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Immunological Evidence for Hypochlorite-Modified Proteins in Human Kidney

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Oxygen radicals and oxidatively modified proteins seem to participate in degenerative vascular and inflammatory diseases. Factors that contribute to the development of atherosclerosis, eg, oxidation of low-density lipoproteins (LDLs), may also contribute to glomerulosclerosis. Although the nature of the* in vivo *oxidants remains unknown, recent findings indicated that the myeloperoxidase (MPO)-H_2O_2-halide system could play an important role in modification of (lipo)proteins in human tissues. MPO, the enzyme responsible for hypochlorite ($HOCl/OCl^-$) formation, is present in human atherosclerotic lesions and in inflammatory conditions. In the present study, MPO was identified by Western blot analysis and immunohistochemical technique in diseased human kidney either with primarily sclerotic or inflammatory lesions. Furthermore, the presence of HOCl-modified proteins was demonstrated in diseased renal tissues using a specific monoclonal antibody (clone 2D10G9), raised against HOCl-modified LDL, that does not cross-react with native LDL or Cu^{2+}-, 4-hydroxynonenal-, or malondialdehyde-modified LDL. The antibody recognized HOCl-modified proteins in glomerular and tubulointerstitial inflammatory and fibrotic lesions and pronounced immunostaining was demonstrated in mononuclear cells. LDL or human serum albumin oxidized by HOCl* in vitro, *but not native LDL or human serum albumin, effectively competed with epitopes in diseased kidney for antibody binding. Western blot analysis in diseased kidney protein samples revealed at least two major proteins recognized by the anti-HOCl-modified protein monoclonal antibody. Densitometric evaluation of immunoreactive bands obtained under these conditions demonstrated that expression of HOCl-modified proteins is tightly coupled to expression of immunoreactive MPO in the same tissue samples. From our studies it is proposed that oxidation of proteins by HOCl might be a leading event in glomerular and tubulointerstitial injury. By this mechanism, mononuclear cells, a permanent source for MPO, may play a key role in the development of nephrosclerosis, glomerulosclerosis, and tubulointerstitial fibrosis, respectively. (Am J Pathol 1997, 150:603–615)

A growing body of evidence suggests that oxygen radicals are important mediators in inflammatory and degenerative diseases.[1] Oxidation of low-density lipoproteins (LDLs), the main carrier of plasma cholesterol, might contribute to the early stages of atherosclerosis.[2–4] Associated with LDL oxidation is the modification of its apoprotein moiety and the generation of a wide range of lipid peroxidation products including hydroperoxides and reactive aldehydes, eg, malondialdehyde (MDA) and 4-hydroxynonenal (HNE). These aldehydes ultimately convert the LDL particle into a high-uptake form for macrophage scavenger receptors leading to the formation of cholesterol-engorged foam cells, which are the hallmark of fatty streaks, the first histologically recognizable phase of atherogenesis.[3,4] Several lines of evidence indicate that oxidized LDL (ox-LDL) and MDA-mod-

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ified LDL do occur *in vivo*. ox-LDL, MDA-LDL, and HNE-modified proteins have been demonstrated in atherosclerotic lesions and subsequently in plasma[5–11] and in extracts of atherosclerotic lesions.[7] Autoantibodies directed against ox-LDL and MDA-LDL have been detected in the plasma of experimental animals and humans.[7,12,13]

Several studies have demonstrated a highly significant relationship between circulating cholesterol levels, glomerular deposits of lipids, and glomerulosclerosis (for review see Refs. 14 and 15). The recruitment of monocytes and the appearance of foam cells in the mesangium seem to be important mechanisms in the development of glomerulosclerosis.[16] Although an analogy has been inferred between the development of atherosclerosis and global glomerulosclerosis, scarce data have been published on oxidative modification of (lipo)proteins in the kidney.[17] In hypercholesterolemic rats with experimental focal glomerulosclerosis, ox-LDLs have been detected by immunohistochemical staining in glomeruli in the kidney.[18] In passive Heymann nephritis, a model for human membranous nephropathy, MDA- and 4-HNE-lysine adducts have been detected in the noncollagenous domain of type IV collagen of glomerular basement membrane.[19] Recently, it has been reported that proliferating mesangial cells are able to oxidize LDL[20] by mechanisms that involve generation of reactive oxygen species (ROS),[21] including free radicals, ie, O_2^-, OH^-, H_2O_2, and HOCl.

Previous findings suggested that the myeloperoxidase (MPO)-H_2O_2-halide system can cause glomerular injury and thus may be important in granulocyte-mediated glomerulonephritis.[22–25] MPO (E.C. 1.11.1.7), a heme protein, is present in azurophil granules of neutrophils and monocytes. After activation of these cells, MPO is secreted into the phagosome or into the extracellular space. H_2O_2 can be generated in parallel by activated phagocytes through a membrane-bound NADPH-oxido-reductase complex. H_2O_2, discharged into the phagocytic vacuole or into the extracellular mileu,[26] may react with MPO and chloride ions to form HOCl[27]; HOCl is approximately 1000 times more toxic than superoxide anion or H_2O_2 itself.[28] Subsequently, HOCl reacts with a wide range of biological target molecules, including lipids, antioxidants, and proteins[29] to form N-Cl derivatives, which in turn are powerful oxidizing agents.

Previous findings have demonstrated that exposure of mouse peritoneal macrophages to HOCl-modified lipoproteins resulted in increased intracellular concentration of cholesterol and cholesteryl esters.[30,31] Recently, HOCl-modified LDLs and HOCl-modified proteins have been detected in atherosclerotic lesions.[32] HOCl and HOCl-modified proteins may thus be important in degenerative renal vascular disease, considering the presumed correspondence between atherosclerosis and glomerulosclerosis, and in inflammatory renal disease. We therefore sought to determine whether HOCl-modified (lipo)proteins are present in human kidney disease. Nephrosclerosis, focal and segmental glomerulosclerosis, and interstitial nephritis were chosen as typical examples of vascular degenerative and inflammatory renal disease. The monoclonal antibody (MAb) used specifically recognized HOCl-modified (lipo)proteins and was not cross-reactive with native LDL and the other forms of modified (lipo)proteins.[33] We report here that this antibody recognized hypochlorite-modified proteins in glomerular and tubulointerstitial inflammatory and fibrotic diseases, raising the possibility that oxidation of proteins by HOCl might be an important event in glomerular and tubulointerstitial injury.

