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Antiproteinuric Therapy while Preventing the Abnormal Protein Traffic in Proximal Tubule Abrogates Protein- and Complement-Dependent Interstitial Inflammation in Experimental Renal Disease

MAURO ABBATE,* CARLA ZOJA,* DANIELA ROTTOLI,* DANIELA CORNA,* NORBERTO PERICO,*† TULLIO BERTANI,*† and GIUSEPPE REMUZZI*†

**Mario Negri Institute for Pharmacological Research, and †Unit of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Bergamo, Italy.*

Abstract. In proteinuric glomerulopathies, the excess traffic of proteins into the renal tubule is a candidate trigger of interstitial inflammatory and immune events leading to progressive injury, and a key target for the renoprotective action of antiproteinuric drugs. Among proteins trafficked to the proximal tubule, the third component of complement (C3) can be activated locally and contribute to inflammation at sites of protein reabsorption. Experiments were performed in rats with renal mass reduction (RMR, 5/6 nephrectomy) with the following aims: (*1*) to study Ig (IgG) and complement deposition in proximal tubules, and interstitial macrophage infiltration and MHC class II expression at intervals after surgery by double immunofluorescence analysis; (*2*) to assess whether lisinopril (angiotensin-converting enzyme inhibitor [ACEi], 25 mg/L in the drinking water, from either day 1 or day 7) limited IgG and C3 accumulation and interstitial inflammation at day 30. In 7-d remnant kidneys, intracellular staining for both IgG and C3 was detectable in proximal tubules in focal areas; C3 was restricted to IgG-positive tubular cells, and there were no interstitial ED-1 macrophage and MHC II-positive cellular infiltrates. In 14-d and 30-d remnant kidneys, proximal tubular IgG and C3 staining was associated with the appearance of interstitial infiltrates that preferentially localized to areas of tubules positive for both proteins. RMR rats given ACEi had no or limited increases in levels of urinary protein excretion, tubular IgG, and C3 reactivity, and interstitial cellular infiltrates in kidneys at 30 d, even when ACEi was started from day 7 after surgery. These findings document that (*1*) in RMR, IgG and C3 accumulation in proximal tubular cells is followed by leukocyte infiltration and MHC II overexpression in the adjacent interstitium; (*2*) ACEi while preventing proteinuria limits both tubular accumulation of IgG and C3 and interstitial inflammation. The data suggest that ACE inhibition can be renoprotective by limiting the early abnormal protein traffic in proximal tubule and consequent deleterious effects of excess protein reabsorption, including the accumulation and local activation of complement as well as the induction of chemokines and endothelin genes known to promote interstitial inflammation and fibrosis.

Proteinuric nephropathies can progress to end-stage renal failure independently of the type of initial insult(s). The results of the most recent studies in this field indicate that proteinuria, commonly taken as a marker of severity, is in fact a major if not the best independent predictor of progression of disease (1,2). Years of basic research on common pathways of injury have also led us to consider the underlying ultrafiltration of proteins into the tubule as a likely trigger of cellular events ultimately responsible for irreversible lesions and decline of function (3). In support of this possibility, together with data of correlations of proteinuria and renal histopathology in humans (4–8), are the results of studies in rat models showing that the disruption of glomerular permselectivity to protein precedes chronic parenchymal damage and that inhibitors of angiotensin-converting enzyme while preventing proteinuria ameliorated renal structural and functional injury (9–15).

Studies to identify cellular processes by which the abnormal trafficking of proteins into the tubule may contribute to the progression of disease have focused on tubulointerstitial lesions, which along with proteinuria well correlated with faster decline of renal function (4,7,16–19). Findings that both proteinuria and protein accumulation in the tubulointerstitium were associated with interstitial inflammation in Adriamycin nephrosis or aging rats (20,21) and that the same effects together were elicited by systemic injections of large amounts of albumin (22) suggested that the increased protein delivery to the tubule may promote recruitment of inflammatory cells into the interstitium. Furthermore, proximal tubular cells cultured *in vitro* were induced by exposure to excess protein to upregulate

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Correspondence to Dr. Mauro Abbate, "Mario Negri" Institute for Pharmacological Research, Via Gavazzeni 11, 24125 Bergamo, Italy. Phone: + 39 (35) 319.888; Fax: + 39 (35) 319.331; E-mail: abbate@irfmn.mnegri.it
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and release chemokines (23,24) and endothelin (25) into the basolateral medium, a polarized type of secretion that *in vivo* would act in turn to promote monocyte and lymphocyte recruitment into interstitium and synthesis of extracellular matrix components by fibroblasts. That this may occur in the initial phase of injury is indicated by findings that in kidneys of rats after 5/6 renal mass ablation, albumin and immunoglobulins accumulate in proximal tubular cells early after surgery, and in the tubular regions preferentially surrounded by inflammatory infiltrates into adjacent interstitium thereafter (26). On the whole, these data suggest that excess protein reabsorption in proximal tubular cells activates pathways of interstitial inflammation and fibrosis in the long term.

Among secondary processes leading to interstitial damage in proteinuric conditions, the activation of complement proteins in the proximal tubule has proinflammatory potential and thus a major role to play (27,28). Complement deposits (C3, C5b-9) were found along the luminal side and in proximal tubular cells in kidneys of proteinuric patients (29), as well as in rat kidneys in which the disease was induced by the primarily nonimmune insults, protein overload, and renal mass ablation (7,22). In rats with aminonucleoside nephrosis, complement is pathogenic to the extent that the treatment with soluble complement receptor presumably by inhibiting C3 activation in proximal tubule attenuated interstitial lesions (27). In addition to activating exogenous complement (30), proximal tubular cells can remarkably synthesize C3 (31,32). Both functional properties are in the same pathway to possibly mediate interstitial inflammation in response to protein load.

