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# Lipoproteins Accumulate in Immune Deposits and Are Modified by Lipid Peroxidation in Passive Heymann Nephritis

Markus Exner,* Martin Susani,* Joseph L. Witztum,† Anny Hovorka,* Linda K. Curtiss,‡ Susanne Spitzauer,§ and Dontscho Kerjaschki*

*From the Division of Ultrastructural Pathology and Cell Biology, Institute of Clinical Pathology,* and Institute of Medical and Chemical Laboratory Diagnostics,§ University of Vienna, Vienna, Austria; and the Division of Endocrinology and Metabolism,† Department of Internal Medicine, University of California, San Diego, and the Scripps Clinic and Research Foundation,‡ La Jolla, California*

***Proteinuria in passive Heymann nephritis is primarily caused by reactive oxygen species that are produced by glomerular cells. Reactive oxygen species apparently exert their damaging effects on the glomerular filter by lipid peroxidation and subsequent adduct formation on matrix proteins of glomerular basement membranes. This raised the question as to the source of polyunsaturated fatty acids required as substrates for lipid peroxidation. Here we have localized by immunocytochemistry rat apolipoprotein E and apolipoprotein B within subepithelial immune deposits. Moreover, apolipoprotein B extracted from isolated glomeruli of proteinuric passive Heymann nephritis rats shows degradation and lipid peroxidation adduct formation, similar to apoproteins of oxidized lipoproteins in atherosclerotic lesions. These data provide evidence that lipoproteins accumulate within immune deposits and suggest that their lipids generate lipid-peroxidation-derived reactive compounds. (Am J Pathol 1996, 149:1313–1320)***

Proteinuria is the clinical hallmark of membranous glomerulonephritis and causes development of the nephrotic syndrome with generalized edema and destabilization of the coagulation system.[1] The range of proteinuria in membranous glomerulonephritis is directly related to the development of end-stage renal failure, and therefore it would be of advantage to reduce proteinuria in patients, even when the underlying glomerular disease cannot be directly influenced at the same time.[2] This, however, requires detailed knowledge of the pathogenesis of glomerular damage, which is difficult to obtain in human patients.

Passive Heymann nephritis (pHN),[3] an established rat model of human membranous nephropathy, is characterized by subepithelial immune deposits in the glomerular basement membrane (GBM) and by heavy proteinuria that commences usually 5 to 7 days after intravenous injection of nephritogenic antibodies such as polyspecific antisera against the crude renal cortical fraction Fx1A[4] or isolated tubular microvilli.[5] Although the pathogenic mechanisms of immune deposit formation are emerging,[6–8] less is known about subsequent molecular events that cause glomerular capillary wall damage. Recent observations attributed a critical role to reactive oxygen species (ROS)[9] that are produced in part by glomerular epithelial cells via the *de novo* synthesized NADPH oxido-reductase enzyme complex, similar to activated neutrophil granulocytes.[10] ROS cause intraglomerular lipid peroxidation (LPO), which generates highly reactive LPO products, such as malondialdehyde (MDA) and 4-hydroxynonenal,[11] that attack matrix proteins in the GBM.[12] LPO adduct formation also plays an important role in the pathogenesis of atherosclerotic lesion in vascular walls, and it was established that locally accumulated low density lipoproteins (LDLs) provide polyunsaturated fatty acids as substrates for ROS and LPO adduct formation.[13] In this study we have explored the pos-

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Address reprint requests to Dr. D. Kerjaschki, Department of Clinical Pathology, University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

sibility that also in pHN lipoproteins were retained within glomeruli and served as substrates for LPO adduct formation.

## Materials and Methods

### Antibodies

Monoclonal anti-lipoprotein antibodies were produced by immunization of mice with delipidated rat apolipoprotein (apo)B (500 μg/mouse) or with rat high density lipoprotein (HDL) (30 μg/mouse). Monoclonal antibodies specific for rat apoB or rat apoE were selected by immunoblotting. Sheep anti-Fx1A sera[14] was obtained from Dr. W. G. Couser (University of Washington, Seattle, WA), and the IgG fraction was purified on a Protein G-Fast Flow column (Pharmacia, Uppsala, Sweden). Monoclonal antibody MDA-2 was raised against murine MDA-LDL and reacts specifically with MDA-lysine adducts.[15] Monoclonal antibody specific for oxidized phosphatidylcholine[16] was a gift from Dr. H. Itabe, Teikyo University, Japan. Goat anti-rabbit IgG conjugated to 10-nm gold particles (Auroprobe) was obtained from Amersham (Buckinghamshire, UK). Affinity-purified rabbit anti-mouse IgG was from Dako (Copenhagen, Denmark), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse $F(ab')_2$ and rabbit anti-goat $F(ab')_2$ were from Jaxell-Accurate (Westbury, NY).

