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Proteinuria and tubulointerstitial lesions in lupus nephritis

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Proteinuria and tubulointerstitial lesions in lupus nephritis.

Background. Response of the renal tubules to proteinuria is implicated in progression of renal disease. Experimentally, proteinuria causes increased tubular synthesis of macrophagic and other chemokines, with increased tubular cellular proliferation and apoptosis, leading to interstitial inflammation and fibrosis. Clinically, diminution of proteinuria leads to the slowing of progression, but whether this leads to reduction in tubular lesions has not been directly demonstrated in humans.

Methods. Initial (Bx1) and systematic six-month biopsies (Bx2) from 71 patients with lupus nephritis were studied, with a subset of 34 biopsies also stained for proliferating cell nuclear antigen (PCNA), the macrophage marker PGM1, and cytokeratins (AE1/AE3), and morphometric cell and tubular profile counts performed.

Results. Positive correlations were found between increasing levels of proteinuria and the following light microscopic parameters: tubular epithelial pyknosis, tubular epithelial nuclear "activation," tubular lumenal macrophages, interstitial inflammation and fibrosis, but not with tubulointerstitial immunofluorescence. Significant positive correlations also were found with the following immunohistochemical parameters: PCNA in epithelial cells ($r = 0.74$) and tubular luminal cells ($r = 0.47$); tubular lumenal macrophages ($r = 0.63$) and tubular epithelial cells with acquired PGM1 staining ($r = 0.36$); and pyknotic tubular epithelial cells ($r = 0.47$). All showed strong correlations with serum creatinine (S_{Cr}) as well. All were reduced at Bx2, generally in parallel to the reduction in proteinuria. Tubulointerstitial immune deposits appear to play only a minor role in the development of tubular epithelial lesions and the progression of renal disease in lupus. They show only limited correlation with S_{Cr} and no correlation with proteinuria. By multiple regression, they are not associated with tubular epithelial lesions, interstitial inflammation or interstitial fibrosis at either biopsy, whereas tubular epithelial lesions are strongly associated with interstitial inflammation at Bx1 and with interstitial fibrosis at Bx2. Cytokeratin correlated strongly with S_{Cr} ($r = 0.53$, $P = 0.002$) but not with proteinuria ($r = 0.27$, NS), and was the sole immunohistochemical parameter to increase at Bx2. It appears to be a sensitive marker for tubular atrophy.

Conclusions. In this study both proteinuria and S_{Cr} showed a hierarchy of correlations with morphologic variables: Tubular epithelial cell changes > tubular macrophages > interstitial inflammation > interstitial fibrosis, corresponding to current experimental models, but not previously demonstrated in humans.

Key words: renal tubules, systemic lupus, erythematosus (SLE), macrophages.

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It has become apparent in recent years that the response of the renal tubules to proteinuria plays a central role in the progression of renal disease [1–4]. Proteinuria causes a host of different chemokines, particularly macrophage chemoattractants [5, 6] and other proteins, including major histocompatibility complex (MHC) antigens [3, 4] and vasoactive substances, such as endothelin-1 [7] to be up-regulated in renal tubular cells, particularly proximal tubules. These proteins, often elaborated on the basolateral aspect of the tubule cells, can then be released into the interstitium where they enchain the appearance of T cells and macrophages, with up-regulation of transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and other chemokines [3, 4, 8]. These lead in turn to fibroblast proliferation, myofibroblastic transformation and ensuing interstitial fibrosis [3, 4, 8]. It is also increasingly apparent that complement plays a role, even in non-immune mediated injury [9, 10]. Experimentally, these proteinuric conditions also are associated with tubular cell proliferation [9, 11–14] and apoptosis/pyknosis [13, 14]. Apoptosis is usually more frequent than proliferation, leading some to suggest that this imbalance is one of the mechanisms of tubular atrophy [13].

However, a number of questions remain to be answered. Relatively limited information exists as to whether the up-regulated proteins and cytokines return toward normal with diminution of proteinuria. Most animal models of proteinuria, such as 5/6 nephrectomy or the various glomerulonephritic models, lead ineluctably to renal failure. These models demonstrate that antiproteinuric treatment leads to lesser lesions compared to untreated animals [15]. However, only puromycin aminonucleoside nephrosis offers the possibility to see the effects of reduction of proteinuria [12, 15]. During the initial proteinuric phase, there is a burst of tubular proliferation [12, 15], accompanied by up-regulation of osteopontin [15], both

of which return to normal levels by 28 to 42 days with disappearance of proteinuria. In humans, there have been relatively few studies correlating morphologic alterations with level of proteinuria. They show a generally good correlation with proteinuria [16–20], but there are no sequential biopsy studies to show whether diminution of proteinuria is attended by regression of tubular proliferative, pyknotic and other lesions.

We studied a series of 71 patients with lupus nephritis with initial biopsies and systematic repeat biopsies after six months of treatment, at a time when the great majority had marked diminution or total disappearance of their proteinuria. We had previously found that tubular lesions offered the best correlations with the current serum creatinine (S_{Cr}) value of any morphologic variable, as well as good correlations with outcome [21, 22], this latter finding confirming numerous other studies [1]. The present study examined in greater depth, not simply the individual lesions, but the interrelationships between proteinuria, S_{Cr}, tubular epithelial lesions, tubulointerstitial immunofluorescence (IF), interstitial inflammation, and interstitial fibrosis to see what light these interrelationships might shed on the pathogenesis of progressive renal disease. A subset of 34 biopsies, representative of the overall series in terms of S_{Cr} and level of proteinuria, also was studied to validate with immunohistochemical techniques several of the morphologic parameters developed on the basis of routine stains of archival material.