If the traffic of circulating proteins and the accumulation of complement components in proximal tubular cells are both linked together to subsequent interstitial injury, then drugs known to limit the abnormal passage of proteins into the tubule should also limit both protein accumulation in proximal tubular cells and interstitial inflammation. The ultimate aim of the present study was to test this hypothesis in a primarily nonimmune model. We performed an immunohistochemical analysis of the remnant kidney after 5/6 renal mass reduction in the rat, with the following specific purposes: (*1*) to assess whether complement (C3) can be detected in proximal tubular cells in regions of ultrafiltered protein (IgG) accumulation, (*2*) to compare the localization of C3 and IgG in proximal tubules with those of monocyte/macrophages and other infiltrating cells bearing MHC II expression into the interstitium, and (*3*) to establish whether treatment with the antiproteinuric drug lisinopril limits the accumulation of ultrafiltered proteins and C3 in proximal tubules and early interstitial inflammation in this model.

Materials and Methods

Animals

Male Sprague Dawley, CD-COBS rats of 240 to 260 g initial body weight were obtained from Charles River SpA (Calco, Italy). Animal care and treatment were conducted in accordance with institutional guidelines that are in compliance with national (D.L. no.116, G.U., suppl 40, 18 Febbraio 1992, Circolare No 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All animals were housed in a constant temperature room with a 12-h dark 12-h light cycle and fed a standard diet.

Disease Model and Experimental Design

Renal mass ablation was performed by surgical removal of right kidney and ligation of two or three branches of the left renal artery (33). Age-matched rats were used as controls after sham operation, consisting of a laparotomy and manipulation of renal pedicles only. Three groups of rats with renal mass reduction (groups I, II, and III) were sacrificed at 7, 14, and 30 d after surgery, respectively; sham-operated controls (group IV) were sacrificed at day 30. To assess the effects of angiotensin-converting enzyme inhibitor, rats with renal mass reduction received lisinopril (25 mg/L in the drinking water) starting from either day 1 (group V) or day 7 (group VI) after surgery and were sacrificed at day 30 ($n = 4$ each group). Lisinopril at this dose in separate experiments was found to cause significant reduction in systolic BP but not hypotension in rats after 5/6 renal mass ablation (34). Twenty-four hour urine samples were collected in metabolic cages both before the time of disease induction and at sacrifice for determination of urinary protein. Proteinuria was determined by the modified Coomassie blue G dye-binding assay for proteins with bovine serum albumin (BSA) as standard (35).

Tissue Preparation

At sacrifice, the animals were anesthetized with sodium pentobarbital solution intraperitoneally (0.1 ml/100 g body wt of a 65 mg/ml solution), and the kidneys were fixed by perfusion via abdominal aorta (36). Kidneys were first perfused with Hanks' solution for 5 min and then with periodate-lysine paraformaldehyde fixative (37) for 10 min, followed by overnight immersion in the same fixative at 4°C. The fragments from remnant kidneys were taken from the center of non-infarcted areas. Fixed tissue specimens were extensively washed with phosphate-buffered saline (PBS) (0.9% NaCl in 10 mM sodium phosphate buffer, pH 7.4) and stored in the same buffer.

Immunohistochemistry

Tissue specimens were immersed in 30% sucrose/PBS for at least 1 h at room temperature, embedded in OCT medium, and frozen in liquid nitrogen. Tissue sections (5 μm thick) were cut using a Mikrom 500 O cryostat (Mikrom, Walldorf, Germany) and either stained immediately or stored at −20°C until further processing. Nonspecific binding of antibodies was blocked with PBS/1% BSA for 15 min (room temperature). The sections were incubated for direct immunofluorescence with FITC-conjugated goat anti-rat IgG (Jackson; 30 μg/ml in PBS), FITC goat anti-rat C3 (whole C3) (Cappel, Cochranville, NC; 20 μg/ml), or Texas Red-anti-rat IgM (Jackson, 30 μg/ml) for 1 h at room temperature. After three washes in PBS, slides were mounted using 100 mM Tris-HCl:glycerol 50:50, 2% N-propyl gallate, pH 8. Sections were examined with a Leika DM-R microscope equipped with epifluorescence and appropriate filters.

Mouse monoclonal antibodies against a cytoplasmic antigen present in rat monocytes and macrophages (ED1) (Serotec, Oxford, United Kingdom) (38) or against rat MHC class II antigen (MHC II) monomorphic determinant (OX-6) (Sera-Lab Ltd, Crawley Down, Sussex, United Kingdom) (39) were used for the localization of infiltrating cells and MHC II expression. Tissue sections were blocked with PBS/1% BSA, incubated overnight at 4°C with primary antibody (ED1, 10 μg/ml; OX-6, 10 μg/ml), washed with PBS, and then incubated with Cy3-conjugated donkey anti-mouse IgG antibodies

(affinity-purified, absorbed with rat IgG, Jackson ImmunoResearch Laboratories, West Grove, PA; 5 μg/ml in PBS) for 1 h at room temperature. Double immunofluorescence labeling was performed by the following sequence: mouse monoclonal (ED1, OX-6), Cy3-anti-mouse IgG, and FITC-anti-rat IgG or FITC-anti-rat C3. In a separate set of incubations on sections of normal kidneys and 30-d remnant kidneys, monoclonal antibody to RECA-1 antigen present on rat endothelial cells (HIS52, Serotec, Kidlington, United Kingdom; cell supernatant, diluted 1:5) was used to assess the relationship between sites of linear peripheral C3 staining with peritubular capillary structures. Primary antibodies were omitted in control experiments. Absence of cross-reactions between antibodies was further confirmed by comparing individual patterns on adjacent sections or after inversion of the incubation sequences.

