Heparan Sulfates in Human Hemodialysis Associated Amyloidosis—*In situ* Detection of *N*-Sulfate-Enriched and *O*-Sulfate-Scarced Portions

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Abstract: β₂-Microglobulin (β₂MG) is a major constituent of amyloid fibrils in hemodialysis-associated amyloidosis (HAA). The present investigation was designed to immunohistochemically examine the localization of fine structural units of heparan sulfates (HS), compared with that of β₂MG in HAA. Amyloid-rich carpal tunnel ligament was obtained surgically from 12 patients on maintenance hemodialysis. Paraffin-embedded sections were examined with an antiserum to β₂MG and two monoclonal antibodies to HS. Both of the monoclonal antibodies specifically recognize the *N*-sulfate-enriched and *O*-sulfate-scarced portions of HS. The results indicated that the accumulation of HS epitopes was of four types, namely, 1) fibrous localization between parallel wavy bundles of collagen; 2) vascular or perineural localization; 3) localization in the vicinity of inflammatory and/or resident cells; and 4) homogeneous localization in the extravascular areas. These patterns of immunolocalization were in agreement with those of β₂MG. Thus, HS having these epitopes may play an important role as co-localized common elements in the amyloidogenesis of HAA.

Keywords: hemodialysis-associated amyloidosis, heparan sulfates, β₂-microglobulin

INTRODUCTION

Carpal tunnel syndrome due to hemodialysis-associated amyloidosis (HAA) is one of the most common and serious complications in patients with chronic renal failure on long-term hemodialysis. Biochemical and immunohistochemical studies have shown that a major proteinous constituent of the amyloid deposits in HAA is β₂MG¹⁻³. Apart from the importance of proteinous constituents, “common elements” that co-localize with amyloid fibers in various forms of amyloidosis may also play an important role in amyloidogenesis. Glycosaminoglycans (GAG) such as heparan sulfates (HS) are well-known common elements⁴⁻⁵. Immunohistochemical studies were performed for GAG in HAA. Agura et al. used a mouse monoclonal antibody to describe the co-localization of certain HS in HAA⁶. In their study, HS epitopes is closely related to the *O*-sulfated and *N*-acetylated glucosamine linked to glucuronic acid⁷⁻¹⁰. HS are molecules that have polymeric structures of enormous complexity and a variety of functions⁸⁻¹⁰. Thus, the examining immunolocalization of epitope structures with different HS monoclonal antibodies in HAA is of interest. In the present report, two monoclonal antibodies that recognize *N*-sulfate-enriched and *O*-sulfate-scarced portions of HS as epitopes were used.

MATERIALS AND METHODS

Materials

Carpal tunnel ligament was obtained from 12 patients with chronic renal failure on maintenance hemodialysis at operative decompression for symptomatic carpal tunnel syndrome. Samples were fixed in 10% buffered formalin for 24 hours and embedded in paraffin.

Antibodies to HS

Two different monoclonal antibodies to HS, designated as HK249 and 10E4, were used. They are well-characterized antibodies and do not react with chondroitin sulfate or other forms of glycosaminoglycans. Although *N*-sulfate-
enriched and O-sulfate-scarced portions of HS\textsuperscript{10,11} are involved in both epitopes, structure details seem to differ as follows:

HK249 (IgM): A 5-week-old Wistar rat was immunized with a mixture of reduced and unreduced perlecan fractions prepared from\textsuperscript{31} Engelbreth-Holm-Swarm tumor (EHS tumor) for production of this antibody. The spleen was harvested and fused with NS-I mouse myeloma cells according to a standard procedure with minor modifications\textsuperscript{12}.

10E4 (IgM): BALB/c mice were immunized with liposome-incorporated membrane haparan sulfate proteoglycans and heparitinase-digested medium heparan sulfate proteoglycans for production of this antibody\textsuperscript{11}. Detailed protocols for hybridoma production and selection were described elsewhere\textsuperscript{13}.

### Staining techniques and immunohistochemistry

Paraffin-embedded sections (4 \( \mu \text{m} \) in thickness) were stained with hematoxylin-eosin and periodic acid-Schiff. For detection of amyloid deposits, 7 \( \mu \text{m} \) sections were stained by the Congo Red method of Puchtler et al.\textsuperscript{14}, or with Thioflavin T. For immunohistochemistry, the streptavidin-biotin method was used: Briefly, after deparaffinization and rehydration of 4 \( \mu \text{m} \) tissue sections, the endogenous peroxidase was inactivated by incubating the sections in absolute methanol containing 0.3% (v/v) hydrogen peroxide for 30 minutes at room temperature. To explore the possibility of epitope masking by another molecule, the sections were preincubated with or without 0.1% (w/v) trypsin (tissue culture grade Gibco Grand Island, NY.). In the control, the sections were treated with a mixture of heparitinase and heparinase (Seikagaku Corp, Tokyo, Japan, 0.1 u/ml each in 50mM Tris HCl, pH 8.0 with 0.5 mM calcium acetate, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide and 10 mM ethylenediaminetetraacetate). Sections were exposed to 10% (w/v) non-fatty milk/3% (w/v) bovine serum albumin in PBS to minimize nonspecific binding. HK249 culture medium was used undiluted, while 10E4, anti-\( \beta_2 \) MG antibodies and two types of mouse anti-CD68 antibodies designated as KP-1 and PG-M1 (DAKOPATTS A/S, Denmark) were used at a concentration of 2 \( \mu \text{g/ml} \). The sections were then incubated with biotinylated antibodies to rat (for HK249 antibody, obtained from Vector Labs; Burlingame, CA), mouse (for 10E4 antibody, obtained from Nichiei Co.; Tokyo) and rabbit (for anti-\( \beta_2 \) MG antibody, obtained from DAKOPATTS A/S, Denmark) immunoglobulins for 90 minutes at room temperature. Finally, the sections were incubated in peroxidase-conjugated streptavidin (Nichirei Co., Tokyo) for 90 minutes at room temperature. Peroxidase reaction products were revealed with 3, 3'-diaminobenzidine (Sigma, St. Louis, MO).