METHODS

Patient population

Renal biopsies and clinical data from 71 patients from four Paris hospitals (Bichat, St. Louis, Broussais, and Henri Mondor) from the period 1986 to 1994 were evaluated. Demographic characteristics have previously been described [21, 22]. Cases were categorized according to 1982 WHO criteria [23]. Initial diagnoses were diffuse proliferative lupus nephritis (WHO Class IV), 55 patients; focal proliferative lupus nephritis (WHO Class III), 9 patients; and mixed membranous and focal or diffuse proliferative lupus nephritis (WHO Class Vc and Vd), 7 patients. All patients had an initial renal biopsy (Bx1) as part of a treatment protocol approved by the appropriate committees at the four hospitals. The six-month induction treatment consisted of monthly intravenous cyclophosphamide combined with prednisone (0.9 ± 0.4 mg/kg · body weight/day for one month tapered to 0.4 ± 0.1 mg/kg · body weight/day at six months) in 58 patients, and corticosteroids alone in 13 patients (1.4 ± 0.3 mg/kg body weight/day tapered to 0.5 ± 0.16 mg/kg · body weight/day at 6 months). Initial treatment was followed at six months by re-evaluation and control renal biopsy (Bx2) to evaluate the effects of therapy. Subsequent biopsies were carried out primarily for clinical indications and are reported elsewhere [24].

Technical methods and record review

Specifics of technical methods are given in a prior communication [21]. Briefly, biopsies were prepared and stained according to standard methods for light and IF microscopy. Electron microscopy is not included in this study. For light microscopic study there were 67 usable biopsies at Bx1 and 71 biopsies at Bx2. Photographs of positive IF were available in 122 (85.9%) of the 142 first and second biopsies. The remaining 20 biopsies had no photographs and/or contained only medulla. This material was graded randomly by one author (GSH), blind to the clinical data. A variety of clinical parameters were evaluated at the time of each biopsy of which the most important for this study were serum creatinine (S_{Cr}, μmol/L), proteinuria (g/24 hours), and hematuria (rbcs/mL). Clinical data were available for all patients for the initial two biopsies, but for six patients the follow-up data as to outcome were incomplete.

The primary outcome parameter measured was doubling of the initial serum creatinine (CRX2) for three months or more. Also evaluated were end-stage renal disease (ESRD), requiring dialysis and/or transplant, and final renal function (RF_{last}), defined as the last S_{Cr}, with an arbitrary value of 500 μmol/L assigned to all patients with ESRD, on dialysis or transplanted.

Morphologic variables

The schema for evaluation of morphologic variables has been reported in detail elsewhere [21]. Lesions were graded semiquantitatively on a scale of 0 to 3+, depending on the proportion of the tubular profiles or interstitium involved: 0 = absence of lesions; 0.5+ = lesions present, but minimal (<5 to 10% of the tubules or interstitium according to the variable); 1+ = lesions involving up to 25%; 2+ = lesions involving 25 to 50%; 3+ = lesions involving >50% of the tubules or interstitium. As best possible, judgments were based on comparing the cells and nuclei in the affected tubule with those in normal profiles of the same nephron segment, that is, proximal, distal, etc. elsewhere in the section. The lesions most germaine to this study are defined as follows.

Tubular nuclear pyknosis (tubpyk). Pyknosis in tubular epithelium was defined as shrinkage of nuclear profiles with condensation of the nuclear chromatin to a solid black, often hyaline appearance, with ultimate fragmentation.

Tubular nuclear "activation" (tubact). Nuclear "activation" was defined cytologically by generalized nuclear enlargement with variation in nuclear size and shape, prominent nucleoli, and irregularities of nuclear chromatin with zones of clearing and clumping on the nuclear membrane.

Macrophages in tubular lumens (macrlum). Macrophages in tubular lumina were identified primarily by their biconcave-to-ovoid nuclei and crisp cell membranes, usually with evident cytoplasmic vacuoles and/or inclusions but occasionally without. Usually these cells were found free in the tubular lumina, but occasionally they could be found attached to the tubular epithelial cells. Every effort was made to distinguish them from sloughed epithelial cells, which generally had pyknotic nuclei and more eosinophilic cytoplasm with ragged cell borders.

Also evaluated and quantitated in similar fashion [21] were tubular necrosis (tubnec), tubular cell flattening (tubflat), and epithelial cells in tubular lumina (eplum).

Tubulointerstitial deposits on IF (tubulif). To evaluate tubulointerstitial deposits on IF, antisera to the following were employed in all cases: IgG, IgA, IgM, C3, C1q, and fibrinogen. Each was graded 0 to 4+ according to the percentage of tubulointerstitium involved, for a maximum possible score of 24 for the 6 antisera. (Although tubular basement membranes often could be distinguished from interstitial staining, this was far from universal, so the tubulointerstitium was considered as a unit.)

Immunohistochemistry

Immunostaining for the macrophage marker PGM1 (Dakopatts, Trappes, France), proliferating cell nuclear antigen (PCNA; Dakopatts) and cytokeratins (AE1/AE3; Dakopatts) was performed on a subset of biopsies from the overall series, selected to be representative of the overall series in terms of S_{Cr} and proteinuria at Bx1. Initially specimens from Bx1 and Bx2 were stained in 18 patients. However, 2 specimens at Bx1 were technically unsatisfactory, leaving a total of 34 biopsies, 16 from Bx1 and 18 from Bx2. They were prepared as follows: Freshly cut paraffin slides were digested with pronase for PGM1 and AE1/AE3, or microwaved for five minutes in EDTA pH 8 solution, followed by blocking with goat serum diluted 1/20 for 20 minutes. They were then incubated 30 minutes with PGM1 diluted 1/200, PCNA 1/100, or AE1/AE3 1/100, washed with TRIS buffer, pH 7.4, followed by 30 minutes' incubation with horse anti-mouse biotinylated antibody (Vector, Burlingame, CA, USA), diluted 1/200 to detect the PGM1 or other marker, washed, then incubated with a preformed avidin-biotinylated alkaline phosphatase complex (Vectastain ABC Reagent; Vector). Development with Vector Red AEC substrate and counterstaining with hematoxylin completed the process.

Cell and tubular profile counts

Slides were examined using an ocular grid with an overall area of approximately 10,000 μ^2 at $\times 40$ magnification. Only cortical parenchyma was evaluated. For each biopsy at least 100 grids of tubulointerstitium were counted, with the exception of several biopsies where limited material prevented this. The minimum number of grids counted was 72. The following morphologic compartments were evaluated: (*a*) for PGM1: (1) tubular lumenal macrophages; (2) cells staining positively for PGM1 within the tubular epithelium; (3) interstitial macrophages; (*b*) for PCNA: (1) tubular epithelial cells; (2) PCNA+ cells in tubular lumens; (*c*) for pyknosis on routine stains: (1) tubular epithelial cells; (2) pyknotic cells in tubular lumens; (*d*) for AE1/AE3, number of tubular profiles containing AE1/AE3+ cells per grid. Tubules with a single AE1/AE3+ cell were counted as positive.