Evaluation of Tubular Staining and Comparison with the Distribution of MHC II-Positive Cells and ED-1 Macrophages

Semiquantitative analysis of proximal tubular IgG or C3 staining compared with cells detected by OX-6 antibody in the same areas was performed by two independent investigators by examining randomly selected fields of view (×400) of cortical areas in double-stained sections (26), using the appropriate filters for FITC and Cy3 fluorescence staining, respectively. The total number of proximal tubules and the number of proximal tubules positive for intracellular IgG or C3 staining were counted in each field (mean number of fields for each animal, 15), and the numbers of OX-6-positive cells in the interstitium were counted in the same fields. Infiltrating ED-1-positive cells were detectable at the sites of OX-6 staining as shown previously in this model (26) and were counted in adjacent sections.

Statistical Analyses

The results are expressed as mean ± SD. Data were analyzed by ANOVA and multiple comparisons. Statistical significance was defined as $P < 0.05$.

Results

Localization of Complement C3 and Comparison with Interstitial Sites of Enhanced MHC Class II Antigen Expression and ED-1 Macrophage Infiltration

The patterns of C3 staining at intervals after 5/6 renal mass ablation are illustrated in Figures 1 and 2, and the results of semiquantitative analysis are represented in Figure 3A. In the cortex of control kidneys, C3 reactivity was confined to a linear interrupted staining at the periphery of proximal tubules and some distal segments. No intracellular C3 was detectable in proximal tubular epithelial cells (Figure 1A). In contrast to control kidneys, intracellular C3 became detectable in remnant kidneys along with IgG in increasing numbers of proximal tubular profiles at each time point after surgery, and this was paralleled by increasingly high levels of urinary protein excretion (mg/d: 7 d, 45.9 ± 24.8; 14 d, 143.4 ± 53.5; 30 d, 218.8 ± 65.9; sham-operated controls, 21.7 ± 2.2). At 7 d, granular C3 staining in the cytoplasm of proximal tubular epithelial cells was weak (Figure 1B), and brush border deposits were found in some tubules. At 14 and 30 d after surgery (Figure 1, C and D), the percentage of proximal tubules positive for C3 and IgG staining further increased ($P < 0.05$ at both time points compared to 7 d). Tubular casts and cell debris also stained for C3 and IgG. The staining for C3 in tubules displayed variable intensity and localization to intracellular sites, brush borders, and cell debris. In addition, there were heterogeneous changes in the linear C3 reactivity at the periphery of the tubules, with partial loss or circumferential staining.

The comparison of intracellular C3 and IgG staining on adjacent sections revealed colocalization to the proximal tubular profiles and overall comparable levels of intensity (Figure 2, A and B). A minor percentage of tubules showing weak or irregular intracellular IgG staining had no C3 reactivity (mean percentage of tubules stained for both IgG and C3: 7 d, 94.9%; 14 d, 95.2%; 30 d, 85.8%). Besides the localization to intracellular sites consistent with subapical and lysosomal compartments that was common to both proteins, an additional finding at high magnification in the sections stained for C3 was the presence of granular or diffuse cytoplasmic staining in the basal region of some proximal tubular cells (Figure 2C). The linear C3 staining along proximal tubules had apparent basement membrane distribution that was not associated with peritubular capillary structures as revealed by double staining for C3 (Figure 2C) and RECA-1 antigen (Figure 2D). In control experiments, sections of remnant kidneys did not exhibit any tubular staining for rat IgM (not shown).

The time course of interstitial infiltration of MHC II-positive cells, as detected by OX-6 antibody, and of ED-1-positive monocytes/macrophages is given in Figure 3B. There were significant increases both in the numbers of OX-6-positive cells and ED-1 monocyte/macrophages in 14-d and 30-d remnant kidneys compared with sham-operated rats. Focal ED-1 macrophage infiltrates were found in the peritubular cortical interstitium in all of the rats at these time points, and they clearly localized to the interstitial areas of high MHC II expression by comparison on adjacent sections. Interstitial cellular infiltrates were also recognized both by OX-6 and ED-1 antibodies in perivascular sites and at the vascular pole of glomeruli.

To compare the distribution of the tubular sites positive for C3 with the localization of MHC II and ED-1-positive cellular infiltrates in peritubular interstitium, sections of remnant kidneys were double-immunostained and analyzed for simultaneous detection using secondary antibodies conjugated with FITC or Cy3 (green and red staining, respectively) (Figure 4). As documented previously for comparison with IgG (26), a strict spatial relationship was found between the regions of C3 accumulation in proximal tubules and the interstitial sites of inflammatory cell infiltration both in the 14-d (Figure 4C; compare with control kidney in Panel A and with 7-d remnant kidney in Panel B) and 30-d remnant cortex (Figure 4E). Infiltrates of MHC II-positive cells and ED-1 macrophages were less dense or absent in other peritubular regions. The comparison of the distribution of IgG staining and ED-1 infiltrating cells (Figure 4D) confirmed the same type of association as found for C3 and MHC II in adjacent sections (Figure 4C). Given the heterogeneous intracellular and/or brush border patterns of C3 within sites of positive staining, it was not possible to establish whether preferential association occurred

