Molecular Mechanisms of Glomerular Injury in Rat Experimental Membranous Nephropathy (Heymann Nephritis)¹

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ABSTRACT
The molecular pathogenesis of human membranous nephropathy (MN) is unknown, despite the relatively high incidence and severity of this glomerular immune disease. Heymann nephritis (HN) in rats is considered an instructive experimental model of MN. This study summarizes current molecular aspects of two key events common to both MN and HN, i.e., formation of characteristic subepithelial immune deposits in the glomerular basement membrane (GBM), and development of glomerular capillary wall damage resulting in proteinuria. In HN, the antigenic targets of immune deposit-forming antibodies were identified in cell membranes of glomerular epithelial cells as a 515-kd glycoprotein (megalin, or gp330), which is a polyspecific receptor related to the low-density lipoprotein receptor family, and an associated 44-kd protein (receptor associated protein, RAP). The epitope was recently narrowed to 14 amino acids in RAP, and several others on megalin/gp330 are under investigation. Proteinuria requires formation of the complement C5b-9 membrane attack complex, which is presumably triggered by antibodies directed against lipid antigens that associate with immune deposits and form immune deposit-forming megalin/gp330 immune complexes. Sublytic C5b-9 attack on glomerular epithelial cells causes upregulation of expression of the NADPH oxidoreductase enzyme complex by glomerular cells, which is translocated to their cell surfaces, similar to activated neutrophil granulocytes in the respiratory burst reaction. Subsequently, reactive oxygen species (ROS) are produced locally, which reach the GBM matrix. Here formation of lipid peroxidation (LPO) adducts is found, preferentially on monomeric and dimerized NC1 domains of covalently crosslinked Type IV collagen. These structural changes within the GBM could be of functional relevance because treatment with the potent LPO-antagonist probucol reduces proteinuria by < 80%. Intact or fragmented apoprotein E-containing lipoproteins were identified as potential sources of the polyunsaturated lipids required for the production of LPO adducts. Lipoproteins accumulate within immune deposits and show signs of oxidative damage, similar to oxidized LDL within atherosclerotic lesions. Collectively, the results obtained so far in HN permit the compilation of a sequence of events, linking formation of immune deposits with proteinuria. However, despite this relatively detailed knowledge of pathogenic events in HN, the bridge to human NM remains to be built.

Key Words: Heymann nephritis, proteinuria, lipid peroxidation

Proteinuria is particularly prominent in membranous nephropathy (MN), a common "classic" autoimmune disease that is caused by formation of subepithelial immune deposits in the glomerular basement membrane (GBM). MN is the most frequent cause of glomerular scarring and chronic renal failure in patients with glomerulonephritis (1). Despite several attempts to halt the course of MN by treatment with high doses of cytotoxic drugs (2), many cases progress chronically to terminal renal insufficiency and cannot be saved from chronic hemodialysis and renal transplantation. Thus, it would be of interest to design specific interventional therapies based on precise knowledge of the pathogenesis of NM and of its "Achilles' heel(s)."

Because molecular dissection of MN is limited by the availability of human material, we and many others have chosen to study Heymann nephritis (HN) (3), an experimental rat disease that faithfully duplicates MN. We have expected that the pathogenesis of immune-deposit formation and glomerular capillary-wall damage could be explored and, eventually, also manipulated. Most experiments mentioned in this review were performed in rats with passive HN (4,5). In this variant of HN, a nephritogenic antibody was injected intravenously into rats, resulting in formation of subepithelial immune deposits within few minutes, and followed by proteinuria 5 to 6 days later.
FORMATION OF IMMUNE DEPOSITS IN HEYMANN NEPHRITIS

The basic pathogenic mechanisms of glomerular immune-deposit formation are now understood in some detail, although not yet sufficient-though detail to specifically interfere with their formation. Because these pathogenetic aspects of HN were recently reviewed elsewhere (6–9), a condensed summary is presented here.