Statistical analyses

In comparing continuous clinical variables (such as S_{Cr}) with semiquantitative variables, such as tubular flattening graded 0 to 3+, with continuous variables, Pearson product-moment correlation coefficients were calculated. For comparisons of semiquantitative variables with one another and for categorical variables (for example, the presence or absence of ESRD), the Spearman rank order correlation test was used. Since these correlations are very extensively cited, to maintain continuity in the text they will usually appear simply in parentheses, with asterisks used to indicate the level of probability: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. Stepwise multiple regression was used in defining the significance of associations between morphologic variables in selected situations.

RESULTS

Immunohistochemical studies

Immunohistochemical studies were carried out to validate the morphologic variables, tubular lumenal macrophages using PGM1, tubular nuclear "activation" using PCNA, and nuclear pyknosis by quantitative counts of pyknotic cells performed on the same slides at the same time as counts of PCNA+ cells. The distribution of cytokeratin was evaluated using AE1/AE3. Correlations between the various parameters and also with S_{Cr} and proteinuria are presented in Table 1.

Macrophages. These have been reported in detail in a previous communication [22]. Briefly, there was a good correlation between tubular macrophages identified on routine stains and those tagged by PGM1 ($r = 0.54**$), and we noted frequent staining of tubular epithelial cells by PGM1, suggesting possible transdifferentiation, although this was not confirmed by double labeling in the present study. Using both routine stain and PGM1, tubular macrophages at Bx2 also had an excellent correlation with later development of renal insufficiency ($r = 0.56*$). Equally importantly from the standpoint of pathogenesis, they had an extremely high correlation with both

Table 1. Correlation betwween various prophologic, immunohistochemical, and clinical parameters

	Proteinuria	S_{Cr}	TubLumMacr (PGM1)	TubCytMacr (PGM1)	PCNA	Pyknosis (count)	Cytokeratin
PCNA-positivity	0.74[a]	0.55[a]	0.89[a]	0.47[a]	—	0.64[a]	0.57[a]
Tubular lumenal macrophages (PGM1)	0.63[a]	0.54[a]	—	0.74[a]	0.89[a]	0.81[a]	0.34
Pyknosis (count)	0.47[a]	0.50[a]	0.81[a]	0.54[a]	0.64[a]	—	0.53[a]
Tubular cytoplasmic PGMI positivity	0.36[a]	0.39[a]	0.74[a]	—	0.47[a]	0.54[a]	0.15
Cytokeratin	0.27	0.53[a]	0.34	0.15	0.57[a]	0.53[a]	—

This subset of 34 biopsies with immunohistochemical studies used Pearson product-moment correlations for the analysis.
[a] Significant at $P < 0.05$

PCNA positivity ($r = 0.98$***) and tubular pyknosis ($r = 0.91$**), but only lesser, nonsignificant correlations with cytokeratin ($r = 0.65$, $P = 0.15$).

Proliferating cell nuclear antigen. It rapidly became apparent that only a minority of cells with characteristics of nuclear "activation" (nuclear enlargement, prominent nucleoli, increased chromatin with clumping on the nuclear membrane, etc.) was positive for PCNA (Fig. 1), in accordance with general cytopathologic experience. Nonetheless, there was a very good correlation between PCNA and tubact ($r = 0.51$**). They, however, differed in their degree of correlation with the level of proteinuria, that of PCNA being very strong ($r = 0.71$***), whereas that with tubact was weaker ($r = 0.27$**).

Proliferating cell nuclear antigen positive cells in the tubular lumens were very much rarer than PCNA+ tubular epithelial cells (35.0 ± 30.1 vs. 0.8 ± 1.4 cells/100 grids), and were present in only 13 of 30 biopsies. However, their presence connoted worse disease, with higher S_{Cr} levels (165 ± 53 vs. 100 ± 30 μmol/L, $P = 0.0004$) and greater proteinuria (6.4 ± 4.7 vs. 2.4 ± 1.9 g/24 h, $P = 0.005$). As might be anticipated, PCNA positivity in tubular cells was significantly higher in the group with PCNA+ lumenal cells (48.9 ± 37.7 vs. 22.3 ± 15.8 cells/100 grids, $P = 0.02$).

Pyknosis. It would have been preferable to study pyknosis/apoptosis by the TUNEL technique [25]. Unfortunately, it could not be made to give reliable results for cell counts on these biopsies because they were fixed in Bouin's fixative containing picric acid. We therefore relied on cell counts of pyknotic cells, performed on the same slides and at the same time as the counts of PCNA+ cells.

There was a very good correlation between counts of tubular epithelial cell pyknosis and tubpyk, estimated semiquantitatively on routine stains ($r = 0.53$**). Pyknotic cells were more frequent in the tubular epithelium (52.6 ± 24.4 cells/100 grids) and in tubular lumina (23.3 ± 13.4 cells/100 grids) than PCNA+ cells in these locations. However, unlike PCNA+ tubular luminal cells, pyknotic cells in the lumina were present to some extent in all cases and correlated only with increasing proteinuria ($r = 0.35$*), not worsening renal function.

Cytokeratin. Cortical staining for cytokeratin in normal kidneys is limited to the distal nephron, from the distal tubules onwards. However, we had noted in other studies that cytokeratin often stains atrophic tubules, so it was studied here. Cytokeratin staining appeared first in individual tubular cells, being distributed particularly near the cell membrane (Fig. 2). At first only one or two cells per tubular profile stain were found in tubules that otherwise showed no evidence of atrophy, that is, with no thickening of the tubular basement membrane, and with intact brush borders in other cells. Gradually, the staining became more extensive both in intensity per cell and in percentage of cells involved, as the standard stigmata of tubular atrophy appeared. However, even fairly late, individual cells negative for cytokeratin could be seen in atrophic tubules, and very rarely, small, shrunken tubules with thickened basement membranes typical of advanced tubular atrophy were negative for cytokeratin. However, the vast majority of obviously atrophic tubules stained uniformly and intensely for cytokeratin. In addition, some cases, usually those with severe disease, showed scattered, isolated AE1/AE3+ cells in the interstitium (Fig. 2).