Materials and Methods

Reagents

Nitrocellulose was purchased from Schleicher & Schuell (Dassel, Germany). Sodium dodecyl sulfate (SDS) was from Serva (Heidelberg, Germany). Human serum albumin (HSA; fatty-acid-free) and goat anti-rabbit immunoglobulin G (IgG; peroxidase-conjugated) were from Sigma (Taufkirchen, Germany). Goat anti-mouse IgG (peroxidase-conjugated) antibodies were from Chemicon (Temecula, CA). Polyclonal anti-human LDL antibodies were from Behring (Marburg/Lahn, Germany), and polyclonal rabbit anti-human MPO IgG fraction was from Dako (Glostrup, Denmark). The primary mouse MAb 2D10G9 (subtype $IgG_{2b\kappa}$) was raised against HOCl-modified LDL (800 molecules of HOCl per LDL particle). Characterization of MAb 2D10G9 revealed it to be highly specific for HOCl-modified LDLs and other HOCl-modified proteins and not to cross-react with native LDL or LDL modified by Cu^{2+}, HNE, MDA, or other lysine modifications of LDL.[33] The MPO-enriched protein fraction from human neutrophils was obtained from J. Schauer (University of Graz, Graz, Austria). All other chemicals were analytical grade reagents obtained from Merck (Darmstadt, Germany).

Table 1. *Age and Sex of Patients and Histopathological Diagnosis of Renal Tissues Taken for Immunohistology and Western Blot Analysis* of HOCl-Modified Proteins*

Histological diagnosis	n	Sex	Age (years)
Renal cell carcinoma*	3	M	57, 59, and 60
Reflux nephropathy and pyelonephritis*	1	M	30
	1	F	47
Idiopathic interstitial nephritis	2	M	63 and 71
Benign nephrosclerosis	5	M	60 ± 7
	2	F	
Minimal change glomerulopathy with focal, segmental glomerulosclerosis	3	M	42 ± 10
	1	F	

M, male; F, female.

HOCl Modification of Proteins

LDL (d = 1.035 to 1.065 g/ml) was isolated by ultracentrifugation as described previously and desalted by size exclusion chromatography on Econopac 10-DG columns (Bio-Rad, Hercules, CA).[33] LDL protein concentrations were determined by the bicinchoninic acid reagent kit (Pierce, Oud Beijerland, The Netherlands) using bovine serum albumin (BSA) as a standard. HOCl solutions were prepared by saturation of a solution of NaOH (3 mol/L) with chlorine until the concentration of NaOH decreased to 0.2 mol/L. HOCl-LDL was prepared as described and HOCl-HSA was obtained in a similar fashion.[30,33,34] Briefly, 1 mg of protein (LDL or HSA) per ml of phosphate-buffered saline (PBS, pH 7.4) was incubated with HOCl solution at 4°C for up to 2 hours at pH 7.4. HOCl was used at a ratio of 800 molecules per LDL particle and 100 molecules per HSA particle, respectively.[33]

Human Renal Tissue

Human renal tissue consisted of tumor-free parts of nephrectomy specimens or of transcutaneous renal biopsies. Nephrectomies were performed because of renal cell carcinoma or because of chronic pyelonephritis and reflux nephropathy. Patients with the latter diagnosis were not actively infected at the time of surgery. Renal biopsy specimens were used in patients with idiopathic interstitial nephritis, nephrosclerosis, and focal and segmental glomerulosclerosis (Table 1). Nontumorous tissue of nephrectomy specimens and renal biopsy specimens were fixed in 4% phosphate-buffered formaldehyde solution (99 mmol/L NaH_2PO_4, 108 mmol/L Na_2HPO_4, 248 mmol/L NaCl, pH 7.35) at 4°C for 24 hours; phosphate-buffered formaldehyde-fixed tissues were then embedded in paraffin. The specimens were primarily used to obtain a clinicopathological diagnosis by performing light microscopy stains, immunohistology for Igs and complement factors on paraffin sections, and transmission electron microscopy. Details of patients and their clinicopathological diagnosis are given in Table 1. Renal diseases other than those listed in Table 1 were excluded. Approval by the local human ethics committee was obtained to use the embedded tissue samples for further investigation.

For identification of HOCl-modified proteins by immunoblotting technique, tumor-free renal kidney parenchyma obtained from nephrectomies (approximately 1 g wet tissue) was frozen in liquid N_2 and homogenized.

Immunohistochemistry

Immunohistochemistry was carried out on 5-μm sections of paraffin-embedded tissue listed in Table 1, including three regular renal tissue samples using either the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique or immunogold-silver enhancement (IGSE) technique.

APAAP Technique

Tissue sections were deparaffinized, rehydrated, and incubated with MAb 2D10G9, used at a 1:2 dilution of the corresponding hybridoma supernatant (containing 63.2 μg of IgG/ml). For competition experiments, the specified competitor was added to the section together with the primary antibody at the concentration indicated. After application of MAbs to the slides for 2 hours at 22°C, a rabbit anti-mouse antibody (Z 259, Dako, Hamburg, Germany) was used at a dilution of 1:40 at 22°C for 1 hour. Alkaline phosphatase mouse MAb (diluted 1:40) was then incubated at 22°C for 1 hour. All dilutions were done in PBS (pH 7.6). For staining, slides were exposed to a solution of sodium nitrite (28 mmol/L), new fuchsin (basic fuchsin; 21 mmol/L), naphthol-AS-BI-phosphate (0.5 mmol/L), dimethylformamide (64 mmol/L), and levamisole (5

mmol/L) in 50 mmol/L Tris/HCl buffer (pH 8.4, containing 146 mmol/L NaCl) for 15 minutes.

Immunohistochemical detection of MPO was done by a rabbit polyclonal anti-human IgG, diluted 1:100, and the APAAP method by adding a mouse anti-rabbit polyclonal antibody (M 737, Dako; diluted 1:50) before addition of rabbit anti-mouse antibody (Z 259).

Sections were counterstained with aqueous hematoxylin before application of gelatin mounting medium.

