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Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies

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Background. In recent studies performed on cultured cells and experimental nephropathies, it has been hypothesized that tubular epithelial cells (TEC), via epithelial-mesenchymal transformation (EMT), can become collagen-producing cells. According to this theory, they should proceed through several activating steps, such as proliferation and phenotype changes, to eventually synthesize extracellular matrix (ECM).

Methods. To evaluate whether EMT operates in human TECs, 133 renal biopsies of different renal diseases were studied, analyzing by immunohistochemistry and in situ hybridization the possible expression of markers of proliferation (PCNA, Mib-1), cellular phenotype (vimentin, α-SMA, cytokeratin, ZO-1) and ECM production (prolyl 4-hydroxylase, HSP47, interstitial collagens).

Results. Independently of histological diagnosis, variable degrees of TEC positivity for PCNA (2.7 ± 2.4 cells/field) and Mib-1 (1.9 ± 2.3) were present. TECs expressing vimentin (1.4 ± 4.7) and α-smooth muscle actin (α-SMA; 0.04 ± 0.4) also were detected. It was possible to observe loss of epithelial antigens from 8 to 10% of the tubular cross sections. Moreover, TECs were stained by prolyl 4-hydroxylase (3.6 ± 4.3), heat shock protein-47 (HSP47; 2.9 ± 5.4), collagen type I (0.2 ± 2.7) and type III (0.3 ± 2.0). Collagen types I and III mRNAs were found in 0.8 to 1.4 cells/field. The number of TEC with EMT features were associated with serum creatinine and the degree of interstitial damage ($P \leq 0.03$), and even considering the 45 cases with mild interstitial lesions, the tubular expression of all markers remained strictly associated with renal function ($P \leq 0.01$).

Conclusions. Our results suggest that, via transition to a mesenchymal phenotype, TEC can produce ECM proteins in human disease and directly intervene in the fibrotic processes. Moreover, the association of EMT features with serum creatinine supports the value of these markers in the assessment of disease severity.

Key words: transdifferentiation, collagen-producing cells, extracellular matrix, fibrosis, cell development, inflammatory injury.

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Epithelial-mesenchymal transformation (EMT) and, more in general, *trans*differentiation are concepts originally belonging to developmental biology and oncology [1]. Cell shifting between epithelial and mesenchymal phenotype, by turning on and off specific genes during early development, is a well recognized process that characterizes the embryonal plasticity [2]. EMT also can be a key element in development of metastasis of tumors derived from epithelial tissues; in fact, mesenchymal cells have the ability to invade and migrate through the extracellular matrix, a property not present in the epithelial lineage [3, 4]. More recent evidence for *trans*differentiation has been observed in almost all mature tissues, where the process seems to be mainly related to wound healing and fibrotic remodeling after inflammatory injury [5–8]. For instance, in the liver, it has been demonstrated that, after adequate stimulation, Ito cells lose their original function of storing vitamin A and become collagen-producing cells [9].

In the kidney, for several years interstitial fibrosis has been considered a common mechanism of disease progression [10, 11] that, at a certain point, becomes independent of the initial cause of disease and is linked to a pathological imbalance between extracellular matrix deposition and degradation, which is stimulated by a variety of cytokines and growth factors [12].

Fibroblasts are the pivotal effector cells in fibrogenesis. Originally identified through light and electron microscopy on the basis of their morphology and only more recently by fibroblast markers, they display a great heterogeneity that is not only organ-dependent but also due to the anatomic location and the microenvironment in the same organ [13]. In the kidney, Bohman identified two distinct populations in the cortex and in the inner medulla [14] that display further morphological and functional differences according to the stage of differentiation or presence of disease [15–17].

Table 1. Histological diagnosis of renal biopsies included in the study

Histological diagnosis	Number of cases
Minimal change disease	9
Membranous nephropathy	20
IgA glomerulonephritis	55
Primary FSGS	19
Membranoproliferative GN	5
Systemic lupus erythematosus	4
Diabetic glomerulosclerosis	13
ANCA-positive renal vasculitis	3
Nephroangiosclerosis	5

Given the complexity of the cell lineage, the origin of renal fibroblasts remains controversial. At present the prevailing theory favors resident interstitial cells [18], but other authors have previously postulated a fibroblast derivation from migrating leukocytes. Recent studies on cultured cells and experimental nephropathies [19–21] have hypothesized that epithelial-mesenchymal transformation can occur in tubular epithelial cells, which should then be able to migrate to the interstitium and produce extracellular matrix proteins.

To evaluate this hypothesis in humans, we used immunohistochemistry and in situ hybridization to study the expression of several markers of possible tubular phenotype transition and extracellular matrix production in 133 kidney biopsies and 10 normal kidneys.

METHODS

Kidney tissue

Kidney tissue was obtained from 133 renal biopsies of different types of renal disease and, for comparison, from 10 cadaver kidneys that could not be grafted because of vascular abnormalities (Table 1).

Tissue samples for light microscopy and in situ hybridization staining were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Routine stainings were performed according to standard techniques.

For immunofluorescence and immunoperoxidase staining the unfixed renal tissue was embedded in OCT compound (Miles Scientific, Naperville, IL, USA), snap-frozen in a mixture of isopentane and dry-ice and stored at −80°C. Subsequently, 5 μm sections were placed on slides and stored at −20°C until immunostaining.