Cytokeratin showed an excellent correlation with semiquantitative estimates of tubular atrophy of $r = 0.56$*** (despite the fact that many tubules with limited/early cytokeratin positivity showed no atrophy by routine microscopy). As with the other tubular markers, cytokeratin here showed a strong correlation with S_{Cr} ($r = 0.53$**). However, unlike the others, it showed no significant correlation with the level of proteinuria ($r = 0.27$, $P = 0.13$). Cytokeratin differs in two other important respects from the other markers studied: (*1*) It shows no correlation with macrophage markers, either tubular lumenal or tubular epithelial (Table 1). (*2*) It increases from Bx1 to Bx2, whereas all of the other cellular markers decline (Fig. 3).

Correlations between markers and serum creatinine and proteinuria. All of the immunohistochemical markers showed strong correlations with S_{Cr}, and all save cytokeratin showed strong correlations with proteinuria (Table 1). In addition, subdivision by level of proteinuria showed that all of the markers increased progressively with increasing proteinuria (Fig. 4), save cytokeratin, and that these changes were greater than the associated modest increase of S_{Cr}. It was further noted that, corresponding

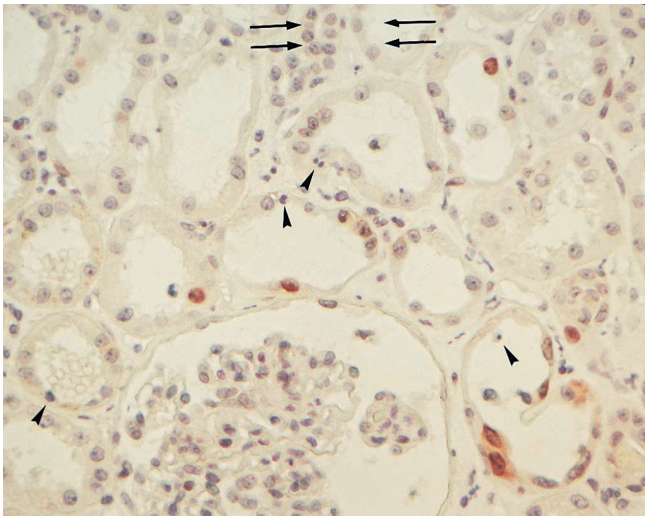

Fig. 1. Staining for proliferating cell nuclear antigen (PCNA). In addition to several brown-staining PCNA+ nuclei, the majority of tubular nuclei show changes of nuclear "activation" with nuclear enlargement and irregularity with prominent nucleoli. For comparison, arrows mark a group of reasonably normal proximal and distal tubular nuclei at the top. In addition, several pyknotic tubular nuclei are indicated by arrowheads. Hematoxylin counterstain, magnification ×350.

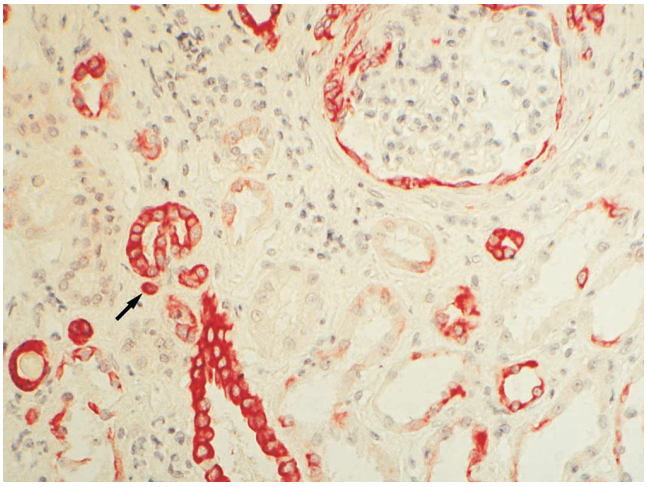

Fig. 2. Staining for cytokeratin (AE1/AE3). A dilated distal tubule (center) as well as several profiles of atrophic tubules stain positively, as does the parietal epithelium lining the Bowman's capsule, a common finding in glomerular inflammatory lesions. Some tubules show individual tubular cell staining for PCNA in the absence of other signs of atrophy, although the tubular lumina are somewhat dilated. An interstitial cell (arrow) is also cytokeratin positive. Hematoxylin counterstain, ×300.