IGSE Technique[35,36]

To block nonspecific binding sites, tissue sections were incubated with skim milk (4%, w/v) in 50 mmol/L Tris buffer (pH 7.6, containing 146 mmol/L NaCl) at 22°C for 25 minutes. After incubation of tissue sections with primary antibody (2D10G9) for 1 hour at 22°C, a biotinylated goat anti-mouse antibody supplied by Jackson ImmunoResearch Labs (West Grove, PA), diluted 1:800 in skim milk (2%, w/v in 50 mmol/L Tris buffer, pH 7.6), was applied at 22°C for 30 minutes, and then a peroxidase-coupled streptavidin (diluted 1:2000 in the same buffer) was used for 30 minutes at 22°C. Finally, an antibody against horseradish peroxidase coupled with 6-nm gold particles was added at 22°C for 45 minutes; the antibody was diluted 1:20 in PBS buffer (pH 7.6) containing 1% BSA, Triton X-100 (0.01%), and Tween 20 (0.01%). After fixation of the section in PBS (pH 7.6), containing 2% v/v glutaraldehyde, a silver enhancement was performed at 22°C for approximately 15 minutes, stopping the reaction depending on the light microscopic appearance of staining intensity. The silver enhancement solution contained citric acid (71 mmol/L), trisodium citrate (12 mmol/L), hydrochinone (22 mmol/L), and silver acetate (12 mmol/L). Thereafter, slides were exposed to developing solution (Kodak Readymatic R, Kodak, Rochester, NY). Kernechtrot (1%) was used as counterstain.

Control experiments for all immunohistochemical assays encompassed immunohistology 1) with nonimmune mouse IgG, 2) with HOCl-modified LDL, HSA, or BSA as competing antigens, 3) without primary MAbs, 4) without secondary antibody, or 5) without alkaline-phosphatase- or immunogold-coupled antibodies.

Polyacrylamide Gel Electrophoresis and Western Blotting Technique

Polyacrylamide gel electrophoresis (PAGE) was performed using 3.75 to 12.5% polyacrylamide gradient gels with electrophoresis at 150 V for 90 minutes in a Bio-Rad mini-blot chamber.[33,37] Homogenized kidney biopsies from nephrectomies (cortex, outer medulla, inner medulla, and papilla) were dissolved in 1 ml of O'Ferral's buffer (3.04 mol/L glycerol, 0.17 mol/L SDS, 0.14 mol/L Tris/HCl buffer, pH 6.8, containing 5% mercaptoethanol) and centrifuged at 13,000 rpm for 10 minutes. From the supernatant, aliquots were diluted with sample buffer (0.1 mol/L Tris/HCl, pH 6.8, 4% SDS, 20% glycerol) in the ratio of 1:1 (v/v) and incubated at 95°C for 5 minutes before application to gels. For Western blotting experiments, proteins were electrophoretically transferred to nitrocellulose membranes. Immunochemical detection of HOCl-modified proteins in renal tissues was performed by MAb 2D10G9 followed by peroxidase-conjugated rabbit anti-mouse IgG (Bio-Rad). The secondary antibody was diluted 1:5.000. To visualize bound antibody, ECL-Western-blotting detection reagents and Hyperfilm-ECL (Amersham Buchler, Braunschweig, Germany) were used according to the manufacturer's suggestions. Films were developed in an automatic processor.

Immunochemical detection of MPO in the same tissue samples was performed with rabbit polyclonal anti-human MPO IgG (Dako) as a primary antibody (diluted 1:100), followed by peroxidase-conjugated goat anti-rabbit IgG as secondary antibodies (dilution 1:8.000).

Some nitrocellulose blots were probed with polyclonal rabbit anti-human LDL antibody (1:2500 dilution) or rabbit anti-human HSA antibody (1:3000 dilution) and peroxidase-conjugated goat anti-rabbit IgGs (1:6000 dilution) to detect apolipoprotein B-100 or HSA.

Results

Hypochlorite-Modified Proteins in Human Kidney

Immunohistochemical Findings

Renal tissues from controls and diseased patients embedded in paraffin were examined for the presence of HOCl-modified proteins *in vivo*. The results in Figures 1 and 2 are representative of the kidney specimens examined and listed in Table 1.

Regular renal cortex (n = 3) did not reveal staining of HOCl-modified proteins by MAb 2D10G9 in pre- and postglomerular vessels, glomeruli, and tubulointerstitium (Figure 1A).

In acute interstitial nephritis, numerous cells in the dense leukocytic infiltrate of the renal interstitium

