Reduction in Glomerular Heparan Sulfate Correlates with Complement Deposition and Albuminuria in Active Heymann Nephritis

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Abstract. In a time-study of active Heymann nephritis, the expression of agrin, the main heparan sulfate proteoglycan in the glomerular basement membrane, was analyzed in relation to deposition of IgG and complement in the glomerular capillary wall and the development of albuminuria. Binding of IgG autoantibodies to the glomerular capillary wall could be detected from 2 wk onward, followed by activation of complement after 6 wk. Progressive albuminuria developed from 6 wk onward to a level of 274 ± 68 mg/18 h at week 12. The staining intensity for the agrin core protein decreased slightly, and the staining intensity for the heparan sulfate stubs that were still attached to the core protein after heparitinase digestion remained normal. From week 6 onward, however, a progressive decrease was seen in the staining of two monoclonal antibodies (mAb) directed against different epitopes on the heparan sulfate polysaccharide side chain of agrin (to 35 and 30% of the control level, respectively, at week 12, both mAb P = 0.016). Moreover, albuminuria was inversely correlated with heparan sulfate staining as revealed by these antibodies (rs = −0.82 and rs = −0.75, respectively, both mAb P < 0.0001). This decrease in heparan sulfate staining was due to a progressive reduction of glomerular heparan sulfate content to 46 and 32% of control level at week 10 and week 12 of the disease, respectively, as measured biochemically. It is speculated that the observed decrease in glomerular heparan sulfate in active Heymann nephritis is due to complement-mediated cleavage of heparan sulfate, resulting in an increased permeability of the glomerular basement membrane to macromolecules.

Active Heymann nephritis (AHN) is an experimental model that closely resembles human idiopathic membranous glomerulonephritis (1,2). Active immunization of rats with a crude extract of renal tubular epithelium (RTE) induces autoantibodies in the circulation. These autoantibodies are predominantly directed against the epithelial glycoprotein megalin, expressed in the microvillous area of the brush border of the proximal tubule and on the glomerular visceral epithelial cell (3,4). Binding of IgG autoantibodies to megalin (gp330) gives rise to the formation of large electron-dense subepithelial immune deposits along the glomerular basement membrane (GBM) and to effacement of podocytes as seen by electron microscopy (5). The immune deposits are often found in association with coated pits and become apparent under the slit diaphragms when increasing in size. The megalin/anti-megalin immune complexes are thought to be shed from the podocyte membrane and attach to the GBM (6).

The alterations in the structure and function of the GBM that lead to the increased permeability of the glomerular capillary wall (GCW) for plasma proteins in AHN have not fully been elucidated. The GCW functions both as a size- and charge-selective barrier for plasma proteins (7–9). The charge selectivity of the GBM is mainly due to the presence of fixed anionic macromolecules, principally the heparan sulfate (HS) polysaccharide side chains of agrin, recently identified as the major heparan sulfate proteoglycan (HSPG) in the GBM (10,11). Several findings indicate that HS plays a role in the maintenance of the permselectivity of the GBM. Enzymatic digestion of HS in the GBM by heparitinase resulted in an augmented passage of native ferritin and albumin into the urinary space (12,13). Furthermore, intravenous injection of a monoclonal antibody (mAb) directed against GBM HS induced an acute, selective proteinuria (14). With antibodies directed against the agrin core protein and the HS side chain, we demonstrated a decrease in GBM staining of HS in various human proteinuric glomerular diseases including idiopathic membranous glomerulonephritis, whereas the staining of the agrin core protein remained unaltered (15). Deposition or in situ formation of immune complexes in the GCW can result in
the disturbance of the structure and sieving properties of the GBM. The formation of immune complexes in the GCW is accompanied by a decrease of anionic sites in the GBM in human and experimental membranous nephropathy (16–19). Whether formation of immune complexes correlates with HS alterations in the GBM or whether a decreased detection of anionic sites may be the result of masking by the immune complexes has not yet been elucidated. The cationic probes used in the previously mentioned studies, however, are not specific for HS, and an immunohistochemical analysis with well-defined mAb to HS or agrin has not yet been performed in membranous nephropathy.

