ORIGINAL ARTICLE

Primary local orbital amyloidosis: biochemical identification of the immunoglobulin light chain κ III subtype in a small formalin fixed, paraffin wax embedded tissue sample

B Kaplan, B M Martin, H I Cohen, J Manaster, Y Kassif, U Rehany, A Livneh

J Clin Pathol 2005;58:539–542. doi: 10.1136/jcp.2004.022517

Background: Amyloidosis refers to a heterogeneous group of disorders associated with the deposition of chemically distinct amyloid fibril proteins. Precise determination of chemical amyloid type has diagnostic, therapeutic, and prognostic relevance. Although immunohistochemical techniques are used routinely to determine the amyloid type, the results can be negative or inconclusive, so that biochemical characterisation is often required. The development and application of new biochemical microtechniques suitable for examination of extremely small tissue samples is essential for precise identification of the deposited amyloid proteins.

Aims: To investigate biochemically the amyloid proteins present in a formalin fixed paraffin wax embedded orbital tissue from a patient with localised orbital amyloidosis in whom immunohistochemistry was not helpful in the determination of amyloid type.

Methods: Extraction of amyloid proteins from fixed tissue and their identification was carried out by a recently developed microtechnique. An extremely small tissue sample was dewaxed and extracted with formic acid. The extracted material was analysed using electrophoresis, western blotting, and amino acid sequencing.

Results: Biochemical examination of the extracted proteins showed the presence of immunoglobulin (Ig) derived amyloid proteins, which were composed of the N-terminal fragments of the Ig light chain κ III subtype (AL- κ III) (16, 8, and 3 kDa).

Conclusions: This is the first chemically proved AL case reported in association with primary localised orbital amyloidosis. The biochemical microtechnique used was useful in achieving a precise diagnosis of amyloid disease, in a case where the results of routine immunohistochemical examination of amyloid were inconclusive.

See end of article for authors' affiliations

Correspondence to:
Dr B Kaplan, Heller
Institute of Medical
Research, Sheba Medical
Centre, Tel Hashomer
52621, Israel; kaplanb@
sheba.health.gov.il

Accepted for publication
26 October 2004

Deposition of amyloid in the eye and its adnexal structures may occur as part of systemic amyloidosis or as a localised form. Local orbital amyloidosis is a rare condition, comprising only 4% of cases of local amyloidosis seen in the head and neck regions.¹ The pathogenic mechanisms leading to local tissue deposition of amyloid are not clear. Because amyloid deposits in orbital tissues are commonly infiltrated with lymphocytes, plasma cells, and foreign body giant cells, and because several authors have demonstrated local clonal proliferations of plasma cells, it has been suggested that locally secreted monoclonal immunoglobulins (Igs) are the direct precursors of the deposited amyloid proteins in the orbit.^{2–7} However, information on the precise chemical structure of amyloid proteins in the orbit is still lacking. Only in one of the approximately 30 reported cases of localised orbital amyloidosis was the deposited amyloid protein extracted and the amino acid sequence determined, thus providing direct evidence of a rare type of amyloid protein composed of IgG heavy chain constant (CH3) domain.⁸ Two reports showed an Ig light chain origin of the amyloid deposits (AL λ) using immunohistochemical methods,^{4,9} but these studies were not supported by direct chemical analysis of the deposited proteins. The lack of chemical examination of the amyloid proteins found in the orbit is probably related to the difficulty in obtaining sufficient amounts of unfixed biopsy material for protein extraction and purification by the established classic methods.^{8–10} Although immunohistochemical techniques are used routinely to determine the chemical nature of amyloid deposits,¹¹ in some instances the results are inconclusive or

negative, therefore requiring a more rigorous chemical analysis.

“Information on the precise chemical structure of amyloid proteins in the orbit is still lacking”

We present here a case of localised orbital amyloidosis, where the routine immunohistochemical techniques could not help provide information on the origin of the deposited amyloid. To obtain this information, the amyloid protein present in a formalin fixed, paraffin wax embedded diagnostic biopsy specimen was extracted and analysed by our recently developed biochemical microtechnique.¹² The immunochemical and chemical examination of the extracted protein allowed the identification of AL proteins of the κ III subtype (AL κ V-III), which had not been previously reported in association with localised orbital amyloidosis.

PATIENT AND METHODS

Patient

A 44 year old patient was first admitted to the hospital at the age of 21 because of cervical lymph node enlargement. The biopsy tissue examination was at that time interpreted as pronounced reactive lymphoid hyperplasia. Two years before our present study, a mass was detected in the left orbit after the patient complained of slight visual disturbances and local

Abbreviations: AL, immunoglobulin light chain derived protein; Ig, immunoglobulin

Figure 1 Haematoxylin and eosin stained section (original magnification, $\times 200$) of the orbital tumour tissue specimen showing deposits of amorphous pink material of amyloid with lymphocytes and foreign body multinucleated giant cells.