### Induction of Passive Heymann Nephritis

Male Sprague-Dawley rats (250 g; n = 6) were injected with 10 mg of sheep anti-Fx1A IgG and were sacrificed after 6 days. A second group of six rats were fed with the 3-hydroxy-3 methylglutaryl coenzyme A reductase inhibitor simvastatin (Merck, Sharp, and Dohme, St. Louis, MO; 1 mg/day) for 21 days before induction of pHN.[12] Kidneys were perfused with phosphate-buffered saline (PBS) and frozen in liquid nitrogen or fixed by perfusion with 4% paraformaldehyde/0.1% glutaraldehyde in 100 mmol/L cacodylate buffer (pH 7.2) for immunoelectron microscopy.

The use of animals for experimental work was authorized by the Austrian Ministry of Science.

### Determination of Proteinuria and Serum Cholesterol

Rats were housed in metabolic cages from day 3 after anti-Fx1A antibody injection until sacrifice, and proteinuria was determined in the 24-hour urine by the Coomassie Plus assay (Pierce, Rockford, IL). Serum was collected at sacrifice, and serum cholesterol was determined by a cholesterol oxidase method.[17]

### Immunohistochemistry

Cryostat sections (3 μm) were prepared from unfixed kidneys of normal, 6-day pHN rats and rats pretreated with simvastatin. Sections were incubated with monoclonal antibodies specific for rat apoB or for apoE (8 μg/ml), followed by affinity-purified FITC-labeled $F(ab')_2$ goat anti-mouse IgG that was depleted of cross-reactivity with rat and sheep IgG by preabsorption with appropriate sera. For negative controls, primary antibodies were omitted or replaced by irrelevant monoclonal IgGs of the same subclass. Sheep IgG was detected by direct immunofluorescence with FITC-$F(ab')_2$ fragments of rabbit anti-goat IgG. Sections were mounted in Geltol (Lipshaw, PA) and examined in an Olympus fluorescence microscope. Slides were examined by two reviewers, and the intensity of glomerular fluorescence was graded from 0 to +++.

### Immunoelectron Microscopy

Blocks of renal cortex from aldehyde-fixed kidneys were dehydrated in ethanol and embedded in K4M Lowicryl.[18] Ultrathin sections were incubated with monoclonal antibodies to rat apoB or apoE, followed by affinity-purified rabbit anti-mouse IgG and goat anti-rabbit IgG/10-nm gold conjugate. For controls, first antibodies were omitted or replaced by irrelevant monoclonal antibodies. Sections were stained with lead citrate and examined in a JEOL 1010 electron microscope.

### Immunoblotting

Glomeruli of normal and 6-day pHN rats were isolated by graded sieving, solubilized in reducing sodium dodecyl sulfate (SDS) sample buffer (3.6% SDS, 10 mmol/L EDTA, 2% β-mercaptoethanol, 20 mmol/L Tris-phosphate buffer, pH 6.8), and the protein concentration was determined.[19] A 50-μg amount of protein was loaded into each slot of 5 to 12% gradient polyacrylamide-SDS gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and stained with 0.5% Ponceau S in 6% trichloroacetic acid. Strips were blocked in PBS/5% dry milk/0.1% Tween 20, followed by incubation with monoclonal anti-apoE and apoB IgG for 12 hours at 4°C in blocking buffer. After incubation

