Microdeposits of amyloid in sclerocalcific heart valves: a histochemical and immunofluorescence study

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SUMMARY Amyloid associated with seven sclerotic and two normal aortic and mitral valves was studied. The sclerotic valve amyloid contained microfibrils with typical random orientation and a fibril width of 9.5–12.5 nm. The amyloid deposits demonstrated permanganate-resistant Congoophilia and contained the amino acid tryptophan. Immunofluorescence studies showed P-component in amyloid deposits of 6 of 7 valves, but none of the sclerotic valves contained amyloid fibril proteins of the AL (primary), AA (secondary), AE_t (medullary thyroid carcinoma) or AS_c (senile cardiac) types. Two non-sclerotic valves, removed from a patient with systemic amyloidosis, showed permanganate-sensitive Congoophilic amyloid deposits which contained amyloid fibril protein AA.

The deposition of amyloid fibrils in the heart valves is a well known occurrence in the primary (AL), myeloma-associated (AL) and senile (AS_c) forms of systemic amyloidosis.¹ Recently, a fourth type of valvular amyloidosis has been reported independently from four laboratories:^{2–5} a localised form of amyloid deposition appearing exclusively and with high incidence in sclerotic and particularly in sclerocalcific valves. The deposits are small and restricted to the areas of scarring and calcification, and their incidence does not correlate with the age of the patients. This form of amyloid is apparently of dystrophic type, but a biochemical marker of the amyloid fibrils has not yet been found. The purpose of this study was to characterise the morphological and chemical nature of dystrophic valvular amyloid.

Material and methods

TISSUES

Heart valves containing amyloid were selected by the macroscopic iodine test. One sample of each positive and negative valve was fixed in 10% formalin and embedded in paraffin. Six μ m sections were then stained with alkaline Congo red, and amyloid was defined by its selective apple green birefringence in polarised light.

Eight mitral and four aortic valves with the following characteristics were studied: seven sclerocalcific amyloid-laden valves; one sclerocalcific mitral valve without amyloid; one mitral and one aortic valve of normal structure, containing small deposits of amyloid from a patient with systemic reactive (secondary) amyloidosis; one completely normal mitral valve; and one completely normal aortic valve. Details of the tissues are provided in Table 1.

An additional specimen of each valve was snap frozen directly, kept at -20°C , washed in cold 0.15 M phosphate-buffered saline (PBS) at pH 7.2, fixed in ethanol and embedded in paraffin.⁶ Six μ m sections were deparaffinised⁶ just prior to conducting histochemical or immunofluorescence studies. Initially, sections made from both frozen and paraffin blocks were studied, but since no differences were observed with the two procedures, all

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Abbreviations used in this paper are in part derived from guidelines adopted at the Third Symposium on Amyloidosis held in Povo de Varzim, Portugal in September 1979: AS_c = amyloid fibril protein from senile cardiac amyloid; AS_oA = senile aortic amyloid; IAA = isolated atrial amyloid; HPA = human prealbumin; AA = amyloid fibril protein A; A λ I, A λ IV, A λ VI immunoglobulin light chain amyloid fibril proteins of the λ I, λ IV and λ VI subgroups; AE_t = amyloid fibril protein from medullary carcinoma of the thyroid; AP = amyloid P-component.

Table 1 Histological findings of heart valves

Tissue no	Sex age (yr)	Valve	Histology	Amyloid*
1	F 22	Mitral	Non-sclerotic	—
2†	M 55	Mitral	Non-sclerotic	— (AA)
3	F 28	Mitral	Chronic rheumatic disease	—
4	F 51	Mitral	Sclerosis and fused commissure, aetiology unknown	+
5	M 49	Mitral	Chronic rheumatic disease	+
6	F 57	Mitral	Chronic rheumatic disease	+
7	F 52	Mitral	Sclerosis and calcification, aetiology unknown	++
8	F 56	Mitral	Sclerosis, aetiology unknown	+
9	M 46	Aortic	Non-sclerotic	—
10†	M 55	Aortic	Non-sclerotic	— (AA)
11	F 51	Aortic	Chronic rheumatic disease	++
12	M 65	Aortic	Calcified bicuspid valve.	+

*Amyloid determined by alkaline Congo red method.

†Tissues no 2 and no 10 were derived from the same patient.

‡Amyloid fibrils detected by electron microscopy.

subsequent studies were carried out with paraffin sections only.

ELECTRON MICROSCOPY

Two sclerotic mitral valves and two sclerotic aortic valves, all containing amyloid deposits, were fixed in 1% buffered osmium tetroxide, stained with uranyl acetate and embedded in Epon. Thin sections were stained with lead acetate, then examined and photographed on a Philips 201 electron microscope.

