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Immunohistochemical identification of heparan sulphate proteoglycan in secondary systemic amyloidosis

B. NORLING, GUNILLA T. WESTERMARK & P. WESTERMARK *Departments of Medical and Physiological Chemistry and Pathology, University of Uppsala and the Department of Pathology, University of Linköping, Linköping, Sweden*

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SUMMARY

The distribution of proteoglycans in kidneys from patients with secondary (AA) systemic amyloidosis was investigated. Antisera reacting with the protein cores of chondroitin sulphate proteoglycan (CSPG), dermatan sulphate proteoglycan (DSPG) and heparan sulphate proteoglycan (HSPG) were used in conjunction with the peroxidase-antiperoxidase (PAP) method. HSPG was the only proteoglycan found to be specifically localized to the amyloid deposits. The staining was most intense on the endothelial side of the deposits in both the glomeruli and in the vessel walls. No staining was observed after absorption of the HSPG antiserum with a fraction of the amyloid preparations, corresponding in size to that reported for glomerular HSPG. The possible role of HSPG and endothelial cells in the pathogenesis of the amyloid deposits is discussed.

Keywords amyloid heparan sulphate proteoglycan immunohistochemistry

INTRODUCTION

Amyloidosis constitutes a group of disorders in which proteins are deposited interstitially in different tissues in a pathological way. Many different amyloidotic syndromes exist, probably with varying pathogenesis, but the end-product, the amyloid, always has a similar morphological appearance with a fine fibrillar substructure. The mechanisms by which certain proteins make up amyloid fibrils are virtually unknown. X-ray crystallographic studies have revealed a β-pleated sheet conformation in native amyloid fibrils and a pure β-pleated sheet model for the fibril has been proposed (Eanes & Glenner, 1968; Glenner *et al.*, 1974). This model has however recently been questioned (Turnell *et al.*, 1986).

Several different, unrelated proteins constitute the fibrils in the different amyloid syndromes, and in spite of this, the morphology of the fibrils is fairly constant (Cohen & Shirahama, 1973; Glenner *et al.*, 1974). It is also possible to create amyloid-like fibrils *in vitro* using different low-molecular-weight proteins such as insulin, immunoglobulin light chains or protein AA (Glenner *et al.*, 1971a; 1974; Pras & Reshef, 1972). These fibrils are, however, often more wavy than the native amyloid fibrils. In the amyloid fibrillogenesis probably both concentration of the fibril protein and modification of the precursor, usually by partial degradation, play roles. It is also possible that other as yet unknown factors are of importance for the formation of the fibrils. Much knowledge regarding the nature of the different subunit proteins in amyloids has been achieved since the first characterization of an amyloid fibril protein in 1971 (Glenner *et al.*, 1971b). Protein AA, which occurs in secondary systemic amyloidosis, is derived from a plasma apolipoprotein SAA (Benditt & Eriksen, 1977), and varies in size between 5 and 10 kD due to variation in splitting position of the precursor (Ein *et al.*, 1972; Levin *et al.*, 1972; Sletten & Husby, 1974; Sletten, Husby & Natvig, 1976; Westermark *et al.*, 1987; 1988).

Although the low-molecular-weight subunit proteins are the predominating constituents in amyloid and possibly solely constitute the amyloid fibrils, other components are more or less constantly present in the deposits. Thus virtually all amyloid forms contain a glycoprotein AP (amyloid P-component), which is a normal plasma protein (serum AP or SAP) (Pepys *et al.*, 1977) and that probably is passively absorbed by and bound to the amyloid fibrils (Skinner *et al.*, 1983). It is unknown whether protein AP is of any importance for the formation of amyloid fibrils. Glycosaminoglycans (GAGs), earlier called mucopolysaccharides, have also been known to be present in most amyloid deposits (Muir & Cohen, 1968). Some of the characteristic staining properties of amyloid, such as metachromasia after treatment with crystal violet, do not depend on the fibrillar structure or on the major subunit protein but on the presence of GAGs in the deposits. The significance of the GAGs in amyloid is still completely unknown. However, it has recently been shown that heparan sulphate (HS), i.e. the predominant GAG of the basement membrane (Gallagher, Lyon & Steward

Correspondence: Per Westermark MD, Department of Pathology I, University Hospital, S-581 85 Linköping, Sweden.

1986), is able to bind to SAP (Pollak *et al.*, 1982). Such an interaction, if occurring *in vivo*, could be of importance both for the formation of amyloid fibrils as well as for the localization of amyloid deposits to certain tissues.