The antigenic target of circulating nephritogenic (auto)antibodies in HN was defined as a large membrane glycoprotein designated gp330 (10). Determination of its amino acid sequence has revealed that it is the largest cloned and sequenced eukaryotic protein so far, with a deduced molecular weight of 515 kd, and therefore, the name “gp330” was appropriately replaced by “megalin,” to emphasize its size (11). Structural similarities were found with the lipoprotein receptor-related protein LRP/α2-macroglobulin receptor, a member of the low-density lipoprotein receptor family (12,13). Similar to LRP (14,15), megalin/gp330 is a polyspecific receptor for a rapidly increasing number of newly recognized diverse ligands (16,17). Examples are Ca++ (18); apolipoproteins E, J (clusterin), and B 100 (19–21); urinary plasminogen activator inhibitor (uPAI) complexes (22,23); cationic nephro- and ototoxic antibiotics (24); lactoferrin (25); and others.

For the pathogenesis of immune-deposit formation, it is relevant that megalin/gp330 is expressed in clathrin-coated pits on the bases of footprocesses of podocytes, where initial immune complexes are formed (26,27). Indirect evidence indicates that megalin/gp330 in this location forms complexes with a 44-kd protein (8), which was designated variably as C14 (28), α2-macroglobulin receptor-associated protein (α2-MRAP), or receptor associated protein (RAP) (29,30). Antibodies specific for RAP were found to induce passive HN (pHN)-like small immune deposits (31,32). It appears that complex formation of this molecule with gp330 inhibits binding of the multiple ligands to megalin/gp330 (29). RAP has attracted interest because several domains of functional significance were recently discovered, such as one required for the binding to gp330. In addition, a high-affinity heparin binding site was also localized (33) and—most interesting in the context of this review—a single epitope was discovered, which participates in the formation of immune deposits in HN (31). This epitope was recently narrowed to 14 amino acids, and specific antibodies directed against a corresponding synthetic peptide were found to induce small immune deposits similar to those found in pHN (34) (Figure 1). It appears, however, that this is only one of several “pathogenic” epitopes required for in situ immune-deposit formation, and that at least one or several more are present also on the megalin/gp330 molecule itself (35). Recently, a binding site for immunoglobulin (Ig) G eluted from glomeruli of pHN rats was localized to the fifth cystine-rich repeat of the second LDL receptor-like domain of gp330/megalin (36), and active and passive HN were induced with an approximately 130 amino acid-long recombinant fusion protein comprising this region of megalin (37).

Collectively, these data establish that the antigenic target of immune deposit-forming antibodies is megalin/gp330 and/or its complex with RAP, designated as the “Heymann nephritis antigenic complex” (HNAC) (38,39). It is realistic to expect that in the near future, the molecular essentials of immune-deposit formation will be understood sufficiently to allow the design of specific immunotherapies for HN, which eventually could serve as blueprints for treatment of human MN.

PROTEINURIA DEPENDS ON C5B-9 FORMATION

Activation of complement plays a direct role in glomerular injury and proteinuria in pHN. The experimental basis for this view is the finding that C5b-9 membrane attack complex was localized within the immune deposits and that depletion of complement by cobra venom factor was associated with lack of proteinuria, whereas formation of immune deposits was not inhibited (reviewed in References 40 and 41).

What causes the formation of C5b-9 within immune deposits? Remarkably, active or passive immunization of rats with polyspecific antibodies raised against complex renocortical fractions (such as isolated tubular microvilli or Fx1A) induced activation of complement and, pari passu, also heavy proteinuria, although nonspecific anti-gp330 antibodies failed to do so (27). This discrepancy raised the suspicion that IgG species directed against complement-activating antigen(s) were contained within polyspecific, fully nephritogenic sera, in addition to immune-deposit-inducing antibodies specific for megalin/gp330 (42). Indeed, evidence was recently obtained for at least one fraction of lipid-specific IgG within polyspecific pHN-inducing sera (43) that caused C5b-9 activation and proteinuria when coincubated with anti-megalin/gp330 IgG. At present, the lipid target(s) for this complement-activating antibody system were not identified.

Apparently podocytes are in command of a defense mechanism to escape from the potentially dangerous membrane insertion of C5b-9; they retrieve C5b-9 and/or inactivated C5b-9-S protein complexes from immune deposits by endocytosis, followed by transport into multivesicular bodies, and finally discharge into the urinary space (44). Presumably this transepithelial transport of C5b-9 accounts also for the appearance of C5b-9 fragments in the urine of patients with MN. The functional implications of C5b-9 activation in tubules remain to be determined (45–47).