HISTOCHEMISTRY

Potassium permanganate treatment of sections prior to alkaline Congo red staining was performed by the method of Wright *et al.*⁷ Tryptophan was detected by the desoxymethylaminobenzene (DMAB) method.⁸ Fluorescence Congo red was performed as described below.

ANTISERA

Antisera to various amyloid fibril proteins were raised in rabbits and absorbed to specificity as previously described.^{9,10} Non-immune rabbit serum was used as a control. Rabbit antihuman prealbumin (HPA) and goat anti-P component (AP) were purchased from Calbiochem-Behring and Atlantic Antibodies Inc, respectively.

IMMUNOFLUORESCENCE

Frozen and paraffin sections were studied by indirect immunofluorescence.⁹ Since sclerotic tissue displayed strong autofluorescence, tissue sections were stained with Congo red prior to incubation with antisera, in order to suppress the background

fluorescence. This procedure was carried out by immersing sections in 0.05% Congo red (in 50% ethanol) for 3 s followed by washing in distilled water. Potassium hydroxide destaining was not used. Slides were then immersed at room temperature in PBS for 15 min, counterstained with 5–10 μ l of appropriate antiserum dilution (generally $1/16$ or $1/32$), washed and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or rabbit anti-goat IgG. This fluorescence Congo red method allowed the initial identification of amyloid by its green birefringence in polarised light and by its bright red fluorescence through a green absorption filter (Zeiss No 487715) in UV light. Amyloid identified in this way could then be examined for specific green immunofluorescence by switching directly to an FITC filter (Zeiss No 487711) using a twin-lamp Zeiss photomicroscope III.

Results

ELECTRON MICROSCOPY

Fibrils with characteristic ultrastructure of amyloid were found in the sclerotic areas of two valves of the four studied. One mitral (no 7) (Fig. 1) and one aortic (no 11) valve contained abundant microfibrils (9.5–12.5 nm width) with typical random orientation. Some fine granular material blending with the fibrils and a few collagen fibres were observed in the same areas. One sclerotic mitral valve (no 8) contained very few small fibrils and one sclerotic aortic valve (no 12) did not show any fibrils in the sections studied.

HISTOCHEMISTRY AND IMMUNOFLUORESCENCE

The results of the histochemical and immunofluorescence studies are presented in Table 2. All amyloid deposits tested contained tryptophan. Congo red staining of amyloid associated with sclerotic valves was resistant to permanganate treatment but, as expected, amyloid of the AA type was permanganate-sensitive.

Anti-AP antiserum did not react with normal valves or the sclerotic mitral valve without amyloid. All amyloid-containing valves studied with anti-AP gave a positive reaction, except for one mitral valve (no 6). The reaction of amyloid with anti-AP was strong at the periphery of the amyloid deposits and showed a linear pattern within the deposits (Fig. 2). Vessel walls were strongly positive, whereas some collagen fibres reacted weakly.

Anti-SAA, an antiserum previously shown to react specifically with amyloid of the AA type,⁹ reacted strongly with amyloid of tissues no 2 (Fig. 3a, 3b, 3c, 3d) and no 10, but failed to react with other heart valves. Antisera specific for amyloid

Fig. 1 Electron micrograph of amyloid fibrils in mitral valve no 7. Note collagen fibre (arrow) × 60 000.

Table 2 Histochemical and immunofluorescence reactions of heart valves

Tissue No	Histology	Histochemistry		Immunofluorescence staining with antisera to							
		KMnO ₄ *	DMAB	AP	SAA	AN	ANIV	ANVI	AScl	HPA	AE _t
Mitral valves											
1	Normal			-							
2	Normal + amyloid (AA)	S	+	+	+		-	-		-	-
3	Sclerotic only			-							
4	Sclerotic + amyloid	R	+	+	-		-	-	-	-	-
5	Sclerotic + amyloid	R		+	-		-	-		-	-
6	Sclerotic + amyloid	R	+	-	-		-	-		-	-
7	Sclerotic + amyloid	R	+	+	-		-	-		-	-
8	Sclerotic + amyloid	R	+	+	-		-	-		-	-
Aortic valves											
9	Normal			-							
10	Normal + amyloid (AA)	S	+	+	+		-	-		-	-
11	Sclerotic + amyloid	R	+	+	-	-	-	-	-	-	-
12	Sclerotic + amyloid†	R	+		-	-	-	-		-	-

*R=permanganate-resistant; S=permanganate-sensitive.
†Insufficient tissue available to study for P-component.
DMAB=desoxymethylaminobenzene.

