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Proteoglycans in haemodialysis-related amyloidosis

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Abstract Changes in extracellular matrices of articular tissue, intervertebral discs and systemic organs in patients with haemodialysis-related amyloidosis were investigated by immunohistochemical and biochemical examination of proteoglycans. Increased staining for chondroitin sulfate (CS) was detected in the amyloid deposits of all patients, ranging from early to advanced stages. Degenerative tissue changes around early-stage amyloid deposits in the intervertebral discs also showed positive staining for CS. Heparan sulfate (HS) was detected in amyloid deposits, especially in the synovial membrane. Biochemical analysis of connective tissues containing amyloid supported the immunohistochemical studies; CS was the major glycosaminoglycan species in these tissues, accounting for 55–81% of the total glycosaminoglycans. Although previous studies have stressed the importance of HS in amyloidogenesis, the present study showed that CS, which increased significantly in articular tissues associated with mechanical stress, also has a close relationship with amyloidogenesis.

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Introduction

In recent years, a newly established form of systemic amyloidosis, seen frequently in patients on long-term haemodialysis(haemodialysis-related amyloidosis), has attracted increasing attention [19,40]. The major constituent protein of this amyloid was identified as β_2-microglobulin (β2M) on the basis of N-terminal sequence analysis [11], and its molecular weight was calculated to be 11,000 Da, which is similar to that of native β_2M. Pathologically, this type of amyloid deposition has been observed to show preferential organ distribution, such as in the synovium and ligaments, rather than in the visceral organs. The reason underlying this predisposition remains unknown.

After pathological studies of systemic tissues, including the intervertebral discs of autopsy cases of patients with long-term haemodialysis, we reported previously that amyloid deposition in the cervical discs occurs in the early years of haemodialysis and precedes clinical symptoms, such as carpal tunnel syndrome and destructive arthropathy [24]. Since the range of spinal movements is largest at the cervical region, intervertebral discs at this site receive severe mechanical stress in daily life [41],and it was thus suggested that tissue changes caused by such stress may accelerate deposition of β2M amyloid in the discs and the periarticular tissues of major joints.

It seems reasonable to speculate that changes in major extracellular matrix components such as proteoglycans, caused by mechanical stress to the discs and joints, might bear a close relationship to β2M amyloid deposition. The present immunohistochemical and biochemical studies were therefore performed on patients with β2M amyloidosis in a wide range of disease stages to investigate changes in the proteoglycans intimately associated

with amyloid deposition. Connective tissues in the degenerative lesions of pre-deposition stage were also investigated.

Materials and methods

This study was performed using autopsy and surgical operation specimens of 44 long-term haemodialysis patients with or without amyloid deposition who had been diagnosed at the Department of Pathology, Toranomon Hospital, Tokyo, from 1976 to 1993. Clinical profiles of these patients and specimen details are summarized in Table 1. In Toranomon Hospital, haemodialysis had been performed using mainly cuprophane membrane.

Fifteen patients who had been on regular haemodialysis for 10 years (nos. 1–15 in Table 1) showed marked nodular amyloid deposition in synovial and ligament tissues. Inflammatory cell infiltration, mainly composed of macrophages, was occasionally observed around the deposited amyloid in these cases, which were assessed to be in advanced stages. Synovial and ligament tissue, removed surgically because of the carpal tunnel syndrome or destructive arthropathy, was processed to frozen blocks and paraffin blocks for diagnosis and immunohistochemical analysis. The efficiency of both frozen and paraffin methods for the present immunohistochemistry was also compared directly. In 3 patients (nos. 1, 5, 10), the quantity of the resected tissue was enough for biochemical analysis, and fresh amyloid-rich periarticular ligament and synovial tissues (1–2 g) were also taken. In the other 12 patients, the quantity of the resected samples was not enough for biochemical

Table 1 Summary of cases and tissue details for haemodialysis-related amyloidosis (*CGN* Chronic glomeluronephritis, *NS* nephrotic syndrome, *DM* diabetes mellitus, *SLE* systemic lupus erythematosus, *PYN* pyelonephritis, *Syn* synovium of carpal tunnel, *Lig* ligament around the knee joint, *Disc* cervical intervertebral disc, *Par* paraffin-embedded specimen, *Fro* frozen specimen, *For* formalin fixation, *Ace* acetone fixation)

No. of cases	Age	Sex	Period of dialysis	Reason for dialysis	Examined tissue	Tissue processing
Haemodialysis-related amyloidosis patients in advanced stage						
1	81	M	10	DM	Lig	Fro/Ace
2	60	M	11	PCK	Syn	Fro/Ace
3	81	M	11	CGN	Syn	Fro/Ace
4	64	M	12	CGN	Syn	Fro/Ace
5	62	M	13	CGN	Syn	Fro/Ace
6	61	M	14	CGN	Syn	Fro/Ace
7	47	M	15	CGN	Syn	Fro/Ace
8	37	M	17	CGN	Syn	Fro/Ace
9	73	M	18	CGN	Syn	Fro/Ace
10	69	M	20	CGN	Lig	Fro/Ace
11	50	M	22	CGN	Syn	Fro/Ace
12	60	F	22	CGN	Syn	Fro/Ace
13	42	M	23	CGN	Syn	Fro/Ace
14	50	F	25	CGN	Syn	Fro/Ace
15	51	F	25	CGN	Lig	Fro/Ace
Haemodialysis-related amyloidosis patients in early stage						
16	76	M	1Y7M	CGN	Disc	Par/For
17	41	M	2Y5M	CGN	Disc	Par/For
18	83	M	2Y5M	NS	Disc	Par/For
19	79	M	3Y2M	DM	Disc	Par/For
20	62	F	3Y3M	Gout	Disc	Par/For
21	65	M	4Y5M	DM	Disc	Par/For
22	56	F	4Y7M	CGN	Disc	Par/For
23	65	M	4Y8M	DM	Disc	Par/For
24	71	M	5Y7M	DM	Disc	Par/For
Haemodialysis-related amyloidosis, systemic						
25	82	M	16Y	CGN	Heart, Stomach	Par/For
26	66	F	16Y	CGN	Heart, Stomach	Par/For
27	37	M	18Y	CGN	Heart, Stomach	Par/For
28	59	M	18Y	CGN	Heart, Stomach	Par/For
29	72	M	19Y	CGN	Heart, Stomach	Par/For
Haemodialysis patients without amyloid deposition						
30	58	M	1M	NS	Disc	Par/For
31	68	M	1M	DM	Disc	Par/For
32	71	M	1M	DM	Disc	Par/For
33	38	F	2M	SLE	Disc	Par/For
34	73	M	3M	NS	Disc	Par/For
35	75	M	3M	NS	Disc	Par/For
36	76	M	5M	CGN	Disc	Par/For
37	66	M	5M	Unknown	Disc	Par/For
38	81	M	6M	Unknown	Disc	Par/For
39	64	M	6M	DM	Disc	Par/For
40	57	M	8M	PYN	Disc	Par/For
41	54	M	11M	CGN	Disc	Par/For
42	65	M	1Y1M	DM	Disc	Par/For
43	44	F	2Y1M	Unknown	Disc	Par/For
44	37	F	2Y5M	SLE	Disc	Par/For

analysis (less than 1 g) and only the immunohistochemical study was performed.

