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Classification of Amyloidosis by Immunohistochemical Method and Potassium Permanganate Reaction: Correlation Between Morphology, Chemical Types and Clinical Features

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=Abstract=

Systemic amyloidosis is a rare disorder, which usually occurs in aged people with a poor prognosis. Forty-five patients with amyloidosis were studied by light microscopy with Congo red staining, potassium permanganate reaction and immunohistochemistry. The biopsy sites were abdominal fat, rectum, lung, bone marrow, liver, pleura, duodenum, gallbladder, colon, oral cavity, larynx, thyroid and skin. The patients were classified according to immunohistochemistry and Congo red staining results after potassium permanganate treatment. Thus, 21 of 45 (46.7%) cases were classified as AA (amyloid A protein), and 24 (53.3%) cases were classified as AL (amyloid light chain) amyloidosis. This study suggests that the association of these two techniques, potassium permanganate reaction and immunohistochemical stain, is more reliable than clinical data alone in distinguishing between AA and AL amyloidosis.

Key Words: AL amyloidosis, AA amyloidosis, Potassium permanganate, Immunohistochemistry.

INTRODUCTION

Amyloidosis has been viewed as a relatively rare disorder, which occured in a variety of clinical symptoms, and was often discovered only at autopsy. Although it might develop in the absence of any recognizable disease process, its appearance was often preceded by long-standing inflammatory disorders such as tuberculosis, chronic osteomyelitis and rheumatoid arthritis (Gerz & Kyle, 1991). These observations gave rise to the concept of "primary" and "secondary" amyloidosis:the former tending to localize in the cardiovascular system and the latter in the kidneys, liver, spleen and adrenal gland. In both amyloidosis, small peripheral blood vessels were often involved were organ distribution overlaped considerably.

The ultrastructural characteristics of amyloids were first delineated in 1959 (Cohen & Calkins). It soon became apparent that an 80 to 100 Å nonbranching microfibrils were the major protein component of all amyloids. These microfibrils characteristically bind to Congo red dye, and yield a distinc-

tive green birefringent color under polarized light.

Over the years, many classifications have been proposed for amyloidosis. Recently, solubilization of amyloid fibrils permitted biochemical study to the amyloid substance and its amino acid sequence (Glenner, 1980). Two major fiber compositions were identified:fibers mainly composed of light chain immunoglobulins and tissue A components. These two types were designated as AL and AA type amyloidosis, respectively. Primary amyloidosis complicating multiple myeloma has been shown to be AL amyloidosis. Secondary amyloidosis with coexisting disease, i.e. familial Mediterranean fever, has been described as AA amyloidosis.

Romhanyi (1972) reported that amyloids in formalin-fixed, paraffin-embedded tissue sections could be subclassfied according to the sensitivity to trypsin digestion by potassium permanganate reaction. These study suggested that the sensitive amyloid to trypsin digestion was probably protein AA, the and the resistant amyloid was in all likelihood composed of protein AL.

The diagnosis of amyloidosis must be established histologically and is usually made by demonstrating deposits, which exhibit characteristic apple-green birefringence in Congo red stained tissue specimens when examined by polarized microscopy. The rectal submucosa has been the traditional biopsy site, with a reported 75% to 85% positive incidence of positive results in known amyloid disease (Gafni & Sohar, 1960; Kyle *et al*, 1966). Invasive biopsy of renal (Cohen, 1967), hepatic (Stauffer *et al*, 1961), splenic (Pasternack, 1974), endomyocardial, or bowel tissue, has consistently shown a high incidence of positive results; however, the biopsies of these sites in amyloid disease carried a risk of bleeding complication. On the other hand, less invasive biopsy of bone marrow (Krause, 1977) or skin (Rubinow & Cohen, 1978) has shown a considerably lower incidence of positive results, approximately 50%.

Westermark & Stenkvist (1973) described a method for the diagnosis of amyloidosis using abdominal fat aspiration biopsy. Amyloid deposits were noted within the connective tissue around the fat cells. Subsequently, Libby *et al* (1983) concluded that the technique matched or exceeded rectal biopsy in its high incidence of positive results, was easy to perform without any serious risks. This study described here analyzed a large series of aspirates from patients known to have amyloid disease and a series of aspirates sent to us for analysis by referring physicians.