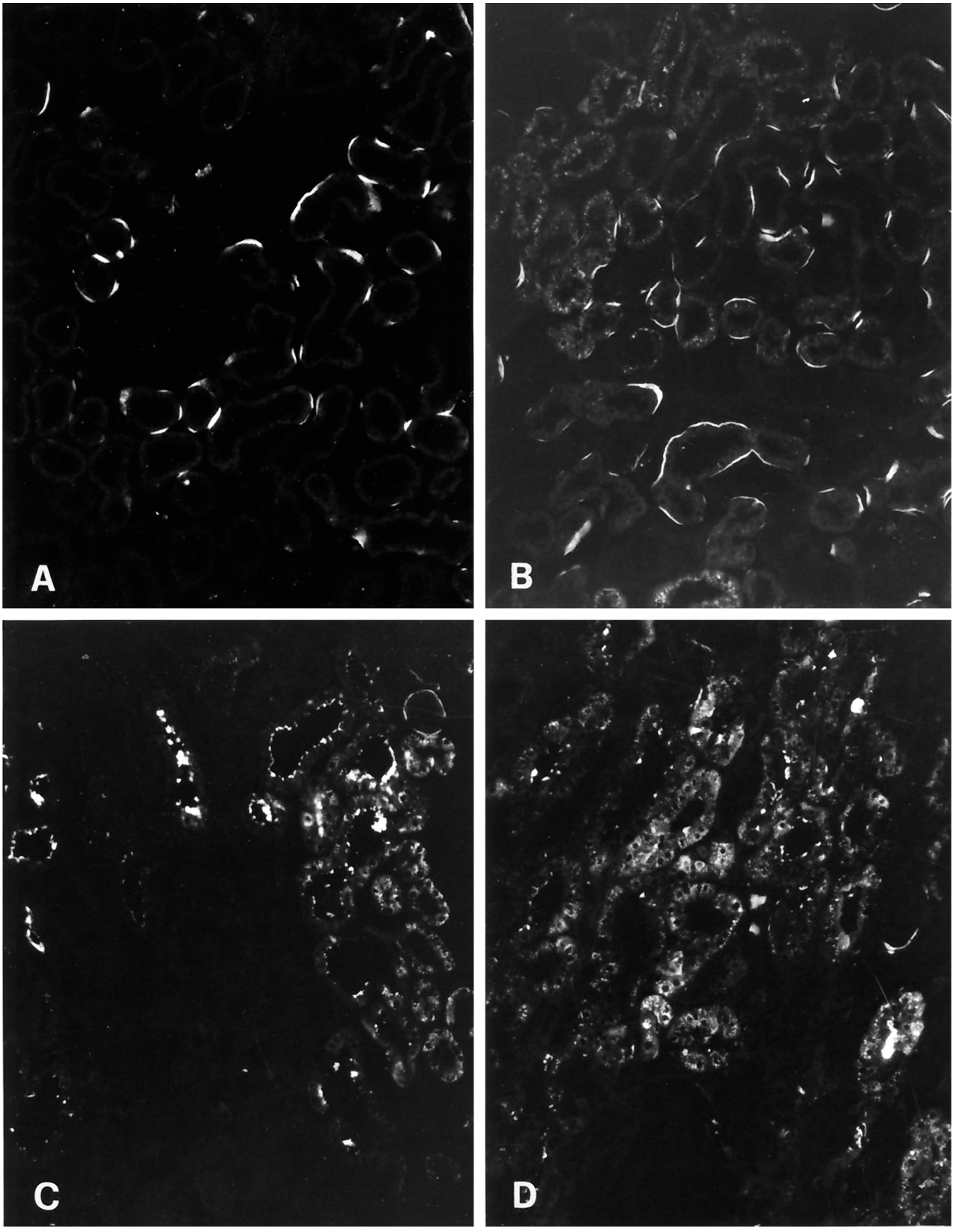

Figure 1. Immunofluorescence analysis of C3 in cryostat sections of normal rat kidney (A) and remnant kidneys after 5/6 renal mass ablation (B through D). (A) Photomicrograph of normal kidney cortex showing interrupted linear C3 reactivity at the periphery of tubules. Note the absence of intracellular staining. (B) In contrast, an area of a 7-d remnant kidney displays intracellular staining in proximal tubular epithelial cells in addition to the peripheral pattern. In areas of remnant kidneys at 14-d (C) or 30-d (D) after surgery, C3 staining is more diffuse in proximal tubules and detectable in brush borders. The linear peripheral staining of most tubules was attenuated or lost in these areas. The kidneys were fixed by perfusion with periodate-lysine paraformaldehyde, and the sections were stained with FITC-conjugated goat anti-rat C3. Magnification, ×250.