Immunoperoxidase labeling

Tubular cells entering the cell cycle were assessed by antibodies directed against proliferating cell nuclear antigen (PCNA; Dako Italia, Milan, Italy) and Mib-1 (Ki-67; Immunotech, Marseille, France). As mesenchymal markers, monoclonal antibodies against vimentin (HistoLine; Zymed, Milan, Italy) and alpha-smooth muscle actin (α-SMA; Zymed) were used. Antibodies against the tight junction protein ZO-1 (Zymed) and cytokeratin intermediate filaments (MNF116; Dako) were applied as epithelial markers. Among various broad-spectrum antibodies against cytokeratins, MNF116 was selected because in normal human kidneys it stained all tubular cross sections. Possible tubular cell production of collagen was tested with antibodies directed against alpha-chain and beta-chain of the enzyme prolyl 4-hydroxylase (Accurate, Chemical & Scientific Corporation, Westbury, NY, USA), and HSP47 (colligin; StressGen Biotechnologies Corp, DBA Italia SRL, Milan, Italy). Moreover, since it is known by collagen IV synthesis that tubular cells intervene in tubular basement membrane formation [22], we tested possible interstitial collagen production through antibodies against interstitial collagens type I (Sigma) and type III (Monosan; Biospa, Milan, Italy). Interstitial leukocyte infiltration was identified by an antibody against leukocyte common antigen (CD45; Immunotech).

An avidin-biotin technique was used as previously described, in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules [23].

Briefly, after incubation with 0.5% avidin (Sigma) and 0.01% biotin (Sigma), to suppress endogenous avidin-binding activity, tissue sections were fixed in a methanol-H_2O_2 solution (to block endogenous peroxidase). After washing, sections were sequentially incubated with the primary antibody, with the secondary biotinylated antibody (Zymed) and with the peroxidase-labeled streptavidin (Zymed). Peroxidase activity was detected with 3,5-diaminobenzidine (DAB) (Sigma), and then sections were counterstained with Harry's hematoxylin (DDK, Milan, Italy), dehydrated and mounted in Permount (DDK). Periodic acid Schiff (PAS) counterstaining was alternatively performed to evaluate the integrity of tubular basement membrane.

Specificity of antibody labeling was demonstrated by the lack of staining after substituting phosphate buffer saline (PBS) and proper control immunoglobulins (Zymed) for the primary antibody.

Double staining

A double staining method was applied to examine the relationship between epithelial and mesenchymal antigens, using the antibody against cytokeratins coupled with mesenchymal or ECM production markers. According to the double staining procedure published by Lan et al [24], sections were treated as for the single stain from the beginning of the procedure to the DAB development, except for the use of TBS instead of PBS.

After washing, sections were then microwaved twice at 750 W for five minutes in citrate buffer. They were sequentially cooled, pre-treated with levamisole, pre-incubated with normal serum, and incubated with the second primary antibody. After washing in TBS, sections were

then labeled with a biotinylated secondary antibody (Zymed) and alkaline phosphatase-conjugated-streptavidin (Zymed). Alkaline phosphatase activity was finally detected with Fast Red (Sigma). After washing in distilled water and counterstaining with Mayer's hematoxylin (Sigma), sections were then mounted with an aqueous mounting medium (Kaiser's Glycerin Jelly; DDK). Specificity of labeling was demonstrated by the lack of staining after substituting TBS and irrelevant immunoglobulins for the first and/or second primary antibody.

In situ hybridization

To identify the cells responsible for interstitial collagen production, in situ hybridization was performed using probes specific for human collagen type I and type III.

Following the method published by Yamawaki et al, tissues were deparaffinized and rehydrated, then permeabilized with saponin-EBSS [25]. Postfixation was carried out with 4% buffered paraformaldehyde. Sections were then acetylated with a solution of acetic-anidride/trietanolamine. After washing, a prehybridization solution, containing standard sodium citrate (SSC) × 10, Denhardt's solution × 10, formamide 50%, Dextran sulfate 10% and salmon sperm DNA 200 μg/mL (all from Sigma), was applied for one hour. Oligonucleotide digoxigenin-labeled probes specific for collagen type I and type III (Roche Molecular Biochemicals, Milan, Italy) were appropriately diluted in the prehybridization solution and applied overnight in a water-saturated atmosphere. After hybridization, stringency washes were done with SSC × 4, SSC × 2–50% formamide at 45°C, and again with SSC × 1 at room temperature for 30 minutes. After incubation in peroxidase quenching solution (3% H_2O_2), specimens were incubated with anti-digoxigenin antibody fluorescein isothiocyanate (FITC)-labeled (Zymed) for one hour. Further amplification was obtained incubating sections with a horseradish peroxidase (HRP)-conjugated monoclonal anti-FITC antibody, according to manufacturer instructions (Zymed). The enzymatic reaction was finally developed with DAB (Sigma). After washing in distilled water, sections were dehydrated and mounted with Permount (DDK). Positive controls were done using digoxigenin-labeled oligos for housekeeping genes (β-actin and G6PDH) and oligo-dT (R&D Systems, Abingdon, UK).

Negative controls were made using irrelevant oligonucleotides (that is, digoxigenin-labeled oligonucleotide for rat insulin; R&D Systems) or sense oligonucleotides (Roche) and substituting the prehybridization solution for the probe solution.

Immunohistochemistry coupled to in situ hybridization

The technique was performed to simultaneously detect interstitial collagen protein and mRNA. Briefly, 5-μm thick renal sections, formalin-fixed and paraffin-embedded, were sequentially treated for in situ hybridization and immunohistochemistry as described earlier in this article for the single stain procedures. To develop the reactions obtaining different colors, sections were pretreated with levamisole, and amplification of in situ hybridization procedure was made by an alkaline phosphatase (AP)-conjugated monoclonal anti-FITC antibody (Dako). Alkaline phosphatase activity was detected with Fast Red (Sigma). At the end of immunohistochemistry, immunoperoxidase activity was detected with DAB, and then sections were not dehydrated, but mounted with an aqueous mounting medium (Kaiser's Glycerin Jelly; DDK).