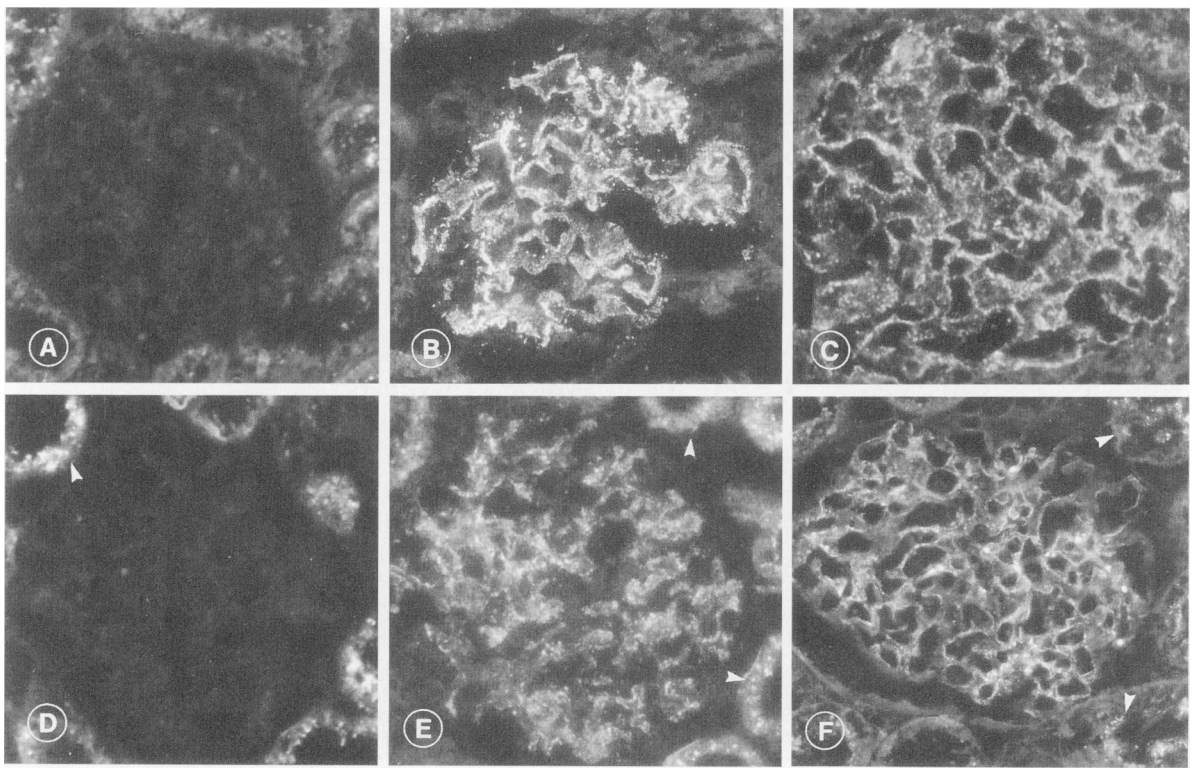


Figure 1. *Localization of apoB* (A, *to* C) *and apoE* (D *to* F) *in rat kidneys by indirect immunofluorescence. In PBS-injected control animals* (A *and* D), *no staining of the glomeruli is detected. In anti-Fx1A-IgG-injected animals, apoproteins are detected in a granular pattern in the periphery of the glomerular capillary loops* (B *and* E). *Granular distribution of apoE is seen in proximal tubule cells* (arrowheads *in* D *to* F). *In simvastatin-treated pHN rats* (C *and* F), *a similar distribution of apoproteins as in untreated pHN rats was observed. Magnification,* ×400.

for 1 hour with horseradish-peroxidase-labeled anti-mouse IgG, strips were developed using enhanced chemiluminescence (ECL-Kit, Amersham, Arlington Heights, IL). Relative concentrations of apoE were assessed by densitometry, using an LKB laser densitometer (Bromma, Sweden).

### *Immunoprecipitation*

Glomeruli of normal and 6-day pHN rats were isolated by graded sieving and incubated in 1% Triton X-100 in Tris-buffered saline, pH 7.4, at 4°C for 1 hour, with sonication in a Bransonic ultrasonication waterbath (Branson, Danbury, CT). Insoluble material was pelleted at 10,000 × *g* in a Microfuge B (Beckmann, Palo Alto, CA). Glomerular lysate containing 200 μg of protein was incubated with 10 μg of monoclonal anti-MDA IgG for 2 hours. Immune complexes were collected on Gamma-Bind G Sepharose (Pharmacia, Uppsala, Sweden) and analyzed by SDS electrophoresis. After electrophoretic transfer onto nitrocellulose membranes, immunoblotting was performed with monoclonal anti-apoB IgG.