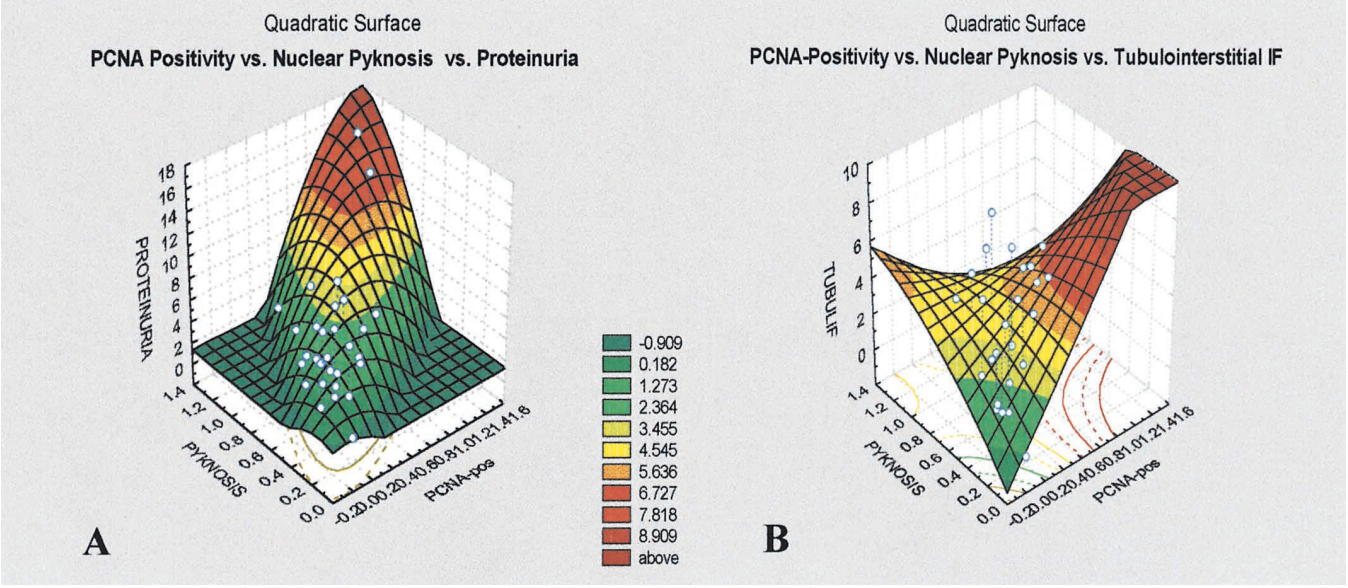

Fig. 5. Quadratic surfaces formed by interaction of correlations between PCNA-positivity and nuclear pyknosis (x and y axes) versus proteinuria (*A*) and tubulointerstitial immunofluorescence (*B*; IF; z axis). In the plot against proteinuria, the quadratic surface forms a clear cut, sharp central peak, highest at the highest values for all three variables, with most points in the center and at or near the quadratic surface. By contrast, on the quadratic surface plotted for tubulointerstitial IF (*B*), there is no central peak and many points are well away from the quadratic surface.

to the decrease in proteinuria between Bx1 and Bx2, there was a parallel decrease, generally statistically significant, in all of the parameters save for cytokeratin (Fig. 3).

Overall series

Correlations with serum creatinine and proteinuria and outcome variables. An interesting pattern emerged when S_{Cr}, proteinuria, and the outcome variables, CRX2 and RF_{last} were compared with tubulointerstitial and chronicity variables in the series as a whole (Table 2). First, a general hierarchy of correlations with these variables could be established, with tubular epithelial variables showing the strongest correlations, slightly weaker correlations for tubular luminal macrophages and interstitial

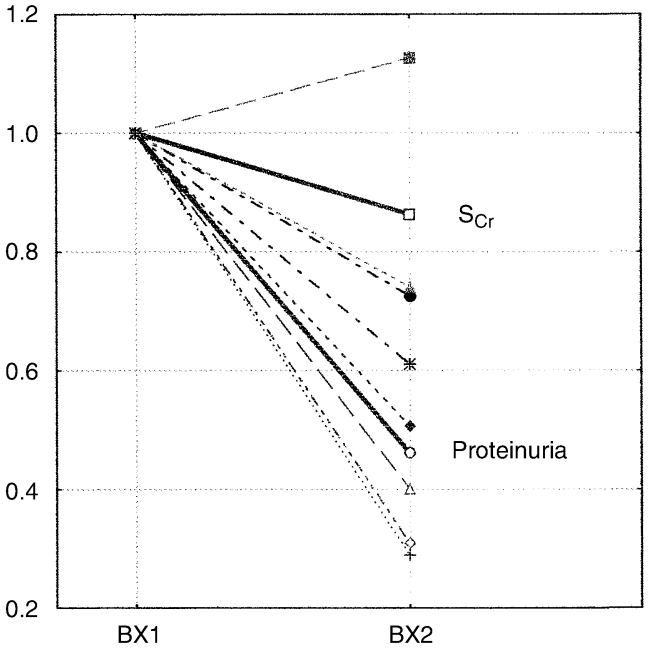

Fig. 3. Changes in mean values for immunohistochemical variables from Bx1 to Bx2, standardized to 1 for initial values. Values fall generally in parallel with proteinuria (bold line), whereas serum creatinine (S_{Cr}) declines by lesser amounts. Cytokeratin is the sole immunohistochemical value to rise between biopsies. Symbols are: (■) cytokeratin; (□) S_{Cr}; (▲) pyknotic tubular cells; (●) interstitial macrophages; (*) pyknotic cells in tubular lumina; (◆) proliferating cell nuclear antigen (PCNA) tubular cells; (○) proteinuria; (△) tubular epithelial PGM-1 positivity; (◇) tubular luminal macrophages; (+) PCNA cells in tubular lumina.

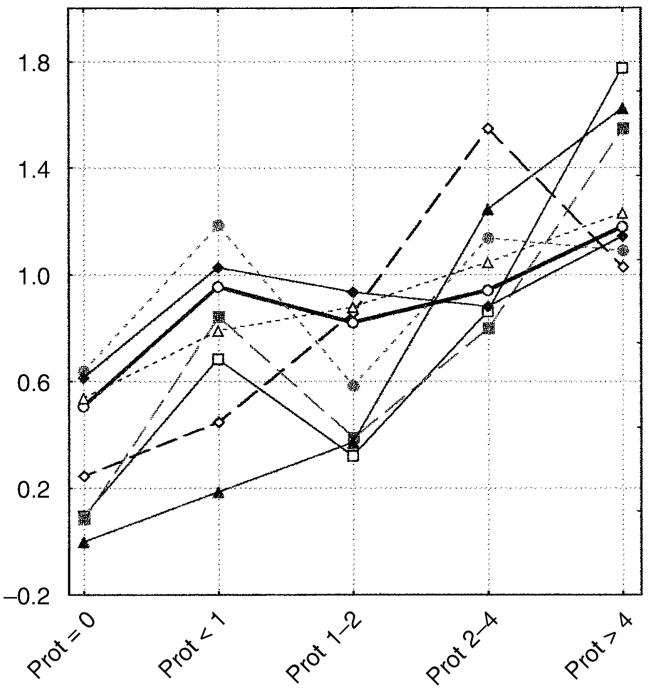

Fig. 4. Staining for various immunohistochemical markers compared to the degree of proteinuria in a subset of 34 biopsies. Values are standardized such that the mean for each parameter equals 1. The various markers increase with the increasing proteinuria, as does S_{Cr}, as indicated with the bold line. However, the S_{Cr} is above normal limits only for Prot >4 g/24 h. Symbols are: (□) tubular luminal macrophages; (▲) PCNA+ cells in tubular lumina; (■) PCNA+ tubular cells; (△) interstitial macrophages; (○) S_{Cr}; (◆) pyknotic tubular cells; (●) cytokeratin; (◇) tubular epithelial PGM1 positivity.

inflammation, and yet weaker, often nonsignificant correlations with tubular atrophy and interstitial fibrosis. Tubulointerstitial IF showed the poorest overall correlations with the above variables, correlating relatively poorly with S_{Cr} and not at all with proteinuria. Second, S_{Cr} showed an even stronger correlation with all these variables than did proteinuria. Multiple regression confirmed that both tubular epithelial variables and interstitial inflammation were significantly related to S_{Cr} at Bx2 ($P = 0.00009$ and 0.0025, respectively), but that tubulointerstitial immune deposits were not ($P = 0.57$).