Study of the tissues in early and predeposition stages allowed assessment of the temporal relationship between changes in extracellular matrix and amyloid deposition. In 9 autopsied patients whose history of haemodialysis was less than 5 years (nos. 16–24), the degree of amyloid deposition located in the degenerated annulus fibrosus of the cervical discs was mild, if present, and inflammatory reactions were rarely observed around the amyloid. The shortest dialysis period for which the deposition of β2M amyloid was detected was 1.6 years. These 9 cases were assessed to be in an early stage of β2M amyloidosis. The duration of haemodialysis of 15 autopsied patients (nos. 30–44) without detectable amyloid deposition was less than 3 years. Changes in the extracellular matrix of cervical discs from these 15 pre-deposition-stage cases were also investigated.

In five autopsied patients (nos. 25 to 29) whose history of haemodialysis was over 16 years, amyloid deposition was also observed in the visceral organs. In general, small vessel walls of the heart and smooth muscle layers of the digestive tracts are most frequently involved in β2M amyloid deposition [6]. Changes in these organs were therefore compared with those in the periarticular tissue and discs.

Specimens taken at autopsy, such as discs, synovium, heart and stomach, were fixed in 10% formalin solution and routinely processed to paraffin blocks. Frozen fresh specimens could not be taken from these early-stage cases or systemic amyloidosis cases because the quantity of the deposited smyloid in each intervertebral disc or visceral organ was small. Paraffin-embedded specimens were also cut serially at 3 or 6 μm in thickness and deparaffinized. Sections cut at 6 μm were stained with Congo red according to the method of Puchtler et al. [28]. Amyloid deposition was confirmed histologically by positive staining for Congo red and characteristic green birefringence under polarized light. Sections cut at 3 μm were subjected to haematoxylin-eosin and immunohistochemical staining to demonstrate the presence of β2M and various kinds of proteoglycans. Foci of positive staining in each case were compared with the site of amyloid deposition. To check the present cases for amyloid heterogeneity, immunostaining for amyloid A protein (AA), κ-light chain, λ-light chain and prealbumin (DAKOpatts, Copenhagen) was also performed [36]. In the present haemodialysis-related amyloidosis cases, amyloid deposition showed positive staining only for ß2M and never showed a positive reaction for AA protein, κ-light chain, λ-light chain or prealbumin.

Monoclonal antibodies and polyclonal antisera used in the present study are listed in Table 2. Monoclonal antibodies HepSS-1(anti-heparan sulfate glycosaminoglycan chain), MAB1948 (anti-perlecan core protein), which are specific for heparan sulfate (HS) proteoglycan, reacted only with frozen sections. The reactivity of ND OG1, which is specific for hyaluronic acid (HA), was weaker in paraffin-embedded tissue sections. Pre-treatment with trypsin was needed for β2M staining in paraffin-embedded tissue sections. Tracheal cartilage was stained as a positive control for chondroitin sulfate (CS), ΔDi-0S, 4S, 6S disaccharides (non-reducing end disaccharides which remain after chondroitinase ABC digestion) and keratan sulfate (KS). Umbilical cord was stained as a positive control for HA; skin tissue was utilized for dermatan sulfate (DS). Kidney tissue acted as the positive control for HS. As negative controls, sections were incubated with normal serum.

Immunohistochemistry

Frozen sections were fixed in acetone for 5 min and subsequently immersed in methanol containing 0.3% (v/v) H_2O_2 for 30 min. They were washed with PBS, incubated in either normal horse or goat serum (DAKO) for 10 min, depending on the species in which the biotinylated antibody had been raised, and then the primary antibody incubation was performed. Paraffin-embedded sections were laid on poly-L-lysine-coated slides, and deparaffinized. Digestion by 0.1% trypsin for 20 min at 37°C was performed, where required. Subsequently, sections were incubated in methanol containing 0.3% (v/v) H_2O_2, washed in PBS, and incubated in normal serum, followed by reaction with primary antibodies for 2 h at room temperature in humidified chambers. Excess antibody was removed by washing with PBS, and the bound antibodies were labelled with biotinylated anti-mouse, anti-rabbit immunoglobulin and streptavidin-biotin complex (DAKOpatts). After three additional washes, bound peroxidase was developed with 0.02% diaminobenzidine (Sigma) at pH 7.6 in 0.05 M Tris buffer plus 0.015% H_2O_2. Immunoreactivity was classified into 4 grades: (-); no reaction, (+-); questionable or weakly positive reaction, (+); moderately positive reaction, (++); strongly positive reaction.

Biochemical analysis

Biochemical analyses were performed on amyloid-rich tissues (1–2 g) from β2M amyloidosis patients. The tissues were finely minced and extracted with 10 ml 4M guanidine hydrochloride and 50 mM sodium acetate, pH 6.0, at 4 °C overnight. Cartilage was carefully removed from the examined specimen, if present, and washed in the saline before homogenization to avoid contamina-

Table 2 Antibodies used in the present study

Monoclonal or polyclonal antibodies: designation of the antibody and description of its specificity	Dilution	Source	Reference
β_2 Microglobulin (poly)	1:50	Dakopatts, Copenhagen Denmark	[13]
Chondroitin sulfate, CS56 GAG chain	1:200	Seikagaku Kogyo, Tokyo Japan	[2]
Chondroitin sulfate, MO-225 D unit chain	1:100	Seikagaku Kogyo	[42]
ΔDi-0S, 1-B-5, unsulfated CS following chondroitinase ABC	1:100	Seikagaku Kogyo	[9]
ΔDi-4S, 2-B-6, 4 sulfated CS	1:100	Seikagaku Kogyo	
ΔDi-6S, 3-B-3, 6 sulfated CS	1:100	Seikagaku Kogyo	
Hyaluronic acid, ND OG1	1:10	Serotec, Oxfrod, UK	
Heparan sulfate, MAB1948 core protein of perlecan	1:200	Chemicon, CA, USA	[15]
Heparan sulfate, HepSS-1 GAG chain	1:200	Seikagaku Kogyo	[20]
Keratan sulfate, 5-D-4	1:100	Seikagaku Kogyo	[8]
Dermatan sulfate, 6-B-6	1:1000	Seikagaku Kogyo	[35]