### *Northern Blotting*

Total RNA of normal rat liver and of isolated glomeruli of normal and proteinuric 6-day pHN rats was isolated,[20] 10 μg of RNA was blotted onto a Zetaprobe membrane (BioRad, Richmond, CA) and hybridized with a 200-bp $^{32}$P-labeled apoE probe, obtained from Dr. W. Schneider, Vienna, Austria. Rat β-actin cDNA was used as internal probe.

## *Results*

### *Apolipoproteins Accumulate within Immune Deposits*

In pHN, apoB and apoE were observed by indirect immunofluorescence in glomeruli in a granular pattern (Figure 1, B and E). By immunogold electron microscopy, both apoproteins were found in highest concentrations within immune deposits (Figure 2, C and D). They were also localized in lysosomes or multivesicular bodies of glomerular epithelial cells (Figure 2D).

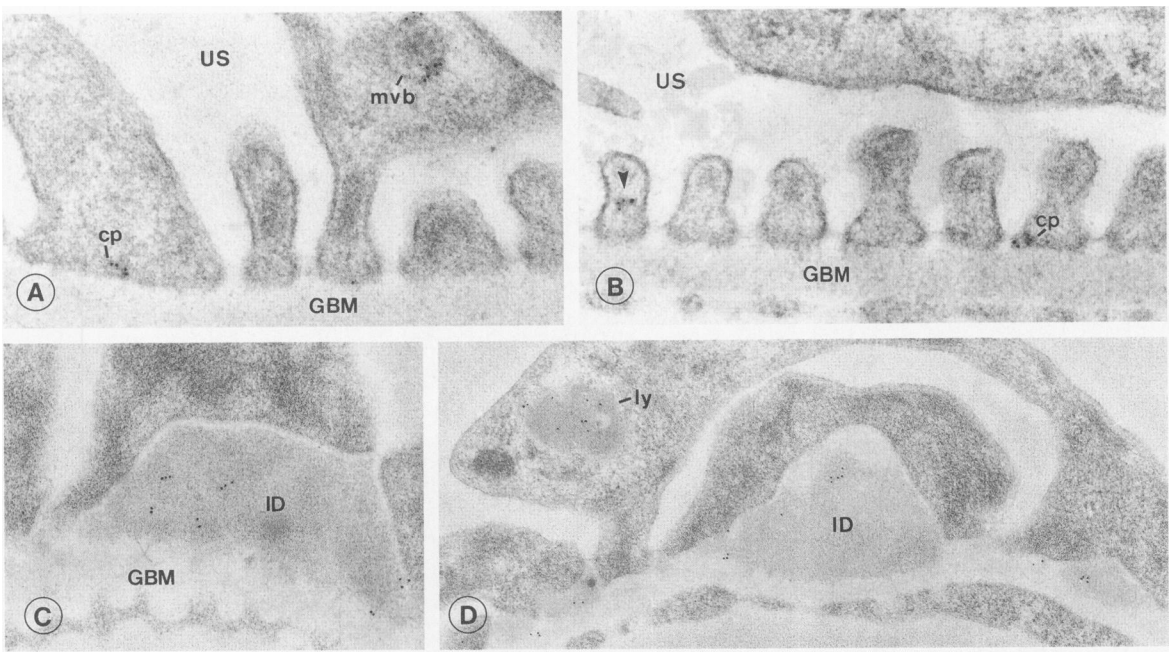


Figure 2. *Localization of apoB* (A *and* C), *and apoE* (B *and* D) *by indirect immunogold histochemistry on ultrathin sections of Lowicryl-embedded kidneys of rats that were injected with PBS* (A *and* B) *or anti-Fx1A IgG* (C *and* D). *ApoB and apoE are detected in multivesicular bodies* (*mvb*), *small intracellular vesicles* (arrowhead *in* B), *and rarely in the coated pits* (*cp*) *of podocytes in normal controls* (A *and* B). *In pHN* (C *and* D), *both apoproteins are located within immune deposits* (*ID*) *and in lysosomes* (*ly*) *in the podocyte's cytoplasm. US, urinary space. Magnification,* ×35,000.

