

Glomerular heparan sulfate alterations: Mechanisms and relevance for proteinuria

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Glomerular heparan sulfate alterations: Mechanisms and relevance for proteinuria. Heparan sulfate (HS) is the anionic polysaccharide side chain of HS proteoglycans (HSPGs) present in basement membranes, in extracellular matrix, and on cell surfaces. Recently, agrin was identified as a major HSPG present in the glomerular basement membrane (GBM). An increased permeability of the GBM for proteins after digestion of HS by heparitinase or after antibody binding to HS demonstrated the importance of HS for the permselective properties of the GBM. With recently developed antibodies directed against the GBM HSPG (agrin) core protein and the HS side chain, we demonstrated a decrease in HS staining in the GBM in different human proteinuric glomerulopathies, such as systemic lupus erythematosus (SLE), minimal change disease, membranous glomerulonephritis, and diabetic nephropathy, whereas the staining of the agrin core protein remained unaltered. This suggested changes in the HS side chains of HSPG in proteinuric glomerular diseases. To gain more insight into the mechanisms responsible for this observation, we studied GBM HS(PG) expression in experimental models of proteinuria. Similar HS changes were found in murine lupus nephritis, adriamycin nephropathy, and active Heymann nephritis. In these models, an inverse correlation was found between HS staining in the GBM and proteinuria. From these investigations, four new and different mechanisms have emerged. First, in lupus nephritis, HS was found to be masked by nucleosomes complexed to antinuclear autoantibodies. This masking was due to the binding of cationic moieties on the N-terminal parts of the core histones to anionic determinants in HS. Second, in adriamycin nephropathy, glomerular HS was depolymerized by reactive oxygen species (ROS), mainly hydroxyl radicals, which could be prevented by scavengers both *in vitro* (exposure of HS to ROS) and *in vivo*. Third, *in vivo* renal perfusion of purified elastase led to a decrease of HS in the GBM caused by proteolytic cleavage of the agrin core protein near the attachment sites of HS by the HS-bound enzyme. Fourth, in streptozotocin-induced diabetic nephropathy and during culture of glomerular cells under high glucose conditions, evidence was obtained that hyperglycemia led to a down-regulation of HS synthesis, accompanied by a reduction in the degree of HS sulfation.

Key words: glomerular basement membrane, protein permeability, extracellular matrix, podocytes, heparan sulfate proteoglycans.

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During glomerular ultrafiltration, the barrier restricting the passage of plasma proteins into the urine is the glomerular capillary wall (GCW). The GCW consists of the fenestrated endothelium, the glomerular basement membrane (GBM), and the foot processes of the glomerular visceral epithelial cells or podocytes with in-between the slit diaphragms. The fenestrae of the endothelial cells allow direct contact of blood with the GBM and hardly restrict passage of macromolecules [1]. Some data show that podocytes are involved in the permeability of the GCW. Cross-linking of the integrin receptors for extracellular matrix (ECM) components on the podocyte resulted in a decreased adhesion of podocytes to the GBM and an increased passage of macromolecules *in vitro* [2]. Furthermore, in saponin-induced podocyte injury in the single nephron model, passage of albumin through the GCW was localized to regions of detachment [3]. An injection of a monoclonal antibody directed against a component of the slit diaphragm resulted in an acute massive proteinuria [4, 5]. Nevertheless, the GBM is probably the most important component of this barrier and restricts the passage of macromolecules by both size and charge. Tracer studies have shown that the GCW behaves like a sieve, through which small molecules, such as inulin, can pass without restriction, whereas with increasing molecular size, the passage into the urinary space decreases progressively [6]. Studies using dextran fractions with similar molecular radii carrying either neutral, anionic (dextran sulfate), or cationic (diethylaminoethyl dextran) charges showed an enhanced permeability to cationic but a restricted permeability to anionic dextran fractions [6–8].

The GBM is organized as a network of fibrils or chords forming a small-meshed sieve. The composition of the GBM is different from most other BMs. Many components are present in the GBM, the most abundant are type IV collagen chains $\alpha 3$, $\alpha 4$, and $\alpha 5$, various laminin isoforms, especially laminin 11 (composed of $\alpha 5\beta 2\gamma 1$ laminin chains) and heparan sulfate proteoglycans (HSPGs) [9–13]. The main BM HSPGs that have been characterized until now are perlecan, agrin, and recently

collagen XVIII, which have a different localization in the glomerulus. Perlecan and collagen XVIII are most abundant in the mesangial matrix, whereas agrin is a major HSPG in the GBM. The glomerular localization and basic characteristics of these BM HSPGs are shown in Figure 1.

Proteoglycans (PGs) consist of a core protein to which GAG side chains are attached. For HSPG, this GAG side chain is HS. HS contains many negative charges, which are important determinants for the charge-dependent permeability of the GBM. HS is an unbranched chain of repeating disaccharide units, which contain an uronic acid (glucuronic or iduronic acid) and the aminosugar glucosamine. After formation of the precursor molecule heparosan, extensive modifications take place starting with *N*-deacetylation/*N*-sulfation of the glucosamine residue, followed by C-5 epimerization of glucuronic acid into iduronic acid, and *O*-sulfation at various positions [36]. For most reactions, several isoenzymes exist that have subtle differences in substrate-specificity; furthermore, these reactions run incompletely, leading to a great variation in the degree of sulfation along the stretch of the side chains. Because of the sequential order of the modification reactions and the substrate specificity of the enzymes involved, the initial distribution of *N*-sulfate groups will greatly determine the location of iduronic acid residues and *O*-sulfate groups. This results in a block-like structure in which alternating *N*-acetylated, mixed *N*-acetylated/*N*-sulfated and *N*-sulfated (heparin-like) domains are found (Fig. 2) [37–42]. Certain functions of HS are restricted to specific sulfation patterns, for example, the binding to antithrombin III or fibroblast growth factor-2 [43–46]. The presence of sulfate groups and carboxyl groups is responsible for the negative charge of HS at physiological pH. The functional relevance of the HS modification is shown by knockout mice lacking the enzyme HS 2-O-sulfotransferase, responsible for 2-O-sulfation of HS. These mice do not develop kidneys and die shortly after birth [47]. This enzyme deficiency has consequences for the interaction of HS with other ECM components, as well as with growth factors, which both are necessary for normal kidney development.