Earlier investigators have demonstrated the presence of GAGs in amyloid either by extraction and chemical analysis (Berenson *et al.*, 1969; Binette *et al.*, 1971; Brandt, Skinner & Cohen, 1974; Kisilevsky *et al.*, 1986; Miyasaki *et al.*, 1979) or by histochemical methods (Kisilevsky *et al.*, 1986; Mowry & Scott, 1967; Snow & Kisilevsky, 1985; Stiller & Katenkamp, 1974). Since, however, these molecules are synthesized on a protein core and exported from the biosynthetically active cell as proteoglycans (PGs) and not as GAGs (Gallagher *et al.*, 1986; Poole, 1986), we were interested in whether or not any intact PG (or fragment thereof) could be found in amyloid. In this study, we have investigated the presence of PGs in the kidneys in secondary (AA) systemic amyloidosis using antisera reacting with the core proteins of chondroitin sulphate PG (CSPG), dermatan sulphate PG (DSPG) and heparan sulphate PG (HSPG).

MATERIALS AND METHODS

Tissues

Kidneys from 10 patients with amyloidosis associated with rheumatoid arthritis and from three non-amyloidotic patients were obtained at autopsy. In six of the patients with amyloidosis, amyloid deposits occurred in glomeruli and vessels (glomerular pattern of infiltration), while in four patients glomerular affection was nearly completely lacking but the vascular infiltration was heavy (vascular pattern). In the latter pattern the amyloid tended to form large, homogeneous areas in and around the vessel walls. Pieces of tissue were fixed in 4% formaldehyde and embedded in paraffin while the rest of the organs were stored at −20°C.

Antisera

The isolation and characterization of CSPG, DSPG, and HSPG from human tissues was done with conventional techniques (Norling & Glimelius, 1988; Norling & Larsson, 1988b). Antisera against these proteoglycans were raised in rabbits using standard procedures. As can be seen in Table 1 only one type of proteoglycan was recognized by each of the proteoglycan antisera. They showed no reactivity towards the two basement membrane specific components laminin and collagen IV. Antiserum to protein AA has been characterized previously (Westermark *et al.*, 1987).

Immunohistochemistry

Immunohistochemistry was performed on sections with the peroxidase-antiperoxidase (PAP) method (Sternberger, 1979). The primary antisera were diluted 1:200–1:800 and the reaction was visualized with 3,3′-diaminobenzidine (Sigma, St Louis, Missouri) or 3-amino-9-ethyl-carbazole (Sigma). Unfixed frozen sections were also studied but with the primary antisera diluted 1:1000–1:5000. For controls, the primary antisera were replaced by normal rabbit serum in appropriate dilution.

For absorption studies, amyloid fibrils isolated from three patients (MB, 859 and 896) with AA-amyloidosis were used. Amyloid fibrils were defatted in chloroform-methanol (2:1), dissolved in 6 M guanidine HCl in 0·1 M Tris/HCl buffer, pH 8·0, containing 0·1 M EDTA and 0·1 M dithiothreitol, and gel-filtered on a Sepharose CL-6B column as described (Westermark *et al.* 1987). Pooled fractions were dialysed against deionized water and lyophilized. The HSPG antiserum was absorbed (100 mg/ml undiluted serum) with material from the different fractions before the immunohistochemical staining. Unabsorbed antiserum was used as control.

Table 1. Specificity of anti-proteoglycan antibody. To test for antiserum specificity, a precipitation assay was used. To 50 μl of the radioactive antigen, 50 μl of antiserum, 30 μl of a protein A Sepharose (Pharmacia Uppsala, Sweden) slurry (50% gel) and 1 ml 0·01 M Tris HCl buffer, pH 8·0 containing 0·15 M NaCl, 0·001 M Na_2SO_4, 0·02% NaN_3 and 0·1% bovine serum albumin was added. After end over end rotation overnight in the cold, the mixture was centrifuged. The supernatant and the gel pellet were separated and their respective content of radioactivity measured. The percent radioactivity in the pellet of total radioactivity is shown in the table as the mean value of at least five experiments.

	Antigens				
Antiserum	HSPG*	CSPG†	DSPG‡	Laminin§	Collagen type IV§
Anti HSPG	49	5	4	5	4
Anti CSPG	6	46	5	5	4
Anti DSPG	4	5	56	4	4
Control	5	4	4	5	4

* Matrix-associated $^{35}SO_4$-labelled HSPG of cultured glial cells isolated as described by Norling *et al.* (1981).