Relationship between tubular epithelial variables, tubulointerstitial IF, and interstitial inflammation and fibrosis. Tubular epithelial lesions and *tubulif* showed no significant association with one another at either biopsy, either in terms of Pearson correlation coefficients (Table 2) or by stepwise multiple regression (Table 3). Further, stepwise multiple regression revealed that tubular epithelial lesions were strongly associated with interstitial inflammation at Bx1 (prior to treatment for most patients), and with interstitial fibrosis after treatment at Bx2, but that *tubulif* was not significantly associated with any of them (Table 3).

Consonant with these correlations, a number of biopsies at both Bx1 (8 patients) and Bx2 (13 patients) showed significant tubular epithelial lesions in the absence of any tubulointerstitial IF staining whatever. Similarly, an impressive number of patients (11 at Bx1 and 18 at Bx2) showed interstitial inflammation in the absence of any tubulointerstitial deposits by IF. Tubular epithelial lesions seemed linked to interstitial inflammation rather than to *tubulif*, in that those with inflammation but no deposits showed means for tubular epithelial lesions close to the group as a whole (3.8 ± 2.4 vs. 4.2 ± 2.5, NS), whereas those with deposits but no inflammation (7 patients at each biopsy) had lower-than-average tubular epithelial lesions (2.4 ± 1.5, $P = 0.03$).

The relative differences in the correlations of the tubular epithelial variables with proteinuria versus their correlation with *tubulif* are perhaps best demonstrated visually. Figure 5 represents the quadratic surfaces formed by the interacting correlations between PCNA-positivity and nuclear pyknosis in the x and y axes with proteinuria (Fig. 5A) or *tubulif* (Fig. 5B) in the z axis. In the plot against proteinuria, the quadratic surface forms a clear-cut, sharp central peak, highest at the highest values for all three variables, with most points in the center at or near the quadratic surface. By contrast, for *tubulif*

Table 2. Correlations between tubulointerstitial, clinical and outcome parameters

	Tubular epithelial	Tubular macrophages	Interstitial inflammation	Interstitial fibrosis	Tubular atrophy	Tubint. IF
Clinical and outcome parameters						
Biopsy 1						
Proteinuria[b]	**0.27**[a]	0.23	0.10	0.19	0.17	0.05
S_{Cr}[b]	**0.35**[a]	**0.51**[a]	**0.35**[a]	**0.27**[a]	**0.28**[a]	**0.31**[a]
CRX2[c]	**0.23**[a]	0.24	0.19	0.14	0.15	0.11
RF_{last}[b]	**0.42**[a]	**0.33**[a]	**0.27**[a]	0.21	0.19	0.12
Biopsy 2						
Proteinuria[b]	**0.33**[a]	−0.01	**0.25**[a]	**0.26**[a]	0.24	0.08
S_{Cr}[b]	**0.49**[a]	**0.43**[a]	**0.52**[a]	**0.47**[a]	**0.47**[a]	0.25
CRX2[c]	**0.23**[a]	**0.39**[a]	0.15	0.15	0.17	**0.36**[a]
RF_{last}[b]	**0.40**[a]	**0.43**[a]	0.20	0.25	0.25	0.20
Biopsy 1 + 2						
Proteinuria[b]	**0.34**[a]	**0.26**[a]	**0.24**[a]	0.08	0.06	0.07
S_{Cr}[b]	**0.48**[a]	**0.51**[a]	**0.45**[a]	**0.32**[a]	**0.29**[a]	**0.30**[a]
CRX2[c]	**0.28**[a]	**0.24**[a]	0.16	0.14	0.16	**0.21**[a]
RF_{last}[b]	**0.42**[a]	**0.36**[a]	**0.23**[a]	**0.23**[a]	**0.21**[a]	0.15
Morphologic parameters						
Biopsy 1						
Tubep	—	**0.59**[a]	**0.55**[a]	**0.41**[a]	**0.44**[a]	0.25
Tubulif	0.25	**0.27**[a]	**0.43**[a]	**0.43**[a]	0.36	—
Biopsy 2						
Tubep	—	**0.39**[a]	**0.33**[a]	**0.57**[a]	**0.45**[a]	0.16
Tubulif	0.16	**0.27**[a]	**0.31**[a]	0.20	0.17	—
Biopsy 1 + 2						
Tubep	—	**0.53**[a]	**0.46**[a]	**0.46**[a]	**0.45**[a]	**0.27**[a]
Tubulif	**0.27**[a]	**0.34**[a]	**0.46**[a]	**0.30**[a]	0.31	—

Abbreviations are: Tubint. IF, tubulointerstitial deposits in immunofluorescence; S_{Cr}, serum creatinine; CRX2, doubling of the initial serum creatinine; RF_{last}, final renal function; Tubep, tubular epithelium (tubep = tubpyk + tubact + tubnec + tubflat + eplum); tubpyk, tubular nuclear pyknosis; tubact, tubular nuclear activation; tubnec, tubular necrosis; tubflat, tubular cell flattening; eplum, epithelial cells in tubular lumens.