In kidneys of normal control rats, apoE was localized by immunofluorescence predominantly in proximal tubular epithelial cells in a granular, lysosome-like pattern (Figure 1D). ApoB was found in similar localization only in traces (Figure 1A). By immunoelectron microscopy, apoB and apoE were encountered in glomerular epithelial cells within coated pits and multivesicular bodies and occasionally also in endothelial cells and the GBM (Figure 2, A and B). ApoE was localized in lysosomes of epithelial cells of proximal tubules, along the brush border microvilli, and in the clathrin-coated intermicrovillar microdomains similar to megalin/gp330[21] (Figure 3).

Localization of oxidized phosphatidylcholine with a specific monoclonal antibody[16] was performed by immunofluorescence. This antibody failed to bind to any structure in cryostat sections of normal and pHN rat kidneys (data not shown).

These data indicate that intact or fragmented, presumably lipid-containing lipoproteins accumulated within immune deposits in pHN.

## *Glomerular Accumulation of Lipoproteins and Proteinuria Are Independent of Plasma Cholesterol*

Normal control rats showed mean plasma cholesterol levels of 2.5 mmol/L and were not proteinuric. Rats with 6-day pHN developed proteinuria, and their plasma cholesterol levels increased significantly (4.3 mmol/L). Rats treated with the 3-hydroxy-3 methylglutaryl coenzyme A reductase inhibitor simvastatin before injection of nephritogenic anti-Fx1A IgG also developed severe proteinuria, similar to untreated pHN rats, but their plasma cholesterol levels were in the range of normal controls (2.3 mmol/L; Table 1). ApoB and apoE were detected in similar distribution and concentration in glomeruli of pHN rats pretreated with simvastatin, as in untreated pHN rats (Figure 1, C and F).

## *Apoprotein B within Immune Deposits Is Modified by Lipid Peroxidation*

ApoB was not detected in SDS-solubilized glomerular proteins of normal rats by immunoblotting (Figure 4, lane A). In pHN glomeruli, however, the anti-apoB antibody bound to several bands over a molecular weight range from ~200 to 500 kd (Figure 4, lane B). This was similar to apoB products formed by non-enzymatic degradation of apoB that occurs with oxidation,[22] however, with the difference that in pHN apoB migrated in several fuzzy bands of high molecular weight rather than in a single streak (Figure 4, lanes B and C).

Direct evidence for LPO adduct formation on apoB was obtained when detergent lysates of isolated glomeruli of pHN rats were first immunoprecipi-