Our study was undertaken to evaluate the diagnostic value of associating histochemical and immunohistochemical methods to distinguish AL and AA type amyloidosis on biopsy materials and to emphasize the value of aspirating abdominal fat as the first choice test for the diagnosis of systemic amyloidosis.

MATERIALS AND METHODS

All 66 biopsies in 45 patients were studied by light microscopy, potassium permanganate method, and immunohistochemical stain using anti-human amyloid A monoclonal antibody (DAKO, Denmark) at Keimyung University Dongsan Medical

Center from 1993 to 1996. Clinical and laboratory data were collected for clinical evaluation. The biopsy materials were examined by light and polarized microscopy to detect the presence of Congophilia and green birefringence.

Light microscopy

All biopsy specimens were fixed in 10% formalin and embedded in paraffin. Then 2-6 μm sections were cut with a knife. Standard stains were used. Amyloid deposits were detected with Congo red staining. Amyloid deposits have a distinctive apple green color when examined under polarized light. When sections were treated by potassium permanganate ($KMnO_4$) before Congo red staining, the disappearance of Congo red staining characterized AA amyloidosis and the persistence of staining favored AL amyloidosis (Van Rijswijk & Meusden, 1979; Wright *et al*, 1977).

Potassium permanganate method

The formalin-fixed, paraffin-embedded tissue sections were cut into 6 μm, deparaffinized in xylol to absolute alcohol and layered with 0.5% celloidin. They were rinsed in running tap water and allowed to air dry. The sections were covered with potassium permanganate (equal parts of 0.3% H_2SO_4 and 5% $KMnO_4$) for 2½ minutes; this solution was removed and replaced with 0.5% oxalic acid until the sections became clear. After rinsing thoroughly in distilled water, the paraffin boat was filled with a trypsin-phosphate solution (2.5 mg/ml in 0.15 M phosphate buffer) and incubated at 37℃ for 1½ hours. Following incubation, the sections were rinsed in tap water and stained with alkaline Congo red. For control purposes, two duplicate sections were processed to the potassium permanganate step. One slide was stained with Congo red; the other was incubated with buffer alone prior to being stained with Congo red. Following the Congo red step the paraffin rim was pulled away and the sections were rinsed in two changes of absolute alcohol to remove the celloidin. All Congo red-stained sections were examined using polarizing microscope as well as conventional microscope.

Immunohistochemical stain for anti-amyloid A

Sections, 4 μm thick, were cut from the formalin fixed, paraffin embedded specimens and deparaffinized routinely. They were treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The slides were placed in a 0.01 M citrate buffer and heated in a microwave oven at 700 W for 5 minutes two times. AA amyloid expression was detected by a standard immunohistochemical procedure of incubating the slides for 30 minutes with monoclonal mouse anti-human amyloid A (DAKO, Denmark) diluted in 1:50 in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). After three washes with PBS, the slides were incubated with secondary biotinylated rabbit anti-mouse antibodies (DAKO) (diluted 1:350) for 30 minutes. They were exposed to avidin biotin peroxidase complex (DAKO). Diaminobenzidine tetrachlororide (DAB) (Sigma, Dorset, UK) was used as the chromogen. Slides were counterstained with Mayer's hematoxylin. For negative controls, the primary antibody was omitted and the anti-amyloid A antibody was substituted with an

unrelated monoclonal antibody of the same concentration.

RESULTS

The mean age at diagnosis was 55 years, ranging from 20 years to 77 years. The median age for the AA type amyloidosis was 54.5 years and that for the AL type was 55.4 years (Table 1). There were 18 males and 27 females of which 11 males and 10 females were AA; 7 females and 17 females and were AL type amyloidosis. Congo red stain of amyloidosis shows orange green birefringence under polarized light microscope (Fig. 1-3). Congophilia of amyloid is lost in AA amyloidosis after pretreatment with potassium permanganate reaction (Fig. 3). Immunohistochemically stained tissue with anti-AA shows positive staining of amyloid in AA amyloidosis (Fig. 4-5).