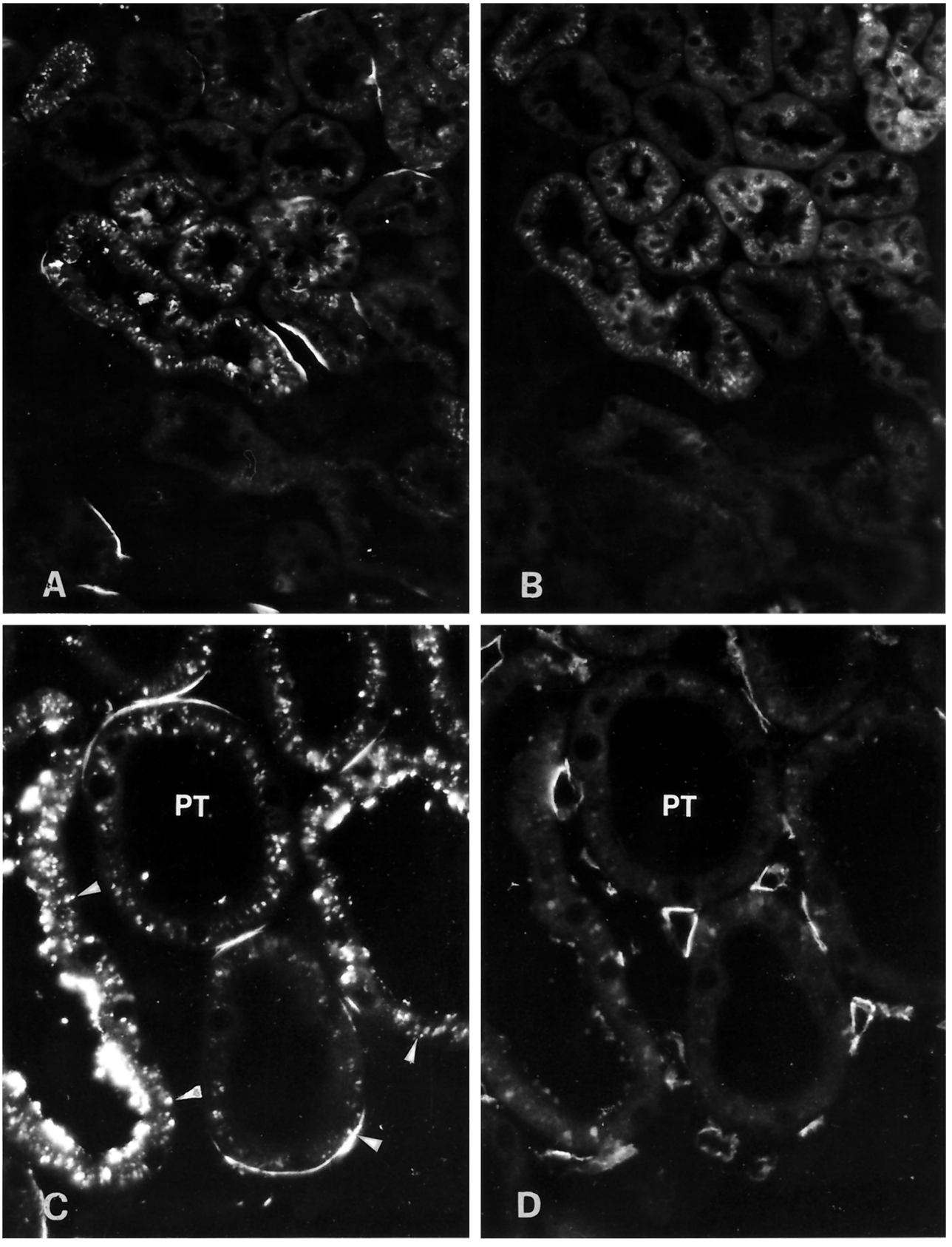

Figure 2. Comparison of C3 and IgG staining in adjacent sections of cortex of a 30-d remnant kidney (A and B), and relationship of C3 peripheral staining with peritubular capillary structures (C and D). Both C3 (A) and IgG (B) are stained in tubular epithelial cells of the same proximal tubules showing prevalent apical or irregular intracellular distribution. (C and D) Linear C3 reactivity at the periphery of tubular profiles is strictly associated with the interstitial aspect of the tubules, and it is not localized to peritubular capillaries in a section that was double-stained for C3 (C) and RECA-1 endothelial cell antigen (D). PT, proximal tubule. Arrowheads in C point to granular staining for C3 in the basal region of the cells. Magnification: ×310 in A and B; ×800 in C and D.

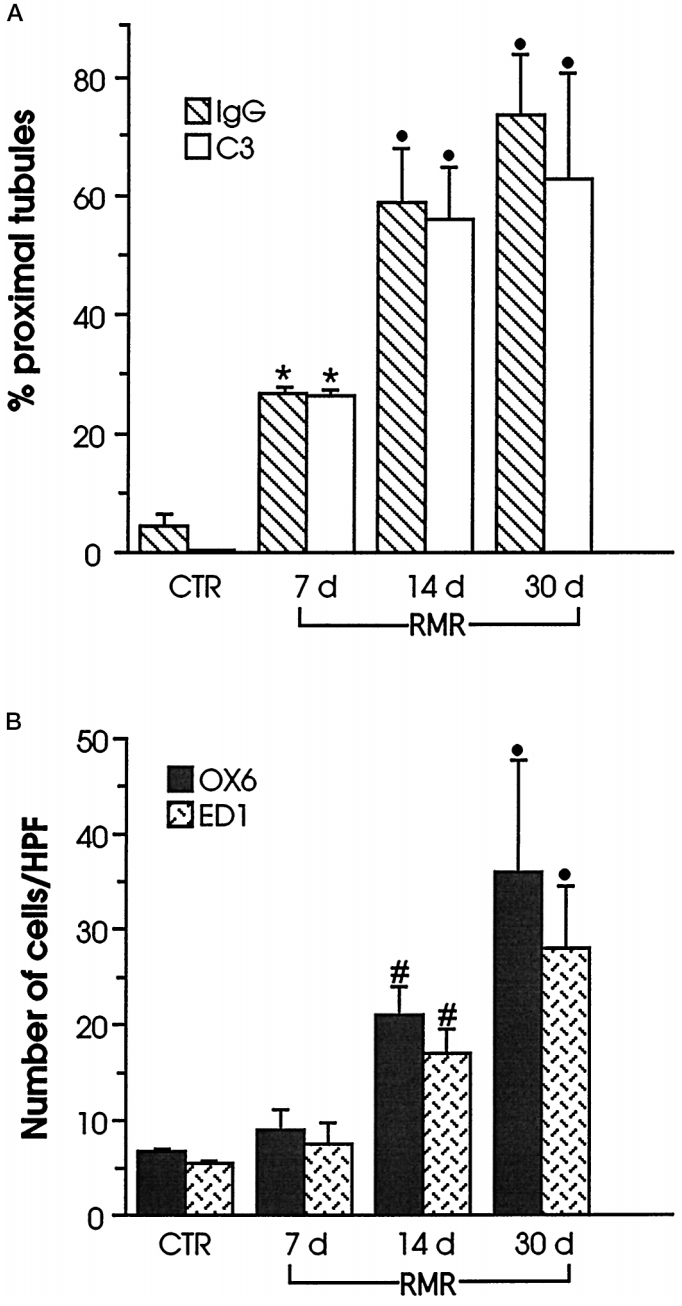

Figure 3. Time course of IgG and C3 accumulation in proximal tubules (A) and interstitial cellular infiltration (B) in 5/6 remnant kidneys. OX-6, MHC II-positive cells; ED-1, monocyte/macrophages; HPF, high-power (×400) field of view; CTR, sham-operated control rats; RMR, rats with 5/6 remnant kidney. $^{*}P < 0.01$ *versus* CTR, $^{\bullet}P < 0.01$ *versus* CTR and 7-d RMR; $^{\#}P < 0.05$ *versus* CTR and 7-d RMR.

with either one of these patterns, although this was not apparent.

