Hydroxyl Radicals Depolymerize Glomerular Heparan Sulfate 
in Vitro and in Experimental Nephrotic Syndrome*

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Heparan sulfate, the polysaccharide side chain of heparan sulfate proteoglycan, is important for the permselective properties of the glomerular basement membrane. In this report, we show a role for hydroxyl radicals in heparan sulfate degradation and an enhanced glomerular basement membrane permeability. First, in enzyme-linked immunosorbent assay, exposure of coated heparan sulfate (proteoglycan) to reactive oxygen species resulted in a 50% decrease of binding of a monoclonal antibody against heparan sulfate, whereas binding of an antibody against the core protein remained unaltered. Second, on polyacrylamide gel electrophoresis, the molecular weight of heparan sulfate exposed to radicals was reduced which indicates depolymerization. Both in enzyme-linked immunosorbent assay and gel electrophoresis, hydroxyl radicals are instrumental for heparan sulfate degradation as shown by the addition of various radical scavengers. Third, in an experimental model for human nephrotic syndrome (Adriamycin nephropathy in rats), glomerular basement membrane staining of two recently described anti-heparan sulfate antibodies (JM403 and KJ865) was reduced by 24 and 43%. Treatment of Adriamycin-exposed rats with the hydroxyl radical scavenger dimethylthioureode both reduced albuminuria by 37% (p < 0.01) and partly prevented loss of heparan sulfate by 24 and 43%. In contrast to the heparan sulfate side chains, the core protein expression and the extent of glycanation did not change in Adriamycin nephropathy. We conclude that glomerular basement membrane heparan sulfate is susceptible to depolymerization by hydroxyl radicals leading to loss of glomerular basement membrane integrity and albuminuria.

Heparan sulfate (HS) is the anionic polysaccharide side chain of heparan sulfate proteoglycan (HSPG) present in basement membranes, extracellular matrix, and on the cell surface of many (if not all) cell types (1-3). Several investigators have shown that HS plays an important role in the permselective properties of the glomerular basement membrane (GBM) (4, 5). Enzymatic digestion of HS by heparitinase resulted in an increased passage of native ferritin and albumin through the GBM (6, 7). Furthermore, intravenous injection of a monoclonal antibody (mAb) directed against HS induces acute, selective proteinuria in rats (8). A reduction in GBM HS-associated anionic sites was found with cationic probes in several human and experimental proteinuric glomerulopathies (9, 10). With recently developed antibodies directed against GBM HSPG core protein and the HS side chain (11, 12), we demonstrated a decrease in HS staining in the GBM in different human proteinuric glomerulopathies, whereas the staining of the HSPG core protein remained unaltered (13). The mechanism responsible for this observation remains to be elucidated.

Previous reports have shown that reactive oxygen species (ROS) are able to degrade proteoglycan core proteins (17, 18) and glycosaminoglycans including HS and heparin (19-23) or can inhibit de novo synthesis of proteoglycans (17, 24). Furthermore, ROS were demonstrated to be involved in experimental glomerulopathies such as Adriamycin (ADR) nephropathy (25), puromycin aminonucleoside nephrosis (26), passive Heymann nephritis (27), and anti-GBM nephritis (28). ROS have been shown to cause proteinuria (29, 30) and loss of HS in the isolated perfused kidney model (29).

These data suggest that loss of GBM HS staining in various types of human glomerulopathies (13) might be secondary to the local production of ROS. To this end, we performed in vitro and in vivo experiments to evaluate the effects of ROS on GBM HSPG and HS and on GBM permeability. As major tools we used a set of four antibodies directed against various epitopes of HSPG as follows: JM403 (12) and KJ865 (31) which are directed against different domains in the HS side chain of HSPG, 3G10 which is directed against an epitope of HS that is generated by digestion of HSPG with heparitinase (32), and BL31 which is directed against the HSPG core protein (33). In enzyme-linked immunosorbent assay (ELISA), we studied the effects of ROS on immobilized HS from bovine kidney or HSPG isolated from rat GBM. On polyacrylamide gel electrophoresis (PAGE), we evaluated the effect of ROS on the molecular weight of HS. Identification of the radical(s) primarily involved in HS alteration was performed by the use of specific oxygen radical scavengers. To investigate the physiological significance of ROS on GBM HSPG expression and GBM permeability, we evaluated HS alterations in the nephrotic syndrome in rats after intravenous injection of ADR (doxorubicin, a member
of the anthracyclin family), since in this model the generation of ROS has been shown (25, 34). The impact of ROS on GBM HSPG and HS expression and albuminuria in this model was investigated by intervention studies with radical scavengers. Our results indicate that hydroxyl radicals are important for both GBM HS degradation and increased GBM permeability in ADR nephropathy in rats.