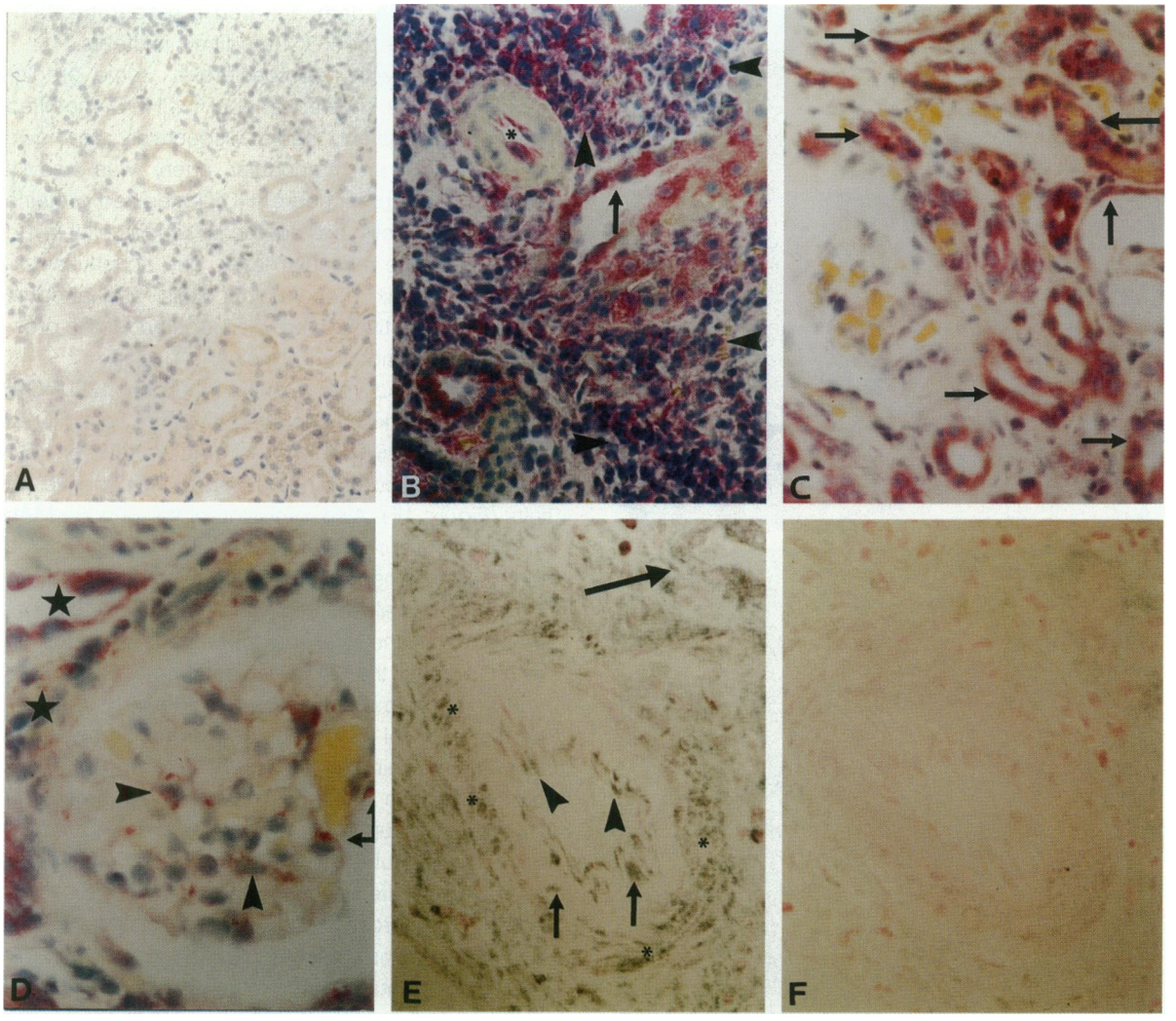
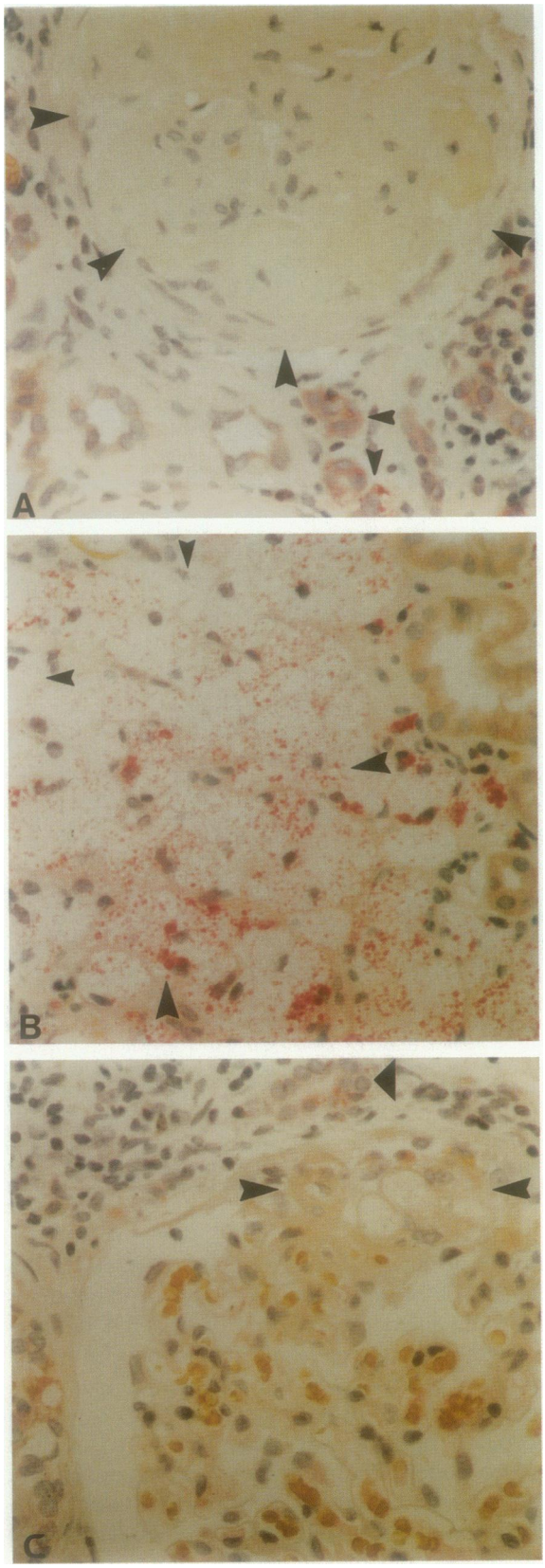


Figure 1. *Immunohistological detection of HOCl-modified proteins in inflammatory and degenerative human kidney diseases. Tissue sections from control and diseased human kidney were incubated with MAb 2D10G9 as a primary antibody (for detailed incubation conditions see Materials and Methods). Staining was performed by either the APAAP method* (**A** *to* **D**) *or the IGSE method* (**E** *and* **F**). *Representative sections of tissues of Table 1 are shown.* **A:** *Regular renal cortex.* **B:** *Acute interstitial nephritis, with staining for HOCl-modified proteins in the dense leukocytic infiltrate of the renal interstitium* (**arrowhead**), *staining of a preglomerular arteriole within the endothelium* (**asterisk**), *and staining of atrophic tubules within epithelial cells* (**arrow**). **C** *to* **E:** *Nephrosclerotic kidney.* **C:** *Staining in atrophic tubules of the cortex* (**arrows**). **D:** *Staining in podocytes* (**arrows**) *and in the mesangium* (**arrowheads**). *Muscle cells of afferent arteriole and distal tubular epithelia demonstrate a distinct positivity* (**asterisk**). **E:** *Staining in an interlobular artery with subendothelial fibrosis in endothelial cells* (**arrowheads**), *in cells of the subendothelial layer* (**arrows**), *and in the muscular layer* (**asterisk**). *Staining was also seen in adventitial extracellular matrix, mononuclear cells, and atrophic tubule* (**long arrows**) . **F:** *A parallel section to that shown in* **E**, *demonstrating no staining for HOCl-epitopes when MAb 2D10G9 was absorbed with excess (molar ratio of 1:100) HOCl-modified HSA.*

were stained by MAb 2D10G9, indicating the presence of HOCl-modified proteins (Figure 1B). Preglomerular arterioles with hyalinosis were stained within the endothelium. Atrophic tubules with broadened basement membrane often had a clear label in epithelial cells (Figure 1B).