To get more insight into the relationship between the HS(PG) expression in the GBM, the development of albuminuria, and the deposition of antibodies in the GCW, we studied these parameters at various time points during the development of AHN. For the evaluation of GBM HS(PG) expression, we used a set of four antibodies directed against different epitopes of HS(PG). Furthermore, we biochemically determined the glomerular HS content. Our results indicate that the loss of HS is primarily associated with complement activation.

Materials and Methods

Experimental Design

AHN was induced in 53 female 8-wk-old Lewis rats (obtained from the animal laboratory of the University of Maastricht, The Netherlands) by subcutaneous immunization in the front foot pads with 5 mg of RTE, purified from kidneys of Wistar rats and emulsified in 100 μl of incomplete Freund’s adjuvant (DIFCO, Detroit, MI) containing 1 mg Mycobacterium tuberculosis (HRa37; DIFCO) and injection of 5 × 10⁶ Bordetella pertussis bacteria (DIFCO) in 50 μl physiological salt subcutaneously in the front shoulders. Animals in the control group were injected with incomplete Freund’s adjuvant, M. tuberculosis and B. pertussis alone.

Before the induction of the disease (week 0) and at weeks 2, 4, 6, 7, 8, 10, and 12, urine was collected during 18 h overnight housing in metabolic cages, and urinary albumin concentration was determined by rocket-immunoelectrophoresis (20). At the same time points, blood samples were taken and serum was frozen at −20°C until use for detection of anti-megalin antibodies in enzyme-linked immunosorbent assay (ELISA). From the RTE-immunized group, five rats were killed on a scale between 0 and 10. No GCW staining was given a score of 0, 10% of the GCW staining positive was given 1, 20% staining positive was given 2, etc., and all GCW loops staining positive was given 10. Therefore, the maximal score for the staining of 25 glomeruli in each section was 250 arbitrary units (AU). The scoring was performed by two independent observers on coded sections, and the mean of these two scores was used for further analysis, so that one observation represents the mean score of the staining of one antibody in one animal at each time point. Five rats were analyzed in each group.

To assess HS(PG) staining of the GBM, the following four antibodies were used (Table 1): two mAb that are directed against different epitopes in the HS side chain, mouse mAb JM403, which is directed against low-sulfated domains of HS containing an N-unsubstituted glucosamine unit (14,23), and mouse mAb KJ865, which is directed against N-acetylated domains of HS (24); goat polyclonal antibody BL31, which is directed against the GBM agrin core protein (25); and mouse mAb 3G10, which is directed against heparitinase-generated HS stubs (26). Non-fixed sections were incubated with 0.25 U/ml heparitinase (heparin-sulfate lyase III; Sigma, St. Louis, MO) in 50 mM Hepes and 10 mM CaCl₂, pH 7.0, for 1 h at 37°C. Then, the sections were incubated with mAb 3G10. By heparitinase digestion, a terminal 4,5-unsaturated uronate residue is formed that is essential for recognition by 3G10. The staining of 3G10 can serve as a general HS marker, independent of the extent of HS modification. Goat anti-human agrin core protein BL31 and mouse anti-rat HS mAb JM403 were used in a double-staining procedure, in which a tetramethylrhodamine isothiocyanate-conjugated secondary antibody was used for JM403 (22). A schematic representation of an agrin molecule and the epitopes recognized by the different antibodies used in this study is shown in Figure 1.

To evaluate the staining of other glomerular PG, we stained kidney sections of control and AHN rats at week 12 with polyclonal anti-serum EY-90 against perlecan (27) and mAb 2D6 against the chondroitin sulfate proteoglycan bamacan (28).

To evaluate the deposition of IgG and complement factor C3c in the GCW, sections were incubated with FITC-labeled goat anti-rat IgG and FITC-labeled goat anti-rat C3c. To evaluate possible involvement of the oxygen/nitrogen radical pathway, sections were incubated with the following antibodies: mouse mAb48 against cytochrome b₅₅₉ (29), rabbit anti-myeloperoxidase, and rabbit anti-nitrotyrosine (peroxynitrite-modified proteins).