swelling. Histological examination of the removed mass showed a massive deposit of pink amorphous material mixed with lymphocytes, plasma cells, and foreign body multinucleated giant cells (fig 1). This amorphous material was Congo red and crystal violet positive, thus confirming the amyloid nature of this tumour. Immunohistochemical staining for κ and λ light chains showed polyclonal populations of plasma cells. The results of immunohistochemistry using the anti- κ and anti- λ antibodies were inconclusive in relation to the presence or absence of extracellular deposits of AL type amyloid. Immunohistochemical staining was negative for amyloid A. No clonal Ig heavy chain rearrangement was detected by polymerase chain reaction analysis of the paraffin wax embedded orbital biopsy material. Morphological and flow cytometry examinations of peripheral blood, bone marrow aspirate, and bone marrow biopsy found no evidence of monoclonal cell populations. In addition, no monoclonal spike was seen in serum/urine protein electrophoresis. Taken together, these findings excluded the presence of an underlying lymphoproliferative disease. Complete blood count, serum biochemical profile, and sedimentation rate were within normal ranges. Computed tomography of the brain was interpreted as normal. There were no clinical or laboratory findings indicating amyloid involvement of other organs. The diagnosis of localised orbital amyloidosis was made. The patient was, and still is, in good health after surgical excision of the mass.

Extraction of amyloid proteins

Amyloid extraction was performed by the procedure described previously.¹² Briefly, the formalin fixed, paraffin wax embedded orbital biopsy specimen was dewaxed at 60°C, washed in xylene, and rehydrated in decreasing concentrations of alcohol. The resulting tissue was homogenised in phosphate buffered saline, centrifuged, and the resulting sediment was suspended in formic acid overnight. The mixture was centrifuged and the supernatant was dried.

Electrophoretic analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using precast 20–25% polyacrylamide LongLife microgels (Gradipore, Frenchs Forest, Australia), as described previously.¹² The material recovered from 2.5–5.0 mg of fixed tissue was applied to each well, run with Tris

Figure 2 Electrophoretic and amino acid sequence analyses of amyloid proteins recovered from the formalin fixed, paraffin wax embedded orbital tissue. Proteins were transferred to a PVDF membrane and stained with Coomassie blue. The excised bands were subjected to N-terminal amino acid sequence analysis. Amino acids are indicated by the single letter code. Lane 1, molecular weight markers; lanes 2 and 3, tissue extracts.

HEPES buffer (Gradipore), and transferred on to Sequi-Blot PVDF membranes (BioRad, Hercules, California, USA) with a Gradipore LongLife Transfer buffer. Proteins were visualised by Coomassie blue R-250 staining.

Western blotting

The electrophoresed samples were transferred on to nitrocellulose (Schleicher and Schuell, Dassel, Germany) and immunostained with polyclonal rabbit antibodies to human Ig κ and λ light chains (Dako, Carpinteria, California, USA) as described previously.^{12–14}

Amino acid sequence analysis

The electrophoresed samples were transferred to Sequi-Blot PVDF membranes and stained with Coomassie blue. Protein bands were excised and subjected to amino acid sequence analysis as described previously.^{12–15}

RESULTS

In our case of localised orbital amyloidosis, routine immunohistochemical examination of the amyloid deposit was not helpful in determining the chemical nature of the protein. Therefore, we undertook a biochemical examination of the amyloid protein. Amyloid protein present in the formalin fixed, paraffin wax embedded orbital tissue was extracted using the microtechnique described above. First, the sample was dewaxed and rehydrated to yield about 15 mg of the tissue, which was subsequently extracted with formic acid and analysed electrophoretically. Three protein bands of approximately 3, 8, and 16 kDa were revealed with

Figure 3 Western blot analysis of amyloid proteins extracted from the formalin fixed, paraffin wax embedded orbital tissue. Proteins were immunostained using antibodies to (A) κ and (B) λ immunoglobulin light chains.

Coomassie blue staining (fig 2). These proteins showed no immunoreactivity with antibodies to Ig λ light chains (fig 3B) on western blot analysis, but they were immunostained with antibodies to Ig κ light chains (fig 3A). Strong immunoreactivity with antibodies to Ig κ light chain was also seen in a higher molecular weight region, and probably resulted from the presence of crosslinked proteins in the formalin fixed tissue.¹⁶ N-terminal sequence analysis of the 3, 8, and 16 kDa bands revealed the EIVLTQSPATLSVSP sequence, belonging to the variable region of the Ig κ III light chain. This sequence was found in each of these three Coomassie blue stained bands (fig 2).