Quantitative evaluation

All peroxidase-stained sections and trichrome-stained sections were evaluated by an electronic image analysis system (ETC3000, Graftek; Villanterio, Pavia, Italy). Images were digitalized using a videocamera (Kappa CF15/2, Gleichen, Germany) connected to a Leitz Diaplan microscope (Leica, Milan, Italy) and to a Pentium 120 computer (Maxwel, Rozzano, Italy) equipped with a frame grabber (Neotech Ltd, Eastleigh Hampshire, UK).

Cell count required a color threshold procedure, followed by filtering and Danielsson algorithm. Tubular positivity for proliferative, mesenchymal and ECM markers were counted as number of positive cells per field (×200). Tubular loss of cytokeratins and ZO-1 were instead assessed as percentage of tubular cross sections. Interstitial infiltration was finally evaluated in consecutive high power fields (×400), and results calculated as the number of positive cells per square millimeter.

Interstitial fibrosis evaluation, assessed on trichrome stained sections, was made by an optical threshold on the extracted hue plane followed by filtering, and was calculated as percentage of the total scanned area.

All results were automatically exported and elaborated using SPSS 9.0 program and expressed as mean and standard deviation of the mean (SPSS Inc., Chicago, IL, USA). Statistical significances ($P < 0.05$) were analyzed using the χ^2 test.

RESULTS

The results are summarized in Table 2.

In normal kidneys rare tubular epithelial cells were labeled by PCNA (0.06 ± 0.8/field) (min 0 − max 1.6), whereas none of them were found positive for Mib-1. All mesenchymal and ECM production markers were completely negative on tubular cells, which expressed cytokeratins and ZO-1 on 100% tubular cross sections. In situ hybridization for interstitial collagens did not reveal any tubular positivity.

Renal biopsies, independently of histological diagnosis, showed variable degrees of tubular positivity for all

Table 2. Immunohistochemistry and in situ hybridization results

Markers studied	Normal kidneys	Renal biopsies	*P* values			
			S_{creat}	U_{prot}	Interstitial infiltrates	Interstitial fibrosis
PCNA	0.06 ± 0.8	2.7 ± 2.4	0.002	NS	0.003	0.001
Mib-1	0	1.9 ± 2.3	0.006	NS	0.002	0.002
Vimentin	0	1.4 ± 4.7	0.03	0.003	0.003	0.003
α-SMA	0	0.04 ± 0.4	0.02	NS	0.0003	0.0003
Cytokeratin loss[a]	0	8.5 ± 12.0	0.003	NS	0.002	0.003
ZO-1 loss[a]	0	10 ± 22.2	0.006	NS	0.003	0.005
P 4-H alpha chain	0	3.6 ± 4.3	0.005	NS	0.0001	0.0001
P 4-H beta chain	0	5.6 ± 4.2	0.009	NS	0.0001	0.0001
HSP47	0	2.9 ± 5.4	0.004	NS	0.0001	0.0001
Collagen type I	0	0.2 ± 2.7	0.01	0.05	0.001	0.001
Collagen type I mRNA	0	1.4 ± 3.3	0.008	0.04	0.0007	0.0006
Collagen type III	0	0.3 ± 2.0	0.02	NS	0.005	0.006
Collagen type III mRNA	0	0.9 ± 2.7	0.009	NS	0.003	0.003

Results are evaluated as number of positive tubular epithelial cells/field (×200). Association with clinicohistological parameters are given (*P* values).
[a] Loss of epithelial antigens are calculated as percentage of tubular cross sections

markers. PCNA stained 2.7 ± 2.4 tubular epithelial cells/field (TEC/f) (min 0 − max 4.8) and Mib-1 was positive on 1.9 ± 2.3 (min 0 − max 3.9; Fig. 1). Tubular vimentin positivity was detected on 1.4 ± 4.7 TEC/f (min 0 − max 6.7), whereas α-SMA was observed on very few cells, being present on 0.04 ± 0.4 TEC/f (min 0 − max 0.9; Fig. 2). A loss of cytokeratins was found on 8.5 ± 12.0% tubular cross sections (min 0 − max 35%) and ZO-1 staining was absent from 10.0 ± 22.2% (min 0 − max 48%; Fig. 3). Labeling for prolyl 4-hydroxylase alpha chain was observed on 3.6 ± 4.3 TEC/f (min 0 − max 8.6), whereas the beta chain of the enzyme was positive on 5.6 ± 4.2 of them (min 0 − max 19.6). HSP47 was detected on 2.9 ± 5.4 TEC/f (min 0 − max 9). A variable number of tubular cells also were labeled by the antibodies raised against collagen type I (0.2 ± 2.7; min 0 − max 4.3), and collagen type III (0.3 ± 2.0; min 0 − max 3.6; Fig. 4).

Especially evident in cases with none or mild interstitial fibrosis, positivity for mesenchymal and ECM antigens and loss of epithelial properties were mainly detected in well preserved tubular structures, without signs of tubular basement membrane (TBM) disruption and possible cell migration into the interstitium, as demonstrated by PAS counterstain (Fig. 5).

By double staining, in addition to normal tubules, only cytokeratin-positive, tubules containing both cytokeratins and mesenchymal or ECM markers were observed (Fig. 6). Tubular cells stained only by mesenchymal or ECM markers were rarely found.