MATERIALS AND METHODS

In Vitro Generation of ROS

To study the effect of ROS on HS in vitro, ROS were generated via the hypoxanthine-xanthine oxidase system and the iron-catalyzed Haber-Weiss reaction according to the method described by Mosley et al. (19). In this system, O$_2^-$, H$_2$O$_2$, and ·OH are formed as is indicated by the following Equations 1–4.

\[
\begin{align*}
\text{Xanthine oxidase} \\
\text{hypoxanthine} & \rightarrow \text{xanthine} + \text{uric acid} + \text{O}_2 \\
2\text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_3 \\
\text{Fe}^{3+} + \text{O}_2^- & \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{2+}
\end{align*}
\]

(Eq. 1)

(Eq. 2)

(Eq. 3)

(Eq. 4)

Hypoxanthine (HX, Sigma) and xanthine oxidase (XO, Sigma, grade III from buttermilk) were dissolved in a buffer containing 50 mM HEPES, pH 7.8, 1 mM EDTA, and (unless stated otherwise) 50 µM Fe$^{3+}$-EDTA chelate and incubated in the wells of an HS-coated micro titer plate for 2 h at 37 °C in the presence or absence of various concentrations of superoxide dismutase (SOD, EC 1.15.1.1 from bovine erythrocytes, Sigma; concentration range from 0.15–2600 units/ml), dimethylthioura (DMTU, Sigma; from 0.003 to 60 mg/ml), or deferoxamine (DFO, Dedem, Diosynth, Oss, The Netherlands). The gel was run at 250 V for 2 h at room temperature. After washing 6 times with PBS, HX and XO were added in an end volume of 100 µl/well for 2 h at 37 °C. Thereafter, the plates were washed 6 times with PBS and incubated with 1% gelatin (Difco) in PBS, 150 µl/well, at room temperature. After washing 6 times with PBS, HX and XO were added in an end volume of 100 µl/well for 2 h at 37 °C. Thereafter, the plates were washed 6 times with PBS and incubated with 1% gelatin (Difco) in PBS, 150 µl/well, overnight at room temperature. Alternately, HSPG, isolated from rat GBM as described earlier (31), was coated at 1 µg/ml in PBS, 100 µl/well, overnight at room temperature. After washing 6 times with PBS, HX and XO were added in an end volume of 100 µl/well for 2 h at 37 °C. Thereafter, the plates were washed 6 times with PBS and incubated with 1% gelatin (Difco) in PBS, 150 µl/well, for 2 h at room temperature to prevent nonspecific binding. After washing 6 times with PBS containing 0.05% Tween 20 (PBS/Tween), the wells were incubated with anti-HS side chain mAb JM403 1:50,000 or anti-HSPG core protein antibody BL31 1:2,000 in PBS/Tween, 100 µl/well, overnight at 4 °C to inhibit nonspecific binding. After washing 6 times with PBS/Tween for 1 h at room temperature. Then, the wells were washed 6 times with PBS/Tween and incubated with either 100 µl of peroxidase-labeled rat anti-mouse Ig (light chain type kappa) (RM19; Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands) 1:1,500 in PBS/Tween or with peroxidase-labeled rabbit anti-goat Ig (Dakopatts, Glostrup, Denmark) 1:2,000 in PBS/Tween for 1 h at room temperature. Then, the wells were washed 9 times with PBS/Tween and incubated with 100 µl of tetramethylbenzidine substrate solution that was prepared immediately before use (SFRI Laboratories, Bergantino, France). After 20 min, the reaction was stopped with 2 M H$_2$SO$_4$, and absorption was measured at a wavelength of 450 nm using a Bio-Rad Multiskan Reader.