Table 3 Immunohistochemical data of haemodialysis-related amyloidosis cases (15 cases in advanced stage, frozen sections, acetone fixation). *ND* Not done, *CS* chondroitin sulfate, *GAG* CS56, type D; MO-225, *HS* heparan sulfate, *Core* MAB1948, *GAG* HepSS-1, *ΔDi-OS* chondroitin disaccharide, *ΔDi-4S* chondroitin 4-sulfate disaccharide, *ΔDi-6S* chondroitin 6-sulfate disaccharide, *HA* hyaluronic acid, *KS* keratan sulfate, *DS* dermatan sulfate

No. of cases	CS		HS		ΔDi-0S	ΔDi-4S	ΔDi-6S	HA	KS	DS
	GAG	Type D	Core	GAG						
1	+	+	±	±	−	+	+	±	±	±
2	ND	++	ND	−	−	±	+	−	±	+
3	++	±	++	+	±	+	+	−	±	±
4	ND	+	ND	±	±	+	±	−	±	+
5	+	+	++	±	±	+	+	−	±	±
6	+	±	+	±	ND	ND	ND	ND	ND	ND
7	+	±	+	±	ND	ND	ND	ND	ND	ND
8	ND	+	ND	−	±	+	+	−	±	±
9	++	+	+	±	+	+	++	±	+	±
10	+	+	±	±	−	±	+	±	+	±
11	ND	+	ND	−	±	±	+	−	±	+
12	++	+	+	±	ND	ND	ND	ND	ND	ND
13	+	+	+	−	±	±	+	−	±	±
14	ND	+	ND	±	±	+	±	−	±	+
15	+	+	−	−	ND	ND	ND	ND	ND	ND

tion by the articular fluid. Insoluble materials were removed by centrifugation. Tissue extracts were then dialysed extensively against H_2O at 4 °C and measured for the total protein content using PROTEIN-GOLD (Integrated Separation Systems) with bovine serum albumin as standard. Samples were digested with Benzonase (60 units/ml) in 0.1 M Tris, 0.1 M acetate, pH 7.3, at 37 °C for 2 h, followed by papain (10 μg/ml) digestion 45 °C for 18 h in the same buffer. Digested samples were applied to Q-Sepharosed column (2 ml bed volume) equilibrated with the same buffer as used for enzyme digestion. The column was step-eluted with 4 M guanidine HCl, 0.05 M sodium acetate, pH 6.0 (4 ml), after extensively washing with 8 M urea, 0.25 M sodium chloride, and 0.5% CHAPS, pH 6.0. Samples were extensively dialysed against H_2O and analysed for glycosaminoglycan content. Concentrations of glycosaminoglycans were measured by dye precipitation assay using Safranin O [21] with CS (Calbiochem) as a standard. Glycosaminoglycans were identified by their susceptibility to specific glycosaminoglycan-degrading enzymes (chondroitinase ABC from *Proteus vulgaris*, chondroitinase ACII from *Arthrobacter aurescens*, heparitinase from *Flavobacterium heparinum* and keratanase from *Pseudomonas* species). Quantities of specific glycosaminoglycans were measured by comparing the concentration of intact glycosaminoglycan (Safranin O precipitable) before and after digestion with specific glycosaminoglycan-degrading enzymes. Composition of CS and HA disaccharides was determined by an HPLC procedure using PA-03 column (YMC) run in an isocratic condition in 16 mM sodium phosphate, pH 7.0, as described [37]. Average molecular sizes of glycosaminoglycans were estimated by Superose 6 Chromatography in 4 M guanidine HCl, 0.05 M sodium acetate, 0.5% Triton X-100, pH 6.0, as described before [43]. Approximately 200 μg of glycosaminoglycans was injected into the column and fractions of 0.4 ml were collected. Glycosaminoglycan content of each fraction was measured by the Safranin O procedure as described above. Recovery of glycosaminoglycans throughout the purification procedures was generally greater than 70%.

Table 4 Biochemical data of haemodialysis-related amyloidosis cases (3 cases in advanced stage)

Sample	1	5	10
Protein (μg)	18	24	34
Glycosaminoglycan (mg)	2	1	3

Glycosaminoglycan composition (% of the total glycosaminoglycan) and molecular weights (*HA* hyaluronic acid, *CS* chondroitin sulfate, *HS* heparan sulfate, *KS* keratan sulfate)

GAG/sample	1	5	10
HA	4	5	3
CS	81	55	78
HS	[a]	13	−
KS	4	10	5
Molecular weight of major GAG, kDa	50	50	61.38[b]

[a] Trace
[b] Minor component

Disaccharide composition of HA and CS (percent of the total disaccharide generated by chondroitinase ABC digestion). *ΔDi-HA* Hyaluronic acid disaccharide, *ΔDi-0S* chondroitin disaccharide, *ΔDi-6S* chondroitin 6-sulfate disaccharide, *ΔDi-4S* chondroitin 4-sulfate disaccharide

Disaccharide/sample	1	5	10
ΔDi-HA	4	8	4
ΔDi-0S	7	10	10
ΔDi-6S	40	12	47
ΔDi-4S	49	70	39

Results

Immunohistochemical results using frozen periarticular tissues from patients with advanced haemodialysis-related amyloidosis are summarized in Table 3. In these advanced cases, the results of the study using frozen sections and paraffin sections were almost identical except for HS (HepSS-1, MAB1948) and hyaluronic acid (HA) (ND 0G1). For HS and HA, a positive reaction was detected only with frozen sections. Regardless of the location of amyloid deposits, such as synovial membrane, the