Sections were fixed in 4% paraformaldehyde in PBS and incubated with rabbit anti-rat inducible nitric oxide synthase (30), followed by peroxidase-labeled secondary antibodies. Endogenous peroxidase activity was blocked with 0.05% H₂O₂ in PBS, and the sections were
incubated with aminoethylcarbimazole and H$_2$O$_2$ as a substrate and counterstained with hematoxylin. Details of all antibodies used are shown in Table 1. Superoxide anion-producing cells were detected using enzyme histochemistry according to the method of Briggs (31).

**Elution of IgG from Cryostat Sections**

To investigate whether normal HS staining could be restored by elution of IgG, non-fixed cryostat sections were incubated with either of the following solutions: 0.02 M citrate buffer, pH 3.2; 0.1 M Tris buffer, pH 11.0; 2 M NaCl, pH 8.0; 2 M KSCN in 0.01 M phosphate buffer, pH 7.6, for 1 h at 37°C; 10 mg/ml collagenase 1A in PBS for 30 min at 37°C; 7 M urea, pH 6.8, in 10 mM Tris buffer; 4 M guanidine HCl, pH 6.8, for 30 min at 4°C; 10 mg/ml heparin in PBS for 4 h at 37°C; 0.12 M glycine, pH 2.8; or 0.1 M glycine/6 M urea, pH 3.5, for 1, 2, 4, or 12 h at 4°C or 37°C (15,32,33). After washing, the sections were stained with JM403 and for rat IgG as described above. Normal rat kidney sections that were incubated with the same solutions were used as a positive control for normal HS staining with JM403.

**Isolation of Glycosaminoglycans from Glomeruli and Quantification of HS**

For the isolation of glomeruli, the kidneys of control rats at week 12 (n = 5), rats with AHN at week 10 (n = 4), and rats with AHN at week 12 (n = 4) were pooled (one kidney per animal). Glomeruli were isolated by the differential sieving procedure (14), weighed, divided into four portions, and counted in quadruplicate, so that the glycosaminoglycan (GAG) purification and quantification procedure could be performed in quadruplicate. The glomeruli were digested with 1 mg/ml papain (from papaya latex; Sigma) in 50 mM NaPO$_4$, 2 mM cysteine-HCl, pH 6.5, and 2 mM ethylenediaminetetra-acetic acid in a volume of 1 ml/100 mg wet weight glomeruli for 16 h at 56°C under constant agitation. Papain was inactivated at 100°C for 5 min followed by centrifugation at 13,000 rpm for 10 min. Proteins were precipitated by addition of 1/3 volume 24% TCA to the supernatant (final concentration, 6%) and incubation on ice for 30 min. This pellet was used for protein quantification according to the method of Lowry (34). After centrifugation for 15 min at 13,000 rpm, the GAG in the supernatant were precipitated by addition of 1/10 volume 3 M NaAc, pH 5.2, and 800 µl of 100% ethanol and incubation overnight at −80°C. After centrifugation for 15 min at 13,000 rpm, the pellet was washed with 75% ethanol, centrifuged, and the resulting pellet was dried and dissolved in 120 µl of water. The isolated GAG were quantified using the modified dimethylmethylene blue assay described by Farndale et al. (35). In the samples, both the total amount of sulfated GAG and the amount after HS degradation by nitrous acid were determined. Used as standards were 0 to 50 µg/ml HS (from bovine kidney; Seikagaku Kogyo, Tokyo, Japan) and chondroitin.
sulfate from whale cartilage (Sigma). Forty microliters of each sample was mixed with 40 μl of the HNO2 reagent at high pH (around 10), 800 μl of dimethylmethylene blue reagent was added, and the absorption at 525 nm was measured immediately in a Perkin-Elmer Lambda 3B spectrophotometer. This HNO2 reagent does not cleave HS and had no effect on absorbance. To degrade HS, 40-μl samples were mixed with 40 μl of HNO2 pH 1.5 reagent and incubated at room temperature for 15 min. Then, the Farnsdael assay was repeated. After incubation with HNO2, pH 1.5, >97% of HS standard was degraded and in the glomerular samples HS was found to be the predominant GAG. The concentration of HS was determined by subtracting Farnsdale values without HS degradation (HNO2 pH around 10) and with HS degradation (HNO2 pH 1.5) and expressed as μg per 10,000 glomeruli.