[a] Value significant at $P < 0.05$ (boldface)

[b] Pearson product-moment correlation

[c] Spearman rank correlation

Table 3. Association between tubular epithelial lesions, interstitial inflammation, interstitial fibrosis and tubulointerstitial immunofluorescence by stepwise multiple regression

Tubular epithelial lesions			Interstitial inflammation			Interstitial fibrosis			Tubulointerstitial IF		
Variable	β	*P*	Variable	β	*P*	Variable	β	*P*	Variable	β	*P*
Biopsy 1											
Intinfl1	0.4849	0.00003	TubEp1	0.4140	0.001	Intinfl1	0.5557	0.00001	Intfib1	0.2701	0.10
Macrlum1	0.3899	0.0005	Intfib	0.3465	0.002	Tubulif1	0.1955	0.10	Intinfl1	0.2554	0.12
			Tubulif1	0.1489	0.13						
			Macrlum1	0.1165	0.32						
Biopsy 2											
Intfib2	0.6070	0.000005	Intfib2	0.7086	0.000008	Intinfl2	0.5433	0.000005	Intinfl2	0.2354	0.14
Macrlum2	0.1821	0.12	Macrlum2	0.2123	0.068	TubEp2	0.4644	0.00004	Macrlum2	0.1594	0.31
			Tubulif2	0.1379	0.21	Macrlum2	−0.1028	0.29			
			TubEp2	−0.1417	0.32						

Abbreviations are in Table 1 and: Intfib, interstitial fibrosis; Intinfl, interstitial inflammation; Tubulif, tubulointerstitial immunofluorescence. TubEp = tubpyk + tubact + tubnec + tubflat + eplum (see *Morphologic variables*). Biopsy 1 comprised 55 untreated patients, and 12 previously treated patients and biopsy 2 comprised 71 treated patients.

(Fig. 5B), there is no such central peak and many points are well away from the quadratic surface.

DISCUSSION

Tubular nuclear activation and pyknosis

Pyknosis. Studies comparing light microscopic identification of pyknosis with TUNEL staining show an exceedingly high correlation between the two ($r > 0.90$), but light microscopy recognizes only 45 to 70% of the cells identified by TUNEL [26–28]. In our material tubular nuclear pyknosis is frequent and shows significant positive correlations with S_{Cr} and proteinuria (Table 1, Figs. 4 and 6), indicating that it increases with disease activity. This increase in pyknosis suggests but does not prove an increase in tubular apoptosis in SLE.

Tubular nuclear "activation." There is a marked increase in cellular proliferation in the tubulointerstitium in systemic lupus erythematosus (SLE) nephritis, as indicated by a marker for proliferation, PCNA [29], and

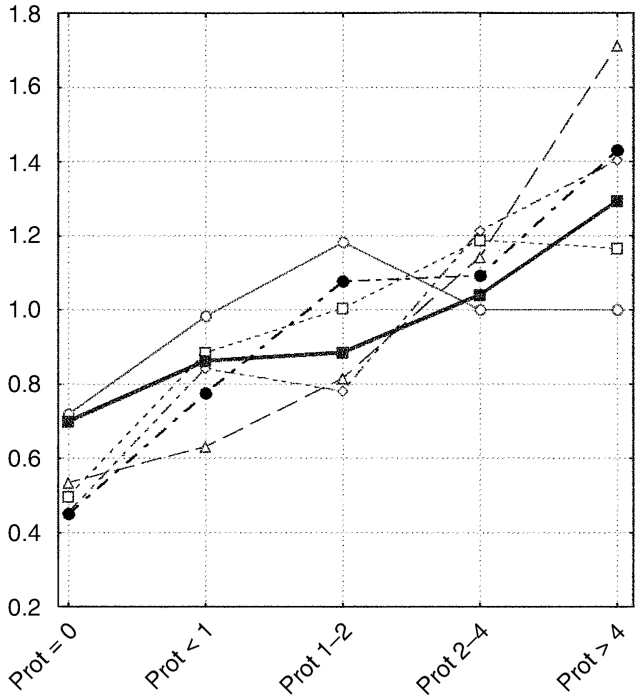

Fig. 6. Comparison of various tubulointerstitial lesions in the overall series, plotted against increasing proteinuria. Serum creatinine (S_{Cr}) mounts with increasing tubular lesions, but is only above normal limits for Prot >4 g/24 h. Symbols are: (△) tubular lumenal macrophages; (●) interstitial inflammation; (◇) tubular nuclear "activation"; (■) S_{Cr}; (□) tubular pyknosis; (○) interstitial fibrosis.

similar increases have been seen in other renal conditions using another proliferation marker, Ki-67 [30]. Our study confirms these observations. However, this study equally made it apparent that PCNA+ cells constituted only a portion of the cells with features of nuclear "activation" that the cytologist associates with increased metabolic activity and protein synthesis.

This study also demonstrates that the levels of both tubact and PCNA+ tubular cells and of pyknotic cells are correlated with the level of proteinuria, and that they diminish with diminution of proteinuria. Although changes in PCNA-positivity have been demonstrated previously in puromycin aminonucleoside nephrosis (PAN) in the rat [15], this is the first demonstration that this occurs in humans, and equally the first demonstration of changes in numbers of pyknotic tubular cells with level of proteinuria. Also, as has been described in other situations [13], here the pyknotic tubular epithelial cells were more frequent than PCNA+ cells. It seems reasonable to assume, as others have proposed [13], that the altered balance between pyknosis and proliferation contributes to the advance of tubular atrophy.

Cytokeratin

Tubular AE1/AE3 staining appears to represent a more advanced stage in the progression of events from proteinuria to renal scarring. It is only poorly correlated with proteinuria, in comparison with PCNA and pyknosis, but does show good correlations with tubular atrophy and fibrosis. It seems, in fact, to be an excellent marker for tubular atrophy. Almost all overtly atrophic tubular profiles, with narrowed lumens and thickened TBMs, in all specimens examined were AE1/AE3 positive, the exceptions constituting certainly no more than 1% of total atrophic tubules. Overall, the amount of AE1/AE3 staining mounted between Bx1 and Bx2, in contrast to decline of all other immunohistochemical variables. Our impression from review of the slides is that minor degrees of cytokeratin staining are reversible, but we have too few cases with declines to demonstrate this conclusively.