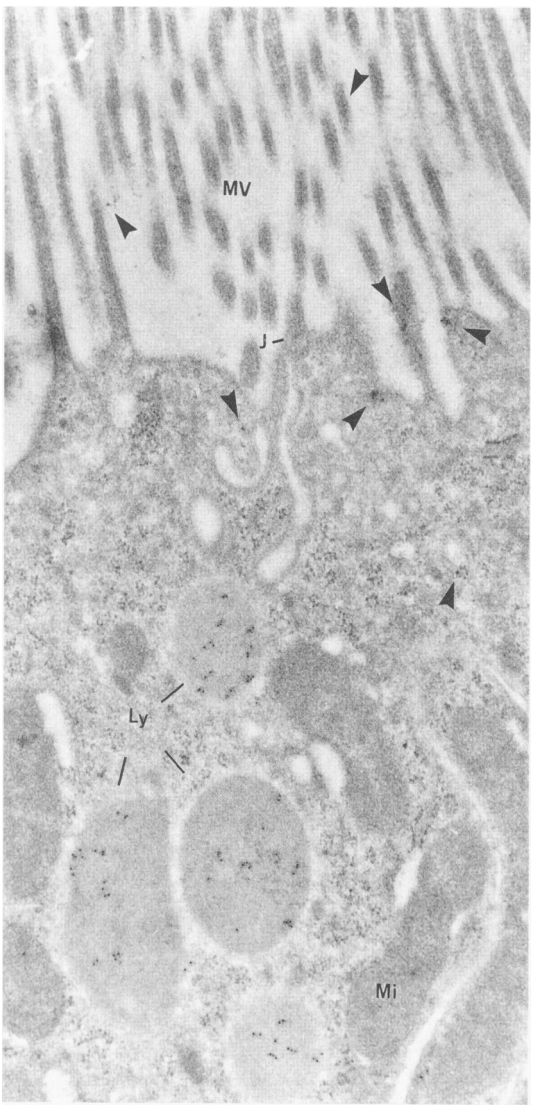


Figure 3. *Localization of apoE in a proximal tubule epithelial cell by an indirect immunogold technique, using K4M Lowicryl sections. Gold particles are found in highest concentration within lysosomes (Ly) and less frequently on the microvilli (MV) of the brush border and the intermicrovillar microdomains* (arrowheads). *Mi, mitochondrion. J, cell junction. Magnification, ×48,000.*

tated with monoclonal anti-MDA IgG and the precipitate subsequently probed with monoclonal anti-apoB IgG by immunoblotting. A similar staining

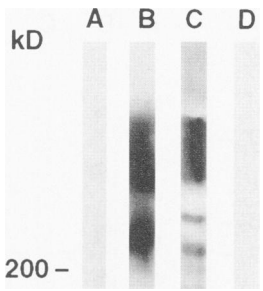


Figure 4. *ApoB in SDS lysates of isolated glomeruli of normal controls* (A *and* D) *and pHN rats* (B *and* C), *detected by immunoblotting with monoclonal anti-apoB antibodies.* A: *Isolated glomeruli of normal rats were lysed and immunoblotted with anti-apoB IgG. ApoB was not detectable in normal controls.* B: *In pHN, apoB formed a streak of >250 kd and a broad band of ~210 to 240 kd.* C *and* D: *Immunoprecipitates were formed from glomerular lysates of pHN* (C) *and normal control* (D) *rats with monoclonal anti-MDA antibody, and the precipitates were probed with anti-apoB antibodies by immunoblotting.* C: *In pHN, a high molecular weight streak is observed, similar to* B. *There are also several distinct bands of lower molecular weight.* D: *No MDA-containing apoB fragments were identified in normal rat glomeruli.*

pattern as with anti-apoB antibody alone was observed (Figure 4, lane C). In controls, similarly prepared immunoprecipitates from normal rat glomeruli failed to bind anti-apoB IgG (Figure 4, lane D). A monoclonal antibody specific for oxidized phosphatidylcholine,[16] a possible indicator for oxidative damage of cell membrane lipids, failed to label any glomerular protein of normal or pHN rats by immunoblotting (data not shown).

These data provide evidence that lipoproteins within immune deposits were oxidized and partially fragmented.

## *ApoE Accumulates within Glomeruli from the Circulation*

Immunoblotting with anti-apoE IgG on SDS lysates of isolated glomeruli of normal control rats revealed an approximately 34-kd band, characteristic for apoE (Figure 5, lane C). When equal amounts of glomerular proteins of normal and pHN rats (Figure 5, lanes A and B) were transferred onto nitrocellulose membranes and used for a quantitative comparison of apoE concentrations by immunoblotting, an approx-

Table 1. *Lack of Influence of Serum Cholesterol Levels on the Deposition of Apoproteins within Immune Deposits*

| Group | Serum cholesterol* (mmol/L) | Proteinuria (mg/24 hours) | IgG† | ApoB† | ApoE† |
|---|---|---|---|---|---|
| Normal controls (n = 6) | 2.50 ± 0.10 | 7.5 ± 0.40 | Ø | ± | ± |
| pHN (Fx1A) (n = 6) | 4.30 ± 0.40 | 130 ± 25 | +++ | ++ | ++ |
| pHN (simvastatin + Fx1A) (n = 6) | 2.30 ± 0.10 | 120 ± 20 | +++ | ++ | ++ |

*Values are mean ± SD.
†Deposition in glomeruli determined by immunofluorescence.
Ø, not detectable.