By in situ hybridization 1.4 ± 3.3 tubular cells/field (min 0 − max 5.6) were positive for collagen type I mRNA, and 0.9 ± 2.7 (min 0 − max 4.2) for collagen type III mRNA. Immunohistochemistry coupled with in situ hybridization for collagen type I and type III showed tubular mRNA expression not only in areas where the protein was clearly accumulated in the interstitium, but also in areas characterized by protein expression limited to the tubular basement membrane (Fig. 7).

Independently of disease diagnosis, all markers were significantly associated with some clinico-histological parameters at time of renal biopsy: serum creatinine ($P \leq 0.03$), interstitial infiltration ($P \leq 0.003$) and interstitial fibrosis ($P \leq 0.003$). The association with urinary proteins was mostly not significant.

Among the diagnostic groups, we did not observe relevant quantitative differences in the expression of all markers. Minimal change disease only was characterized by the highest percentage of cases resembling normal kidneys (8 of 9 cases, corresponding to 89%), without any sign of possible EMT in tubular cells. IgA nephritis accounted for a number of cases ($N = 55$) sufficient to perform a statistical analysis that confirmed the association of immunohistochemical and in situ hybridization results with serum creatinine and tubulointerstitial damage.

To evaluate the potential usefulness of these markers in the assessment of tubular cell alterations, independently of well established tubulointerstitial lesions, we selected among all cases 45 biopsies on the basis of their mild interstitial infiltration (less than 250 CD45 + cells/mm^2) and fibrosis (less than 15%). In this group of biopsies the expression of EMT markers was significantly associated with serum creatinine ($P \leq 0.01$).

DISCUSSION

Taken together, our results suggest that in human kidney diseases tubular epithelial cells, via their transition to a mesenchymal phenotype, by themselves can produce extracellular matrix proteins and directly intervene in fibrotic processes.

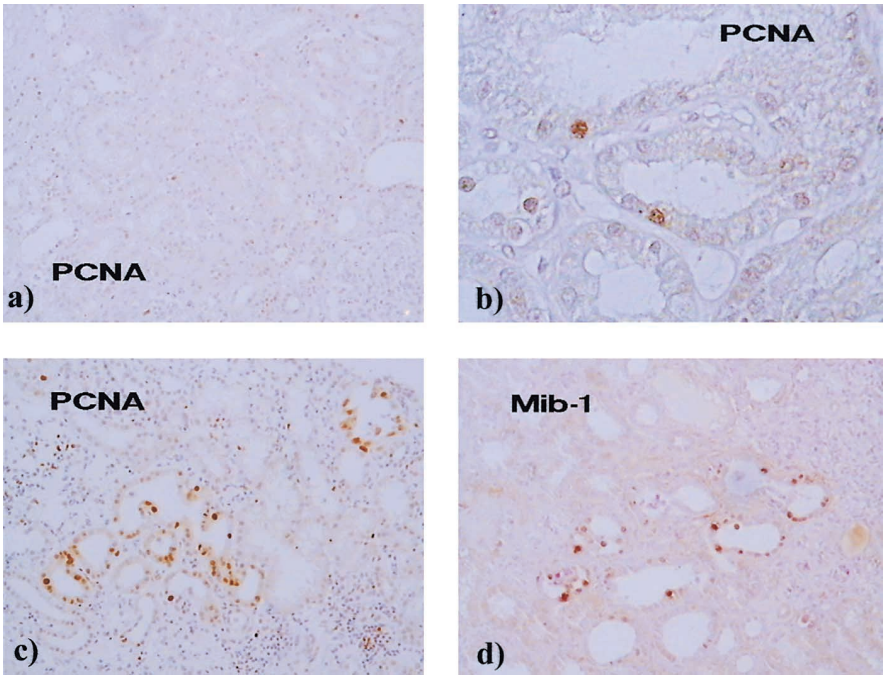


Fig. 1. Normal kidney and membranoproliferative glomerulonephritis. In a normal kidney proliferating cell nuclear antigen (PCNA) stains few interstitial cells (*a*, IPX, ×100). At higher magnification (*b*), it is evident that the few PCNA positive cells are tubular epithelial cells (IPX, ×400). Membranoproliferative glomerulonephritis in a kidney showing several tubules positive for proliferative markers, both PCNA (*c*) and Mib-1 (*d*) (IPX, ×100).