The relevance of HS for the charge-dependent permeability is documented by several experimental data [48, 49]. First, enzymatic digestion of HS by heparitinase caused an enhanced permeability of the GBM for native ferritin and albumin [50, 51]. Second, an acute, selective proteinuria could be induced in rats by a single intravenous injection of a monoclonal antibody directed against GBM HS [52]. Third, a reduction in GBM HS-associated anionic sites was demonstrated with cationic probes in several human and experimental proteinuric glomerulopathies [53–57]. Fourth, in several human and experimental glomerulopathies, an inverse correlation has been found between albuminuria and the GBM HS stain-

ing [58–61]. Apart from its role in the charge-dependent permeability, HS may also play a role in the size-dependent permeability. HS is able to bind other components of the GBM, such as collagen and laminin, whereas the core protein of agrin contains a laminin-binding site and probably contributes to the molecular architecture of the GBM [29, 62–65].

Besides the role in the glomerular filtration, HS may have several other functions in the GBM. It functions as an anchorage molecule and storage depot for various proteins, such as platelet-derived growth factor, basic fibroblast growth factor-2 and its receptor, antithrombin III, hepatocyte growth factor, interferon- γ , lipoprotein lipase, and platelet factor 4 [44, 66]. HS has anticoagulant properties, preventing coagulation at the endothelial surface of the GBM [67]. Moreover, HS inhibits mesangial cell proliferation [68–71]. Therefore, it may affect inflammation, mesangial proliferation and expansion, and other processes involved in glomerular pathology.

In different human proteinuric glomerulopathies, such as systemic lupus erythematosus (SLE), minimal change disease, membranous glomerulonephritis, and diabetic nephropathy, we demonstrated with antibodies directed against the agrin core protein and the HS side chain [72, 73] a decrease in HS staining in the GBM, whereas the staining of the agrin core protein remained unaltered [58–61]. This suggested that in certain forms of glomerular pathology, changes occur in the HS side chains of agrin that may be related to the development of proteinuria. This review focuses on four different mechanisms that are identified up to now for these changes in the HS polysaccharide side chain, masking by immune complexes, depolymerization by radicals, degradation by proteolytic enzymes or metabolically-induced biochemical changes in the HS structure.

MASKING OF HEPARAN SULFATE BY IMMUNE COMPLEXES

Systemic lupus erythematosus is an autoimmune disease characterized by the formation of autoantibodies, mainly directed against nuclear antigens. Currently, it is clear that this autoimmune response is T-cell dependent and antigen driven. A major candidate autoantigen for this response is the nucleosome, the building unit of chromatin, consisting of histones and DNA [74]. Besides serving as an immunogen for T- and B-cell responses, nucleosomes are also important for the development of glomerulonephritis in SLE. The observation that anti-nuclear antibodies could bind to HS started investigations on the mechanism for the development of glomerulonephritis [74–76]. It was shown that the binding of antinuclear antibodies to HS was not due to cross-reactive binding, as initially thought, but was mediated by nucleosomes [77]. Renal perfusion studies made it clear that

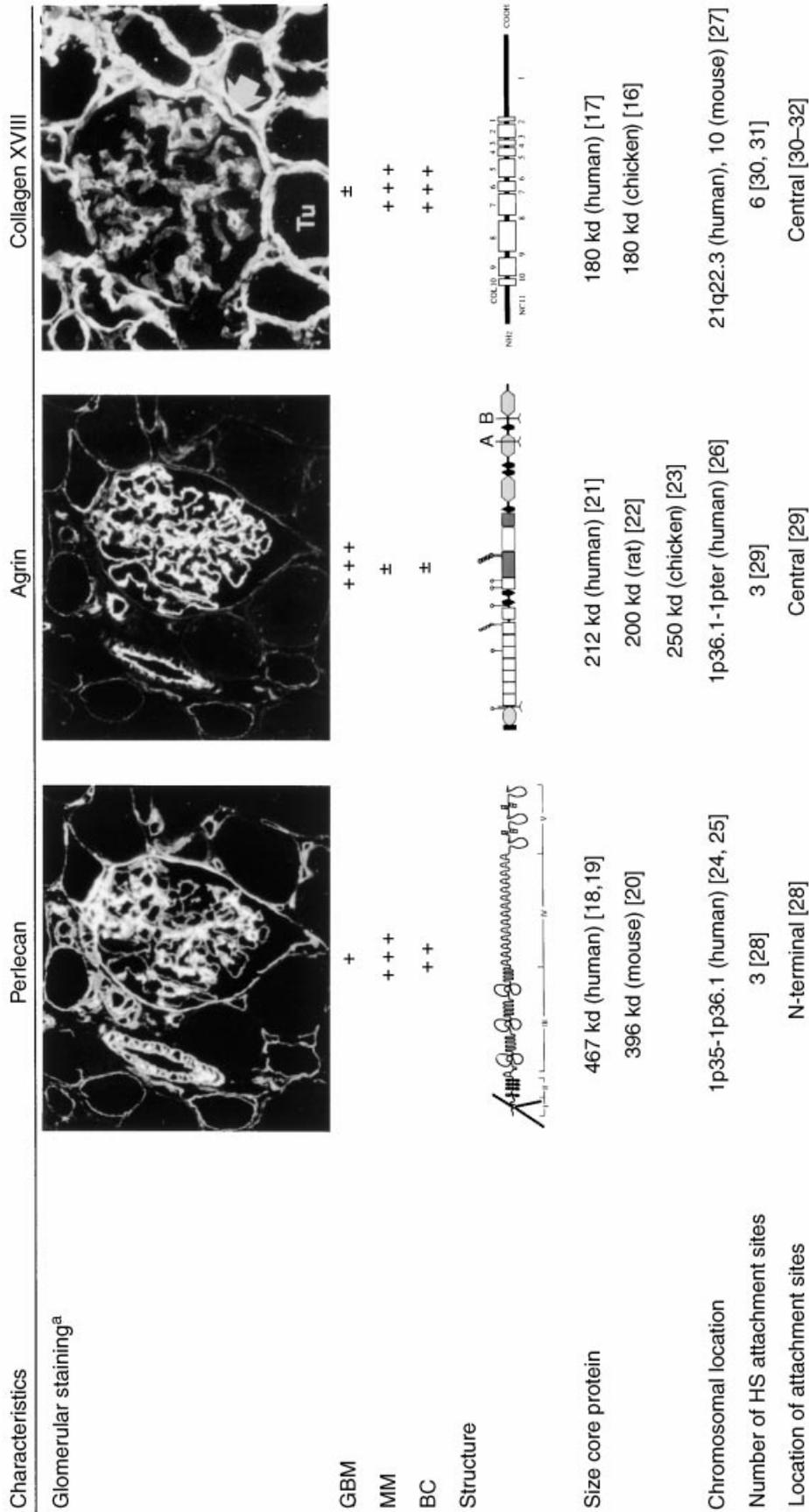
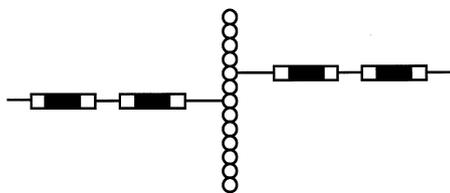


Fig. 1. Glomerular staining pattern, structure, and basic characteristics of perlecan, aggrin and collagen type XIII. Abbreviations are: GBM, glomerular basement membrane; MM, mesangial matrix; BC, Bowman's capsule; HS, heparan sulfate. The structure diagrams are reprinted with permission: for perlecan, from the International Society of Nephrology [34]; for aggrin, from *EMBO J* [29]; and for collagen type XVIII, from *Biochem Biophys Res Comm* [35]. ^aIndirect immunofluorescence on normal rat kidney tissue with mouse anti-rat perlecan monoclonal antibody (mAb) 11B4 [33], or hamster anti-rat aggrin mAb MI-90 [15], on normal human kidney tissue with rabbit anti-human collagen type XVIII (reproduced with permission from the *Am J Pathol* [17]).



