposits around subcutaneous fat cells and subsequently described the technique of fat pad aspiration [16]. Several studies have confirmed the value of subcutaneous fat biopsy in the diagnosis of systemic amyloidosis [10,16,17]. The aim of this study was to describe a method for typing of systemic amyloidosis using SDS-PAGE and Western blot analysis.

The Western blot analysis method that was described in this study makes it possible to subtype the most common types of systemic amyloidosis with use of subcutaneous fat tissue. This method is an important component of the clinical diagnostic evaluation where the type of amyloidosis may determine further evaluation and therapy of the patient. The greatest advantage of this method is the accessibility of the subcutaneous fat tissue, which obtained...
with minimal risk for the patient and it is a simple procedure [18]. Another advantage of fat biopsies is their relative robustness and resistance to autolysis. The advantages with the Western blot method include the limited equipment needed and that it is technically simple and fast. In our study we used in-house raised antisera, produced in rabbit by standard methods because commercially available antibodies often fail in analysis of amyloid fibril protein and thereby the sensitivity and reliability of the method is increased. Some of these antibodies are also used in immunohistochemistry [19]. There is a need for rapid, standard method for diagnosis of systemic amyloidosis. The Western blot method that was developed in Rudbeck laboratory and described in this study has been to be a rapid, sensitive and reliable method for diagnosis of systemic amyloidosis and could hopefully be a valuable method for diagnosis of amyloidosis also in other clinical laboratories.

We also investigated the prevalence of amyloid in intima by immunohistochemistry and found amyloid deposits in 50% of the cases. Amyloid deposits in the aortic intima are common in association with atherosclerosis and aging. Previous studies have show that the amyloid localized to the aortic intima may be a biochemical entity different from other form of localized amyloid. Intimal amyloid differed from medial amyloid (medin) both in morphologic characteristics and its association with atherosclerosis [20]. In one patient intimal amyloid characterized to be derived from apolipoprotein A1 although it is still not certain that it always has this biochemical composition. This patient, however, later turned out to carry a mutation in the apo A1 gene [13,14]. In our study we used an antiserum against medial amyloid (medin), which react specifically with medin but did not react with intimal deposits and then we could exclude medin. The questions still remained to be explained, which protein precursor caused intimal amyloid in ageing aorta? More research in this area is needed in order to hopefully found a answer.
REFERENCES