In later stages of HN and in parallel with development of proteinuria, C5b-9 was also found inserted into cell membranes of podocytes by freeze fracture electron microscopy, presumably causing sublytic damage (44). It is possible that, under these conditions, the scavenging mechanisms of podocytes be-
Figure 1. Immune deposits (ID) induced by injection of synthetic peptide P31-53-specific rabbit antibody, directed against the sole nephritogenic epitope of the 44-kd receptor-associated protein RAP. ID are visualized by direct immunofluorescence on 1.5-μm frozen sections. There are numerous fine granular ID in the peripheral glomerular capillary walls. The mesangium is also labeled in a granular pattern (M). Several large intracellular vacuoles of glomerular epithelial cells (arrowheads) contain rabbit immunoglobulin (Ig) G. (Original magnification, ×1200.) (Reproduced with permission from Reference 34.)

came overloaded and inefficient. It will be interesting to examine whether clearing of C5b-9 from glomeruli could be related to the regression of disease observed in some patients in MN, and to which extent C5b-9 influences the pathogenic local immunological processes within glomeruli.

These data raised the question as to the functional consequences of complement attack on podocytes. Sublethal C5b-9 attack in cultured glomerular epithelial cells was found to trigger metabolic changes and induction of de novo synthesis of prostaglandins, proteases (48,49), etc. Recently, evidence was obtained that podocytes also become "activated" in vitro (50), as they initiate synthesis of several presumably functionally relevant membrane proteins that are not produced at all or only in low concentrations in their resting state. Examples are metalloprotease 9 (51), basic fibroblast growth factor and one of its receptors (52), and cytochrome b558, an integral component of the NADPH oxidoreductase complex (53).

FORMATION OF RADICAL OXYGEN SPECIES AND PROTEINURIA

Proteinuria in HN critically depends on the formation of radical oxygen species (ROS), because interventional application of oxygen radical scavengers drastically reduced proteinuria, although formation of immune deposits was not affected (54-55). The source of ROS in HN, however, remained obscure, especially because "professional" ROS-producing inflammatory cells were absent from glomeruli in pHN. This raised the question whether ROS could be produced by intrinsic glomerular cells. In keeping with this hypothesis, cultured mesangial cells produced ROS in vitro when challenged with C5b-9 (56) presumably by activation of the NADPH oxidoreductase enzyme complex (57), similar to activated neutrophil granulocytes. In HN the podocytes were found to express relatively large amounts of the membrane enzyme cytochrome b558 (53), an essential component of the neutrophil respiratory burst NADPH oxidoreductase complex (58). When complement activation was abolished by pretreatment of pHN rats with cobra venom, the enzyme was detected only in low levels, similar to levels in normal control rats. Cytochrome b558 was localized by immunoelectron microscopy in podocytes within cytoplasmic membrane vesicles and also on the cell surfaces, in particular, along the basal aspect of the foot process membranes. These morphologic data

were confirmed by biochemistry, indicating a significant increase of cytochrome b$_{558}$ in HN with proteinuria by quantitative immunoblotting (53) and by increase of specific mRNA by approximately 30 times in proteinuric rats (unpublished data). Because almost immediate oxidant production by mesangial cells was induced in vitro by sublytic amounts of C5b-9 (56), it remains to be determined whether the NADPH oxidoreductase system is involved in this scenario, or if alternate means of ROS generation are activated. It is also possible that modulation of the antioxidant defense—such as the enzymes catalase, superoxide dismutase, and glutathion peroxidase—are affected in this setting (59). Collectively, these results provided evidence that in pHN, podocytes synthesized and exteriorized at least one essential component of the respiratory burst oxidoreductase enzyme complex, similar to activated neutrophil granulocytes (60). Functionality and completeness of the oxidoreductase complex in glomeruli was demonstrated by localization of one of its products, H$_2$O$_2$, by a cytochemical technique, based on specific precipitation of H$_2$O$_2$ with Ce$^{+++}$ (Figure 2) (60). H$_2$O$_2$ was found evenly distributed throughout the GBM in pHN rats with proteinuria only, but not in control rats (53). This suggested that the NADPH oxidoreductase complex on the podocyte's surfaces indeed generated ROS, which diffusely flooded the entire GBM and its matrix proteins. These unexpected findings have raised the question of whether ROS-mediated chemical modifications of GBM matrix proteins could be detected in proteinuric pHN rats.