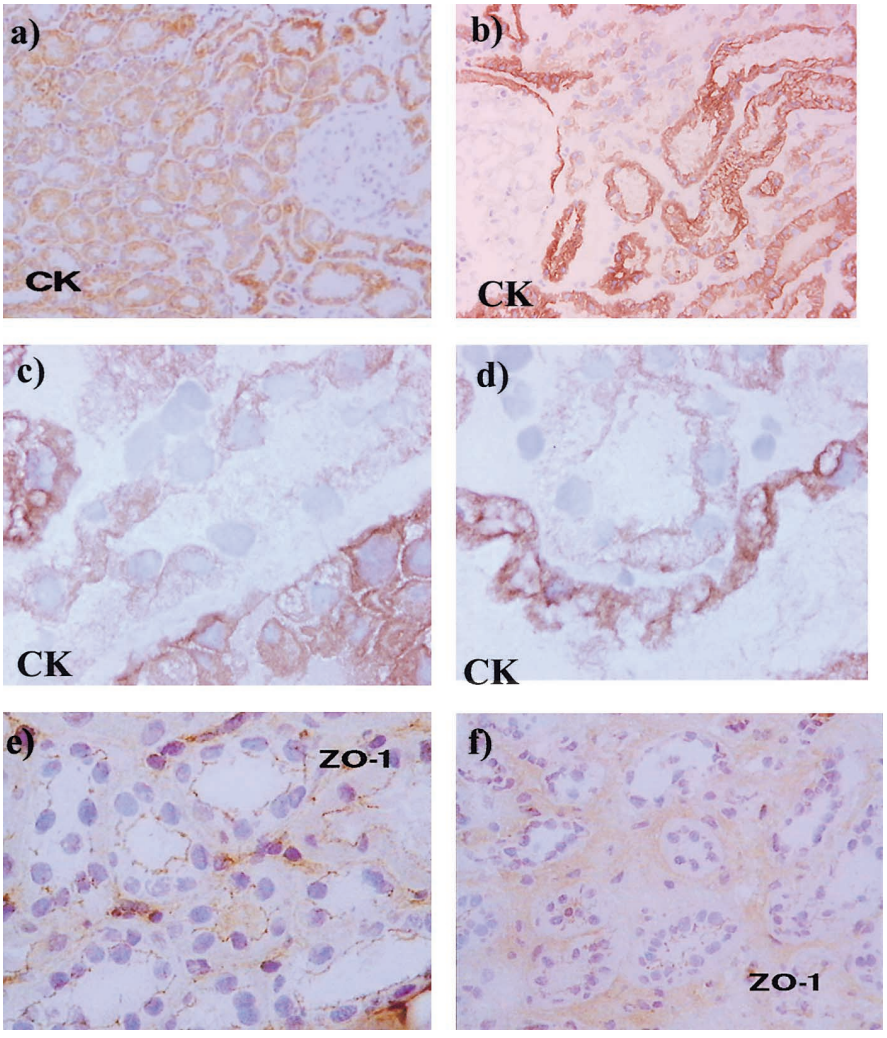


Fig. 3. Normal kidney and lupus nephritis. In a normal kidney, the anti-cytokeratin antibody MNF116 (*a*; IPX, ×100) and the tight junction protein ZO-1 (*e*; IPX, ×400) are present in all tubular cross sections. In the kidney with lupus nephritis, some tubuli do not stain for cytokeratins (*b*; IPX, ×200), and at higher magnification the loss appears disomogeneous (*c* and *d*; IPX, ×1000), while some tubular cross sections are negative for ZO-1 (*f*; IPX, ×200).

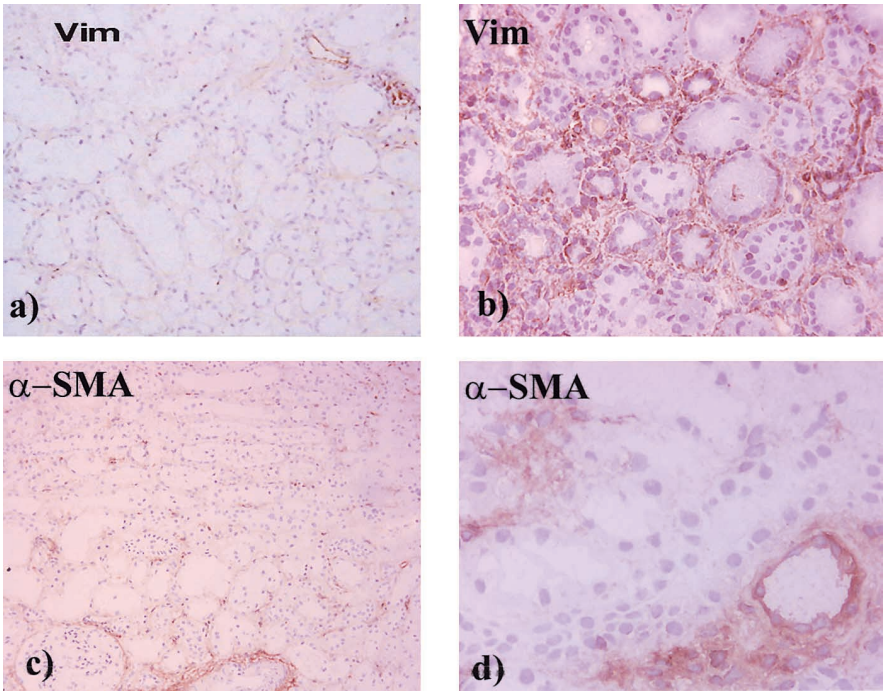


Fig. 2. Normal kidney and primary focal and segmental glomerulosclerosis. The mesenchymal markers vimentin (*a*) and α-smooth muscle actin (α-SMA) (*c*) are completely negative in tubular cells of a normal kidney (IPX, ×100). Primary focal and segmental glomerulosclerosis (*b*, *d*) where several vimentin-stained tubular epithelial cells are present (b; IPX, ×200) and a tubular cross section contains α-SMA positive cells (d; IPX, ×400).