Effects of Lisinopril on C3 and IgG Tubular Cell Accumulation and Interstitial Inflammatory Cell Infiltration

As shown in Table 1, rats given lisinopril from either day 1 or day 7 after surgery had levels of urinary protein excretion at 30 d comparable to sham-operated controls, in contrast to the high levels found in the untreated rats with 5/6 remnant kidneys at this time. This effect was associated with limited increases in the percentage of proximal tubular profiles showing positive staining for IgG and C3 to levels that were significantly lower in the 5/6 remnant kidneys of rats treated with lisinopril compared with untreated rats with 5/6 remnant kidneys at this time. Thus, the patterns of staining were comparable to those of sham-operated controls in 50% of rats treated with lisinopril from day 1 (Figure 4F, compare with 5/6 remnant kidney of untreated rat in Figure 4E), as well as in one of the rats treated from day 7. In the other kidneys, abnormal staining for IgG and C3 was present in variable percentages of tubules and it was overall similar to remnant kidneys at 7 d after surgery as described in the above section. In the rats treated with lisinopril, numbers of MHC II-positive cells and ED-1 monocytes/macrophages in remnant peritubular interstitium at 30 d were also significantly lower than in remnant kidneys of untreated rats, and they were reduced to control values in the rats given lisinopril from day 1 after surgery.

Discussion

The results of the first set of experiments in the present study show that in rats with proteinuria of glomerular origin due to 5/6 renal mass ablation, intracellular C3 staining was detectable in proximal tubules in focal areas as early as 7 d after surgery and was still restricted to proximal tubules at subsequent time points. At variance, in normal kidneys C3 reactivity was confined to a linear interrupted staining at the periphery of tubules and not detectable within the cells. That the abnormal C3 staining in proximal tubules of remnant kidneys was associated with ultrafiltered protein was indicated by evidence of C3 and IgG colocalization to the same tubules in adjacent sections. This was also supported in the same experimental model by detection of protein reabsorption droplets in remnant glomeruli (26), reflecting early events of altered glomerular protein traffic. Thus, the combined pattern is consistent with tubular accumulation of ultrafiltered complement as suggested by studies of other rat models of proteinuric nephropathy. Deposition of C3 in proximal tubules was found in rats with protein overload proteinuria induced by the parenteral administration of heterologous albumin, that develop acute tubulointerstitial nephritis (22). Similarly, in rats with aminonucleoside nephrosis, complement components C3 and C5b-9 were found on the luminal side of proximal tubular cells as early as 7 d after injection of puromycin aminonucleoside (27). Recently, deposition of rat C3 and C5b-9 was also observed at the luminal surface and within absorption droplets of proximal tubular cells in the kidneys of proteinuric rats with mesangial proliferative glomerulonephritis (28).

C3 is an essential component of both the classical and alternative pathways of complement activation. In normal kidney, small amounts of C3 are activated by the cleavage of its internal thiolester bond by the reaction with ammonia (40). The activated C3 can bind to factor B, which is cleaved by factor D to form C3 convertase. Although C3 convertase has the ability to cleave the C3 molecule into C3a and C3b, further reaction is