-  Core protein of HSPG molecule
 N-acetylated domain in HS (contains no N-sulfates, almost no O-sulfates)
 Mixed N-acetylated and N-sulfated domain in HS (contains some N- and 6-O-sulfates)
 High sulfated (heparin-like) domain in HS (contains N-, 2-O- and 6-O-sulfates)

Fig. 2. Schematic representation of an heparan sulfate proteoglycan (HSPG) molecule.

nucleosomes could also mediate the binding of anti-nuclear antibodies to the GBM *in vivo* [78]. The cationic histone part of the nucleosome is responsible for the binding to the anionic HS in the GBM [79], as evidenced by a number of observations. First, the removal of HS from the GBM by renal perfusion with the HS-degrading enzyme heparinase partly prevented binding of subsequently perfused nucleosome antinuclear antibody complexes [78]. Second, masking of the cationic charges on histones in the nucleosome by antihistone antibodies decreased the nephritogenicity of nucleosome autoantibody complexes [80]. Third, treatment of MRL/*lpr* mice with heparin or noncoagulant heparinoids prevented the deposition of complexes in the GBM and ameliorated glomerulonephritis [81]. That the HS side chains serve as the binding sites within the HSPG molecule was already suggested by the observation that the formation of immune complexes within the GBM in (NZB \times NZW) F1 mice was not influenced by the administration of antibodies directed against the core protein of GBM HSPGs [82]. The relevance of nucleosome-mediated autoantibody binding for lupus nephritis in mice and patients with SLE was substantiated by the identification of antinucleosome antibodies in glomerular eluates in murine lupus [83] and the presence of nucleosomes within glomerular deposits in patients with diffuse proliferative (World Health Organization class IV) lupus nephritis [84]. Using monoclonal antibodies against the HSPG core protein and HS side chain [72], we found a strong decrease or even total absence of staining for the HS side chain in patients with lupus nephritis, whereas the staining for the HSPG core protein was unaltered [58]. This observation extended previous reports in which a decrease of anionic sites within the GBM was reported in lupus nephritis [85, 86]. Binding of neutrophil- or platelet-derived cationic proteins to the GBM was already suggested as a mechanism for the decreased number of anionic sites in lupus nephritis [87, 88]. The mechanisms responsible for the decrease in HS staining were further analyzed in the MRL/*lpr* lupus mouse model. In these mice, the decrease in HS staining correlated with Ig deposits in the GBM and albuminuria. This decrease in staining of HS was not due

to the loss of HS from the GBM, as quantitation of HS isolated from glomeruli with an absent HS staining revealed a normal HS content [59]. Nucleosome-complexed autoantibodies were shown to inhibit dose dependently the binding of anti-HS monoclonal antibody (mAb) to HS in an ELISA system, whereas noncomplexed autoantibodies had no effect. A similar mechanism seemed to occur *in vivo*, as a significant correlation was found in human lupus nephritis between decrease in HS staining of the GBM and histone deposition (Fig. 3) [84]. Furthermore, treatment with heparin(oids), which prevented immune complex deposition in the GBM and the development of glomerulonephritis, also prevented the loss of HS staining [81].

Therefore, in SLE nephritis and possibly also in other glomerular diseases in which immune complex deposition in the GBM is known to play a role in the pathogenesis, this deposition may lead to masking of HS and its anionic charges, resulting in an enhanced permeability of the GBM for macromolecules. Further research must be accomplished to reveal which domains of the HS molecule serve as ligands for these immune complexes.

DEPOLYMERIZATION OF HEPARAN SULFATE BY RADICALS

Generation of oxygen and nitrogen radicals in the glomerulus

The first cells identified in glomerulopathies to produce radicals were infiltrating phagocytes [polymorphonuclear neutrophils (PMNs), monocytes, and macrophages], which generate radicals upon activation. Therefore, in forms of glomerulonephritis in which inflammatory cells infiltrate the glomerulus, such as anti-GBM nephritis (discussed later in this article), reactive oxygen species (ROS) can locally be released by infiltrating phagocytes. Later it was shown that intrinsic renal cells are also able to generate radicals under various circumstances. Glomerular epithelial cells in culture produce ROS after exposure to adriamycin [89], puromycin aminonucleoside [90], and adenosine 5'-triphosphate (ATP) [91]. Cultured mesangial cells generate ROS during phagocytosis