Biopsy sites of the involved organ were as follows (Table 2): abdominal fat (Fig. 1) in 36 patients (14 AA and 22 AL type), rectal

Table 1. Age distribution of amyloidosis patients

	<20	20-29	30-39	40-49	50-59	60-69	70-79	>80	total
AA type	0	2	1	3	7	6	2	0	21
AL type	0	2	2	3	6	8	3	0	24
Total	0	4	3	6	13	14	5	0	45

Table 2. Biopsy sites and types in amyloidosis patients

	AA type	AL type	Total
Abdominal fat	14	22	36
Rectal mucosa	7	2	9
Kidney	6	1	7
Lung	2	0	2
Bone marrow	2	0	2
Liver	1	0	1
Pleura	1	0	1
Duodenum	1	0	1
Gallbladder	1	0	1
Colon	0	1	1
Oral cavity	0	1	1
Larynx	0	1	1
Thyroid	0	1	1
Blood vessel	0	1	1
Skin	0	1	1
Total	35	31	66

mucosa (Figure 2) in 9 patients (7 AA an 2 AL type), kidney (Figure 3) in 7 patients (6 AA and one AL type), lung in 2 patients (AA type), bone marrow in 2 patients (AA type), liver (Figure 4), pleura, duodenum and gallbladder in every AA amyloid patient and colon, oral cavity, larynx, thyroid, blood vessel and skin in each one AL patient. In 36 of the 45 patients with known amyloidosis, amyloid was identified in the abdominal fat. So, the accuracy of the abdominal fat aspiration biopsy was 80%. False positive was in 5 (11.1%) patients and false negative was in 4 (8.9%) patients.

Chief complaints of amyloidosis were similar to both AA and AL type (Table 3). Weakness or fatigue, and weight loss were the most frequent initial symptoms. Weight loss occurred in 14 patients. Edema, particularly in the periorbital and facial areas, was noted in 12 patients. Gastrointestinal symptoms, such as nausea/vomitting or diarrhea,

Table 3. Chief complaints in amyloidosis patients

	AA type	AL type	Total
Arthralgia	7 (%)	3 (%)	10 (%)
General weakness	7 (%)	7 (%)	14 (%)
Edema	6 (%)	6 (%)	12 (%)
GI symptom	2 (%)	2 (%)	4 (%)
Dyspnea	1 (%)	2 (%)	3 (%)
Hoarseness	0 (%)	1 (%)	1 (%)
Neck mass	0 (%)	1 (%)	1 (%)
Total	23	22	45

GI: gastrointestinal.

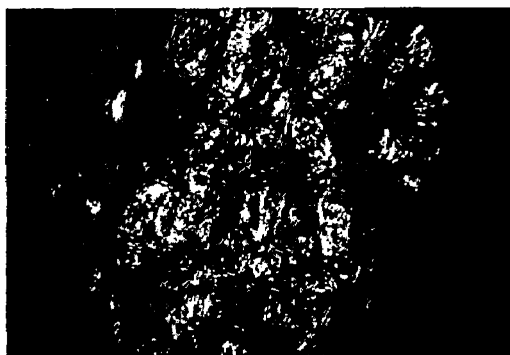

Figure 1. Light micrograph shows green birefringent staining when examined under polarized light. Congo-red stain, x100.

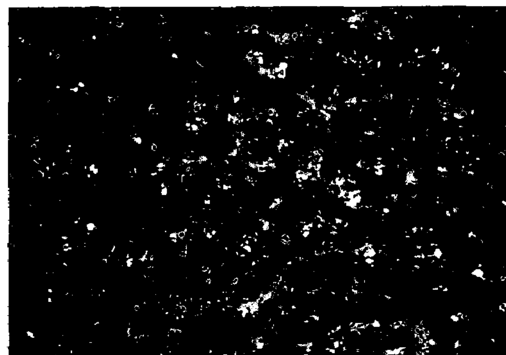

Figure 2. Light micrograph of the liver tissue shows green birefringent amyloid deposits scattered in the periportal areas under polarized light. Congo-red stain, x200.