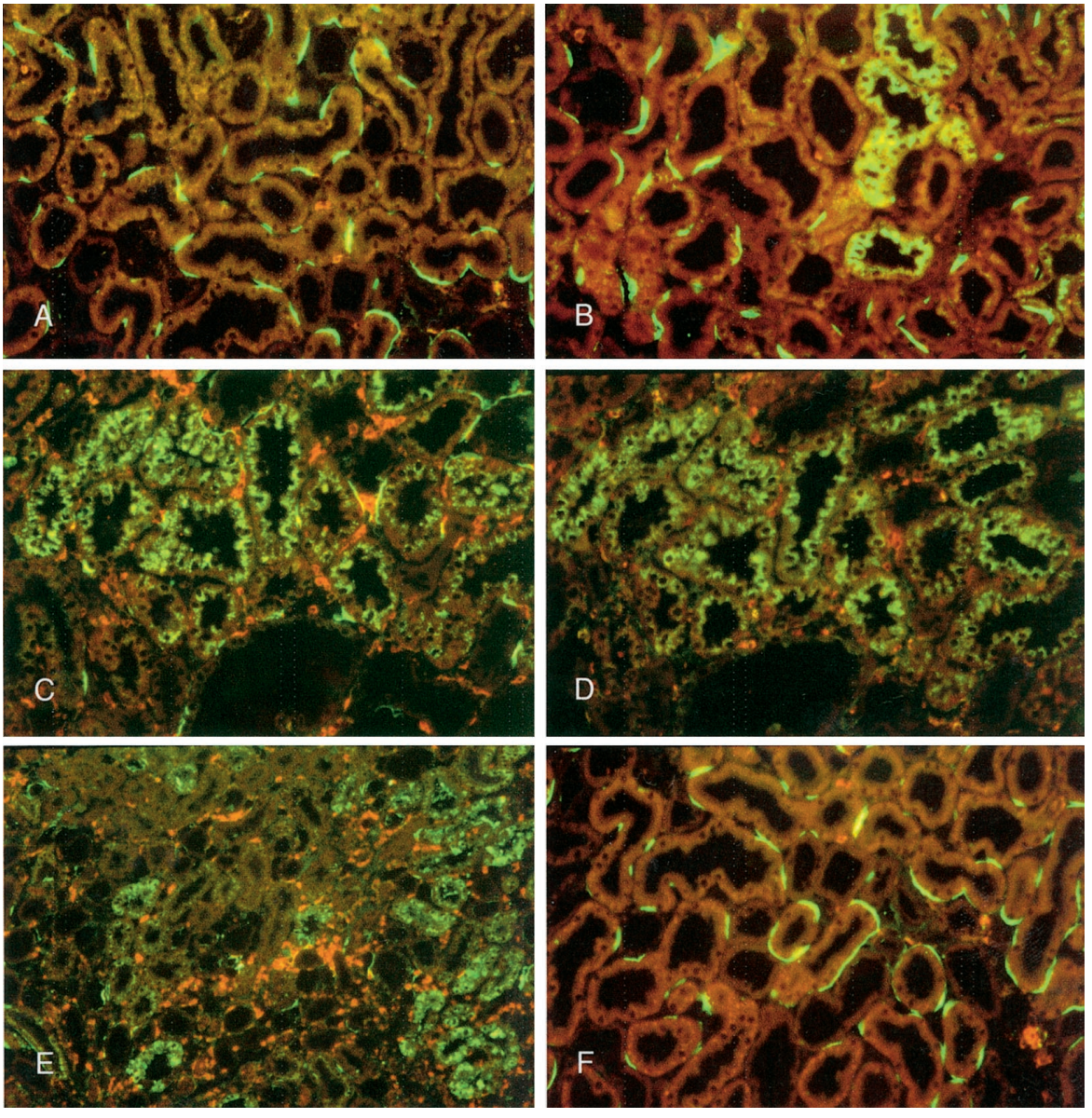

Figure 4. Comparison of C3 staining with interstitial sites of enhanced MHC class II antigen expression (OX-6-positive cells) and ED-1-positive macrophage infiltration, and effects of angiotensin-converting enzyme (ACE) inhibitor on C3 protein accumulation (FITC, green fluorescence) and interstitial inflammation (red fluorescence). Panels A, B, C, and E are representative of results of double immunofluorescence staining for C3 and MHC II antigen in control kidney (A) and remnant kidneys at 7 d (B), 14 d (C), or 30 d (E) after surgery. OX-6-positive cellular infiltrates were present in the peritubular interstitium of 14-d and 30-d remnant kidneys, and had preferential localization at the sites of C3 protein accumulation. Panel D shows the same area from Panel C at 14 d in an adjacent tissue section stained for IgG and ED-1 macrophages. As shown in Panel F, patterns of C3 and OX-6-positive staining were comparable to control in the 5/6 remnant kidney of a rat treated with ACE inhibitor from day 1 and sacrificed at day 30 after surgery. Magnification: ×250 in A, B, C, D, and F; ×125 in E.

usually inhibited by the complement regulatory proteins present on the cell membrane or in plasma (41). However, regulatory proteins active at the level of C3 convertase were not detectable at the luminal surface of the proximal tubular cells in the normal kidney (42,43). Thus, it is possible that complement components, which are otherwise absent in the tubular lumen, are activated on the apical surface of proximal tubular cells in the proteinuric condition, such that C3 activation may not be effectively modulated by decay accelerating factor, which is locally induced (43). *In vitro* evidence is actually available that proximal tubular cells activate complement via the alternative pathway (30) leading to fixation of the C5b-9 MAC neoantigen on the cell surface (44). This, in addition, was followed by cytoskeletal alterations, superoxide anion and hydrogen peroxide production, and synthesis of proinflammatory cytokines such as interleukin-6 and tumor necrosis factor-α (45). Although the generation of C5b-9 on the apical surface can contribute to functional impairment of proximal tubular cells linked to proteinuria, the C3 component may have additional and independent proinflammatory actions

Table 1. Effect of ACE inhibitor on proteinuria, accumulation of IgG and C3 in proximal tubules, and interstitial infiltration of MHC II-positive cells and ED-1 macrophages in remnant kidneys after 5/6 renal mass ablation[a]

Group	Proteinuria (mg/d)	IgG (% proximal tubules)	C3 (% proximal tubules)	OX-6 (MHC II) (No. of cells/HPF)	ED-1 (No. of cells/HPF)
30-d RMR	218.8 ± 65.9	73.5 ± 12.0	62.7 ± 18.9	36.0 ± 12.9	28.2 ± 7.2
30-d RMR + ACEi-(day 1)	14.3 ± 5.1[b]	6.7 ± 7.1[b]	3.2 ± 3.7[b]	9.2 ± 4.3[b]	8.0 ± 3.1[b]
30-d RMR + ACEi-(day 7)	34.4 ± 18[b]	30.7 ± 22.9[b]	7.0 ± 6.1[b]	13.0 ± 1.8[b]	8.6 ± 1.1[b]
30-d control	21.7 ± 2.2[b]	4.3 ± 1.9[b]	0	6.6 ± 0.9[b]	5.5 ± 1.3[b]

[a] Values are mean ± SD. ACE, angiotensin-converting enzyme; RMR, renal mass ablation; ACEi, ACE inhibitor.
[b] $P < 0.01$ *versus* 30-d RMR. ACE inhibitor treatment was started at day 1 or day 7 up to day 30 after RMR.