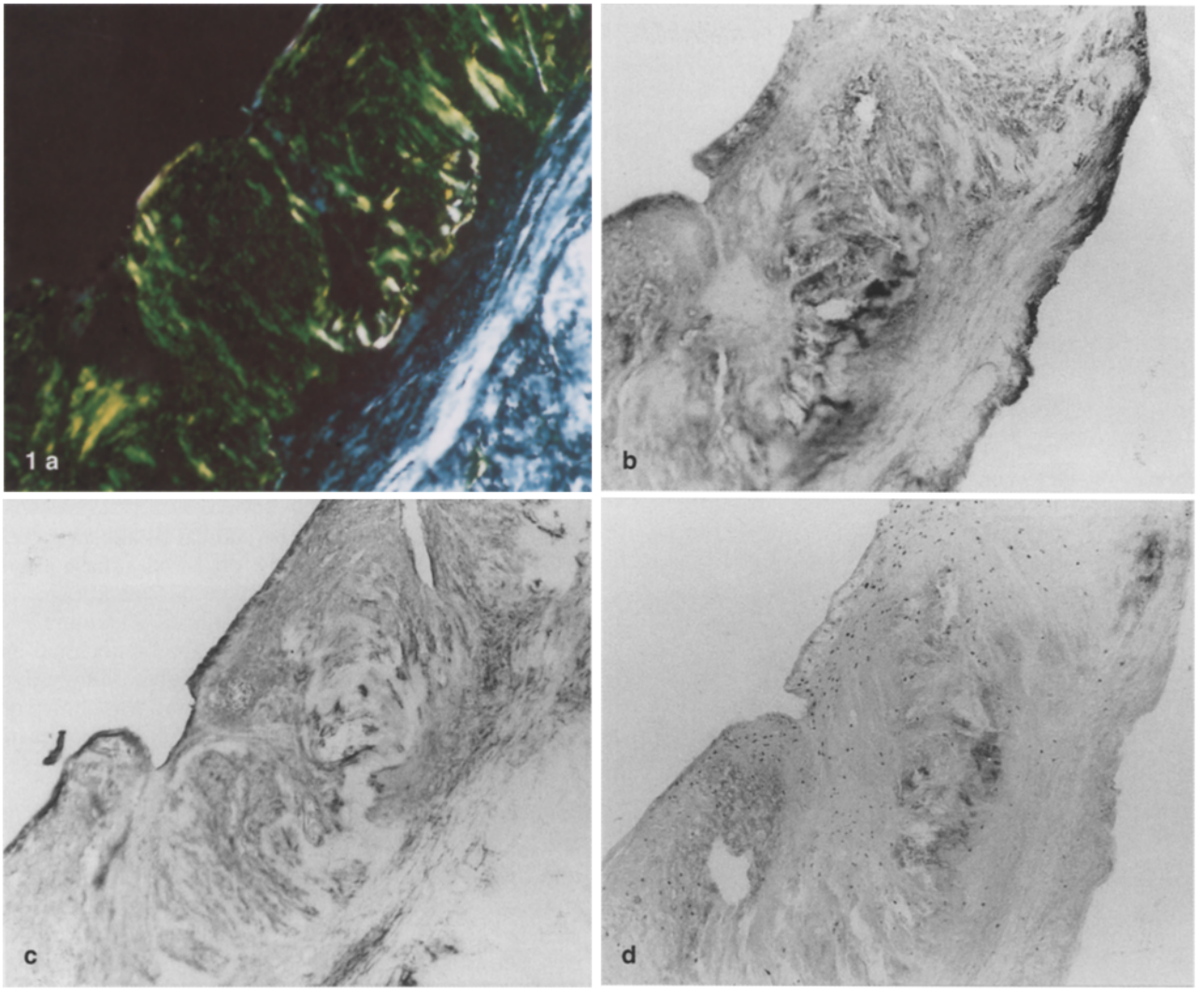
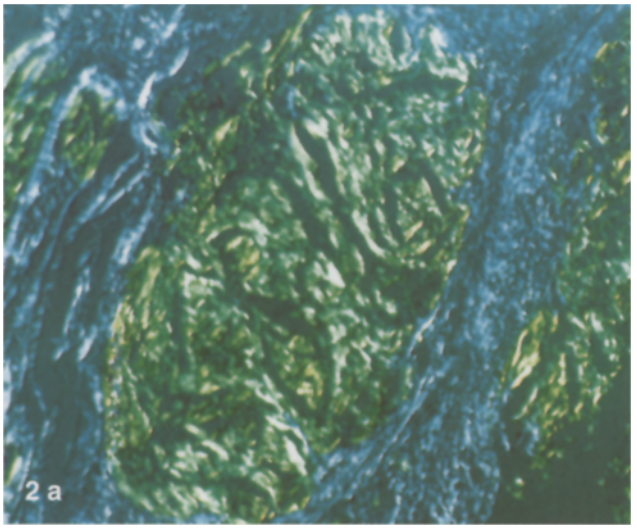
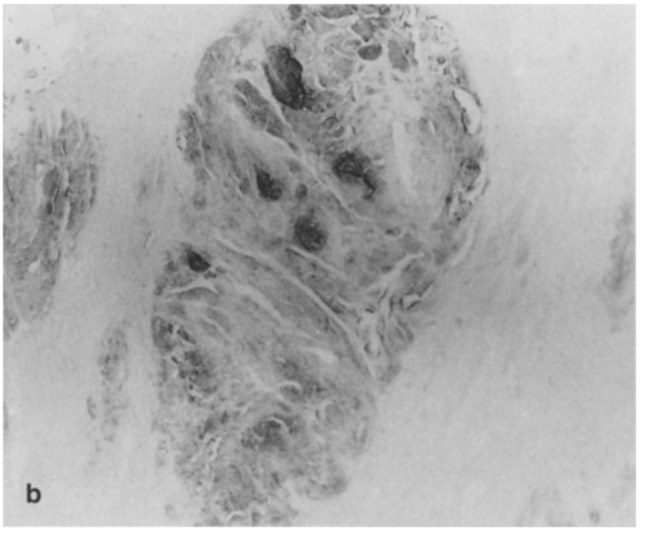


Fig. 1a–d Advanced haemodialysis-related amyloidosis (case 9, dialysis period: 19 years), synovial membrane, frozen sections. **a** Congo red staining (×25). **b** Immunostaining of CS, CS56 (×25). **c** Immunostaining of HS, MAB1948, anti-core protein of HS proteoglycans (perlecan; ×25). Band-like amyloid deposits in the synovial membrane are positively stained for CS and HS core protein. Stainability for these antibodies is almost equal. **d** Immunostaining of HS, HepSS-1, anti-GAG chain of HS (×25). Stainability is weak in comparison with MAB1948 (×25)

connective tissue of the synovial interstitium, and the collagen bundles of the ligament, moderate-to-strong reaction for CS antibody (CS56, MO-225) was detected at the amyloid deposits (Figs. 1b, 2b). Areas stained positively by CS were generally in accordance with the area stained positively by Congo red and β2M. In synovial connective tissue, a positive reaction for CS-56 antibody was also detected in the interstitium around the amyloid deposits; however, reactivity was generally much weaker than in amyloid deposits. In cases with severe inflammation, amyloid deposits were fragmented and replaced by marked infiltration of inflammatory cells composed mainly of macrophages. In these cases, the fragmented amyloid deposits and surrounding interstitium showed almost the same positive reactivity for CS. Reaction to MO-225, CS disaccharide D unit common to antigenic CS, was weaker than that of CS56, but a positive reaction in the surrounding interstitium was rarely observed with MO-225. In the amyloid deposits, especially in the synovial membrane, chondrocyte-like round mesenchymal cells with abundant, clear cytoplasm, surrounded by basophilic mucoid substance were occasionally observed (Fig. 3a). Amyloid deposits in the synovial membranes, especially around the mesenchymal cells, showed strong positive staining for CS (Fig. 3b). With regard to the disaccharides after the digestion of chondroitinase ABC, the reaction to ΔDi-4S and ΔDi-6S disaccharides was generally stronger than that of ΔDi-0S disaccharide (chondroitin).