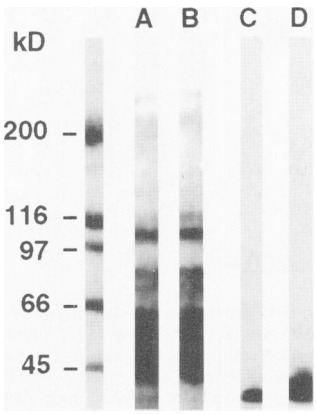


Figure 5. *Detection of apoE (34 kd) in isolated rat glomeruli by immunoblotting. Glomerular protein (50 μg) of normal controls (A and C) and proteinuric pHN rats 6 days after injection of anti-Fx1A antibodies (B and D) were transferred onto a nitrocellulose membrane and probed with antibodies specific for rat apoE (C and D). An approximately tenfold increase of apoE was noted in pHN (D).*

imately tenfold increase of apoE over normals was observed in pHN (Figure 5, lane D).

To distinguish between increased biosynthesis of apoE by glomerular cells or uptake of apoE from the circulation, levels of apoE-specific mRNA were compared by Northern blotting between normal controls and pHN rats. Only traces of apoE mRNA were detected in normal glomeruli, and there was no increase in pHN (Figure 6). The validity of the apoE cDNA probe was confirmed by blotting onto rat liver mRNA, which revealed an approximately 1.2-kb band (Figure 6). This indicated that the relatively large concentrations of apoE found in immune deposits were derived from the bloodstream and not synthesized by glomerular cells.

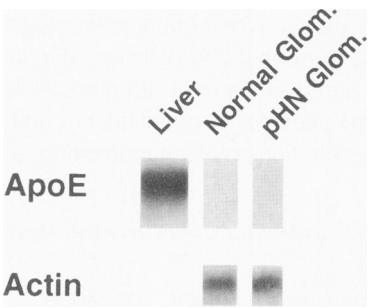


Figure 6. *Quantitative determination of the concentration of glomerular mRNA of apoE in glomerular RNA preparations of normal and pHN rats by Northern blotting. There was barely any signal obtained in normal glomeruli, and there was no up-regulation of specific mRNA levels in pHN. Similar amounts of glomerular RNA were loaded in normal and pHN groups, as indicated by blotting with a β-actin cDNA probe. ApoE mRNA showed up as a strong band of 1.2 kb in a preparation of rat liver RNA.*

## *Discussion*

Intraglomerular production of ROS[9] and formation of LPO adducts[12] were identified as principal pathogenic factors of proteinuria in pHN,[9] primarily because intervention with antioxidants and the potent LPO antagonist probucol[23] reduced proteinuria by ~90%. We have provided evidence that in pHN ROS are generated largely *in situ* by glomerular (epithelial) cells in the absence of intraglomerular neutrophil granulocytes and macrophages.[10] ROS apparently initiate local LPO,[12] generating a large variety of biologically active molecules including highly reactive compounds, such as MDA and 4-hydroxynonenal,[11] which cross-link proteins via Schiff base formation with lysil residues. In GBMs of proteinuric rats with pHN, MDA-lysine adducts were found predominantly on type IV collagen that was cross-linked presumably via its lysine-rich NC1 domains.[12] In this study we have addressed the possibility that lipoproteins could provide a source of polyunsaturated fatty acids required for LPO, similar to their role in the development of atherosclerotic lesions.[24] We have localized oxidized lipoproteins within immune deposits of proteinuric rats with pHN, suggesting that they could contribute to the formation of LPO adducts and indirectly also to the development of proteinuria.

A major result of this study was that apoE and apoB were found in high concentrations within immune deposits in pHN by immunohistochemistry, using monoclonal antibodies specific for rat apoE and apoB. This raised the question of whether the presence of these apoproteins indicated intact lipid-containing lipoproteins or only soluble lipid-free apoprotein fragments. As it is established that apoB and most of its fragments are extremely hydrophobic and therefore are found only in conjunction with lipids,[25] its presence within immune deposits suggests lipid-intact or -fragmented lipoproteins.

By contrast, apoE is a hydrophilic protein that is not only abundant in β-VLDL and LDL in the rat[26] but is also produced in soluble, lipid-free form by many cells, such as macrophages.[27] We have excluded the possibility that apoE was locally produced by glomerular cells in pHN by Northern blotting, suggesting that apoE mRNA detected previously in renal cortex[28] was primarily derived from extraglomerular sources. Localization of apoE within immune deposits could be consistent with filtration of free soluble apoE from the circulation, as its size (34 kd) is smaller than albumin; it is, however, more likely that apoE is part of apoB-containing intact or fragmented rat lipoproteins.