† Medium-derived $^{35}SO_4$-labelled CSPG of cultured glial cells isolated as described by Norling *et al.* (1984), method III.

‡ Isolated from human umbilical cord as described by Norling & Glimelius (1988) and labelled with ^{125}I using the chloramine-T method (Hunter & Greenwood, 1962).

§ Purchased from Bethesda Research Laboratories, Rockville, MD and labelled with ^{125}I as above.

RESULTS

Proteoglycans

The immunohistochemical staining patterns of frozen sections of normal human kidney with antisera to the different proteoglycans will be described elsewhere. Briefly, the staining for CSPG and DSPG was concentrated in the interstitium and the tubular cells in contrast to the staining of only the basement membrane of glomeruli, tubular cells and vessels with the HSPG antiserum. When paraffin-embedded material was used instead the staining patterns for CSPG and DSPG were the same but weaker. However, the staining for HSPG was substantially lacking indicating that under these conditions not enough of the HSPG antigen could be recognized by the antiserum. In contrast to the latter, when routinely formaldehyde-fixed material from patients with AA-amyloidosis was used, the HSPG antiserum reacted with the amyloid, both in the glomeruli and the vessels with no staining elsewhere (Figs 1 and 3). Often there was a considerably stronger reaction on the endothelial side of the deposits both in the glomeruli and in the vessel walls (Figs 1 and 3). This pattern contrasted to the staining when antiserum to protein AA was used where the deposits were more evenly stained (Fig. 2). The intensity of the staining reaction varied

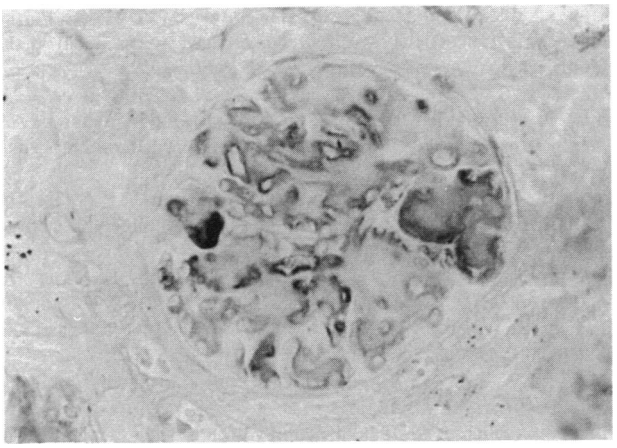

Fig. 1. Glomerulus showing reactivity with antiserum to HSPG core protein. The reaction is especially strong along the endothelial sides. Formaldehyde-fixed kidney tissue. PAP visualized with DAB. × 320.

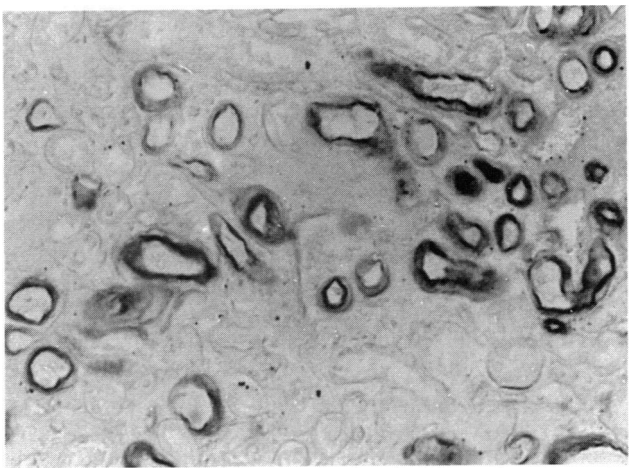

Fig. 3. Vasa recta with amyloid deposits showing reaction with the antiserum to HSPG. The staining is stronger along the endothelial sides. The same tissue as shown in Fig. 1. PAP visualized with DAB. × 320.

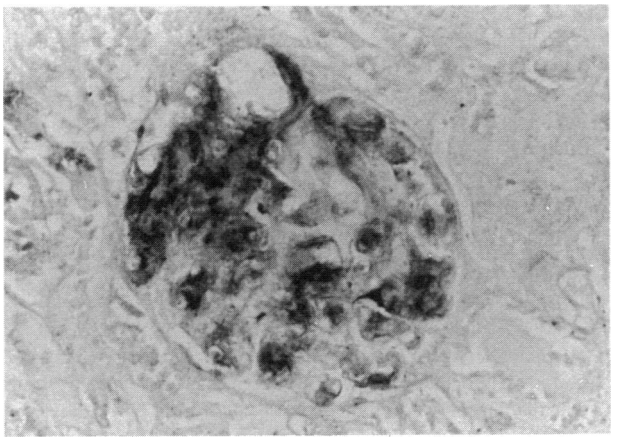

Fig. 2. Glomerulus immunohistochemically stained with antiserum to protein AA. The reaction products show a distribution as in Fig. 1 but the staining is more even. The same tissue as shown in Fig. 1. PAP visualized with DAB. × 320.

between the patients and in sections of one patient, the anti-HSPG antiserum only gave a very weak reaction with the amyloid. However, this tissue showed signs of severe autolysis.