Animals

For all experiments, we used male Wistar-Unilever rats that were bred at our animal laboratory and weighed 150–200 g at the start of the experiments. The animals were fed standard food and tap water ad libitum.

Experimental Design

Time Response Study — In a previous study (36), we compared different doses of ADR. We found that a dose of 5 mg/kg body weight (BW) Adriamycin® (Adriablastina; Farmitalia, Milan, Italy) was optimal to induce proteinuria. To study the time course of ADR nephropathy, 40 animals were injected with 5 mg of ADR/kg BW and after either 1–5 or 9 weeks 8 rats were sacrificed, and their kidneys were snap-frozen in liquid nitrogen and used for IF. Urine and serum samples were col-

FIG. 1. Schematic representation of an HSPG molecule. The binding sites of four antibodies directed against different domains of HSPGs are indicated. O, HSPG core protein; , HSPG stub after heparitinase digestion; •, N-acetylated domain in HS; , mixed N-acetylated and N-sulfated domain in HS containing N-unsubstituted glucosamine residues; ■, high sulfated (heparin-like) domain in HS.

FIG. 2. Effect of various doses of hypoxanthine and/or xanthine oxidase on HS. Coated HS was incubated for 2 h at 37 °C with hypoxanthine (□), xanthine oxidase (▲), or both (●) in various dilutions starting with 7.36 mM HX and 50.4 milliunits/ml XO. Then, ELISA was performed with anti-HS mAb JM403.

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Fig. 3. Effect of ROS on HS and HSPG in vitro. Coated HS (A, B, and C) or HSPG from rat GBM (D, E, and F) were incubated with hypoxanthine and xanthine oxidase for 2 h at 37 °C in the presence of various concentrations of superoxide dismutase (A and D), dimethylthiourea (B and E), or deferoxamine (C and F). Then ELISA was performed with anti-HS mAb JM403 (A–F, ○) and anti-HSPG core protein antibody BL31 (D–F, □).

Treatment with ROS Scavengers—To study the involvement of ROS in ADR nephropathy, 22 rats received an injection of 5 mg of ADR/kg BW and were treated with ROS scavengers. One group (n = 8) received an initial intraperitoneal injection of 500 mg of DMTU/kg BW dissolved immediately before use in saline 6 h before the ADR injection, followed by intraperitoneal injections of 125 mg of DMTU/kg BW twice daily (at 8.00 and 20.00 h) for 7 days, starting 6 h after ADR injection. The other 2 groups (both n = 7) received either 52,600 units of SOD/kg BW dissolved immediately before use in saline or the same volume of saline intraperitoneally twice daily (at 8.00 h and 20.00 h) for 7 days starting 6 h before ADR injection. These doses are in accordance with other studies, where they were found to be effective (26, 37–39).

Urine was collected every week to determine albumin and IgG concentrations. Clearance of albumin was calculated as urine albumin excretion per 24 h/serum albumin concentration, and selectivity index was calculated as clearance IgG/clearance albumin.

Determination of Urine and Serum Albumin and IgG Concentration

Urinary albumin excretion was measured in urine collected for 24 h in metabolic cages. Urine and serum concentrations of albumin were determined by rocket immunoelectrophoresis (40), with goat anti-rat albumin and rat albumin as a standard (both from Nordic, Tilburg, The Netherlands). Urine and serum IgG concentrations were determined by ELISA (8).
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Assessment of HS and HSPG Staining in the GBM by Indirect IF