###RESULTS

**Amyloid deposition and immunolocalization of \( \beta_2 \) MG**

Twelve cases of confirmed carpal tunnel syndrome in patients on maintenance hemodialysis were analyzed for the presence of amyloid deposition by the Congo Red staining method. As shown in Fig. 1, all of them were positive for Congo Red. Congo Red positive sites showed birefringence when viewed under polarized light (data, not shown).

Thioflavin T fluorescence failed to demonstrate amyloid deposition in this tissue because collagen fibers were also positive for Thioflavin T (data, not shown). \( \beta_2 \)-Microglobulin immunostaining basically corresponded to the sites of amyloid deposits that were determined by Congo Red staining. As shown in Fig. 2A and B, \( \beta_2 \) MG accumulation was of four types: (1) small nodular and fibrous localization between bundles of collagen (arrows in Fig. 2A), (2) vascular localization (arrow head in Fig. 2A), (3) localization in the vicinity of inflammatory and/or resident cells (arrows in Fig. 2B), and (4) extravascular amorphous deposits (arrow heads in Fig. 2B).

**Immunolocalization of N-sulfate-enriched and O-sulfate-scarced portions of HS**

These portions of HS were detected with HK-249 and 10E4 antibodies. As in the case of \( \beta_2 \) MG, the immunolocalisation corresponded in principle to the sites of amyloid deposits that were determined by Congo Red staining. Fig. 3 shows that HK249 antibody, a rat monoclonal antibody to this portion of HS, immunostained the sites between bundles of collagen. This staining pattern was completely identical with that of 10E4 antibody, a mouse monoclonal antibody to this portion of HS (data, not shown). Fig. 4A and 4B show perineural and
vascular staining with these antibodies. Homogeneous staining that was indicated by arrows was observed with these antibodies, but this pattern of staining was never observed in sections stained with Congo Red (data, not shown). It is possible that Congo Red staining is not sensitive enough to detect a small amount of amyloid in the above sites. Another possibility, i.e., that amyloid is not present in these sites cannot be completely excluded. Intensely stained areas indicated by arrows and asterisks in Fig.4 correspond to blood vessels and Schwann’s sheaths. Fig. 5 shows the staining patterns in more advanced lesions, in which 10E4 antibody immunoreacted to areas in the vicinity of cells (arrow head). At least some of them are infiltrated or residual macrophages because some of them are positive for CD68 detected with KP-1 (DAKOPATTS A/S, Denmark) and PG-1 (DAKOPATTS A/S, Denmark) antibodies (data, not shown). Fig.5 also shows that 10E4 antibody stains extravascular areas in a homogeneous (arrow) and/or small nodular (asterisks) manner.

**DISCUSSION**

The present study has indicated that immunolocalization of N-sulfate-enriched and O-sulfate scarced portions of HS was of four types, namely, 1) fibrous localization between parallel wavy bundles of collagen; 2) vascular or perineural localization; 3) localization in the vicinity of inflammatory and/or resident cells; and 4) homogeneous localization in the extravascular areas. Identical results were obtained when frozen sections were used. Furthermore, various pretreatment conditions of the paraffin-embedded sections (such as formic
acid and 7 M urea treatment) did not seem to alter the staining patterns. We therefore concluded that the immunohistochemical techniques used in the present study were reliable. HS have polymeric structures of enormous complexity. Aruga et al. used mouse monoclonal antibodies to other portions of HS (O-sulfated and N-acetylated glucosamine linked to glucuronic acid) in their immunohistochemical study and showed that HS was immunostained at sites where β2MG was deposited. Although there was no description of fibrous localization between parallel wavy bundles of collagen, their results and ours were basically identical. Although different forms of HS accumulation co-existed in a tissue section, the interrelation between the different distributional patterns also remains to be clarified. It is of interest that these forms of immunoreactivity for HS is very similar to that for β2MG which is described in the present investigation, or reported by other investigators.

In general amyloid deposition and inflammation are two different pathological changes. However, HAA may be distinguished from other forms of amyloidosis in that inflammatory changes (such as cellular infiltration) co-locate at sites of amyloid deposition. For example macrophage-like cells, detected in situ with KP-1 and PG-1 antibodies, were observed in the vicinity of HS-positive sites, but the roles of these cells in the amyloidogenesis of this tissue remains to be clarified. One hypothesis is that these cells are stimulated and secrete inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), which in turn stimulate fibroblasts to secrete collagenase in order to accelerate amyloid formation. This hypothesis is supported by reports indicating that a) β2MG in HAA is modified by advanced glycation end products (AGE), b) AGE-modified protein can induce macrophage to synthesize TNF-α, and c) TNF-α can induce fibroblasts to produce collagenase.

HS are immunostained in amyloid deposits of not only HAA but also other forms of amyloidosis. Moreover, infusion of rats with perlecan (an HS-containing macromolecules) with a 39-42 amino acid peptide termed AB facilitated the development of fibrillar AB amyloid in brain. The precise mechanism by which HS promotes amyloidogenesis remains to be investigated. One possibility is that HS protects amyloid fibrils from degradation. An analogous role of HS in protecting macromolecules...
from protease attacks has been described in the case of basic fibroblast growth factor bound to HS20.

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