The amyloid in the vascular pattern of infiltration exhibited a weaker reaction compared to the amyloid in kidneys with glomerular pattern. When formaldehyde treated sections were stained immunohistochemically with the antisera to CSPG and DSPG no or only background reaction was obtained in the amyloid deposits whereas the interstitium and the tubular cells were stained as in the normal kidney.

Absorption studies

In order to further characterize the staining reaction for HSPG, different fractions obtained from the purification procedure, leading from isolated amyloid fibrils to purified protein AA, were screened for their possible inhibitory activity. Absorption of the anti HSPG antiserum with alkali degraded preparations of AA fibrils (DAM, Pras *et al.*, 1969) abolished the reaction with the amyloid while absorption with purified protein AA or protein AP had no effect. Gel chromatography on Sepharose CL-6B of dissolved amyloid fibrils from three patients with AA-amyloidosis resulted in each case in two major protein peaks, the first corresponding to the void volume material and the second to protein AA. Between these, one or two small peaks occurred. Absorption with material from these three separations abolished or reduced markedly the reaction with the amyloid only when fractions eluted shortly after the void volume peak were used, i.e. with K_{av} values between 0·15–0·35. Proteins from the void volume material had no or very little effect.

DISCUSSION

Many studies have shown an increased amount of glycosaminoglycans in tissues infiltrated with amyloid (Berenson *et al.*, 1969; Binette *et al.*, 1971; Brandt *et al.*, 1974; Miyasaki *et al.*, 1979; Mowry & Scott, 1967; Snow & Kisilevsky, 1985; Stiller & Katenkamp, 1974). The type of predominating glycosaminoglycan found has varied but in most studies, HS has been the major fraction (Berenson *et al.*, 1969; Binette *et al.*, 1971; Kisilevsky *et al.*, 1986) although both CS and hyaluronic acid has been found. All studies so far have focused on the glycosaminoglycans and to the best of our knowledge, nothing has been documented on the proteoglycan core proteins in amyloid. In this report, we describe that also the core protein of HSPG is present within the amyloid deposits. We also found that only HSPG seemed to occur in increased amounts in amyloid. This was especially evident in sections of formaldehyde-fixed material which showed an intense immunostaining in the amyloid only when antiserum to HSPG was used while apparently normal tissue was unreactive. Furthermore, the hydrodynamic size of the amyloid fractions from Sepharose CL-6B gel filtration, which abolished this staining, is the same as that reported for the predominant HSPG isolated from the glomeruli of several animal species (Kanwar, Hascall & Farquhar, 1981; Parthasarathy & Spiro, 1984; Stow *et al.*, 1983). The significance of HSPG for formation of amyloid is still uncertain. However, appearance of glycosaminoglycans has been reported to occur simulta-

neously with deposition of experimentally induced AA-amyloid fibrils (Kisilevsky *et al.*, 1986; Snow & Kisilevsky, 1985) and this may indicate that the presence of HSPG is not only a secondary phenomenon. Interestingly enough the strongest HSPG immunoreactivity was seen on the endothelial side of the amyloid deposits (Figs 1 & 3). Since endothelial cells earlier have been shown to synthesize and deposit HSPG in their microenvironment, at least *in vitro* (Norling, Glimelius & Wasteson, 1981; Oohira, Wight & Bornstein, 1983), this increased staining could be the result of an altered HSPG metabolism by these cells.

Protein AA is heterogeneous mainly due to varying split positions of the precursor molecule SAA, yielding subspecies that vary considerably in isoelectric points (Westermark, 1982; Westermark *et al.*, 1987). Since some of these subspecies are positively charged at a neutral pH, the highly anionic HSPG can hypothetically bind these basic molecules in tissues. It is thus possible that such a bound AA-molecule could serve as a nidus for fibril formation. Further studies on the binding properties between proteoglycans and amyloid proteins are however necessary to deduce which part of the HSPG (the glycan and/or protein part) that is responsible for its localization to the amyloid deposits.

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