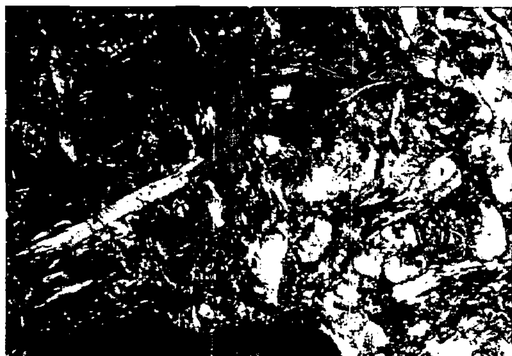

Figure 3. Abdominal wall biopsy.
A. Fat tissue fragments contain Congo red positive fibrils with green birefringence under polarized light.

Figure 3. Abdominal wall biopsy.
B. Section stained with Congo red after pretreatment with potassium permanganate. Congophilia of amyloid is lost.

Figure 4. Intestinal wall biopsy.
A. Positive Congo red staining of rectum.

Figure 4. Intestinal wall biopsy.
B. Immun-ohistochemical stain for anti-AA shows positive staining of amyloid in the lamina propria and blood vessel walls.

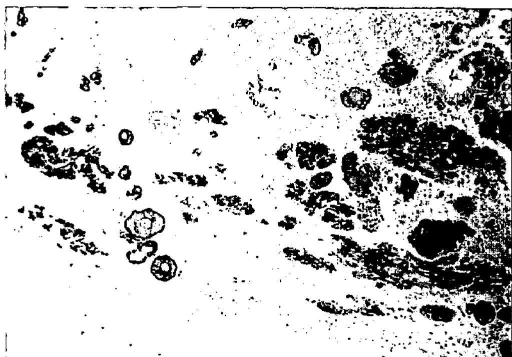

Figure 5. Immunohistochemical staining of the intestine.
A. Nodular deposits of AA amyloid.

Figure 5. Immunohistochemical staining of the intestine.
B. Brown reaction product is seen in the wall of medium-sized artery. Rabbit anti human AA.

were found in four patients. Dyspnea and edema were frequent in patients with congestive heart failure. Hoarseness or change of voice was noted in one patient.

DISCUSSION

Systemic amyloidosis is a rare disorder, which usually occurs in aged people. The mean age of onset was 65 years, and there was a slight male preponderance in a large series 182 patients of the primary type and 47 patients with myeloma-associated amyloidosis (Kyle & Greipp, 1984). In a report of 64 patients with secondary systemic amyloidosis, the median age of onset was 51 years in males and 64 years in females (Gerz & Kyle, 1991). In our study, the mean age of onset was 56.2 years which is consistent with other reports. Children of 12 and 14 years old with primary systemic amyloidosis have occasionally been reported (Pick *et al*, 1977; Hake *et al*, 1976), but these are extremely rare cases.

Distinguishing AA and AL type amyloidosis is important for therapeutic or investigative purposes. Clinical features are not always evident or sufficient for such classification. Definitive classification of different amyloid types has required biochemical analysis including amino acid sequence. However, this approach requires large amounts of fresh samples and special equipment for isolation, purification, and analysis. Thus, less complex methods, such as the potassium permanganate-trypsin treatment of Romhanyi (1972) have been limitedly used to identify AA protein in permanent sections. Immunofluorescence methods and enzyme-labeled antibody methods, using antiserum raised against different types of amyloid fibril proteins, have been used to differentiate amyloid types in tissue sections (Cornwell III *et al*, 1977).

The ability of amyloid, in formalin-fixed tissue sections, to retain staining affinity for Congo red and to exhibit dichroism under polarized light after prior incubation with trypsin or brief exposure to potassium permanganate varied according to the type of amyloid protein present (Wright *et al*, 1977). Amyloid predominantly composed of protein AA appeared to lose Congo red affinity and polarization characteristics following such treatment. These deposits actually appeared to dissolve during trypsin incubation. Although both histochemical methods yielded comparable results when applied to amyloid of known amino acid composition, the permanganate method was much less time consuming and yielded better preserved histologic details. Wright *et al*, (1977) supported these results and described a modification of the trypsin method that was simple to perform as a routine staining procedure and could be applied to even the smallest biopsy tissue. The method was reproducible, correlated well with amyloids of known amino acid composition, and could be employed in virtually any histology laboratory. The mechanism of potassium permanganate action on protein AA is, unknown but it probably results from altering the β-pleated sheet conformation and hence the loss of Congo red affinity. This denaturation apparently then renders the amyloid protein AA susceptible to trypsin digestion.