**LIPID PEROXIDATION CONTRIBUTES TO PROTEINURIA**

GBM are highly crosslinked complex protein meshworks, and direct effects of ROS, such as protein cleavage or polymer formation, would be very difficult to detect. Therefore we have chosen to study indirect effects of ROS on GBM matrix proteins, and in particular, to study lipid peroxidation (LPO), a common means of ROS-induced tissue damage (61,62) causing generation of highly reactive compounds by the action of ROS on polyunsaturated fatty acids. Highly reactive dialdehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (which crosslink proteins by Schiff base formation with lysyl residues) are frequent LPO products. LPO and adduct formation in vascular walls were recognized as important components of atherosclerotic lesions, and monoclonal antibodies specific for MDA-lysyl Schiff bases were generated in these studies (61,63). When these antibodies were used as markers for LPO, MDA adducts accumulated in glomeruli of proteinuric HN rats (Figure 3), although they were barely detectable in nonproteinuric control rats (64). In addition, specific labeling with this antibody of isolated GBM of proteinuric rats was found, in contrast to GBM of normal control animals. These data provided evidence that LPO occurred within glomeruli with proteinuria in pHN, and that adduct formation was found in the GBM matrix (64).

**LPO-ADDUCT FORMATION OCCURS ON GBM TYPE IV COLLAGEN**

Exposure of the GBM to ROS and LPO products raised the question of whether adduct formation af-

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**Figure 2. Localization of H$_2$O$_2$ within the glomerular capillary wall of a proteinuric rat with passive Heymann nephritis (induced by intravenous injection of anti-Fx1A IgG 7 days before euthanization).** Isolated kidneys were perfused with a medium containing cerium ions, $\beta$-NADPH, and glucose. (A) The formation of precipitates of the locally formed H$_2$O$_2$ and the cerium ions was only seen in proteinuric rats, and it was found in a diffuse distribution within the glomerular basement membrane (GBM), including the immune deposits (ID). This suggests that a soluble product of glomerular epithelial cells could induce diffuse damage to the glomerular basement membrane. A mitochondrion (M) of the epithelial cell is devoid of the reaction product. (B and C) Within glomerular epithelial cells also, lysosomes (ly) contain H$_2$O$_2$-cerium precipitates. (Original magnification, x12,000.) (Reproduced with permission from Reference 53.)
Figure 3. Localization of (A) sheep anti-rat Fx1A in immune deposits, and (B) MDA adducts in glomeruli from proteinuric rats with 7-day passive Heymann nephritis (PHN) and (C) MDA adducts in glomeruli from 7-day PHN rats pretreated with probucol (Immunofluorescence microscopy on 1-μm frozen sections. (A) Granular deposits of immunoglobulin G are shown around the peripheral glomerular capillary loops in the typical pattern of PHN. (B) MDA adducts are shown prominently in glomerular epithelial cells in a fine granular pattern extending around the glomerular capillary loops, within the GBM, and throughout the mesangium (M). (C) In a probucol-pretreated rat, there is a marked reduction in glomerular staining for MDA-adducts, when compared to that of unmanipulated PHN rats in (B). (Original magnification, ×700.)

This preferential, nonrandom formation of MDA adducts posed the question of the identity of the 220-kd and the 440-kd bands. To approach this intriguing problem. GBM were isolated from proteinuric rats with PHN, and H3 was introduced into Schiff bases by incubation with H2-cyanoborohydride. The GBM were solubilized in SDS, and their radiolabeled proteins were immunoprecipitated with antibodies to matrix components. It was found that only antibodies to Type IV collagen bound H3-labeled proteins, whereas antibodies specific for laminin, heparan sulfate proteoglycan core protein, and fibronectin failed to form precipitates (Figure 4). The apparent molecular weights of the radiolabeled, Schiff-base-containing proteins immunoprecipitated by anti-Type IV collagen antibodies were approximately 220 kd and 440 kd. These results were interpreted as evidence that MDA-adduct formation was preferentially concentrated on Type IV collagen in the GBM of proteinuric PHN rats, and that, presumably, dimerization by the highly reactive dialdehydes occurred in this setting (64). This view is in agreement with previous in vitro experiments in which isolated GBM were chemically crosslinked by the bifunctional compound dimethylmalonimidate (65). Evidence was obtained which showed that relatively low amounts of crosslinks caused increase in permeability for albumin and immunoglobulins, whereas massive crosslinking resulted in clogging of the GBM filter, suggesting a fibrillar, functionally relevant substructure of the GBM (66).