Also in nephrosclerosis, tubules with damaged, flattened epithelial cells, which were surrounded by a fibrotic interstitium with a sparse mononuclear inflammatory cell infiltrate, had a focal staining for HOCl-modified proteins (Figure 1C) comparable to the reaction of damaged tubules in interstitial nephritis. In several cases, protein droplets in the lumina of proximal tubular epithelia stained for HOCl-modified protein. In kidneys with nephrosclerosis, immunohistological evidence for HOCl-modified epitopes was also obtained in glomeruli. In glomeruli, a weak staining pattern for HOCl-modified proteins was observed in mesangium and in podocytes. In Figure 1D, a glomerulus in a kidney with nephrosclerosis, shown representatively, was slightly stained by MAb 2D10G9 in podocytes and mesangium. In the same specimen, muscle cells of afferent arteriole and distal



tubular epithelia demonstrated a moderate staining by 2D10G9 for HOCl-modified proteins.

In biopsies with nephrosclerosis, some preglomerular arteries showed a label for HOCl-modified proteins in intima and media, respectively. As shown in Figure 1E, an interlobular artery was heavily stained for the presence of HOCl-modified proteins in endothelial cells, cells of the subendothelial layer, and cells of the muscular layer. The adventitial extracellular matrix, mononuclear cells, and atrophic tubules, respectively, were also labeled for the presence of HOCl-modified proteins.

Competition of the primary antibody (2D10G9) with authentic HOCl-modified proteins, eg, HOCl-LDL or HOCl-HSA, but not with native LDL or HSA prevented antibody binding and gave negative results (Figure 1F). Also, omission of the primary MAb or replacement of 2D10G9 with either $IgG_{2b\kappa}$ isotype control or a nonspecific polyclonal mouse IgG fraction eliminated all staining (data not shown), demonstrating that the staining observed was due to 2D10G9 rather than nonspecific effects.

In addition to results shown in Figure 1, C–E (ie, the presence of HOCl-modified proteins in nephrosclerosis), staining with MAb 2D10G9 was also performed in tissue sections from benign nephrosclerosis with pronounced vascular lesions, glomerular sclerosis, and tubular atrophy. There were, however, no apparent differences in staining patterns except that totally sclerosed glomeruli did not show HOCl-modified proteins within the scar (Figure 2A). In patients with minimal-change glomerulopathy and focal segmental glomerulosclerosis, very faint or no positivity for HOCl-modified protein could be demonstrated within the segmental sclerosis containing foam cells (Figure 2B); this finding was in contrast to the strong label of some but not all interstitial foam cells in these cases (Figure 2C).

Figure 2. *Immunohistological detection of HOCl-modified proteins in inflammatory and degenerative human kidney diseases. Tissue sections were incubated with MAb 2D10G9 as a primary antibody (for detailed incubation conditions see Materials and Methods). Staining was performed by the APAAP method. Representative sections of tissues of Table 1 are shown.* **A:** *Nephrosclerotic kidney. A totally sclerosed glomerulus* (**arrowheads**) *without label of HOCl-modified protein is shown. Atrophic tubules are positive* (**small arrowheads**). **B** *and* **C:** *Focal, segmental sclerosis in minimal change glomerulopathy.* **B:** *Interstitial group of foam cells, positive in cytoplasma for HOCl-modified proteins* (**arrowheads**). *Some foam cells were not labeled by HOCl-epitope-specific antibody* (**small arrowheads**). **C:** *Segmental sclerosis in a glomerulus with no immunohistochemical stain for HOCl-modified proteins in sclerotic focus and foam cell* (**arrowheads**). *Atrophic tubular epithelia have a faint positivity* (**broad arrowhead**).

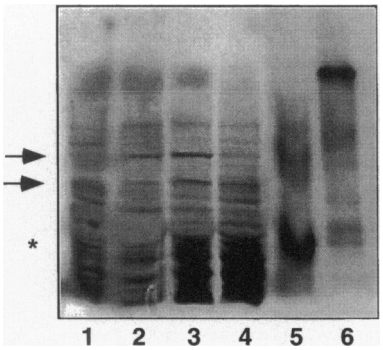


Figure 3. *Immunoblot experiments for the presence of HOCl-modified proteins in diseased renal tissues. Antigens (total protein kidney homogenate, 6 mg/lane* (**lanes 1** *to* **4**)*, and HOCl-modified proteins* (**lanes 5** *and* **6**) *were separated on 3.75 to 12.5% nonlinear polyacrylamide gels under denaturating conditions. Proteins were transferred to nitrocellulose and detected using 2D10G9. Immunoreactive bands were visualized with rabbit anti-mouse IgGs using the ECL-Western blotting detection system. The antigens used were as follows:* **lane 1**, *control kidney;* **lane 2**, *interstitial nephritis;* **lane 3**, *reflux nephropathy and pyelonephritis;* **lane 4**, *nephrosclerosis;* **lane 5**, *1.5 μg of HOCl-HSA; and* **lane 6**, *1.5 μg of HOCl-LDL.* **Arrows** *indicate the position of the 125- and 140-kd immunoreactive proteins, respectively. The* **asterisk** *indicates the position of the 67-kd nonaggregated HOCl-HSA. Competition experiments with MAb 2D10G9 preabsorbed with HOCl-HSA at a molar ratio of 1:20 prevented antibody binding to the bands indicated.*