Immunohistochemistry plus $KMnO_4$ technique can provide differential diagnosis between AL and AA amyloidosis on biopsy

specimens, if the results of anti-AA antiserum by immunohistochemistry and of $KMnO_4$ test are undisputable and concordant. In some patients, it was not possible to determine the nature of amyloidosis by immunohistologic studies. In a few of these patients, the $KMnO_4$ test could not be made, maybe due to the small size of amyloid deposits on the fragment studied with this technique. Larger deposits could possibly have led to definite diagnosis. Application of this knowledge to an amyloidosis patient as encountered in the clinic, may be inhibited by several factors. The amounts of amyloid accumulating in most cases are too small to permit extraction, isolation and chemical characterization of the amyloid protein. Even if technically possible, the analytic procedures are sufficiently complex and expensive rendering their application to large groups of patients highly impractical. In some patients, AA detection or Congo red staining after $KMnO_4$ was only partially positive or negative. These patients were excluded in this report. In this connection it must be recalled that in amyloidosis, fragments of light chains in AL and tissue A component in AA amyloidosis are not the only components of the amyloid substance, and their relative importance can differ from patient to patient (Glenner, 1980); moreover, it has been shown that light chain-derived amyloidosis also often contains some AA protein. It must be stressed that even in the limited number of amyloid samples studied to date, it can be shown that a single focus of amyloid may contain more than one type of amyloid fiber protein. For example, most amyloid deposits which contain protein AA as the major fibrillar component also contain detectable amounts of protein AL (Glenner & Page, 1976). Conversely, in some amyloid deposits in which protein AL predominates small amounts of protein AA has also been identified (Husby *et al*, 1973); but this appears to be the exception rather than the rule. In a few patients, immunohistochemistry and $KMnO_4$ gave opposite results. These cases were also excluded in this report. Given the concurring results of amyloid tissue A component and $KMnO_4$ in most patients, a technical error or unreliable method is an unlikely reason to explain the results. In these patients, in addition to a possible heterogeneity of the amyloid substance, there may exist another type of amyloid, non-AA and non-AL. Indeed, if positive staining with anti-AA antiserum is specific for AA-containing amyloid, the persistence of Congo red staining after $KMnO_4$ treatment only signifies a non-AA amyloid substance.

In most cases, amyloidosis is diagnosed on biopsy but it is not possible to extract the abnormal substance. Thus, it is necessary to have simple techniques for characterizing amyloidosis on biopsies. Westermark (1971), and Westermark & Stenkvist (1971) described that interstitial fibrillar amyloid deposits frequently occur in subcutaneous fat, especially in the abdominal wall, in secondary (AA) amyloidosis. Our analysis of fat aspiration samples from patients known to have systemic amyloidosis demonstrated an overall accurate result rate of 80%, indicating a high degree of sensitivity when disease was known to be present. This supports the usefulness of fat aspiration as a diagnostic tool and compares favorably with the reported rate of 75-85% positive results for

rectal biopsy samples (Kyle *et al*, 1966; Gafni & Sohar, 1960). Subsequently, all these patients underwent biopsy of other organs that confirmed the presence of amyloidosis. False-positive results were 11.2% and false-negative results were 8.9%. The usefulness of this test in patients of unknown clinical status appears to rest on its excellent predictive value, i.e. a positive test result is indicative of amyloid disease.

In summary, to characterize AL or AA amyloidosis, we applied immunohistochemistry with amyloid A protein antisera, together with $KMnO_4$ technique on tissue biopsies and found that the association of these two techniques is very reliable in distinguishing between AA and AL amyloidosis. Subcuta-neous fat aspiration is recommended as the diagnostic procedure of choice in the evaluation of possible amyloid disease. A positive result may obviate further expensive and invasive diagnostic testing. Use of subcutaneous fat surpasses other biopsy sites because it requires no subspecial consultation nor coagulation testing prior to the procedure, and it is a virtually risk-free of procedure.

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