Indirect IF was performed as described previously (11, 13) on 2-μm cryostat sections of rat kidneys that were fixed during 10 min in acetone at 4 °C (except for the sections that were digested by heparitinase to allow staining with 3G10). All antibodies were diluted in PBS containing 1% BSA and 0.1% sodium azide (IF buffer) and were incubated for 45 min at room temperature. Sections were incubated with mAb JK305 diluted 1:20, followed by FITC-labeled goat anti-mouse IgM Fe-specific (Nordic, Tilburg, The Netherlands), diluted 1:50 in IF buffer containing 4% normal rat serum. To evaluate the GBM HS staining with a mAb directed against an epitope of HS that is generated by digestion with heparitinase, non-fixed sections were incubated for 1 h at 37 °C with 0.25 units/ml heparitinase (heparin-sulfate lyase, EC 4.2.2.8, Sigma) in 50 mM HEPES and 10 mM CaCl₂, pH 7.0. Next, the sections were incubated with mAb 3G10 diluted 1:200 in IF buffer, followed by incubation with FITC-labeled goat anti-mouse IgG2b (Southern, Birmingham, AL) diluted 1:100 in IF buffer containing 5% normal rat serum. Sections were double-stained with goat anti-human GBM HSPG core protein BL31 and mouse anti-rat GBM HS mAb JM403. Sequential incubations were performed as follows: 1) goat anti-HSPG core protein BL31, diluted 1:400; 2) FITC-labeled rabbit anti-goat IgG (De Beer Medicals, Diessen, The Netherlands), diluted 1:500 in IF buffer containing 4% normal rat serum; 3) mouse anti-HS mAb JM403, diluted 1:800; 4) TRITC-labeled goat anti-mouse IgM Fe-specific (Nordic, Tilburg, The Netherlands), diluted 1:250 in IF buffer containing 4% normal rat serum. The double staining experiments included the following controls: 1) omitting BL31 or JM403 or both; 2) incubating BL31 or JM403 with the conjugate used for the detection of the other antibody in the double staining.

After incubation with antibodies the sections were washed with PBS and embedded in Vectashield mounting medium H-1000 (Vector Laboratories Inc., Burlingame, CA) and examined with a Zeiss Axioskop microscope equipped with an epi-illuminator. The staining intensities of the antibodies in the GBM were scored in 25 glomeruli on a scale of 0-10, 0 being no staining and 10 being a maximal staining intensity. The maximal score for the staining in each section was 250 arbitrary units (AU). The scoring was performed by two independent observers on coded sections, and the mean of the two scores was used for further analysis. As described previously the inter-observer variation is low (41).

Statistical Analysis

For statistical analysis, the Mann-Whitney U test and the ANOVA for repeated measurements were used for inter-group comparisons. To test correlations, the Spearman rank correlation test was used. A p value of less than 0.05 was considered significant.

RESULTS

Effect of ROS on HS(PG) in Vitro — To find optimal concentrations of HX and XO, concentration ranges from 0.002 to 7.36 mM HX and from 0.01 to 50.4 milliunits/ml XO were tested on coated HS in ELISA (Fig. 2). The optimal concentrations of HX and XO were chosen such that both components alone had no effect on the ELISA signal, and the combination resulted in a maximal decrease of JM403 signal on coated HS. The optimal concentrations were 1.84 mM HX and 12.6 milliunits/ml XO, resulting in a decrease of ELISA signal of JM403 of 58 ± 6%. These concentrations (1:4 dilution in Fig. 2) were used for all further experiments.

The addition of SOD to the HX and XO mixture dose-dependently prevented loss of the anti-HS signal in ELISA (Fig. 3A). Since SOD inactivates O₂⁻, the first oxygen radical formed in the cascade of reactions, no additional ROS (H₂O₂ and ·OH) will be formed. This indicates that ROS are indeed responsible for the decrease in HS recognition by mAb JM403. This experiment, however, does not answer the question which of the radicals is primarily responsible. Therefore, we also added DMTU to HX-XO. Now, O₂⁻ and H₂O₂ are still formed, but ·OH radicals are scavenged. DMTU also dose-dependently prevented loss of the anti-HS signal in ELISA (Fig. 3B). This was corroborated by the finding that chelation of Fe³⁺ by DFO (Fig. 3C), thus anticipating the formation of ·OH radicals by the Haber-Weiss reaction, also prevented loss of the anti-HS signal. When HS was exposed to HX and XO in an iron-free buffer, the anti-HS signal remained normal as well. These data clearly indicate that primarily ·OH radicals are involved in HS alteration in vitro.