According to the monoclonal antibody MAB1948, which reacts to the core protein of the HS proteoglycan (perlecan), amyloid deposits in the synovial tissue of most cases showed moderate positive staining (Fig. 1c); however, stainability for MAB1948 ranged case by case. In Fig. 2 amyloid deposits show an almost negative reaction. In cases 1, 10 and 15, amyloid deposits in the collagen bundles of the ligament showed only a weak positive





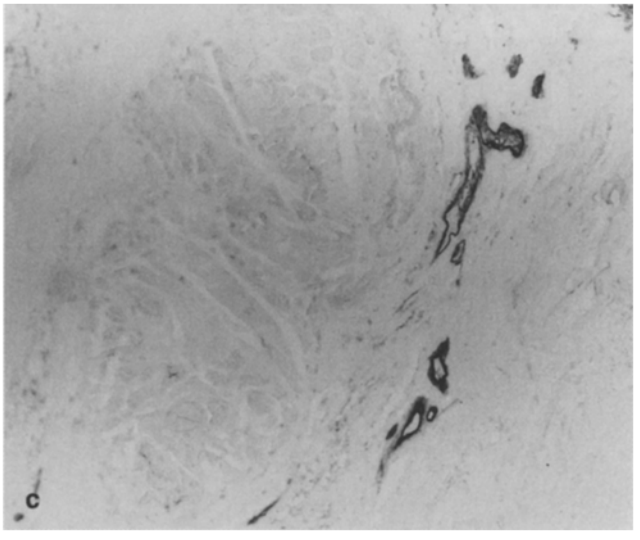


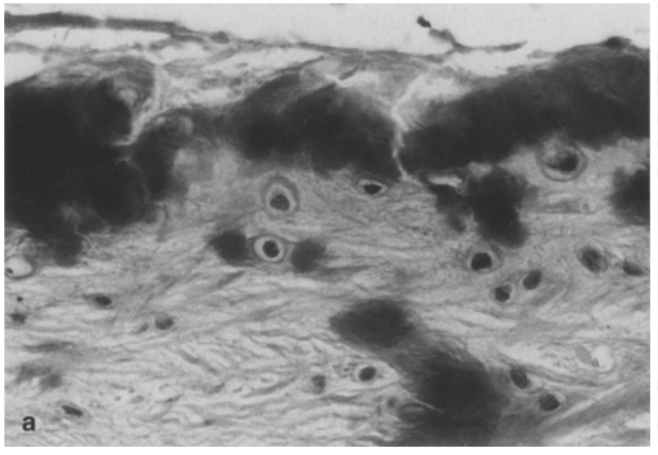
Fig. 2a–c Case 9 (same case as Fig. 1), synovial interstitium, frozen sections. **a** Congo red staining (×25). **b** Immunostaining of CS, MO-225 (×25). **c** Immunostaining of HS, MAB1948 (×25). Amyloid nodules in the synovial interstitium are positively stained for CS, but in this site stainability for HS is almost negative whereas small vessel walls are positively stained

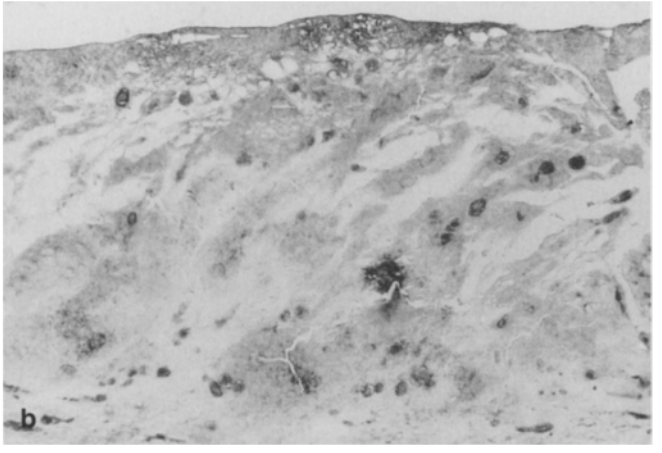
reaction. In the amyloid-rich synovial tissue, an increase of small vessels was usually observed. These normal small vessels and normal synovial membrane also showed mild-to-moderate positive staining for MAB1948. However, according to the monoclonal antibody HepSS-1, which reacts to the glycosaminoglycan chain of the HS, clearly positive staining was shown only in one case (no. 3), and in the other cases the reaction was generally weak (Fig. 1d).

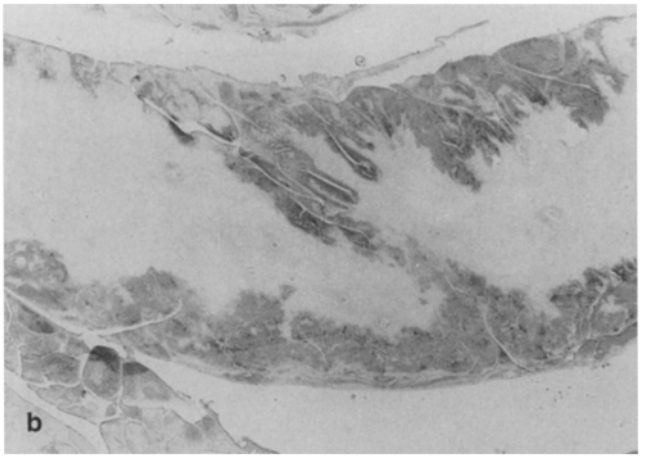
HA was weakly stained in the amyloid deposits in the synovial membrane and synovial interstitium. DS and KS showed generally weak positive reactions in the amyloid, but only at the same levels or weaker than in the surrounding tissue.

The results of biochemical analysis using amyloid-rich periarticular connective tissues are summarized in Table 4. CS content ranged from 55 to 81% of the total

Fig. 3a, b Advanced amyloidosis (case 8, dialysis period: 17 years), synovial membrane, paraffin-embedded sections. **a** Haematoxylin and eosin staining (×40). Band-like amyloid deposits are located on the synovial membrane. Chondrocyte-like mesenchymal cells with clear and abundant catoplasm are observed around the amyloid. **b** Immunostaining of CS, MO-225 (×25). Amyloid deposits and chondrocyte-like mesenchymal cells show positive staining for CS