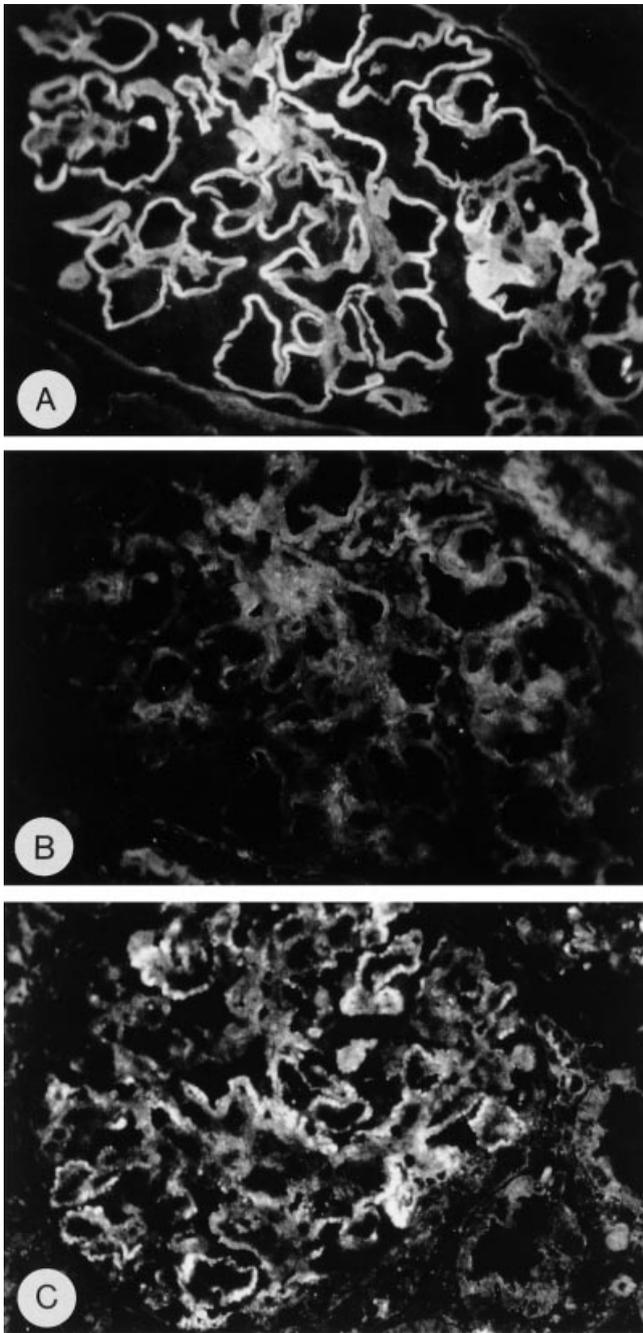


Fig. 3. Glomerular capillary wall staining of the agrin core protein, heparan sulfate (HS), and histones in human systemic lupus erythematosus (SLE) nephritis. Immunofluorescence with anti-grin core protein mAb JM72 (A) anti-HS side chain mAb JM403 (B) in a double staining, and antihistone 3 polyclonal antiserum (C) in a patient with lupus nephritis (magnification $\times 350$). Reproduced with permission (A and B) from van den Born et al and the International Society of Nephrology [58] and (C) from Van Bruggen et al and *Nephrol Dial Transplant* [84].

of extracellular material [92], after complement activation at the cell surface, after interaction with the C5b-9 membrane attack complex [93], after binding of immune complexes [94], and after stimulation by cytokines such

as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) [95]. During ischemia and subsequent reperfusion, ROS are produced by endogenous xanthine oxidase [96]. In the isolated perfused kidney, a partial ischemic system, indeed an increase in xanthine oxidase expression was observed [97]. Besides ROS, vascular endothelial cells, PMNs, and monocytes can serve as a source of nitric oxide (NO).

Various processes can lead to the formation of O_2^- , such as reperfusion after ischemia, redox cycling of certain drugs, and high-energy irradiation. Also during inflammation, invading immune cells can form and excrete O_2^- (Fig. 4). Upon an initial formation of superoxide anion (O_2^-), a cascade of reactions can occur. O_2^- can react with H_2O and form H_2O_2 , and in the presence of metal ions, such as Fe^{3+} , hydroxyl radicals ($\cdot OH$), can be formed in the so-called Haber-Weiss reaction. These hydroxyl radicals can damage proteins, carbohydrates, membrane phospholipids, and DNA [69, 70, 98, 99]. Furthermore, myeloperoxidase (MPO) can halogenate proteins by catalyzing the reaction of H_2O_2 with one of the halides Cl^- , I^- , or Br^- forming hypohalous acids (HOCl, HOI, or HOBr). Hypohalous acids are able to damage proteins by halogenation [100]. NO can react with O_2^- to form peroxynitrite ($ONOO^-$), which is able to cause nitration of tyrosine residues in proteins and lipid peroxidation [101–103].

Radicals increase glomerular permeability

There is compelling evidence that radicals increase the permeability of the glomerulus and the GBM both *in vitro* and *in vivo*, as summarized in Table 1. In several models of glomerulopathy, oxygen radicals have been shown to be involved in the development of albuminuria. Adriamycin nephropathy and puromycin aminonucleoside nephropathy in rats are experimental models for the nephrotic syndrome and are characterized by heavy albuminuria and hypoalbuminemia [122–124]. Adriamycin can be subjected to redox-cycling by which superoxide (O_2^-) is formed [125–127]. Puromycin aminonucleoside is metabolically degraded to hypoxanthine, which can serve as a substrate for xanthine oxidase, resulting in O_2^- formation. In rats with passive Heymann nephritis, the formation of immune complexes and activation of complement are thought to be involved in ROS formation and development of proteinuria. In inflammatory glomerular diseases, ROS are produced in the mitochondria of PMNs via cytochrome b_{558} , which is part of the NADH/NADPH oxidoreductase complex of the respiratory burst. In rats with passive Heymann nephritis, it is suggested that in response to immune complex formation and complement activation, glomerular epithelial cells express enzymes of the respiratory burst, ROS are generated resulting in damage of the GBM and proteinuria [128]. In the heterologous phase of passive anti-GBM nephritis,

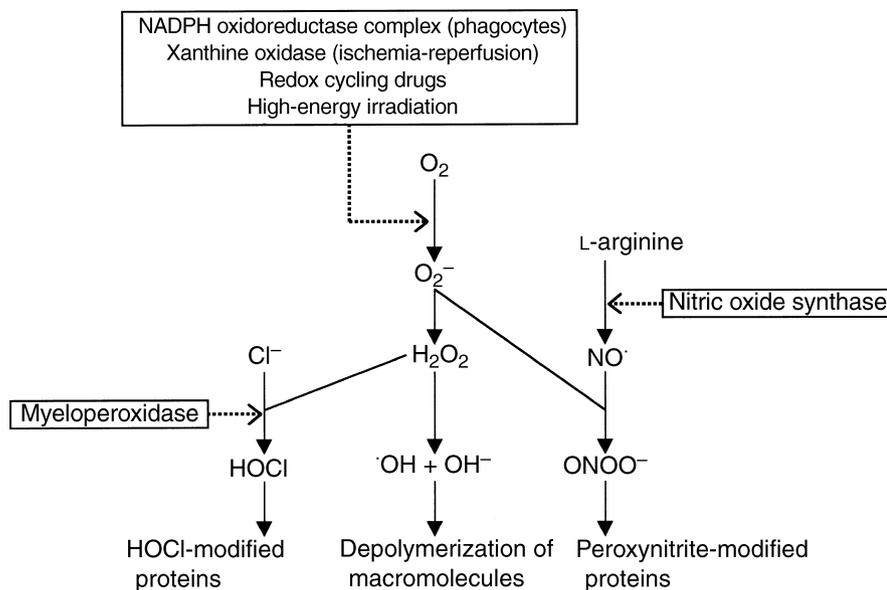


Fig. 4. Major reactions involved in the formation of oxygen/nitrogen radicals.