DISCUSSION

Amyloidosis refers to a heterogeneous group of disorders characterised by the extracellular deposition of fibrillar Congo red positive proteins in various tissues of the body. Although amyloid proteins found in different clinical forms of amyloidosis share common morphological and tinctorial features, these proteins vary with respect to their primary structure (25 different types of amyloid proteins have been described so far) and extent of tissue/organ involvement (systemic versus localised). Amyloid deposition in the orbit may appear as a localised phenomenon or as a part of systemic amyloidosis. Amyloidosis in the orbit is thought to be associated with B cell dyscrasia, either benign or malignant.^{2 17 18} It is extremely important to differentiate between the local and systemic form, and between the primary and reactive forms of amyloidosis, and to determine precisely the chemical type of the deposited protein, because the prognosis and the treatment are based strictly on these distinctions. The diagnosis of primary localised orbital amyloidosis made here was supported by clinical and laboratory investigations, which excluded the presence of underlying B cell systemic disease. Routine immunohistochemical techniques were used to determine the type of the deposited amyloid protein, but unfortunately the results were inconclusive. Therefore, we focused on determining the chemical type of the amyloid using our recently developed biochemical microtechnique. The applied method allowed unequivocal identification of the amyloid type by showing the deposition of Ig light chain derived proteins (AL) in the orbit. In fact, the results of the western blot analysis of the extracted proteins agreed with the data obtained by the amino acid sequencing, where Ig fragments of κ III light chain variable domain were revealed.

"This technique may allow the biochemical analysis of amyloid proteins in a larger number of biopsy specimens than was previously possible, thus contributing to the precise diagnosis of amyloid diseases and to a better understanding of their pathogenesis"

We were also interested in elucidating the primary structure of the deposited amyloid because of the fact that only a few studies on localised ocular/orbital amyloidosis have characterised the amyloid proteins biochemically. These included identification of amyloid composed of heavy chain CH3 domain in the orbit,⁸ AL λ proteins in the eyelid,¹⁰ and lactoferrin type amyloid in the cornea.¹⁹ In these studies, the amyloid extraction and purification techniques were modified to overcome the difficulties related to the small quantities of tissue available. Our case posed an additional obstacle for biochemical examination because the small sample available in our study was formalin fixed and paraffin wax embedded. This sample was dewaxed and extracted using our new microtechnique, allowing recovery of amyloid proteins from small fixed biopsy specimens and their

Take home messages

- We report a microtechnique for the biochemical characterisation of amyloid proteins in amyloidosis that can be used with extremely small samples of paraffin wax embedded tissue
- This technique is useful in achieving a precise diagnosis of amyloid disease in cases where the results of routine immunohistochemical examination of amyloid are inconclusive
- Using this technique, we have demonstrated the first chemically proved case of immunoglobulin light chain derived protein associated with primary localised orbital amyloidosis

biochemical identification.¹² To the best of our knowledge, this is the first report in which the presence of AL deposits in a primary localised orbital amyloidosis was chemically proved.

Compared with other biochemical micromethods used previously for the identification of amyloid proteins in formalin fixed biopsy tissues,^{16 20} the microtechnique used here is simpler, less expensive, and suitable for the analysis of different types of amyloid proteins in extremely small biopsy specimens. This technique may allow the biochemical analysis of amyloid proteins in a larger number of biopsy specimens than was previously possible, thus contributing to the precise diagnosis of amyloid diseases and to a better understanding of their pathogenesis.

Authors' affiliations

B Kaplan, A Livneh, Heller Institute of Medical Research, Sheba Medical Centre, Tel-Hashomer, 52621, Israel and Tel-Aviv University, Sackler School of Medicine, Israel

B M Martin, Laboratory of Neurotoxicology, National Institute of Mental Health, Bethesda, 20892-1262 MD, USA

H I Cohen, Department of Pathology, Western Galilee Hospital, Nahariya Medical Centre, 22100 Israel

J Manaster, Haematology Unit, Western Galilee Hospital

Y Kassif, U Rehany, Department of Ophthalmology, Western Galilee Hospital

The patient gave full permission for this case to be published.

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ECHO

CA IV may be target antigen in autoimmune pancreatitis

Please visit the *Journal of Clinical Pathology* website (www.jclinpath.com) for a link to the full text of this article.

Japanese researchers have uncovered a serum antibody that may prove to be a marker for disease affecting multiple exocrine glands—like autoimmune pancreatitis (AIP) and Sjögren’s syndrome (SS). The target antigen—carbonic anhydrase isozyme IV (CA IV)—is commonly found in exocrine gland epithelia.

The antibody was significantly more common among patients with definite (27%) or probable (43%) AIP and SS (45%) than among those with pancreatic cancer (14%) or alcoholic chronic pancreatitis (13%), when compared with healthy controls in an ELISA screen with truncated recombinant CA IV antigen. The same trend occurred in a parallel screen with a synthetic peptide of CA IV. Reactivity of in vivo CA IV—the entire protein in its native configuration—remains to be confirmed, as does its potential role in pathogenesis.

Fifteen patients had definite AIP and 14 probable AIP; 15 had alcoholic chronic pancreatitis and 14 pancreatic cancer; 20 patients had SS; and there were 30 controls.

AIP shows lesions in other organs that are redolent of other diseases—SS in salivary glands; extrahepatic sclerosing cholangitis; and, occasionally, ulcerative colitis—with epithelial inflammation as the common feature. This suggests an autoimmune reaction to a common antigen. Previous studies have shown serum antibodies to CA I and II in idiopathic chronic pancreatitis and SS, though these do not cross react. This study looked for another cross reacting target antigen—and checked for serum antibody to CAs IV, IX, and XII expressed in pancreatic duct epithelia.

▲ Nishimori I, et al. *Gut* 2005;**54**:274–281.