Cytokeratin staining of renal tubules has not received a great deal of attention in the literature, most studies using cytokeratin markers as markers of the level of nephron in studies of renal cystic disease or renal tumors. However, Moll et al performed a detailed study of intermediate filaments in fetal, adult, and diseased kidneys in which they described the neo-expression of cytokeratins 7 and 19 in damaged and atrophic tubules at all levels of the nephron, as well as vimentin in all save distal tubules [31]. They interpreted these findings as an expression of dedifferentiation. Coexpression of vimentin and cytokeratin also has been described in damaged tubules in Balkan nephritis [32], as well as in experimental renal ischemia [33]. Relative ischemia is a common thread in a number of types of nephropathy. Diminution and disappearance of the peritubular capillary network in chronic renal disease is an old observation [34], recently resurrected [14, 35, 36]. However, in addition to simple ischemia in the relative dedifferentiation of these tubular epithelial cells, it seems likely that nonischemic inflammatory processes will be found to be involved as well.

Tubular epithelial lesions and proteinuria and serum creatinine

The present study confirms the relationships between proteinuria and tubular epithelial lesions, with their entrained interstitial inflammation and fibrosis. As seen in Tables 1 and 2, tubular epithelial lesions have a significant correlation with proteinuria at both time periods, as well as with outcome. Further, these elements show a definite increase with increasing levels of proteinuria, and at the lower levels of proteinuria this increase occurs in patients whose S_{Cr} values are still normal (Figs. 4 and 6).

However, tubular epithelial lesions, interstitial inflammation and fibrosis are even more strongly correlated with S_{Cr} than with proteinuria (Table 2). This is likely related to tubular dysfunction, either as a result of the noxious effects of proteinuria or by independent mechanisms. Tubular dysfunction occurs in many renal diseases, including SLE [37], as manifest by increased enzymuria

during the active phases [38–40], and experimentally correlates with the degree of tubular damage [38, 39].

The strong correlation between tubular epithelial lesions and S_{Cr} is of particular interest. Over the years it has been shown that in a variety of glomerular diseases, including SLE [41], impairment of renal function correlates more closely with the extent of interstitial fibrosis than with glomerular lesions [35, 42, 43]. In addition, there is an inverse correlation between S_{Cr} and the numbers of postglomerular interstitial capillaries [35], and in some diseases a direct correlation between S_{Cr} and the degree of interstitial inflammation [44].

However, our study suggests that the correlations of interstitial inflammation and interstitial fibrosis in these situations with S_{Cr} are in part secondary to their association with tubular epithelial lesions. As Table 2 reveals, interstitial fibrosis is more weakly associated with S_{Cr} at the time of biopsy and with outcome variables than are tubular epithelial lesions, tubular lumenal macrophages or interstitial inflammation. Multiple regression [21] confirms these impressions, indicating that neither glomerulosclerosis nor interstitial fibrosis is significantly associated with S_{Cr} at either biopsy, whereas tubular nuclear "activation," tubular necrosis, tubular macrophages, and interstitial inflammation are significantly associated. The only previous study to deal directly with tubular measurements found that in a variety of glomerular diseases there was a strong association between creatinine clearance and proximal and distal tubule area percentages [45].

Relative roles of tubular epithelial lesions and tubulointerstitial immune deposits

This study suggests that tubulointerstitial immune deposits play only a minor role in the development of tubular epithelial lesions, and that proteinuria is more important in the origin of the latter. First, there is no significant correlation between tubular epithelial lesions and tubulif at either biopsy, nor any association between them on multiple regression (Tables 2 and 3). Second, tubular epithelial lesions were significantly correlated with proteinuria (Table 2), and worsened with increasing proteinuria (Figs. 4 and 6) whereas tubulif was not correlated with proteinuria.

This study also suggests that tubular epithelial lesions are more important than tubulointerstitial deposits in the progression of renal disease, in that they were more strongly associated with interstitial fibrosis, particularly at Bx2 (Table 2). Further, stepwise multiple regression indicated that tubular epithelial lesions were strongly associated with interstitial inflammation at Bx1 and with interstitial fibrosis at Bx2, whereas tubulointerstitial deposits were not significantly associated with either variable at either biopsy (Table 3).

Relatively few human biopsy data are available on this subject. Park et al found no correlation between the presence of tubulointerstitial deposits and interstitial inflammation [46]. In our study, there was a significant correlation between the two, but there were numerous exceptions in both directions as indicated above. Two other studies examining immune complexes and interstitial inflammation in lupus came to the conclusion that interstitial deposition of immune complexes may play a pathogenic role in interstitial inflammatory processes, but considered that its prognostic significance is minor [20, 47]. A recent report, however, describes eight cases, most associated with lymphoproliferative disorders, in which massive tubulointerstitial deposits were associated with tubulointerstitial nephritis in non-lupus patients [48]. Thus, a potential role for tubulointerstitial deposits in lupus cannot be eliminated, but rather simply be diminished in importance.

Diminution of interstitial fibrosis

Our most recent study found that interstitial fibrosis may actually diminish in response to therapy, (presumably because of an altered balance between the enzymes responsible for degradation and their inhibitors, which would lead to its accumulation), and such cases are attended by an excellent prognosis [24]. The cases in which fibrosis diminishes show striking diminution of proteinuria and its consequent tubular lesions, whereas those cases where interstitial fibrosis increases are marked by continued high level proteinuria and tubular lesions.

Conclusions

The above data allow us to construct a rough hierarchy in the relationship between proteinuria and the various morphologic lesions (Tables 2 and 3). Tubular epithelial lesions, particularly PCNA-positivity and pyknosis, are the most closely related; inflammatory lesions, both intratubular and interstitial, are somewhat less closely related; and interstitial fibrosis and tubular atrophy, both directly and as manifest by AE1/AE3 positivity, the least closely connected. This hierarchy is in accord with the sequence of events proposed in current models of proteinuria-related renal damage [2–4]. These data support the notion that interstitial fibrosis and decline in renal function are secondary to the tubular epithelial lesions. Specifically in lupus nephritis, they suggest that the tubular epithelial lesions are primarily secondary to proteinuria and not to tubulointerstitial immune deposits, which appear to play a minor role. It may be helpful to conceive of the tubulointerstitial lesions in terms of their reversibility, with nuclear activation/proliferation and pyknosis being completely reversible, and lesions such as interstitial fibrosis (and possibly cytokeratin positivity) being potentially partially reversible. These data thus provide a missing link at the midpoint in the chain of evidence extending from experimental to clinical data, implicating proteinuria in the pathogenesis of renal failure.

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