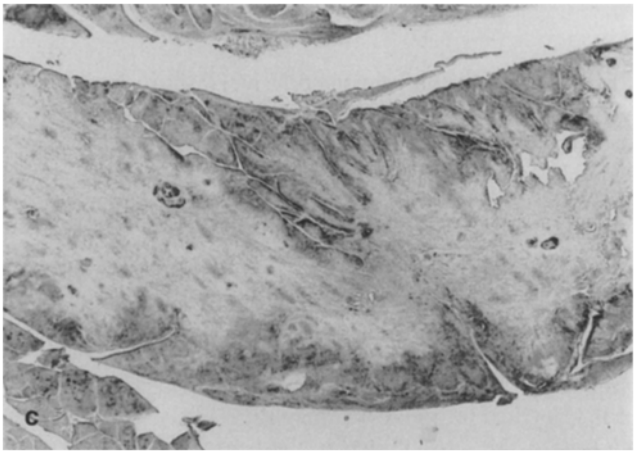
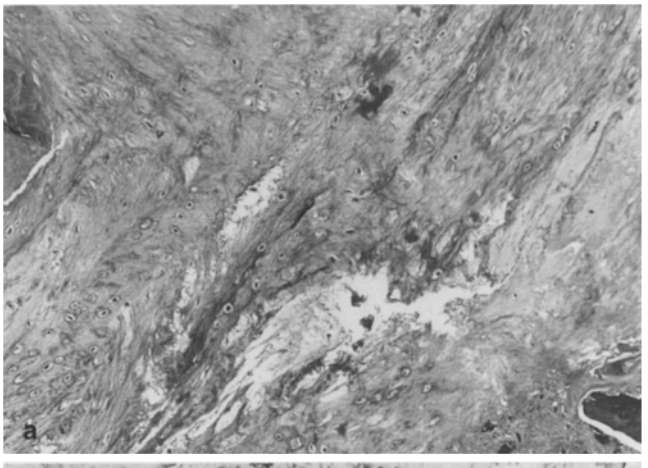
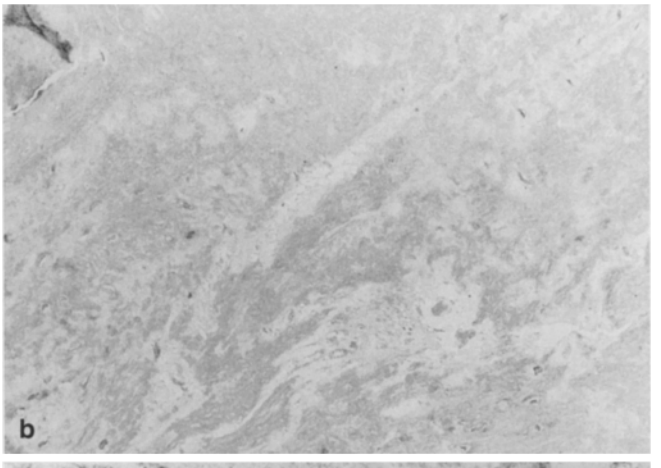
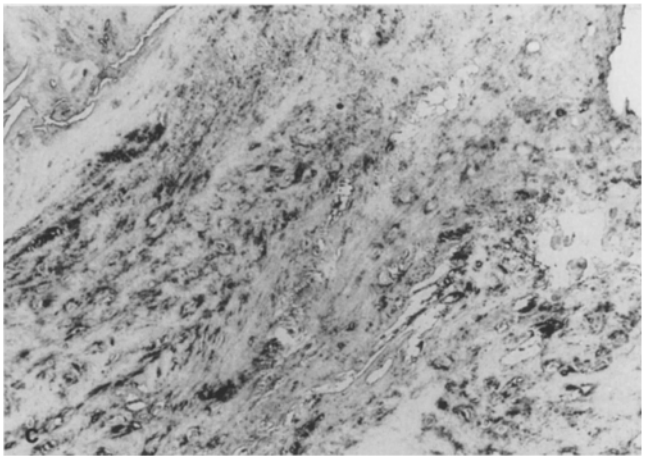


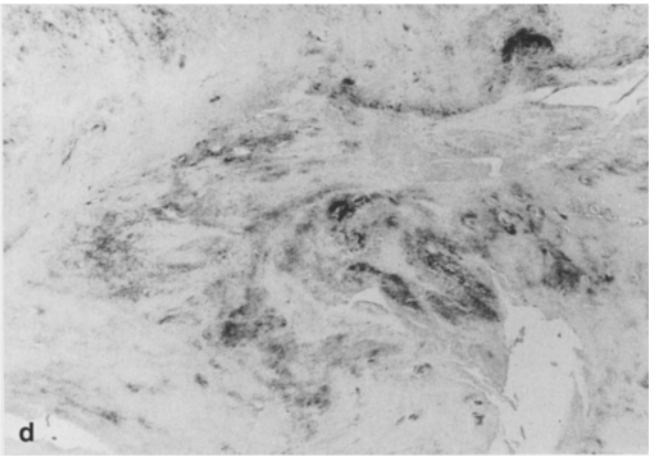
Fig. 4a–c Early-stage amyloidosis case (case 18, dialysis period: 2 years, 5 months), cervical disc tissue, paraffin-embedded sections. **a** Congo red staining (×25). **b** Immunostaining of ß2M (×25). **c** Immunostaining of CS, MO-225 (×25). Small amyloid deposits along the degenerated fissure, which are positively stained for ß2M, show positive reaction for CS

Fig. 5a–d Degenerative lesion around the amyloid deposition in the cervical vertebral disc (case 18, dialysis period: 2 years, 5 months). **a** Haematoxylin and eosin stain (×25). Around the amyloid deposits along the fissure, various kinds of degenerative change, such as interstitial oedema and chondrocyte proliferation, are observed. **b** Immunostaining of ß2M (×25). ß2M amyloid is not detected in the degenerative area. **c** The same site as **a**. Immunostaining for CS, MO-225 (×33). Degenerative change lesions show a strong positive reaction for CS. **d** Pre-deposition-stage case (case 42, dialysis period: 1 year, 1 month), cervical disc tissue, paraffin-embedded sections. Immunostaining of CS, MO-225 (×25). Degenerative foci with fissure formation oedema and chondrocyte proliferation also show positive staining for CS