Table 1. Mechanisms and consequences of radical generation in various experimental model systems and glomerular diseases

Produced radical	Experimental model	Scavengers	Mechanism	Consequence
O_2^-	Isolated glomeruli [104]		TNF α release by activated macrophages/PMNs [105]	GBM permeability \uparrow
$\cdot OH$	Renal H_2O_2 perfusion [106]	CAT, DFO		proteinuria \uparrow
$\cdot OH$	ADR nephropathy [61, 107, 108]	SOD, DMTU	O_2^- release by redox cycling of ADR [125–127]	proteinuria \uparrow
$\cdot OH$	PAN [109–111]	SOD, DMTU, DFO, Na-benzoate, allopurinol, α -tocopherol + ascorbic acid	O_2^- formation via degradation of hypoxanthine	proteinuria \uparrow
$\cdot OH$	PHN [112, 113]	DMTU, sodium benzoate, DFO	increased cyt b_{558} expression by podocytes [128]	proteinuria \uparrow
$\cdot OH$	Anti-GBM [114,115]	CAT, DMSO	activated PMNs	albuminuria \uparrow
HOCl	In vitro GBM filtration [116]			GBM permeability \uparrow
HOCl	In vitro GBM [117]		MPO- H_2O_2 -halide release by activated PMNs	GBM degradation
Hypohalous acids	Isolated glomeruli [104, 118]		activated macrophages/PMNs	GBM permeability \uparrow
Hypohalous acids	Renal perfusion MPO- H_2O_2 [119]		GBM halogenation	GBM permeability \uparrow
Hypohalous acids	Renal perfusion con A/anti-con A [120]		PMN activation, GBM halogenation	proteinuria \uparrow
$ONOO^-$	MPO immunization, perfusion with lysosomal PMN extract and H_2O_2 [121]		PMN/monocyte influx	$ONOO^-$ -modified proteins

Abbreviations are: ADR, adriamycin; PAN, puromycin aminonucleoside; PHN, passive Heymann nephritis; GBM, glomerular basement membrane; MPO, myeloperoxidase; con A, concanavalin A; PMN, polymorphonuclear neutrophil; CAT, catalase; DFO, deferoxamine; SOD, superoxide dismutase; DMTU, dimethylthiourea.

activated PMNs attached to the GBM secrete ROS and lysosomal enzymes, which are responsible for the glomerular damage. In Table 1, the effects of hypohalous acids and peroxynitrite on glomerular permeability are also summarized. Taken together, several *in vitro* and *in vivo* experiments indicate that hydroxyl radicals are the most harmful in affecting normal glomerular permselectivity. Furthermore, the MPO- H_2O_2 -halide system has the capacity to disturb glomerular filtration, but it is unclear if

this can be also prevented by treatment with scavengers. Only few data are available on the pathogenic role of NO and peroxynitrite in glomerular permeability.

Radicals depolymerize heparan sulfate

The increase of the permeability of the GBM by radicals can be caused by damage of many macromolecules in the GBM, including degradation of HS. Several investigators have reported the susceptibility of HS, other

GAGs, and PGs to radicals *in vitro* [61, 129–136]. All of these studies point to the hydroxyl radical as the most harmful ROS. The susceptibility of different GAGs to depolymerization by ROS is dependent on the degree of sulfation. High-sulfated GAGs are more resistant to depolymerization than low-sulfated or nonsulfated GAGs [130, 131, 134]. HS in the GBM is composed of mixed high- and low-sulfated regions and contains large *N*-acetylated stretches [137], suggesting that degradation will predominantly occur in these low-sulfated or nonsulfated parts. The isolated perfused kidney, in which ROS are known to be produced as result of the ischemia, is also associated with an increased protein excretion and partial degradation of HS [138]. Both of these effects can be prevented by the addition of allopurinol or a mixture of mannitol, superoxide, and catalase, again suggesting that hydroxyl radicals are responsible for HS degradation and proteinuria in this model [97]. This production of ROS during ischemia may be the result of the release of xanthine oxidase into the circulation, as shown in the hemorrhagic shock model of ischemia reperfusion. In this model, xanthine oxidase is shown to bind to GAGs of vascular cells, with subsequent degradation of HS [96]. Also, other studies show xanthine oxidase binding to HS, which localizes the enzyme to cell surfaces and increases catalytic activity [139, 140]. We have shown that the HS polysaccharide side chains of rat agrin *in vitro* can be depolymerized by ROS produced by the hypoxanthine-xanthine oxidase system in the presence of Fe^{3+} , whereas the PG core protein remained intact. This depolymerization could be prevented by superoxide dismutase and dimethylthiourea, indicating that $\cdot\text{OH}$ were responsible for this effect (Fig. 5A) [61]. In adriamycin nephropathy in rats, a model in which ROS are known to play a role [127], we found that the degree of albuminuria was correlated with a decrease in staining of GBM HS, whereas the staining for the agrin core protein remained normal. Both albuminuria and the decrease in GBM HS staining could be partially prevented by treatment of the rats with dimethylthiourea, which supports the role of $\cdot\text{OH}$ in the depolymerization of HS and the development of albuminuria *in vivo* (Fig. 5 B, C) [61].

Besides to ROS, NO gas or NO derived from cultured endothelial cells can also degrade HS and heparin *in vitro* via the formation of HNO_2 at a pH below 5 [141]. Because NO is produced by endothelial cells, PMNs and monocytes after inflammatory stimulation and these inflammatory cells create local acidic conditions, it may be involved in HS depolymerization and the development of albuminuria in glomerular inflammation. NO can react with superoxide to form peroxynitrite, which is probably also capable of GAG degradation, as it is shown to degrade hyaluronic acid [142].

These studies clearly show that HS is susceptible to depolymerization by radicals, providing an important

mechanism by which radicals can affect the permeability properties of the GBM.