Since various HS preparations differ in the degree of N- and/or O-sulfation and uronate C-5 epimerization, it was still uncertain whether GBM HS is also susceptible to ROS. Moreover, it is not known whether ROS can also alter the core protein of GBM HSPG. For that reason, ELISA experiments were repeated with HSPG isolated from rat GBM. We used anti-HS mAb JM403 to evaluate the effect of ROS on the HS polysaccharide side chain and a polyclonal antibody (BL31) against the core protein to assess ROS-mediated damage to the core protein. Incubation with HX and XO also resulted in a decreased JM403 signal on rat GBM HSPG. This decrease could also be prevented dose-dependently by adding SOD, DMTU, and DFO to HX-XO (Fig. 3, D-F) and by incubating HX and XO in an iron-free buffer. Oxygen radicals had no effect, however, on the recognition of the HSPG core protein by BL31 (Fig. 3, D-F). These data indicate that ·OH radicals are responsible for the decrease of anti-HS binding to GBM HSPG.

Theoretically, two different mechanisms might explain this finding. First, ·OH radicals alter the biochemical composition of HS, thereby changing the epitopes recognized by mAb JM403. Second, ·OH radicals depolymerize HS chains into oligosaccharides. Therefore, we evaluated the molecular size of native and ROS-exposed HS (from bovine kidney) on PAGE. In addition, HS was exposed to ROS in the presence of various scavengers. On PAGE, native HS forms a band in the top of the gel (Fig. 4, lane 2). After treatment with HX-XO, however, the band was lost (Fig. 4, lane 3). The control samples incubated in an iron-free buffer (Fig. 4, lane 1) and the control incubated with HX-XO only (Fig. 4, lane 4) behaved like native HS. These data indicate that ·OH radicals are responsible for the decrease of anti-HS binding to GBM HSPG.
After incubation with 1.84 mM HX and 12.6 milli-units/mL XO, this pattern changes to a smear down to the level of the octasaccharides, indicating depolymerization of HS (lane 7). Adding either DMTU, SOD, or DFO to HX-XO or incubating HX-XO in an iron-free buffer prevented the reduction in molecular mass (lanes 8–11). HX or XO alone had no effect on HS (lanes 5 and 6). From this experiment, we conclude that 'OH radicals depolymerize HS into oligosaccharides.

**In Vivo Experiments**—These results (degradation of HS but no effect on the core protein of HSPG) resembled our 1P finding in various human glomerular diseases (13). To investigate an possible role of ROS in vivo, we decided to induce a nephrotic syndrome into rats by intravenous injection of ADR, since ROS have been suggested to play a role in this disease.

**Time Response Study**—The characteristics of ADR nephropathy are shown in Table I. The injection of 5 mg/kg BW ADR induced a heavy albuminuria and a decrease in serum albumin levels. The induced proteinuria was selective, since the selectivity index (clearance of IgG/clearance of albumin) was 0.17 ± 0.0017 for the ADR group, whereas for the saline group it was 0.88 ± 0.0018. These data suggest that, besides other postulated mechanisms (14–16), depolymerization of GBM HS by ROS could play a role in other glomerulopathies as well. This is substantiated by the beneficial effects of ROS scavengers, not only in ADR nephropathy (34, 39) but also in various other experimental models of glomerulopathy (46), such as purine aminonucleoside nephropathy (26), passive Heymann nephritis (27) and anti-GMnephritis (28). Therefore, depolymerization of GBM HS by ROS could be a common mechanism responsible for HS loss and proteinuria in these diseases.

In this study, we have shown that in vitro exposure to ROS of both HS and HSPG isolated from rat GBM results in alterations of HS leading to a decreased recognition by a specific anti-HS mAb in ELISA. PAGE revealed that HS was depolymerized by ROS. Addition of specific scavengers of hydroxyl radicals prevented this depolymerization both in ELISA and in PAGE. This indicates that hydroxyl radicals are responsible for this effect. Other investigators have also shown that ROS can cause depolymerization of glycosaminoglycans (19–23). Most studies focused on hyaluronic acid and heparin which are indeed depolymerized by ROS (21–23, 42–44), but little is known about the susceptibility of HS to ROS.

We observed in ADR nephropathy a progressive albuminuria that was accompanied by an increase in selectivity, compatible with the loss of negative charges from the GBM. The number of HSPG core molecules and HS side chains was unaltered, but there was a decrease of the GBM staining by two anti-HS mAbs which recognize different domains in the HS side chain. In addition, this loss of HS staining correlated with albuminuria.