**LPO ADDUCT FORMATION IS CONCENTRATED IN THE NC1 DOMAINS OF TYPE IV COLLAGEN**

Because NC1 domains of Type IV collagen are particularly rich in cationic lysyl residues (67), it was interesting to determine whether they could serve as targets for adduct formation with MDA. Indeed it was found that NC1 domains purified from GBM of proteinuric rats contained MDA adducts in high density (64). These findings raised the possibility that lipidperoxidation products, such as MDA and other dialdehydes, bind to Type IV collagen, in particular to the NC1 domain, and that dimerization of the fibrous Type IV collagen molecules could be mediated primarily by crosslinking of the NC1 domains.

It is intriguing that lesions in the NC1 domains of GBM Type IV collagen were previously found also in other glomerular diseases that cause proteinuria. I.e., Goodpasture's syndrome (68,69) and the hereditary Alport disease (70). Synoptically, these findings suggest a common final pathway toward proteinuria for pathogenetically unrelated glomerular lesions.
Figure 4. Identification of Type IV collagen as a major protein modification by MDA adducts in 7-day PHN rat GBM. Glomeruli from normal and 7-day PHN rats were extracted with deoxycholate, labeled in their Schiff bases using \( ^3\)H-cyanoborohydride, followed by immunoprecipitation with antibodies specific for extracellular matrix proteins. (A and B) Coomassie blue-stained gels of extracts from normal (A), and proteinuric 7-day PHN (B) rat GBM, showing that the same amount of protein was loaded in each preparation. (C and D) Autoradiographs of Lanes A and B, showing \( ^3\)H-labeled bands in the GBM of normal control rats (C), and 7-day PHN rats (D). The major bands labeled correspond to a approximately 220 kD and a 440 kD protein, as well as a approximately 250-kD protein (marked by dots). In proteinuric rats, the same pattern of proteins is labeled, but more specific activity is detected, indicating that more Schiff bases are present in proteinuric GBM than in control rats. (E) Autoradiograph of the Immunoprecipitate of detergent lysisates of \( ^3\)H-labeled GBM from 7-day PHN rats, obtained with anti-Type IV collagen antibody, which specifically immunoprecipitates proteins with apparent molecular weights of approximately ~220 and 440 kD. (F and G) Immunoprecipitates with antibodies specific for laminin and heparan sulfate proteoglycan-core protein, which fail to bind to any \( ^3\)H-labeled GBM protein. This indicates that Type IV collagen contains most Schiff bases in GBM of proteinuric rats with PHN, and that this molecule is also dimerized by the bifunctional aldehydes generated as a consequence of LPO. The left lane shows globular protein molecular weight standards.

THE LPO INHIBITOR PROBUCOL REDUCES PROTEINURIA

Is the formation of LPO-adducts on GBM matrix proteins of pathogenic relevance for proteinuria, or just an epiphenomenon? This question was answered in an interventional approach by treating rats 3 wk before induction of HN with the potent inhibitor of lipid peroxidation, probucol (71,72). This regime resulted in reduction of proteinuria by approximately 80% of that of untreated HN rats (Table 1). Probucol-induced side effects of lowering cholesterol levels were not of relevance, because treatment with the cholesterol-lowering drug simvastatin (which does not act as a radical scavenger) failed to influence proteinuria (Table 1). These results indicated that LPO-induced formation of adducts in the GBM are a major cause of damage of the glomerular filtration barrier, and could be causally related to proteinuria (64).