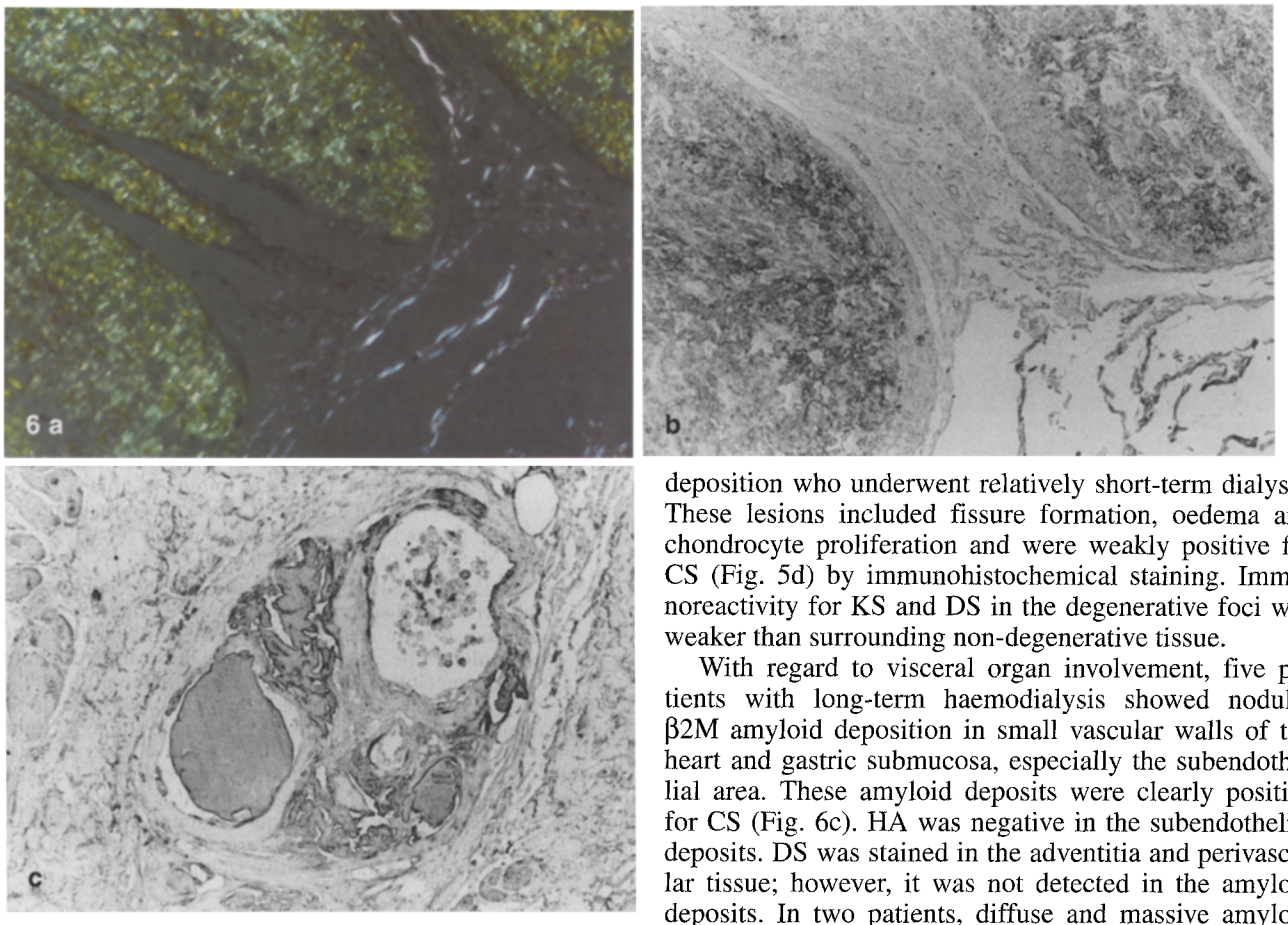


Fig. 6a–c Systemic amyloidosis case (case 29, dialysis period: 19 years), gastric wall (**a**, **b**) and heart (**c**) tissue, paraffin-embedded sections. **a** Congo red staining (×25). **b** Immunostaining of CS, CS56 (×25). Diffuse amyloid deposits in the proper muscular layer show positive staining for CS. **c** Immunostaining of CS, MO-225 (×40). Subendothelial nodular deposits in the small vessel wall are positively stained for CS

glycosaminoglycan. HS was detected in one case, and the content was 13%. The HA content ranged from 3 to 5% and of KS from 4 to 10%. The disaccharide composition of CS and HA after the digestion of the chondroitinase ABC showed ΔDi-4S and ΔDi-6S to be the major components, compatible with the results of the immunohistochemical study.

In the early-stage amyloidosis patients whose dialysis period ranged from 1.6 to 5.6 years, CS staining was positive at almost all sites of amyloid deposition (Fig. 4c). Amyloid deposition was observed mainly along degenerative fissures in the annulus fibrosus and was accompanied by focal proliferation of chondrocytes and focal interstitial oedema. CS was also positively stained in the degenerative lesions around the amyloid deposits (Fig. 5c). No HA proteoglycans could be detected in the amyloid deposits associated with degenerated disc tissues.

Degenerative changes in cervical discs such as those seen in patients with the early-stage amyloidosis were also occasionally observed in patients without amyloid deposition who underwent relatively short-term dialysis. These lesions included fissure formation, oedema and chondrocyte proliferation and were weakly positive for CS (Fig. 5d) by immunohistochemical staining. Immunoreactivity for KS and DS in the degenerative foci was weaker than surrounding non-degenerative tissue.

With regard to visceral organ involvement, five patients with long-term haemodialysis showed nodular β2M amyloid deposition in small vascular walls of the heart and gastric submucosa, especially the subendothelial area. These amyloid deposits were clearly positive for CS (Fig. 6c). HA was negative in the subendothelial deposits. DS was stained in the adventitia and perivascular tissue; however, it was not detected in the amyloid deposits. In two patients, diffuse and massive amyloid deposition was seen in the smooth muscular layers of the gastrointestinal walls, and this was also positively stained for CS, as seen in the small vessel walls (Fig. 6b). As for visceral organ involvement, HS (MAB1948 and HepSS-1) was not tested, because fresh frozen specimens could not be obtained in the present study.

Discussion

Previous biochemical studies have identified several different proteins as major constituents of the various kinds of amyloid that meet the same criteria, that is, Congophilia and green birefringence under polarized light, a distinctive fibrillar ultrastructure and a cross-β X-ray diffraction pattern. It has been well documented that the anatomical distribution of β2M amyloid is unique with preferential deposition in periarticular tissues and intervertebral discs, as other types of amyloidosis show characteristic distribution of amyloid deposition according to their constituent proteins. However, it is difficult to explain the mechanism underlying such a characteristic distribution of β2M amyloid only on this basis.

A glycoprotein called amyloid P component and extracellular matrix materials such as proteoglycans are known to co-exist around the amyloid fibrils [3–5, 10, 16, 17, 23, 26, 27]. The major component of the in-

creased acidic glycosaminoglycans in experimentally induced AA amyloidosis was revealed to be HS [18], which was found in association with other basement membrane components, such as laminin, fibronectin and type-4 collagen [22]. Co-deposition of HS proteoglycans in amyloid deposits has also been confirmed for human AA and AL amyloid, β-protein amyloid in Alzheimer's disease, and prion amyloid in the Creutzfeldt-Jacob disease [18, 33, 34], while Shinoi et al. have reported that Alzheimer's β-protein amyloid is closely associated with CS proteoglycan [30]. In our laboratory, positive staining for HS in the AA and AL amyloid deposits was confirmed, and positive staining of CS was not detected or was only weak (unpublished data). Ultrastructual observation using cationic reagents, such as Ruthenium red and Cuprolinic blue, showed the HS proteoglycans to be mainly distributed on the surface of experimentally induced AA amyloid fibrils [32].