in proteinuric settings (46). In this respect, we found evidence of granular C3 staining in the basolateral region of proximal tubular cells, in addition to intracellular sites consistent with subapical and lysosomal compartments. Such reactivity together with the linear peripheral C3 staining may reflect polarized secretion of newly synthesized C3 into the interstitium, a pathway that would reinforce the role of proximal tubular cells as a trigger of tubulointerstitial injury. Previous *in vitro* studies did show that proximal tubular epithelial cells synthesize complement components including C3 (32). Moreover, exposure of human proximal tubular epithelial cells in culture to serum proteins at the apical surface upregulated C3 mRNA expression and enhanced the secretion of the protein predominantly at the basolateral site (47). Exactly how excess protein exposure may activate a C3-dependent inflammatory pathway in proximal tubular cells has been theoretically linked, among multiple mechanisms of activation, to excess peptide degradation and consequent ammonia production, in turn leading to activation of alternative pathway, cleavage of unreacted C3, and additional generation of reactive complement components (46). Amidated C3 is one such molecule that can bind to CR1 receptors of monocytes and stimulate phagocyte metabolism (6).

Whatever the mechanisms of C3 accumulation in the proximal tubules, the present study indicates temporal and spatial relationships of intratubular C3 accumulation with events of interstitial inflammation. Abnormal C3 staining was found in proximal tubular epithelial cells in remnant kidneys at 7-d after surgery in a stage closely preceding the appearance of inflammation. Indeed, interstitial infiltrates of MHC class II-positive cells and ED-1 monocytes/macrophages were first detectable in the peritubular interstitium of 14-d remnant kidneys and then more evident at 30 d. More importantly, in double-stained sections, the infiltrating cells concentrated almost exclusively in regions containing C3-positive proximal tubules, and they were less frequent or absent in other peritubular areas. These findings provide morphologic evidence to implicate complement C3 activation in the proximal tubule as an early process with potential role in the local recruitment of inflammatory cells at tubular sites of accumulation of filtered plasma proteins.

Our findings also strengthen the general role of complement in the early stages of induction of tubulointerstitial injury as shown in other models (22,27,28). However, complement is not the single factor responsible for the full manifestation of leukocyte recruitment and tubulointerstitial injury even in the immune or toxic models in which the latter was limited by anticomplement reagents (27,28). We suggest that complement proteins may add their pathogenic potential to other protein-dependent mechanisms of proximal tubular cell activation in proteinuric disease leading to interstitial inflammation. Actually, in cultured proximal tubular cells, IgG, albumin, or transferrin caused concentration-dependent increases in the transcription of NF-κB-dependent genes for vasoactive and inflammatory mediators (48) and in the rate of synthesis of endothelin-1 (25), a peptide with potent vasoactive and chemotactic properties. Similarly, albumin and transferrin stimulate transcription of the gene for monocyte chemoattractant protein-1 in these cells (23). Albumin and IgG also stimulate the production of RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) (24), an immunoregulatory cytokine with chemotactic properties for monocytes and memory T cells (49).

If the interstitial inflammatory reaction were the consequence of proximal tubular cell uptake of filtered proteins including C3, limiting protein filtration and reabsorption should prevent tubular cell activation and C3 accumulation and eventually preserve tubulointerstitial structure. Drugs that limit the synthesis or biologic activity of angiotensin II have attracted great interest as a result of their unique property of slowing the rate of progression of renal disease (48,50). Consistent experimental and clinical evidence (14,51) tends to suggest that one mechanism for the beneficial effect of ACE inhibitors is related to their ability to reduce glomerular protein traffic. Here we found that the ACE inhibitor lisinopril given to rats with renal mass ablation prevented or stabilized urinary protein excretion, respectively, when treatment was started from day 1 or day 7 after surgery. This functional effect was associated with reduction of C3 and IgG staining in proximal tubules to comparable levels of sham-operated control rats, and with the normalization of the number of MHC class II-positive cells and monocyte/macrophages in the interstitium at least for animals given lisinopril from day 1 after surgery. These findings offer additional support to the potential role of protein traffic to the induction of tubulointerstitial inflammation in proteinuric renal disease, at least in part via activation of

proximal tubular cells leading to complement accumulation and new synthesis of proinflammatory molecules including the C3 component itself. The contribution of tubular C3 complement is supported by recent data in rats with aminonucleoside nephrosis showing that a soluble form of recombinant human complement receptor type 1 (sCR1), which inhibits complement activation by decay acceleration of C3 convertase, limited tubulointerstitial cell infiltration and injury (27). Similar effects were observed in rats given cobra venom factor to deplete serum complement (27). Interference with complement activation of C3 convertase with soluble CR1 presumably by local inhibition at sites of tubular deposition was also effective in reducing the severity of lesions in rats with mesangial proliferative glomerulonephritis (28). Our findings do not exclude the possibility that additional mechanisms of tissue injury can be interrupted by ACE inhibitor while exerting its salutary action, mainly those linked to hemodynamic changes in this model or to the fibrogenic potential of angiotensin II (52).

In summary, we have shown that in rats with renal mass ablation (*1*) proteinuria is associated with enhanced protein traffic and C3 complement accumulation in proximal tubules in the early stage of progressive disease; (*2*) abnormal accumulation of complement and possibly basolateral secretion of newly synthesized C3 are followed by macrophage infiltration and MHC II overexpression into the peritubular interstitium and may have role in the inflammatory response; (*3*) inhibition of ACE with lisinopril while limiting the abnormal traffic of complement and other proteins abrogates interstitial inflammation.

Given the role of tubulointerstitial injury in subsequent scarring and renal function impairment, strategies to prevent or retard progression of tubulointerstitial lesions in humans should be focused on the reduction of proteinuria. Future approaches, however, may consider intracellular congestion of proteins and downstream cellular signaling as well as other secondary protein-independent pathways as possible target mechanisms, particularly when permselective properties of glomerular capillary barrier are irreversibly compromised.

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