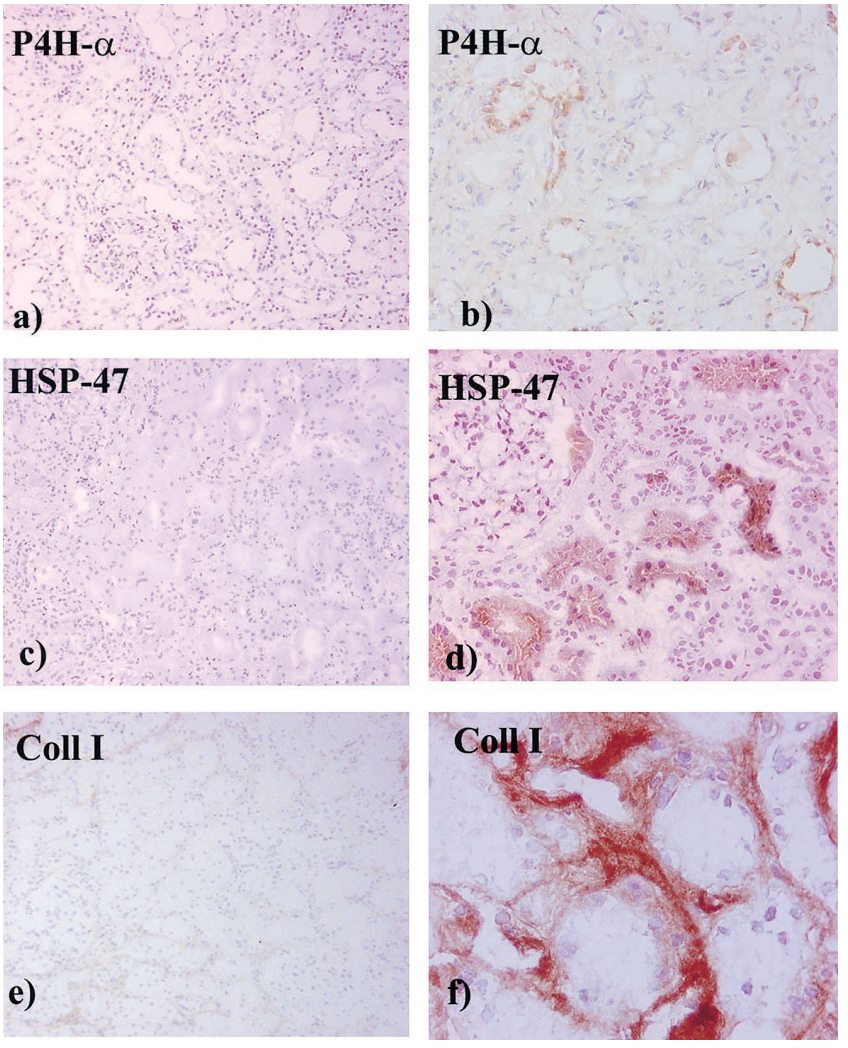
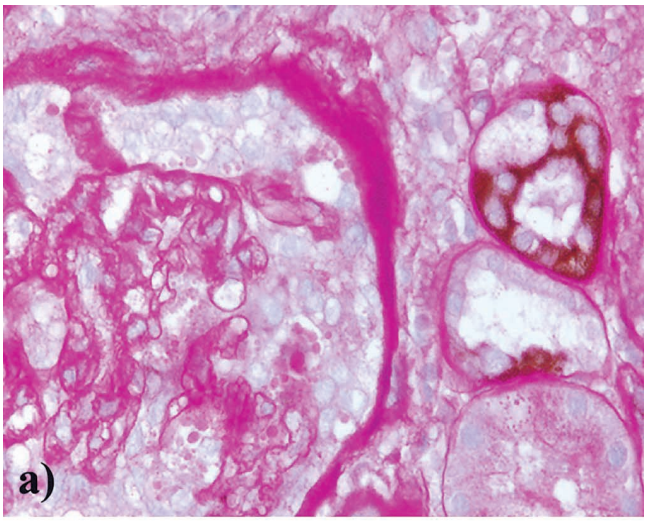


Fig. 4. Normal and membranous nephropathy kidneys. In a normal kidney, the proteins involved in collagen synthesis, prolyl 4-hydroxilase-α chain (P4H-α; *a*) and heat shock protein 47 (HSP47; *c*) are completely negative, while collagen type I does not stain tubular epithelial cells and is scarcely expressed in the interstitium (*e*; IPX, ×100). In the kidney with membranous nephropathy, several tubules appear P4H-α and HSP47 positive (*b* and *d*; IPX, ×200), while tubular cell positivity for collagen type I is associated to an increased positivity among tubules (*f*; IPX, ×400).



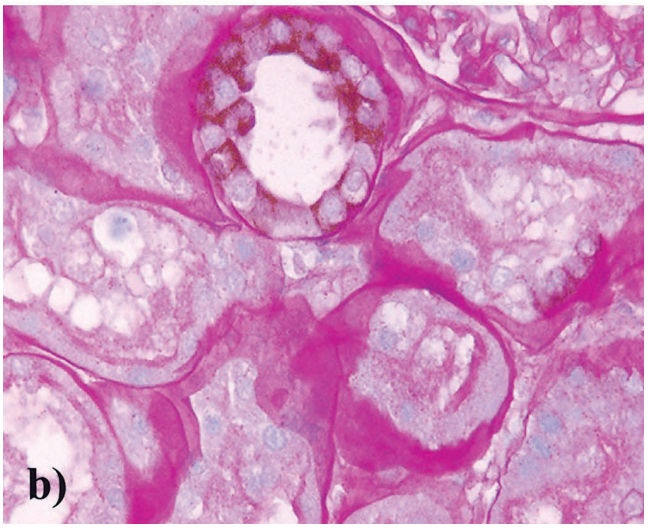


Fig. 5. Lupus nephritis. P4H-α positivity involves two tubular cross sections (*a*; IPX, PAS counterstain, ×200). It is evident, especially at higher magnification (*b*) that the tubular basement membrane surrounding the positive tubule is thickened, but not disrupted (IPX, ×400, PAS counterstain).