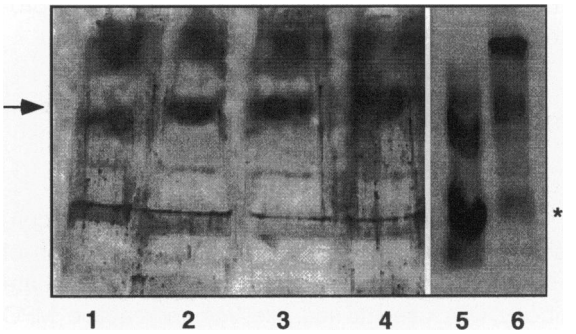


Figure 4. *Immunoblot experiments for the presence of HOCl-modified proteins in different renal compartments in chronic interstitial nephritis. Antigens (total protein kidney homogenate, 6 mg/lane* (**lanes 1** *to* **4**)*, and HOCl-modified proteins* (**lanes 5** *and* **6**) *were separated on 3.75 to 12.5% nonlinear polyacrylamide gels under denaturing conditions. Proteins were transferred to nitrocellulose and detected using MAb 2D10G9. Immunoreactive bands were visualized with rabbit anti-mouse IgGs using the ECL-Western blotting detection system. The antigens used were as follows:* **lane 1**, *inner medulla;* **lane 2**, *papilla;* **lane 3**, *cortex;* **lane 4**, *outer medulla;* **lane 5**, *1.5 μg of HOCl-HSA; and* **lane 6**, *1.5 μg of HOCl-LDL. The* **arrow** *indicates the position of immunoreactive 240-kd protein. The* **asterisk** *indicates the position of the 67-kd, nonaggregated HOCl-HSA.*

Western Blotting Experiments

To strengthen the immunohistochemical findings on HOCl-modified proteins in kidney biopsies (Figures 1 and 2), protein homogenates from nephrectomies from control and diseased kidney tissues were subjected to SDS-PAGE under reducing conditions. In Western blotting experiments using MAb 2D10G9 as a primary antibody, no significant stain due to the presence of HOCl-modified proteins was observed in control kidney (Figure 3, lane 1). In diseased renal tissues, several diffuse immunoreactive bands occurred under our conditions. These bands were not present when the antibody was preabsorbed with HOCl-modified HSA, in contrast to other nonspecific bands also seen in lane 1. In tissues from pyelonephritis (Figure 3, lanes 2 and 3), a highly immunoreactive band with an approximate molecular mass of 125 kd was observed. The same band was almost absent in renal tissues obtained from nephrosclerosis (Figure 3, lane 4).

When studying the presence of HOCl-modified proteins in different regions of a kidney with reflux nephropathy and pyelonephritis (Figure 4), MAb 2D10G9 recognized a broad band with an approximate molecular mass of 220 kd in papilla, outer medulla, and cortex, respectively (Figure 4, lanes 2 to 4); staining of the inner medulla of the same kidney biopsy with MAb 2D10G9 revealed a less pronounced immunoreactive band (Figure 4, lane 1) with a somewhat lower molecular mass (approximately 200 kd).

However, MAb 2D10G9 recognized several additional, unidentified proteins that were present in homogenates of diseased human kidney. In some tissue samples, a broad band with an apparent molecular mass slightly higher than 500 kd, together with a band in the stacking gel was observed. However, the latter protein bands were not recognized by anti-human apolipoprotein-B-100 antibodies. When the same kidney samples (as shown in Figures 3 and 4) were stained with MAb 1B10A11, an antibody specifically recognizing HOCl-LDL but not other HOCl-modified proteins,[33] no significant staining in diseased kidney tissue samples was observed. This suggests that HOCl-modified proteins other than HOCl-LDL are likely formed during development of sclerosis.

The heterogeneity in immunoreactive bands was probably due to cross-linked HOCl-modified proteins. In some tissue samples, a diffuse smear indicated the presence of HOCl-modified proteins in diseased human kidney. Some bands, stained with MAb 2D10G9, were also recognized by anti-human albumin antibodies, which suggested that part of the HOCl-modified proteins could be HOCl-modified albumin.

Competition experiments with MAb 2D10G9 preabsorbed with HOCl-HSA at a molar ratio of 1:20 prevented antibody binding, demonstrating

that staining was specific for HOCl-modified proteins.

Identification of MPO in Human Kidney

Immunohistochemical Findings

The presence of MPO in human kidney was considered to be a prerequisite for the generation of HOCl-modified proteins. Therefore, a polyclonal antibody was used to localize immunoreactive MPO protein in paraffin-embedded tissue sections prepared from those formaldehyde-fixed renal tissues that were taken for immunohistochemical detection of HOCl-modified protein. Immunohistology in kidney biopsies with interstitial nephritis revealed extensive staining for MPO in inflammatory cells, which suggested large amounts of MPO present in human leukocytes (Figure 5A). In tubular epithelial cells, immunohistochemical staining for MPO was faint and mostly diffuse in the cytoplasm of the cells; sometimes a granular cytoplasmic label could be demonstrated (Figure 5A). This immunochemical staining, however, could clearly be differentiated from background staining when nonspecific rabbit IgG instead of anti-human MPO IgG was used as a primary antibody (data not shown). In kidney biopsies obtained from patients with nephrosclerosis, some mononuclear cells in the interstitium and cells present in the media and adventitia of arteries were stained by anti-human MPO IgG (Figure 5B).

Western Blotting Experiments

To verify the presence of MPO protein in human renal tissue, frozen tissue sample homogenates (the same as shown in Figure 3) were used for Western blot analysis. After detergent-soluble proteins were subjected to SDS-PAGE, immunoblotting with the rabbit antibody revealed an immunoreactive protein of approximately about 58 kd that co-migrated with authentic MPO (Figure 6, lane 5). All of the different kidney specimens examined by Western blotting, including controls (Figure 5, lane 1) and diseased renal tissues (Figure 5, lanes 2 to 4), contained active MPO to serve as a catalyst for hypochlorite modification of proteins. The intensity of immunoreactive 58-kd MPO protein in tissues from interstitial nephritis and reflux nephropathy/pyelonephritis (Figure 5, lanes 2 and 3) was much more pronounced (5- to 10-fold) in comparison with controls. Densitometric evaluation of the immunoreactive 58-kd MPO band revealed similar intensities in tissues obtained from controls and patients with nephrosclerosis (Figure 5, lanes 1 and 5). Omission of the primary antibody or the use of nonimmune rabbit IgG as a primary antibody resulted in a lack of detectable stain in detergent-soluble protein homogenates.