Statistical Analyses

For statistical analyses, the Mann–Whitney U test and the Wilcoxon test were used to perform intergroup and intragroup comparisons, respectively. To test correlationships, the Spearman rank correlation test was used.

Results

Autoantibody Production and Albuminuria in Active Heymann Nephritis

Anti-megalin autoantibodies were detectable in the serum of immunized rats from week 2 onward and reached maximum levels at week 4 (P < 0.0001 week 4 versus week 0, P < 0.0001 immunized versus control at week 4) and subsequently declined to control levels (Figure 2A). Rats with AHN started to develop albuminuria 6 wk after immunization, which increased to 274 ± 68 mg/18 h at week 12 (P = 0.029 week 12 versus week 0, P = 0.016 immunized versus control at week 12). Control rats had an albumin excretion of less than 0.5 mg/18 h throughout the experiment (Figure 2B).

IgG and C3c Staining in the GCW

The development of albuminuria was preceded by granular deposition of IgG in the GCW that could be detected progressively from 2 wk after induction of AHN, whereas no IgG could be detected in the GCW of control animals (Figure 2C). The production of anti-megalin autoantibodies coincided with the deposition of IgG in the GCW. The decrease in autoantibody titer after week 4 is presumably due to increased deposition of IgG in the GCW. IF staining for the complement factor C3c in the GCW of immunized rats first became positive at week 6, concomitantly with the onset of proteinuria, and remained detectable in the glomeruli throughout the experiment, whereas no C3c was found in the GCW of the control rats (Figure 2D).

Expression of Agrin, HS, and Other Proteoglycans in the GBM

In normal kidneys, anti-agrin core protein antibody BL31 stains the GBM in a bright, linear way. Bowman’s capsule and the tubular basement membrane (TBM) are also stained by BL31, but in a weaker and more variable pattern (Figure 3B). After heparitinase digestion, mAb 3G10 directed against the HS stubs stains most basement membranes in a bright, linear way. The TBM and basement membranes of peritubular capillaries are stained with weaker intensity than the GBM, Bowman’s capsule, and basement membranes of muscle cells in arteries and arterioles (Figure 3A). mAb JM403 against low-sulfated domains in HS side chain stains the GBM in a bright, linear way. A more segmental staining was observed in Bowman’s capsule. Most TBM are weakly stained (Figure 3C). mAb KJ865 against N-acetylated domains in HS stained the GBM in a bright, linear pattern and showed segmental staining of Bowman’s capsule. Basement membranes of muscle cells in
arteries and arterioles are stained as well, and the TBM are stained very weak or not at all (Figure 3D). In AHN, there was no significant alteration in staining intensity with the anti-HS stub mAb 3G10 (222 ± 617 AU at week 0 and 224 ± 24 AU at week 12) (Figure 3E), but the staining of the anti-agrin core protein antibody BL31 decreased by 25% (248 ± 63A U at week 0 versus 187 ± 28 AU at week 12, P < 0.016) (Figure 3F). All capillary loops, however, remained positively stained, and there was only a decrease in intensity of staining. Furthermore, the staining pattern in the GBM changed from a linear to a slightly irregular, granular staining, which sometimes had a moth-eaten aspect. In some tubuli, the TBM showed a segmental increased thickness, which was positive for agrin. The same holds true for Bowman’s capsule. This staining pattern was comparable to our observations in a previous study with an mAb against the human agrin core protein on biopsies of patients with membranous nephropathy (15). From week 6 after induction of AHN onward, there was a progressive decrease in the number of positively stained capillary loops as evaluated with anti-HS side chain mAb JM403 and KJ865 as well as a reduction in the intensity of staining (Figure 3, G and H, and Figure 4, A and B), concomitantly with C3c positivity in the GCW (compare with Figure 2D). Staining with JM403 and KJ865 diminished progressively to 35 and 30% of control levels at week 12, respectively, while in the control animals there was no decrease in HS staining (P = 0.016 AHN [n = 4] versus control [n = 5] at week 12 for both mAb) (Figure 4, A and B). A significant inverse correlation was found between albuminuria and GBM JM403 staining (r = -0.82, P < 0.0001) (Figure 4C) or GBM KJ865 staining (r = -0.75, P < 0.0001) (Figure 4D). Although there was a clear decrease in the MBM staining of KJ865, Bowman’s capsule and regions of interstitial fibrosis stained positive for this mAb. This increased interstitial staining by KJ865 was earlier described for biopsies of renal allografts with chronic vascular rejection (24). It was also observed that the glomeruli of rats with AHN were