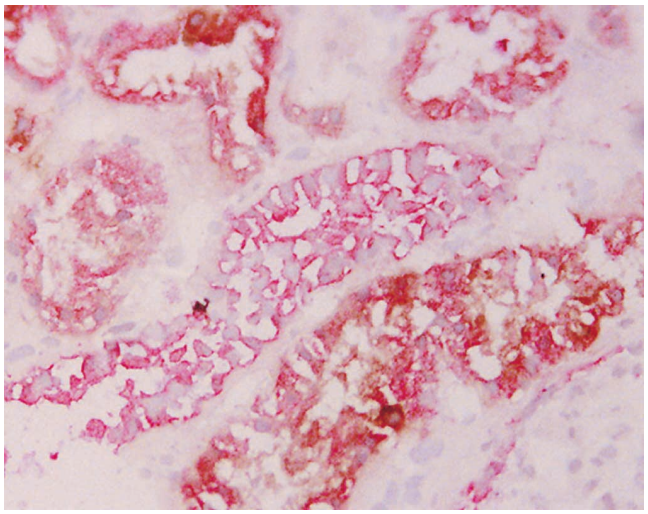


Fig. 6. Diabetic glomerulosclerosis. A tubule that is only cytokeratin positive (in red) is surrounded by several tubules double-stained by cytokeratins (in red) and P4H-α chain (in brown; IPX-AP, ×400).

For several years it has been well known that tubular epithelial cells, far from being innocent bystanders of renal damage, actively participate to the interstitial events that occur during both immunologically- and non-immunologically–mediated renal disease [26–28]. First evidence for their direct implication was obtained with the discovery that they can act as antigen presenting cells through the expression of HLA class II antigens and adhesion molecules (reviewed by Rubin-Kelley and Jevnikar) [29]. Moreover, in the last years, a growing body of evidence has documented their ability to produce a variety of chemokines, cytokines and growth factors that further contribute to interstitial inflammatory and reparative events [12, 21]. Very recently it has been hypothesized that tubular epithelial cells can *trans*differentiate to a mesenchymal phenotype, migrate to the interstitium and behave as proper fibroblasts [19–21, 30].

Generally speaking, the process of epithelial-mesenchymal transformation implies various activating steps, including proliferation, phenotype changes, and ability to produce extracellular matrix proteins [9].

Increased proliferation of tubular cells has been documented especially in culture after activating stimuli [31–33], but has been demonstrated in vivo as well [34, 35]. Interestingly, Nadasdy et al observed that a high tubular proliferation rate characterizes atrophic-appearing tubules that show a higher proliferation index than normal and damaged but non-atrophic tubular cells [35]. Our results provide evidence for an increased tubular cell proliferation in human biopsies taken from patients with different renal diseases, in which both of the antigens studied (PCNA and Mib-1) were found to be positive in a greater amount of tubules compared to normal kidneys. These aspects suggest an attempt to repair the damage, as it occurs after ischemic injury [36].

Phenotypic modulation is common among renal cells and not surprising because of the common derivation (apart from collecting duct cells) from the metanephrogenic mesenchyme. Glomerular endothelial as well as mesangial and epithelial cells, both in cultures and in animal experimental models, have been shown to undergo phenotypic changes as consequence of damage [37–39]. In the absence of specific fibroblast markers, except for FSP1 [19], which unfortunately is not specific for human fibroblasts, most authors have focused on the appearance of mesenchymal markers in tubular epithelial cells.

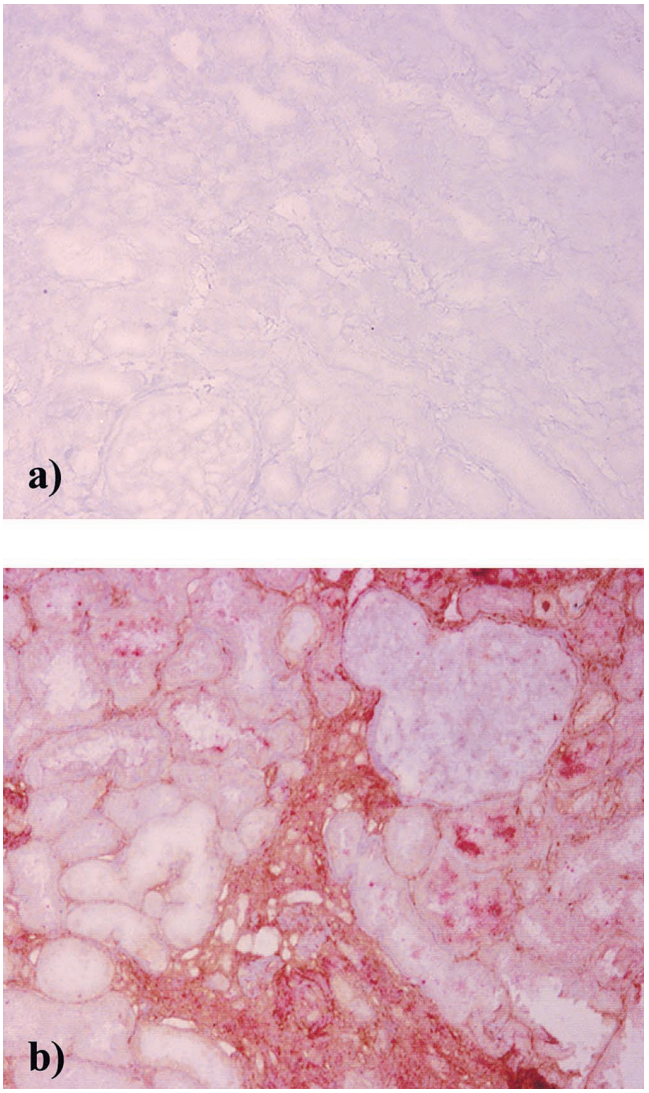


Fig. 7. Nephroangiosclerosis and IgA nephropathy. (*a*) In situ hybridization negative control with nephroangiosclerosis performed using a sense oligonucleotide for collagen type I demonstrates a complete negativity (digoxigenin-anti-digoxigenin-IPX, ×100). (*b*) IgA nephropathy shows collagen type I in situ hybridization (in red) followed by collagen type I immunohistochemistry (in brown). An interstitial area shows a strong expression of both the mRNA (in red) and the protein (in brown). Some mRNA positive tubules (in red) are present also in areas characterized by less interstitial protein accumulation, where the protein appears to stain only the tubular basement membrane (digoxigenin-anti-digoxigenin-AP/IPX, ×100).