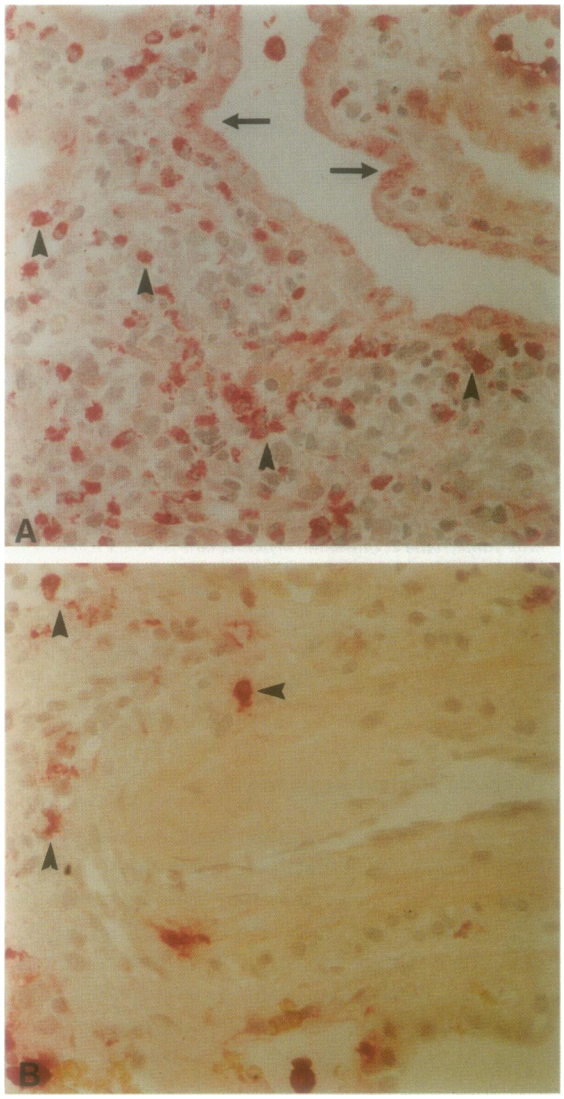


Figure 5. *Immunohistological detection of human MPO in interstitial nephritis and nephrosclerosis. Tissue sections from diseased human kidney were incubated with polyclonal anti-human MPO as a primary antibody (for detailed incubation conditions see Materials and Methods). Staining was performed by the APAAP method.* **S:** *Leukocytes were strongly positive* (**arrowheads**); *also, in tubular epithelia a faint, sometimes granular cytoplasmic label could be shown* (**arrows**) *that could clearly be differentiated from background staining in control sections with substitution of the rabbit anti-human MPO antibody by nonspecific rabbit IgG.* **B:** *In kidney with nephrosclerosis, some interstitial mononuclear cells surrounding arteries with pronounced subintimal fibrosis and cells in media arteries demonstrated a positive staining for MPO* (**arrowheads**).

Subsequently, immunoblot analysis of MPO was performed in different anatomic areas of the kidney. Specimens obtained from the patient with reflux nephropathy and pyelonephritis (shown in Figure 4) revealed the 58-kd MPO protein present in medulla

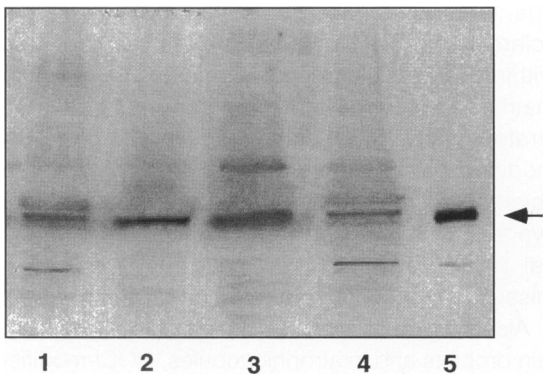


Figure 6. *Reactivity of polyclonal anti-human MPO IgG with renal tissues as analyzed by Western blotting. Antigens (6 mg of total protein kidney homogenate* (lanes 1 *to* 4) *and 1.2 μg of MPO-enriched protein fraction* (lane 5) *were separated on 3.75 to 12.5% nonlinear polyacrylamide gels under denaturating conditions. Proteins were transferred to nitrocellulose, detected using rabbit anti-human MPO, and visualized with goat anti-rabbit IgGs with ECL-Western blotting detection system. The antigens used were as follows:* lane 1, *control kidney;* lane 2, *interstitial nephritis;* lane 3, *reflux nephropathy and pyelonephritis;* lane 4, *nephrosclerosis; and* lane 5, *MPO-enriched protein fraction isolated from human neutrophil granulocytes). The* arrow *indicates the position of immunoreactive MPO (56 kd).*

(inner and outer), cortex, and papilla at similar intensities (data not shown).

Discussion

A proposed analogy between atherosclerosis and glomerulosclerosis suggests that factors that contribute to the development of atherosclerosis, ie, oxidatively modified (lipo)proteins, may also participate in glomerular injury.[14,15,17,36,38]

LDLs can be taken up by glomerular epithelial cells[39] and mesangial cells[40,41] and may induce mesangial cell proliferation and production of macrophage chemotactic factors.[42] Macrophages and perhaps mesangial cells oxidize LDL,[43] thereby leading to the formation of cholesterol-enriched foam cells *via* uptake of modified LDL.[44,45] In animal experiments, oxidized lipoproteins have been demonstrated in the glomerulus of rats with focal and segmental glomerulosclerosis.[17] High concentrations of autoantibodies against oxidatively modified HSA or LDL, eg, ox-LDL and MDA-LDL, in patients with chronic renal failure undergoing hemodialysis[46] support the occurrence of enhanced *in vivo* oxidation of LDL. An enhanced *in vitro* oxidation of LDL in uremic patients[47] or patients with renal failure or renal transplants[48] also suggested that oxidatively modified lipoproteins may play a role in the progression of atherosclerosis and renal disease.