Figure 2. (A) Time course of serum anti-megalin antibody titer in active Heymann nephritis (AHN). Rats were immunized with renal tubular epithelium (RTE) in adjuvant (■) or with adjuvant alone (□). Values are expressed as percentage of positive control. (B) Time course of albuminuria in AHN. Rats were immunized with RTE in adjuvant (■) or with adjuvant alone (□), and albuminuria is expressed as mg/18 h albumin excretion. (C) Time course of glomerular capillary wall (GCW) IgG staining. GCW IgG staining was evaluated in direct immunofluorescence (IF) with anti-rat IgG-FITC on kidney sections of rats with AHN (■) or control rats (□). Maximal score is 250 arbitrary units (AU). (D) Time course of GCW C3c staining. GCW C3c staining was evaluated in direct IF with anti-rat C3c-FITC on kidney sections of rats with AHN (■) or control rats (□). Maximal score is 250 AU.
Figure 3. Agrin core protein and HS staining of control rats and rats with AHN. Indirect IF on kidney sections of control rats at week 12 (A through D) and rats with AHN at week 12 (E through H) with antibodies directed against the following epitopes of heparan sulfate proteoglycan (HSPG): A and E, anti-HS stub 3G10 after digestion with heparitinase; B and F, anti-agrin core protein BL31; C and G, anti-HS monoclonal antibody (mAb) JM403 in a double staining with BL31; D and H, anti-HS mAb KJ865. Magnification, ×260. Exposure time, 15 s.
somewhat larger than those of control rats at the same time point but showed no mesangial expansion (Figure 3).

To compare the staining of the core protein of agrin with the staining for perlecan and the chondroitin sulfate proteoglycan bamacan, we stained kidney sections of control and AHN rats at week 12 with antibodies for these PG. The polyclonal antibody against perlecan and the mAb against bamacan stained most BM in a linear way. Both antibodies stained the mesangial matrix, Bowman’s capsule, TBM, basement membranes of smooth muscle cells in arterioles and arteries, and basement membranes of endothelial cells in blood vessels. The polyclonal antibody against perlecan stained the GBM with a lower intensity than the other basement membranes, whereas the mAb against bamacan did not stain the GBM at all. The differential staining pattern in the GBM between perlecan and agrin is already described (11,36). However, there were no differences in glomerular staining patterns of these antibodies between control rats and rats with AHN at week 12 (data not shown).

**Effect of IgG Elution on HS Staining in the GBM**

To investigate whether masking by IgG depositions was responsible for the decrease in HS staining, we eluted IgG from cryostat sections of AHN kidneys. Elution with 0.12 M glycine, pH 2.8, and 0.1 M glycine/6 M urea, pH 3.5, for 1, 2, or 4 h resulted in a 50 to 75% reduction of IgG staining intensity, but all capillary loops remained weakly positive for IgG. The staining of JM403, however, was not restored by either of these treatments. All other elution conditions resulted in either no change of IgG depositions or a reduced staining of HS in normal rat kidneys. These experiments make it unlikely that in AHN the decrease of HS staining is due to masking of HS by autoantibodies. This is in line with the finding that in a double staining for HS and megalin, megalin positivity in immune