Fig. 2 Amyloid deposits in sclerotic mitral valve no 8. (a) Congo red fluorescence reaction. Original magnification $\times 250$. (b) Immunofluorescence reaction with anti-AP antiserum. Original magnification $\times 250$.

Fig. 3 Amyloid deposits in the fibrosa of non-sclerotic mitral valve no 2. (a) alcoholic Congo red reaction in ordinary light $\times 250$. (b) in polarised light $\times 250$. (c) Congo red fluorescence reaction utilizing green absorption filter (Zeiss no 487715) $\times 300$. (d) immunofluorescence reaction with anti-SAA antiserum utilizing FITC filter (Zeiss no 487711) $\times 300$.

fibril proteins A λ I, A λ IV, A λ VI, AS α and AE α were non-reactive with all amyloid deposits tested. Anti-HPA was similarly free of reactivity.

Discussion

The localised amyloid deposits associated with sclerotic heart valves have several characteristics

which are common to most forms of amyloid studied thus far. The deposits demonstrated typical permanganate-resistant birefringence in polarised light when stained with Congo red. To date, permanganate sensitivity has been observed only in amyloid of the AA type⁷ and in amyloid associated with seminal vesicles.¹¹ The ultrastructure of amyloid microfibrils showed typical random orienta-

tion with a fibril width of 9.5 to 12.0 nm. These fibrils are somewhat larger than those reported in other forms of amyloid, but are similar in size to those recorded recently in other studies of sclerotic heart valves^{2,4} and in senile plaques associated with Alzheimer's disease.¹²

This type of amyloid contained tryptophan, an amino acid which has thus far been found in all types

of amyloid except those associated with insulinoma, pancreatic islets,¹¹ medullary carcinoma of the thyroid,¹³ senile cardiac amyloid of the isolated atrial type,¹⁴ seminal vesicles¹¹ and some forms of cerebral amyloid.¹⁵ In addition, dystrophic amyloid contained P-component (AP), a pentagonal substance which has been found to be associated with all types of amyloid studied to date. The peripheral

pattern of AP associated with dystrophic valves has been observed in other types of localised amyloidosis.¹⁶ P-component is also present in vessel walls, a finding consistent with observations that this substance binds to the microfibrillar mantle of elastic fibres in the skin and blood vessels of normal adults.¹⁷ The absence of reactivity to anti-AP of the amyloid deposition in one of the mitral valves (case 6) is not understood, since neither the clinical nor the histological findings in this patient differed significantly from those of other patients with amyloid positive sclerocalcific valves.

Sclerotic heart valve amyloid appeared chemically distinct from amyloid containing fibril proteins AA, AL (λ I, λ IV, λ VI), AE_I and AS_Q. These results confirm and extend the recent study of Iwata *et al*⁵ on amyloid deposits in heart valves. Using the peroxidase-antiperoxidase method, these authors concluded that dystrophic valvular amyloid lacked fibril proteins AA and AL. Since it is known that antisera produced to whole immunoglobulin light chain may fail to detect λ or κ light chain fragments in amyloid, specific antisera produced from three subclasses of amyloid fibril protein AL were used in these studies. The lack of reaction with anti-HPA indicates that the amyloid of sclerotic heart valves differs from the generalised form of senile cardiac amyloid. The latter form of amyloid has been shown to contain the fibril protein AS_Q, which possesses structural¹⁸ and immunological¹⁹ similarity to HPA.

Autofluorescence of amyloid deposits is a well known phenomenon, especially following formalin fixation. However, with the exception of mature plaques of senile dementia,²⁰ ethanol-fixed fresh or frozen tissues generally show little or no background fluorescence. Sclerocalcific heart valves, on the other hand, showed a high degree of autofluorescence, presumably related to the presence of scar tissue. This problem was eventually overcome by using fluorescence Congo red prestaining and specific FITC immunofluorescence on the same tissue sections. This method has proved useful for locating small foci of amyloid in a background of dense fibrous tissue prior to assessing immunofluorescence reactivity.²¹

The discovery of a dystrophic form of heart valve amyloidosis raises the possibility that the same type of amyloid deposition could be associated with other degenerative lesions. The uniform presence of amyloid in the aging aorta^{21,22} suggests a mechanism of amyloidogenesis similar to that associated with atherosclerosis.²² The presence of amyloid in calcified porcine bioprotheses implanted in humans (Goffin *et al*, unpublished observation) may represent yet another example of amyloid deposition as an integral process in the development of, or the

response to, degenerative change.

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