Treatment with the hydroxyl radical scavenger DMTU resulted in an amelioration of albuminuria and a partial prevention of the loss in GBM HS staining. This is in line with our in vitro findings that hydroxyl radicals can induce depolymerization of HS. On the basis of these results, we postulate that the ensuing albuminuria is (at least partly) due to the depolymerization of HS, leading to loss of HS from the GBM. In various human and experimental proteinuric glomerulopathies, loss of HS staining in the GBM has been observed as well (13–15, 45). Moreover, the correlation between albuminuria and loss of HS staining is not unique for ADR nephropathy, since we found a similar correlation in murine lupus nephritis (14), active Heymann nephritis (45), and in patients with diabetic nephropathy (15). This suggests that, besides other postulated mechanisms (14–16), depolymerization of GBM HS by ROS could play a role in other glomerulopathies as well. This is substantiated by the beneficial effects of ROS scavengers, not only in ADR nephropathy (34, 39) but also in various other experimental models of glomerulopathy (46), such as purine aminonucleoside nephropathy (26), passive Heymann nephritis (27) and anti-GMnephritis (28). Therefore, depolymerization of GBM HS by ROS could be a common mechanism responsible for HS loss and proteinuria in these diseases.

**Discussion**

Apart from degradation of HS, ROS could also exert a direct toxic effect on the glomerular epithelial cell resulting, for example, in a structurally altered HS. Indeed, an increase in the extent of sulfation of HS could be an alternative explanation for the decrease in GBM HS staining that we found. Both anti-HS mAbs that we used in our study recognize exclusively low- or non-sulfated domains in the HS side chain. mAb JM403 is directed against a low-sulfated domain of HS containing an N-unsubstituted glucosamine unit (12), whereas mAb KJ865 is directed against N-acetylated domains of HS (31). Increase of HS sulfation greatly diminishes recognition by both mAbs and thus might explain loss of GBM staining. HS oversulfation,
Fig. 5. GBM HSPG staining of normal rats or rats with ADR nephropathy treated either with saline or dimethylthiourea. Indirect IF on kidney sections of normal rats (A–D), saline-treated ADR rats (E–H), or dimethylthiourea-treated ADR rats (I–L) with antibodies directed against the following epitopes of HSPG: A, E, and I, HSPG core protein BL31; B, F, and J, anti-HS mAb JM403 in a double staining; C, G, and K, anti-HS mAb KJ865; D, H, and L, heparitinase-created HS-stub (mAb 3G10) (magnification: 150 x).
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Fig. 6. A, time course of GBM HS staining in ADR nephropathy. Rats received a single intravenous injection of 5 mg/kg ADR at week 0. GBM HS staining was evaluated in indirect IF with anti-HS mAb JM403. Maximal score is 250 arbitrary units. B, correlation between albuminuria and GBM HS staining in ADR nephropathy. 

What could be the physiological meaning of HS susceptibility to ROS? GBM HS contains large N-acetylated stretches (recognized by KJ865), which are very sensitive to ROS (23). This could mean that HS functions as an antioxidant in the GBM, such that other molecules are protected from degradation by ROS. This antioxidant effect has also been shown for heparin and hyaluronic acid (42, 43, 53). One can only speculate on the physiological effects of the (by depolymerization) released HS oligosaccharides. It is known that HS can function as an anchorage molecule for growth factors, such as basic fibroblast growth factor (54). After degradation of HS, these growth factors would be set free and activate glomerular epithelial and/or mesangial cells. Released HS oligosaccharides could also exert a local impact on the coagulation, whereas in the GBM loss of HS could lead to clogging (55).

Our present study provides evidence that ROS-mediated HS depolymerization plays a role in ADR nephropathy and is correlated to GBM permeability. Since HS interacts with other basement membrane components, such as collagen IV and laminin, it is an important factor in the assembly and integrity of basement membranes and is consequently a determinant for the charge- as well as for the size-dependent permeability (55–57). Since ROS are generated by various mechanisms in proteinuric glomerular diseases, it is probable that this mechanism is operative in different glomerulopathies resulting in an increased permeability to macromolecules.

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