LIPOPROTEINS ACCUMULATE WITHIN IMMUNE DEPOSITS AND PROVIDE LIPIDS FOR PEROXIDATION

Development of atherosclerotic vascular lesions is driven by LPO adduct formation, which is powered by ROS produced primarily by inflammatory cells and by LDL particles that provide polyunsaturated lipids as substrate for ROS (reviewed in References 62 and 73).

TABLE 1. Proteinuria in passive Heymann nephritis (PHN): Response to pretreatment with probucol or simvastatin

<table>
<thead>
<tr>
<th>Groupa</th>
<th>Serum Cholesterol</th>
<th>Proteinuria</th>
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<tbody>
<tr>
<td></td>
<td>Start PHN (mM/L)</td>
<td>At 7 days (mM/L)</td>
</tr>
<tr>
<td>Normal Controls ( (N = 10) )</td>
<td>2.28 ± 0.04</td>
<td>2.22 ± 0.06</td>
</tr>
<tr>
<td>PHN ( (N = 6) )</td>
<td>2.26 ± 0.08</td>
<td>4.27 ± 0.02</td>
</tr>
<tr>
<td>Probul + PHN ( (N = 11) )</td>
<td>1.97 ± 0.01</td>
<td>1.95 ± 0.04</td>
</tr>
<tr>
<td>Simvastatin + PHN ( (N = 11) )</td>
<td>1.95 ± 0.01</td>
<td>2.38 ± 0.22</td>
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a Treated rats were given probucol or simvastatin for 21 days before induction of PHN. Values are mean ± SD.

Formation of glomerular ROS and LPO may be less intense in pHN, but, nevertheless, requires lipid donors. Cell membranes of glomerular cells are unlikely candidates because oxidized phosphatidylcholine, an indicator for oxidative cell membrane damage, was not detected in glomeruli of proteinuric pHN rats (74). By contrast, large amounts of apoE- and apoB-containing intact or fragmented lipoproteins were detected within immune deposits, and indirect evidence for oxidative damage of glomerular apoB was provided.
Thus, it appears that lipoproteins cross the GBM, and are retained within immune deposits, possibly by megalin/gp330, which serves in vitro as receptor for apoE (16) and apoB 100 (21). Because uptake of small amounts of apoE and apoB by clathrin-coated pits of podocytes in vitro (75) and in vitro (76) were also discovered in normal glomeruli, it was speculated that megalin/gp330 could serve as lipoprotein receptor in housekeeping of normal podocytes. It remains to be determined why lipoproteins accumulate within immune deposits in pHN and are not rapidly endocytosed and degraded by podocytes.

SUMMARY AND OUTLOOK

This brief review summarizes recent aspects of a putative chain of sequential pathogenic events in pHN (Table 2). Attention was focused here on recent hypotheses of molecular mechanisms of glomerular capillary wall damage and proteinuria. Intriguingly, the results obtained so far suggest several common elements of pathogenesis of atherosclerotic lesions (73) and of acute glomerular capillary wall damage and proteinuria in pHN.

Building on established findings that formation of ROS is essential for development of glomerular damage, evidence supporting the idea that LPO is a major effector of ROS action on the GBM matrix has been reviewed. However, only a small number of the large spectrum of ROS-induced reactions has been recorded. Several of the presented findings actually raise more questions than answers. For example, it is not clear whether or not matrix proteins other than Type IV collagen are affected in the GBM, and it cannot be excluded that the modifications on the collagen molecules could be functionally less important than ROS- and/or LPO-induced changes on other, less abundant GBM matrix proteins. These also include glomerular integrins (77), which apparently contribute to the cohesion of the glomerular tuft and attachment of podocytes to the GBM (78). A major unanswered question is how the LPO-related chemical modifications could physically alter the porosity of the GBM. It is conceivable that crosslinking of Type IV collagen could distort the texture of the GBM, which then could have some influence on its permeability (65). The role of endogenous glomerular proteinases in this context is unknown. It is possible that all of these reactions converge in a concerted action and together cause dramatic functional changes of the GBM, without, however, changing its morphologic appearance, at least by conventional techniques of light and electron microscopy.

It remains to be seen whether this putative sequence of pathogenic events of HN, which couples immune-deposit formation to development of proteinuria (Table 2), is of relevance for other experimental systems, and especially for human MN.

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