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**Figure 4.** Time course of glomerular basement membrane (GBM) HS staining with mAb JM403 and mAb KJ865 and correlation with albuminuria. GBM HS staining was evaluated in indirect IF with mAb JM403 (A) and mAb KJ865 (B) on kidney sections of rats with AHN (■) or control rats (□). Per animal, the GBM staining intensity of 25 glomeruli was scored on coded sections on a scale between 0 and 10, so that the maximal score is 250 AU, and the mean of the scores of two independent observers was used (for details, see Materials and Methods). $n = 5$ at each time point. In AHN rats, JM403 staining decreased from 220 ± 8 AU at week 0 to 76 ± 35 AU at week 12 and KJ865 staining decreased from 220 ± 31 AU at week 0 to 66 ± 51 AU at week 12 (both mAb, $P = 0.016$), whereas in the control rats the JM403 and KJ865 scores at week 12 were 216 ± 31 AU and 219 ± 40 AU, respectively. Correlation between albuminuria and GBM HS staining by mAb JM403 (C) and mAb KJ865 (D). For JM403, $r_s = -0.82$, $P < 0.0001$ and for KJ865 $r_s = -0.75$, $P < 0.0001$. 

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complexes was not associated with a decrease of HS staining (data not shown).

**Quantification of HS Content in Glomeruli**

To investigate whether the decrease in GBM HS staining was due to a decreased amount of glomerular HS, the total GAG content and total GAG minus HS were determined in glomeruli of control rats at week 12 and AHN rats at weeks 10 and 12 in the Farndale assay with and without HNO$_2$ pH 1.5 treatment. The HS content of control rats at week 12 was 3.0 μg/10,000 glomeruli and was decreased in AHN rats to 46% of control at week 10 and to 32% of control at week 12 (Figure 5). These results indicate that the decrease in GBM HS staining that we observed in AHN is due to an absolute reduction in glomerular HS content.

**Detection of Components of the Reactive Oxygen/Nitrogen Pathway**

Because we have shown in a previous study that hydroxyl radicals can cause depolymerization of HS (22), we hypothesized that oxygen radicals and/or nitrogen radicals are also involved in HS loss in AHN. In Figure 6, the reactions and several enzymes involved in the formation of hypochlorite, hydroxyl radicals, and peroxynitrite are shown. We stained kidney sections of immunized and control rats at weeks 6 and 12 for cytochrome b$_{558}$, myeloperoxidase, inducible nitric oxide synthase, and peroxynitrite-modified proteins. However, there were no differences in staining intensities between immunized and control animals with these antibodies. We also could not detect O$_2^-$ producing cells by enzyme-histochemistry in control or AHN rats (data not shown). Although these experiments suggest that in AHN hypochlorite radicals and peroxynitrite are not involved, a role for hydroxyl radicals cannot be excluded, since we could not measure hydroxyl production due to its short half-life.

**Discussion**

Immunization of rats with RTE induced subepithelial antibody depositions and subsequent development of heavy albuminuria as also found in previous studies (2,21). However, the precise mechanism by which proteinuria develops in AHN (and in the passive variant of Heymann nephritis [PHN]) has not been elucidated.

In this study, we observed intense staining of IgG along the GCW from 2 wk onward after the immunization with RTE. The formation of IgG deposits precedes the development of albuminuria, as was also found by others (2), and coincided with detection of anti-megalin autoantibodies in the serum. The GCW was positive for C3c staining from 6 wk after immunization, concomitantly with the development of albuminuria and decrease of GBM HS staining. The staining of C3c in the GCW reflects ongoing disease activity and complement activation as shown in PHN (37) and AHN (38,39). Activation of complement plays a major role in the injury of the glomerular epithelial cell (40). Proteinuria of complement component C6-deficient rats with AHN, however, was similar to that of non-complement-deficient rats with AHN, and it was shown that the size of the immune complexes correlated to the levels of proteinuria in this study (41). Initially, these immune complexes were shown to be localized in the clathrin-coated pits. Later on, when the immune complexes increase in size, they become detached from the podocyte and bind to the GBM (6); however, the mechanism by which this occurs has not been elucidated.

Because HS is thought to be important for the permeability properties and the integrity (42) of the GBM, we studied GBM HS(PG) expression in AHN. We previously showed agrin to be the major HSPG in the GBM (10,11). The staining intensity for the HS stubs that were still attached to the agrin core protein after heparitinase digestion remained unaltered, but the staining for the agrin core protein decreased in the course of the disease. A decrease in agrin core protein staining would be expected to coincide with a decrease in staining for HS stubs; however, we observed no alteration in the staining for the HS stubs. BL31 is specific for the agrin core protein (25), whereas mAb 3G10 is not agrin-specific and therefore it may stain HS stubs of other HSPG as well. Therefore, a decrease of agrin in the GBM may not necessarily lead to a decrease in HS stubs.