In haemodialysis-related amyloidosis, co-deposition of acidic glycosaminoglycans in amyloid tissue has been reported, and their intimate distribution on the surface of β2M amyloid fibrils has been demonstrated ultrastructually by periodic acid-silver methenamin staining [23]. Ohishi et al. examined the glycosaminoglycan content of different amyloid-rich tissues and purified amyloid fibrils by two-dimensional electrophoresis [25]. They showed a high content of CS and HA in synovial tissues of two β2M amyloidosis patients, while HS and DS predominated in the case of purified AL and AA amyloid fibrils. However, Aruga et al. reported a specific accumulation of HS in the β2M amyloid deposits by immunohistochemical staining using HepSS-1 antibody, and they considered the positive reaction for CS (CS56) to be non-specific [1]. They also argued that the biochemical analysis by Ohishi et al. was unreliable because their samples might be contaminated by articular fluid.

In the present study, we detected a marked accumulation of CS in β2M amyloid deposits by immunohistochemical staining regardless of the organ and tissue and confirmed an intimate anatomical relationship between amyloid sites and increased proteoglycans. Using CS56 antibody, a weak positive reaction was also observed in the loose synovial connective tissues around the amyloid deposits. However, we considered that the positive reaction in the background was due to the high sensitivity of CS56 antibody. We also postulated that the production of CS proteoglycans was elevated in the surrounding interstitial connective tissue. By MO-225 antibody, the positive reaction in the amyloid deposits was generally weaker than CS56, but the positive staining in the background was rarely detected. This result by MO-225 supported the interpretation that the positive staining for CS56 was specific. In the synovial membrane and interstitial connective tissue, we confirmed the accumulation of HS at the same site as amyloid deposits by immunohistochemical staining using MAB1948 monoclonal antibody, which detects the core protein of HS proteoglycan (perlecan). Aruga et al. [1] showed accumulation of HS using HepSS-I antibody, which detects GAG chains of HS proteoglycans; however, sensitivity of HepSS-1 was weaker than that of MAB1948 in our data, and only one case showed a clear positive reaction in the amyloid deposits. Unstable antigenic structures of GAG chains of HS proteoglycan may produce weak immunoreactivity. In three cases, staining by MAB1948 was weak or negative in the amyloid deposits. In these cases, amyloid deposits were located in the dense collagen bundles of the ligament tissue at a short distance from the synovial membrane, and proliferation of small vessels was not as clear as amyloid-rich synovial membrane found in other patients. There is a possibility that accumulation of HS proteoglycans is closely associated with an increase in synovial cells or small vessels around the amyloid deposits.

In three cases, we quantified the proteoglycan content in amyloid-rich tissues by a biochemical method different from that of Ohishi et al. We also tried purification of the β2M amyloid fibrils; however, the amount of total proteoglycans associated with the purified amyloid fibrils was too little to quantify each proteoglycan (data not shown). Our data, like those of Ohishi et al. [25], indicated that the CS proteoglycans were the major component and HS proteoglycans were minor in the amyloid-rich tissues. Our biochemical analyses were also in good agreement with the immunohistochemical study.

Snow and Kisilevsky assessed the temporal relationship between changes in the extracellular matrix and amyloid deposition, using an experimentally induced AA amyloidosis model [31]. In both the usual model and the model where rapid induction occurs with an amyloid-enhancing factor, an amyloid-associated HS glycosaminoglycan appears in the tissue together with AA protein. In the present study, examination of the cervical discs in relatively short-term haemodialysis patients (less than 5 years) revealed a similar close connection, although the glycosaminoglycan was CS in this case. In these early-stage amyloidosis patients, reaction of the HS antibodies was not tested because all tissues were fixed in formalin solution. The present results indicate that in haemodialysis-related amyloidosis, changes in proteoglycans also take place at the same time or before the amyloid deposition, as seen in experimental AA amyloidosis rather than as a secondary phenomenon. Inflammatory reactions, rarely seen in early-stage amyloidosis, did not appear to influence the increase in CS.

We have previously examined the anatomical distribution of β2M amyloid in the intervertebral discs and its temporal progression in long-term haemodialysis patients. The results showed that the most severely stressed sites in the vertebral column, the lower cervical discs, are most susceptible to amyloid deposition [24]. Various kinds of degenerative changes were frequently observed around the amyloid deposits in the early stage, and thus it was suspected that mechanical stress could be directly related to the microenvironment necessary for β2M amyloid deposition. Experimental examination of the relationship between mechanical stress and metabolism of extracellular matrices in periarticular tissue demonstrat-

ed CS content to increase in load-bearing joints, while it decreased in non-load-bearing joints [7]. In human articular tissue, the proteoglycan content is known to decrease generally with age, although foci of degenerative changes show a local increase in acidic glycosaminoglycan content [12, 29]. In haemodialysis patients in both early-stage amyloidosis and pre-deposition stages, degenerative changes in disc tissue showed positive staining for CS. Thus, the increase in CS content takes place before amyloid deposition in the degenerative foci due to mechanical stress, possibly providing a convenient microenvironment for β2M amyloidogenesis. Chondrocyte-like mesenchymal cells, which are usually seen in advanced-stage amyloidosis, and increased chondrocytes in the degenerated disc tissue are both positively stained for CS, which may be related to the local production of CS. As for the deposition in the visceral organs, the "mechanical stress theory" cannot explain the mechanism of the amyloidogenesis directly. Factors that affect the increase of CS and other proteoglycans in the small vessels, especially the influence of mechanical injury on the intima, should be investigated further.

A high affinity of β2M for collagens has been reported, whereas that for proteoglycans has been reported to be lower [14]. Kisilevsky proposed a hypothesis that acidic glycosaminoglycans primarily affect the three-dimensional structure or polymerization of the pre-protein of amyloid [18]. In β2M amyloidosis, the role of increased CS and HS proteoglycans cannot be explained by their high-affinity absorption of native β2M from the serum or interstitial fluid, but they may influence the process of fibrillogenesis through negative charges of molecules.

In summary, the present study revealed that CS proteoglycan is always increased in β2M amyloid deposits regardless of the site of deposition or period of haemodialysis, and HS proteoglycans increase in synovial membrane and interstitial connective tissue. A relationship between amyloidogenesis and local degenerative changes due to mechanical stress is postulated that could be mediated by an alteration in the metabolism of CS proteoglycans. The exact role of the increased proteoglycans remains unclear, but it is possible that they might affect the formation of three-dimensional structure of the amyloid or polymerization of the constituent proteins. We believe that the relationship between β2M amyloidogenesis and metabolism of proteoglycans, such as CS and HS proteoglycans, warrants further attention.

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