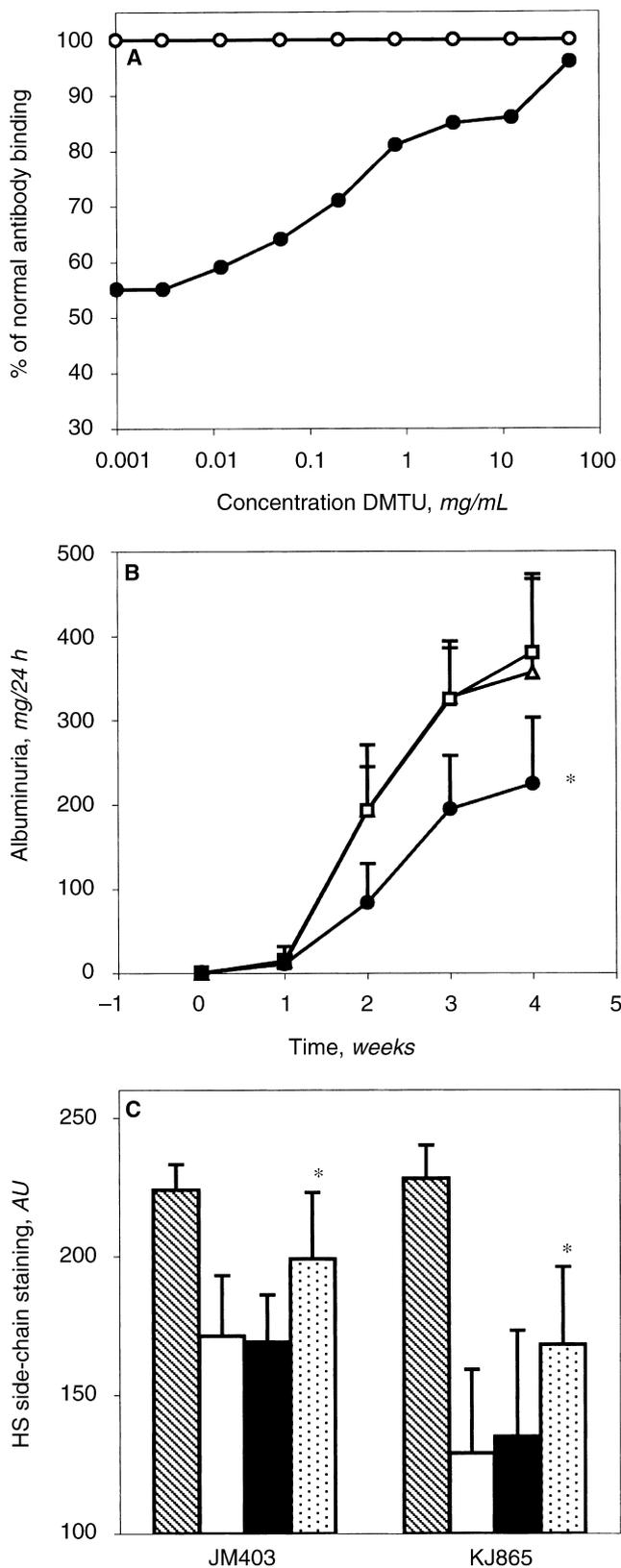
Physiological significance of the susceptibility of heparan sulfate to reactive oxygen species

In vitro experiments indicate that heparin and hyaluronic acid act as antioxidants and decoy molecules [143–145]. This is probably the mechanism by which heparin treatment of patients with diabetic nephropathy results in a decrease of albuminuria [146, 147]. In adriamycin nephropathy, streptozotocin (STZ)-induced diabetic nephropathy in rats and murine lupus nephritis heparin treatment prevented the loss in HS-associated anionic sites in the GBM and ameliorated proteinuria [81, 148–150]. This suggests that HS can also serve as an antioxidant in the GBM to protect other molecules from degradation. By depolymerization of HS, oligosaccharides are released, which could have pathophysiological relevance, although this is rather speculative. Because HS can function as an anchorage and storage molecule for growth factors, such as fibroblast growth factor-2 [45, 46], these can be set free upon depolymerization of HS and activate glomerular epithelial and/or mesangial cells. In addition, the release of HS oligosaccharides could impair local anticoagulation and may lead to clogging of the GBM [67]. Further research is needed to answer these questions.

CLEAVAGE OF HS(PG) BY ENZYMES

Neutral serine proteinases

Among the enzymes that are released by activated PMNs are the cationic neutral serine proteases elastase and cathepsin G. Infusion of elastase or cathepsin G results in the binding of these cationic proteinases to the GBM (probably to the anionic HS) and subsequently to proteinuria [151]. The involvement of these proteinases in the development of albuminuria is further substantiated by the observation that beige mice, which have a deficiency for PMN elastase and cathepsin G, do not develop albuminuria after the administration of anti-GBM antibodies, although a comparable influx of PMNs to control mice is seen [152]. Furthermore, *in vivo* perfusion of elastase in rats results in proteinuria and decrease in GBM HS staining and to a lesser extent of agrin core protein staining, whereas other ECM components such as S-laminin, fibronectin, and collagen type IV were not affected [153]. *In vitro* experiments showed that elastase, which is highly cationic at physiological pH, can bind to the anionic HS. Next, elastase cleaves agrin near the HS attachment sites, and HS side chains bound to small peptide fragments are released [132, 153]. A comparable result was obtained by intratracheal instillation of elastase in rats, which leads to pulmonary emphysema. The BMs in alveoli showed a decrease in HS staining and to a lesser extent of the agrin core protein staining, whereas



no alteration was observed for laminin, fibronectin, and type IV collagen [154]. Also, endothelial cell-surface HSPG is susceptible to PMN-derived elastase and to human elastase [155]. The role of elastase is further confirmed by the observation that treatment of rats with anti-GBM nephritis with an inhibitor of elastase results in a reduced proteinuria, despite an equal amount of IgG binding [156]. These studies show that although elastase (and probably also cathepsin G) is not a specific HSPG-degrading proteinase, it has a relative preference to cleave HSPGs above other ECM components, thereby inducing proteinuria [153, 154].

Heparan sulfate-specific endoglycosidases

The HS-specific endoglycosidase heparanase can be released during inflammation by activated PMNs and platelets, leading to HS degradation [157, 158]. Also, activated macrophages, mononuclear cells, mast cells, and T cells are able to release a heparanase that can cleave and release HS from endothelial cell-derived ECM [159–162]. Finally, a synergistic action is suggested for heparanase and several other enzymes. The effect of heparanase can be enhanced by elastase. Probably, minor proteolysis of HSPG making the substrate more accessible to other enzymes is necessary for heparanase for further degradation of HS [157, 158]. Other studies show that several enzymes, such as plasminogen activator and thrombin, which themselves do not degrade HS, can work synergistically with heparanase [163, 164]. This suggests that during inflammation and activation of the coagulation cascade, the activity of the released heparanase is enhanced by enzymes formed during coagulation. Taken together, these studies indicate that heparanases released during inflammation are able to degrade HS and could therefore play a role in increasing the permeability of the GBM during inflammatory glomerulopa-

Fig. 5. (A) Effect of reactive oxygen species (ROS) on heparan sulfate (HS) and heparan sulfate proteoglycans (HSPG) in vitro. Coated HSPGs from rat GBM were incubated with hypoxanthine, xanthine oxidase, and Fe^{3+} in the presence of various concentrations of 'OH scavenger dimethylthiourea, then ELISA was performed with anti-HS mAb JM403 (●) or the goat anti-agrin core protein antibody BL31 (○). (B) Effect of ROS scavengers on albuminuria in adriamycin nephropathy. Rats with adriamycin nephropathy were treated with either dimethylthiourea (●), superoxide dismutase (△), or saline (□). * $P < 0.01$ vs. saline, analysis of variance for repeated measurements. (C) Effect of ROS scavengers on GBM HS staining in adriamycin nephropathy. Indirect immunofluorescence on kidney sections of normal rats (▨) and rats with adriamycin nephropathy treated with either saline (□), superoxide dismutase (■), or dimethylthiourea (▤). Indirect immunofluorescence was performed with anti-HS side chain mAbs JM403 (left) and KJ865 (right). Twenty-five glomeruli were scored on a scale between 0 and 10, such that the maximal score is 250 arbitrary units (AU). * $P = 0.03$ versus saline-treated rats. Reproduced with permission from Raats et al and *J Biol Chem* [61].

thies. The importance of these mechanisms remains to be determined.