Do fatty acids of lipoproteins provide substrates for ROS and produce LPO adducts within immune deposits? Direct proof requires extraction and chemical analysis of lipids from immune deposits, which, however, is quantitatively not feasible. Therefore, indirect approaches were chosen to answer this question, capitalizing on the finding that in atherosclerotic lesions oxidation of LDL caused breakdown of polyunsaturated fatty acids and liberated MDA, which formed Schiff bases with lysine residues of apoB.[29] Here we show that apoB extracted from isolated glomeruli of rats with pHN contained MDA-Schiff bases, using a combination of immunoprecipitation with monoclonal anti-MDA and immunoblotting with anti-apoB antibodies. These findings indicated that apoB had undergone LPO-mediated adduct formation in glomeruli of pHN rats, similar to LDL in early vascular lesions in atherosclerosis.

Another manifestation of oxidation is the non-enzymatic degradation of intact ~500-kd apoB protein,[30] resulting in a characteristic broad streak ranging from ~80 kd to 500 kd in SDS gels. Here we report that fragmentation occurred also on apoB extracted from isolated glomeruli in pHN. However, high molecular weight fragments were detected that migrated as multiple discrete, fuzzy bands. This type of fragmentation can be seen in early stages of LDL oxidation,[15,30] but could also be due to degradation by proteases that presumably were up-regulated in podocytes in pHN, as recently examplified by metalloproteinase-9.[31] Lipids of glomerular cell membranes of proteinuric pHN rats apparently failed to serve as alternative or additional lipid donors for LPO, because oxidatively modified phosphatidylcholine, an indicator of cell membrane LPO, was not detected in pHN glomeruli by a specific monoclonal antibody.[16] Collectively, these results provide indirect evidence that lipids carried by lipoproteins within immune deposits could provide the source of LPO adducts.

Proteinuria in pHN is associated with significantly increased serum cholesterol levels, raising the possibility that accumulation of lipoproteins within glomeruli could be caused by passive insudation. This, however, appears unlikely, because reduction of serum cholesterol levels of pHN rats to those of normal controls by the 3-hydroxy-3 methylglutaryl coenzyme A reductase inhibitor simvastatin failed to influence the amount of apoproteins within immune deposits.

Previous studies indicated that cultured human glomerular epithelial cells are endowed with receptors for lipoproteins, in particular for those rich in apoE.[32] In the context of pHN, it is of interest that apoE was identified in vitro as the ligand of megalin/gp330 (reviewed in Ref. 7), the antigenic target in immune deposits.[8] Megalin/gp330 is an approximately 515-kd glycoprotein that is structurally related to the LDL receptor family[33] and serves as a polyspecific receptor for other ligands besides apoE, such as human apoB 100, $Ca^{2+}$, small peptide hormones, cationic antibiotics, etc.[34] It remains to be determined whether specific interaction of apoE and megalin/gp330 also occurs in vivo in pHN and contributes to binding of apoE-containing lipoproteins within immune deposits. Because small amounts of apoproteins were also detected in glomeruli of normal controls, it is possible that uptake of lipoproteins by glomerular epithelial cells occurs as a regular housekeeping mechanism, and it remains to be determined whether alterations of this pathway in pHN are related to accumulation of lipoproteins within immune deposits.

Collectively, the results of this study suggest that in pHN lipoproteins accumulate within immune deposits and that their fatty acids presumably provide a substrate for LPO, which in turn is largely responsible for glomerular damage and proteinuria.[12] Previous investigations have sought to establish analogies between pathogenic mechanisms of atherosclerosis and chronic renal damage, such as glomerulosclerosis.[35] Here we report that also in acute proteinuria in pHN similar pathogenetic elements occur as in early stages of atherosclerotic lesions. As apoproteins were recently localized also within glomerular immune deposits in membranous glomerulonephritis[36] (own unpublished data) it is possible that similar mechanisms of proteinuria operate also in this human disease.

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