Despite much interest in this area of research, little progress has been made in the identification of the definitive *in vivo* oxidant. HOCl is a potent oxidant that predominantly modifies reactive protein groups.[30] However, LDL when exposed to MPO-generated tyrosyl radicals may undergo also lipid peroxidation, a process that is independent of free metal ions.[49] Enzymatically active MPO, which colocalizes with tissue monocyte-derived macrophages, has been identified as a component of human atherosclerotic plaques.[50] Subsequently, the presence of hypochlorite-modified (lipo)proteins in human atherosclerotic lesions has been undoubtedly demonstrated.[32]

A series of experimental models, eg, ischemic acute renal failure, renal graft rejection, acute glomerulonephritis, and toxic renal diseases implied that ROS in general are pathogenetic factors in the development of renal diseases. ROS may degrade the glomerular basement membrane and alter glomerular and tubular cell function.[51–53] Other potential mechanisms of ROS-mediated renal injury include increase of glomerular permeability to proteins, alterations in glomerular hemodynamics, and formation of chemotactic and cytotoxic substances, thereby enhancing the inflammatory and profibrotic state within the injured glomerulus. ROS can be released from either isolated glomeruli or resident glomerular cells (eg, mesangial cells); however, the main source for ROS synthesis in renal tissues *in vivo* seems to be infiltrating polymorphonuclear neutrophilic leukocytes and monocytes. With that respect, one important question is the cellular origin of MPO, the enzyme responsible for HOCl formation in the presence of ROS. MPO represents up to 5% of neutrophil protein, although the enzyme is less abundant in monocytes but still represents ~1% of total protein.[50]

The renal effects of the radical substance HOCl have also been investigated in animal experiments. Previous studies suggested that H_2O_2 can cause glomerular injury by reaction with halides in the presence of MPO to form oxidants, ie, HOCl, which can oxidize and halogenate tissues.[22–25] We have obtained several lines of evidence that the MPO-H_2O_2-Cl^- system is functional *in vivo* in human kidney. First, HOCl-modified proteins were recognized by MAb 2D10G9, a specific MAb for identification of HOCl-modified proteins. Immunoreactive HOCl-modified epitopes were detected in renal tissues obtained from patients suffering from acute or chronic kidney disease by immunostaining and immunoblot analysis. Apparently due to cross-linking of modified proteins, the nature of HOCl-modified proteins detected in human kidney could not be definitively analyzed. HOCl-modified LDL or HSA effec-

tively competed for antibody binding to the vascular structure and tubulointerstitium of human kidney. Second, MPO, the relevant enzyme for HOCl generation, was demonstrated in renal tissue. The 56-kd MPO protein that was immunoreactive with rabbit polyclonal antibodies for MPO was present in detergent extracts (Figure 6) and detected by immunocytochemistry in all human kidney biopsies examined (Figure 5); the apparent molecular mass of the immunoreactive protein in different kidney tissues and the MPO present in neutrophils was indistinguishable by Western blotting (Figure 6). The measurement of MPO as a marker for quantification of infiltrated inflammatory cells has proven to be of value in different tissues.[54–56] Third, our present findings further demonstrate that in renal tissue the expression of immunoreactive HOCl-modified proteins is apparently linked to the extent of expression of immunoreactive MPO.

The most pronounced staining of immunoreactive MPO and HOCl-modified proteins was found for kidneys obtained from patients with pronounced interstitial inflammatory infiltrates (interstitial nephritis and pyelonephritis/reflux nephropathy). Interestingly HOCl-modified proteins were found in inflammatory as well as in degenerative diseases, although generation of HOCl catalyzed by MPO was primarily expected at sites of inflammatory cell infiltration by MPO-rich neutrophilic leukocytes. However, also in rats, very small quantities of MPO were localized in the glomerular capillary wall without demonstrable injury, and subsequent exposure to H_2O_2 resulted in severe glomerular damage associated with halogenation of glomerular basement membrane.[22] These observations corroborate the present findings of MPO in normal kidney and in kidneys with nephrosclerotic vascular damage (Figure 5).

In kidneys with nephrosclerosis, immunohistological evidence for HOCl-modified epitopes was obtained in glomeruli. In glomeruli, a weak staining pattern for HOCl-modified proteins was observed in mesangium and in podocytes. In labeled mesangial areas it could not be definitely ascertained whether HOCl proteins were located in the extracellular matrix or situated in mesangial cells. It is not known whether HOCl-modified proteins were produced by podocytes or only taken up by them. The glomerular HOCl-modified proteins could be synthesized during the degeneration of glomeruli and thus be a byproduct, not pathogenetically important for glomerular sclerosis. On the other hand, it might be speculated that HOCl-modified proteins may directly cause glomerular cell damage. In fully developed scars of global glomerulosclerosis, no HOCl-modified protein was detected within the acellular densely packed extracellular matrix. Although interstitial foam cells demonstrated a focally accentuated positivity for HOCl-modified proteins, foam cells within a glomerular sclerosis were only very weakly positive or negative for HOCl-modified protein. The apparently focal positivity within foam cells may have been missed in the sections of glomerular foam cells.

Also, in proximal tubules within cytoplasmic protein droplets and in atrophic tubules, HOCl-modified proteins were seen by immunohistology. Cellular changes leading to atrophy may cause the generation of HOCl; the generation of ROS is believed to be a general phenomenon in aging contributing to atrophy and cellular death.[56–59] Tubules positive for HOCl-modified protein were surrounded by MPO-positive cells and sometimes even demonstrated MPO label in tubular epithelia.

From our present findings it cannot be precisely concluded whether HOCl-modified proteins may actively participate in the degeneration of tubules, as biopsies do not lend themselves to interpretation of dynamic processes. There is evidence that tubular epithelial cells may participate in the pathogenesis of renal injury *via* formation of ROS. In primary cultures of rabbit proximal tubule, cortical collecting duct, and papillary collecting duct, both a time- and concentration-dependent synthesis of superoxide anion and H_2O_2 in response to different stimuli has been measured.[60] HOCl-modified proteins synthesized in glomeruli by phagocytes could also be excreted into primary urine and taken up by tubular epithelial cells. These oxidized/modified proteins could be cytotoxic to tubular epithelia, as demonstrated for oxidized lipoproteins in cultured tubular epithelial cells,[61] and thus may actively participate in the irreversible damage to tubular segments.

From our studies it may be concluded that HOCl, a naturally occurring powerful oxidant formed *via* the MPO-H_2O_2-halide system *in vivo*, causes modification to proteins in human kidney disease. This process might be a leading event in glomerular and tubulointerstitial injury. From our findings we may conclude that HOCl-modified proteins take part in the degenerative scarring process of glomeruli, vessels, and tubulointerstitium.

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