Alternatively, due to the high intensity of the staining of mAb 3G10, there may be a decrease in HS stubs that is not observed. We observed no differences in staining of perlecan and bamacan in control and AHN rats at week 12. The used antibodies, however, stained the GBM with minor intensity (perlecan) or...
not at all (bamacan). Recently, it was shown that agrin is the major HSPG in the GBM and that large parts of the GBM are negative for perlecan (11). These results suggest that the changes in HS staining that we observed are specific for the HS side chains of agrin in the GBM. The staining of HS in the GBM decreased considerably in the course of AHN, as evaluated by two mAb directed against different domains in the HS side chain. This is in line with the observation that a loss of GBM anionic sites occurs both in PHN and AHN (16–19). The staining for HS in the GBM was inversely correlated with albuminuria, which suggests a functional relationship between these pathologic changes. This inverse correlation between albuminuria and GBM HS staining is not unique for AHN, since it was also observed in adriamycin nephropathy, murine lupus nephritis, and diabetic nephropathy (22,32,43). Quantification of glomerular HS showed a clear reduction in the amount of HS, corresponding to the reduced staining of HS in the GBM. This indicates that in AHN, glomerular HS content decreases during progression of the disease compatible with an increased permeability of the GBM for macromolecules. Theoretically, several mechanisms could account for the decrease in GBM HS and albuminuria in AHN. First, GBM HS could be masked by immune complexes as was also suggested earlier for loss of anionic sites in AHN (16,18,19) and for lupus nephritis (32). It has been shown that the receptor-associated protein (RAP), but not megalin, binds to heparin (44). The C terminus of RAP includes a region with a highly positive charge density. The immune deposits could bind via RAP to HS in the GBM. RAP, however, does not bind to HS (45). Furthermore, AHN and PHN can be induced by megalin alone, or anti-megalin antiserum, respectively, independently from RAP (21,46). Loss of HS staining due to masking by IgG is also unlikely because deposition of IgG preceded HS loss by several weeks and elution of 50 to 75% of the IgG did not result in restoration of HS staining. Moreover, biochemical quantification of glomerular HS clearly showed a reduction of HS content in AHN, which make a reduced staining due to masking unlikely. Second, oxygen and/or nitrogen radicals could be responsible for HS degradation. Reactive oxygen species (ROS) are also thought to play a role in human membranous nephropathy, AHN, and PHN (29,47–49). Furthermore, the ROS scavengers dimethylthiourea, sodium benzoate (both scavengers of the hydroxyl radical), and deferoxamine (which chelates iron) reduced albuminuria in PHN (49). In the present study, however, we could not show involvement of hypochlorite radicals or peroxynitrite in AHN. We could not measure the production of hydroxyl radicals, which can depolymerize HS from rat agrin (22). Therefore, it is still possible that in the present study an increased production of hydroxyl radicals is induced (by complement activation), which depolymerizes HS and leads to albuminuria in AHN. A third mechanism that may be responsible for the decreased GBM HS is complement-mediated cleavage of HSPG. In a model for hyperacute rejection, Platt and coworkers observed that HS is enzymatically cleaved from porcine endothelial cells after incubation with human serum (50). The binding of natural antibodies, the generation of the complement component C5a, and the production of oxygen radicals are essential for this cleavage. Since we observed that hydroxyl radicals can depolymerize HS in vitro (22), this mechanism of complement-mediated ROS production might be involved in the decrease of HS that we found since C3c staining occurred simultaneously with the decrease in HS staining.

In summary, this study shows that in AHN albuminuria and the presence of complement in the GCW are associated with a decrease in the GBM content of HS. This decrease is possibly the result of complement-mediated cleavage of HS.

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Figure 6. Reactions and enzymes involved in the synthesis of oxygen and nitrogen radicals. (Immuno)histochemistry was performed on the components that are in boldface.
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