CHANGES IN HEPARAN SULFATE PROTEOGLYCAN SYNTHESIS

Besides masking, depolymerization and degradation of HS, there is evidence for a fourth mechanism that can lead to a reduction of functional HS in certain glomerulopathies: a changed biosynthesis that results in an abnormal glomerular HS content and/or alterations in the structure or sulfation of HS.

As mentioned before, many investigators have studied the GBM distribution of HS-associated anionic sites in human and experimental glomerulopathies by using cationic dyes such as ruthenium red, polyethyleneimine, poly-L-lysine, cationic ferritin, and cuproline blue. It is assumed that each anionic site, visible as a granule or filament (depending on the dye used), represents the equivalent of a proteoglycan (PG) monomer [165]. Thus, a reduction in the number of GBM anionic sites might reflect a reduced HSPG content, whereas a change in the size or morphology of the anionic sites is interpreted as a change in the HS structure (size/charge). Other studies, however, show that changes in the number or appearance of anionic sites may also be related to biochemical changes in the surrounding ECM [166]. It is clear that this methodology can only hint at changes in HS content and/or structure and that immunohistochemical and biochemical analysis can provide more definitive answers.

Glomerular heparan sulfate proteoglycan synthesis in diabetic nephropathy

Functional studies on glomerular permeability in patients with insulin-dependent diabetes mellitus (IDDM), using uncharged polydisperse dextrans or tracer molecules with equal molecular weight, but with different charges, indicated both an increased porosity of the GBM, as well as a reduced charge-dependent permeability, in all probability related to loss of GBM anionic charges [167–170]. Therefore, the significance of GBM HSPG for the development of incipient and overt diabetic nephropathy has been analyzed in several studies. A relative decreased GBM content of HS and HSPG has been found in patients with IDDM [171–173]. We found a decreased staining for HS in the GBM in kidney biopsies from diabetes patients (both IDDM and non-IDDM), which correlated with the fractional protein excretion, whereas the GBM staining for the core protein of HSPG was unaltered [58, 60]. Others, however, also found a decreased GBM staining for the core protein of HSPG in diabetic kidneys [174, 175]. Because of the obvious limitations of the study of human material, investigations were also performed in experimental models

of diabetic nephropathy. Most of these studies have been carried out in the STZ-induced diabetes model in the rat. Biochemical quantitation of HS in the GBM, glomeruli, or renal cortex revealed a decreased [176–179] or unaltered [166, 180] HS content in these rats. Studies on HS synthesis in the GBM or glomeruli in this model by *in vivo* ³⁵S-sulfate incorporation revealed either a decreased [178, 179, 181] or normal [182, 183] synthesis of HS. Studies using ³⁵S-sulfate incorporation in *ex vivo* systems (isolated perfused kidney) or *in vitro* (short-term culture of glomeruli) suggested a decreased HS synthesis [184, 185], although this is not found by all investigators [182]. A clear interpretation of these results is hampered by the use of different rat strains, differences in diabetes duration, the absence or incomplete description of the development of a progressive diabetic nephropathy, and most importantly, by the use of different techniques to evaluate the HS content. A disadvantage of the incorporation studies is related to the rather indirect method to identify the individual GAGs by either GAG-degrading enzymes or nitrous acid treatment. In addition, it is stressed that the rate of HS synthesis yields no information about the actual glomerular HS content and that the PG synthesis of isolated glomeruli in tissue culture is different from that *in vivo* [186]. Moreover, one should realize that GBM thickening and mesangial matrix expansion contribute to a reduction in HSPG density, although the absolute HSPG content was not changed [187]. In a few reports, a semiquantitative analysis was performed on the mRNA content coding for perlecan in renal cortex or glomeruli of diabetic and control mice/rats. In one study [188], a temporary decrease was found, which normalized after six months. In both other studies, no change in perlecan mRNA expression was found [189, 190]. It should be noted, however, that it is not perlecan but agrin that is the most abundant HSPG in the GBM [14, 15]. Because perlecan is predominantly localized in the mesangial matrix (Fig. 1), its relevance for glomerular filtration is highly questionable.

Glomerular heparan sulfate structure in diabetic nephropathy

In vitro studies have shown that the extent of biosynthetic modification affecting the number, length, and substitution patterns of the modified domains, as well as their position along the HS chain may differ among cell types [191], alter during proliferation [192], cell transformation [193–196], infection [197], age [198], differentiation [199–201], and in response to a variety of agents [202–205]. As indicated at the beginning of this article, structural HS changes might have important consequences for protein binding and other functions of HS.

With respect to diabetes, an undersulfation of HS has been described in STZ-induced diabetes in the rat. Biochemical analysis of liver HS purified from diabetic ani-

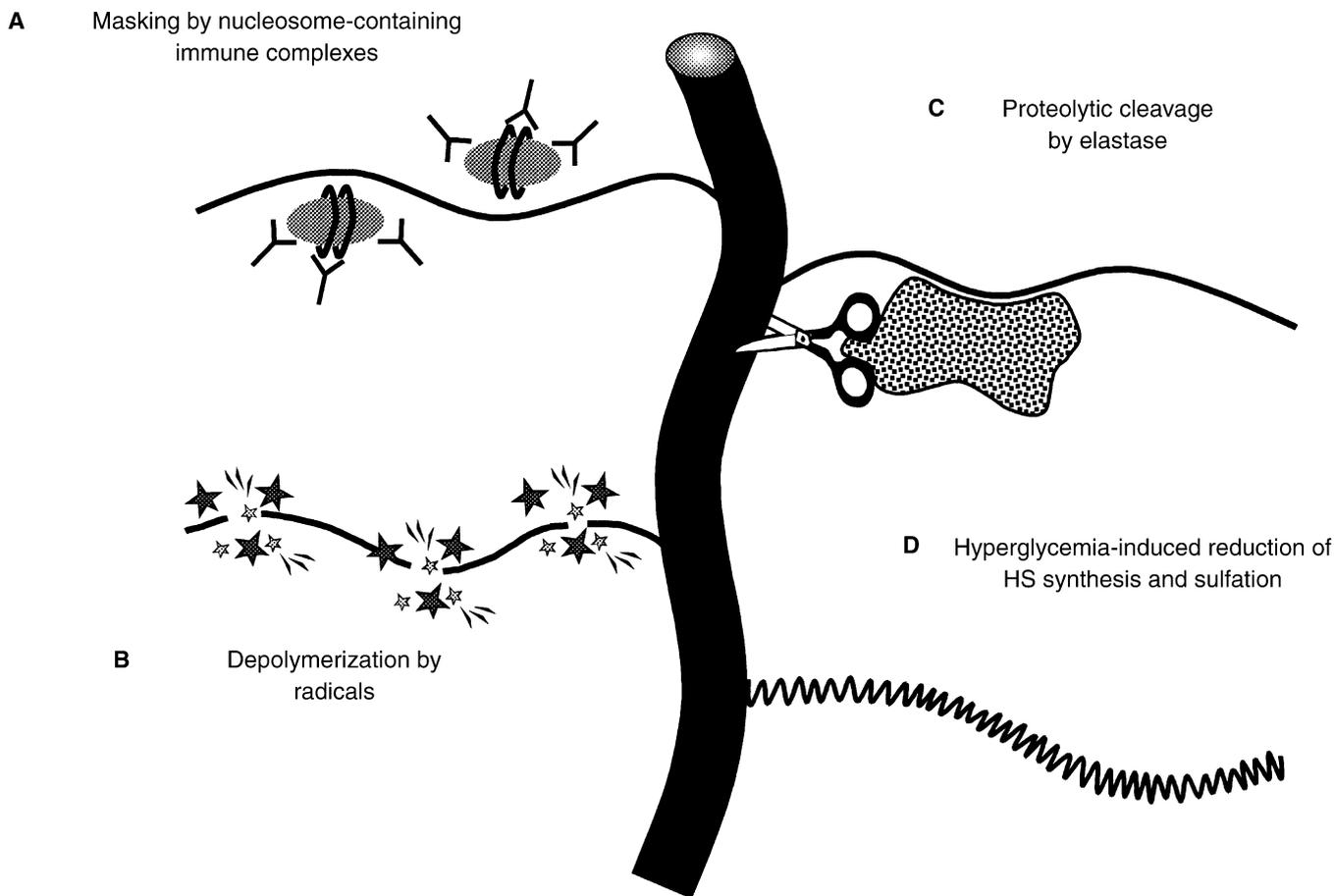


Fig. 6. Schematic representation of the four described mechanisms leading to reduced heparan sulfate (HS) function. (A) Positively charged amino acids in the N-terminal regions of the four core histones present in nucleosome-containing immune complexes bind to HS in the GBM, thereby neutralizing the anionic charge of the GBM. (B) Oxygen and/or nitrogen radicals, formed during glomerular injury and/or inflammation, are able to depolymerize HS, leading to loss of HS in the GBM. (C) By virtue of its positive charge, elastase binds to the HS side chains and subsequently cleaves the HSPG core protein close to the HS attachment site. (D) Hyperglycemia can induce alterations via a reduction of the HS synthesis by podocytes. Moreover, the degree of sulfation can be reduced, leading to a diminished GBM anionic charge.

mals showed an elution from a cation exchange column at a lower salt concentration compared with control liver HS [206]. This indicates a lower negative charge density (sulfation) of diabetic HS. Also, for glomerular HS, an undersulfation was found, as indicated by lower sulfate-to-uronate ratio [180]. Biochemical analysis of radiolabeled HS isolated from intestinal cells from diabetic and control rats showed a reduction of both *N*- and *O*-sulfation of the diabetic HS sample, which again points to diabetes-induced undersulfation [207]. Culture of mesangial cells on mesangial matrix that was nonenzymatically glycosylated or prolonged exposure of mesangial cells to elevated glucose levels leads to a decreased production of HS, which, in addition, is undersulfated [208–210]. This was also found after stimulation of mesangial cells with angiotensin II, which is assumed to be increased in diabetes [211]. This undersulfation of HS could be due to a decreased activity of the enzyme glucosaminyl *N*-deacetylase/*N*-sulfotransferase, the key enzyme in HS

sulfation. Indeed, in hepatocytes and glomeruli of STZ-induced diabetic rats, a reduced activity of this enzyme was found [190, 212, 213]. In conclusion, although not found by all investigators, many studies in experimental diabetes suggest a down-regulation of HS synthesis, which is accompanied by a reduction in the degree of sulfation.

Because the diabetic condition affects all body tissues, the Steno hypothesis assumes that this metabolic dysregulation of HS will also be found in other tissues than the kidney [214]. Indeed, immunohistochemical quantitation of HS in skeletal muscle capillary BM [215] and in the dermal-epidermal junction of the skin of IDDM patients [216] clearly demonstrates a loss of HS staining in these structures, analogous to our findings in the GBM. In the STZ diabetes model in the rat, we found an association between an increase of the transcapillary passage of albumin and a decreased HS content in various tissues [217],

which again suggests a functional role of HS in vessel wall permeability.

Heparan sulfate proteoglycan changes in nondiabetic glomerular diseases

The number of studies dealing with HS(PG) synthesis in nondiabetic renal diseases is very limited. A higher charge density, corresponding with an oversulfation, was found for glomerular HS originating from rats with PAN nephrosis (a model for the nephrotic syndrome) [218]. On the other hand, a reduced charge density was found for glomerular HS in rabbits with membranous glomerulonephritis [219]. However, no detailed HS analyses were performed. Elucidation of HS alterations in glomerular diseases can become an active area of research. Recently, phage display-derived single chain antibodies to HS were developed [220]. Given the limited amount of tissue obtained by renal biopsy, these antibodies could be important to probe structural HS changes. Moreover, the availability of the laser dissection technique allows the glomerular quantitation of several enzymes involved in HS modification by real-time polymerase chain reaction (PCR).

CONCLUSION

Until now, different mechanisms for alterations of HS have been identified as leading to an impaired function of the GBM. Alterations in GBM HS are associated with proteinuria in many glomerular diseases. This review showed how masking by immune complexes, depolymerization by radicals, degradation by enzymes, and biochemical changes can lead to reduced HS function and proteinuria. These four mechanisms are schematically depicted in Figure 6. Formally, it cannot be excluded that in certain situations the HS alterations are secondary to proteinuria, associated with an effect of plasma proteins on podocytes. Because HS is not only important for the glomerular permeability, but also for the binding of growth factors and their receptors and for the prevention of coagulation in the GBM, HS alterations may aggravate glomerulopathy by enhancement of inflammation, clogging, mesangial expansion, and proliferation.

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