Vimentin filaments are the hallmark of cells derived from mesenchymal tissues. Constitutively present in cultured tubular epithelial cells [40], they have been found in vivo only in diseased kidneys of both animals [41, 42] and humans [43]. In agreement with Essawy et al, who demonstrated tubular positivity for vimentin in human biopsies of kidneys with fibrotic lesions [44], we found this marker to be positive in many of our tissues.

A very rare finding, at least in our experience, was the positivity of tubular cells for α-SMA, a marker widely studied in renal pathology, whose interstitial expression currently is considered one of the best predictors of disease progression [44–47]. α-SMA characterizes the so-called myofibroblast, a cell morphologically intermediate between fibroblast and smooth muscle cell [48], and has been detected in tubular cells of 5/6-nephrectomized rats by Ng et al [49]. The authors describe tubular labeling by α-SMA increasing with time after nephrectomy and in strict correlation with tubular basement membrane disruption and signs of possible tubular cell migration toward the interstitium. However, the latest article from the same group showed that α-SMA positivity also was in intact tubules in humans [50]; our results are in complete agreement with their study.

Besides the presence of mesenchymal markers, a good criterion to evaluate possible signs of epithelial-mesenchymal transition is the disappearance of intermediate filaments of keratin type, which has been already documented in cultured mouse tubular cells [20] and recently in human biopsies [50]. Moreover, a decrease in cell-cell adhesion with the reduction of junctional proteins has been described by Healy et al [30, 51]. Our results confirm that both these events characterize tubular cells during renal damage.

In our biopsies, the presence of tubules showing only mesenchymal markers was a rare finding. The great majority of tubules with mesenchymal antigen positivity were also cytokeratin positive by double staining, confirming in our opinion the existence of different steps of phenotype transition. Previous data obtained in culture cells and experimental nephritis clearly demonstrated this concept. In fact, a transient and completely reversible phenotypic modulation was observed after exposure to a mild and transient initial insult, whereas a permanent transition was present in rat kidneys with severe progressing glomerulonephritis [38].

To our knowledge never studied in renal cells, prolyl 4-hydroxylase is a crucial enzyme for synthesis of collagen and of proteins with collagen-like sequences [52]. It catalyzes in fact the formation of 4-hydroxyproline, and not only enables the cell to collagen production, but also controls its correct release from the endoplasmic reticulum [53]. The active protein is a tetramer composed by two alpha and two beta chains, with an exceeding synthesis of beta chain that also possesses an independent disulfide isomerase activity [53, 54]. The presence of the alpha chain is essential for tetramer assembly and activity, containing both the binding and the catalytic sites. In our experience normal kidneys did not show tubular cell labeling for the alpha nor the beta chain. Instead, positivity for both the enzyme chains was evident in some tubules of most renal biopsies, and showed a larger number of cells stained by the beta chain, in agreement with the enzyme synthesis pathway. These results were supported also by a corresponding tubular positi-

vity for HSP47, recognized as a collagen specific molecular chaperone that plays a pivotal role in the biosynthesis, processing, and secretion of procollagen from endoplasmic reticulum [55].

Consistent with these findings, tubular epithelial cells were positive for interstitial collagens, both protein and mRNA. Interestingly, by in situ hybridization coupled with immunohistochemistry, we detected mRNA positive tubules not only in areas of interstitial collagen accumulation, but also in areas where the corresponding protein was only expressed by the tubular basement membrane.

In summary, although the theory of transdifferentiation implies that after phenotypic changes tubular cells migrate into the interstitium where they become proper fibroblasts, we found immunohistochemical and in situ hybridization features of *trans*differentiation also in cells belonging to still well-recognizable tubular structures, in which no apparent signs of possible migration toward the interstitium were present. These aspects were especially evident and prevalent in cases with no or only mild signs of tubulointerstitial damage, and obviously we do not exclude a possible subsequent interstitial migration of *trans*-differentiated tubular cells. Nevertheless, from our data we consider another hypothesis: after injury, some tubular cells undergo proliferation and dedifferentiation in the attempt to repair the damage, and this gives them the ability to produce not only collagen type IV, but also interstitial collagens, as has been observed not only in vitro [56–58], but also in vivo [50, 59]. It is already well known that basement membrane composition has a profound influence on epithelial cell behavior [60] and alterations of the TBM can induce tubular cell transdifferentiation [22]. For these reasons we believe that interstitial collagen deposition into the TBM, by altering its composition, can create a vicious circle that perpetuates and enhances the damage.

In our study, tubular phenotype alterations were strictly associated with the degree of renal functional impairment, with the number of interstitial leukocytes and with the extent of interstitial fibrosis independently of histological diagnosis, confirming the hypothesis that from a certain point tubulointerstitial events become independent of the cause of renal disease and lead to progression of damage [12, 27, 28, 30]. However, we found signs of EMT in cases with mild interstitial inflammatory and fibrotic damage. In these biopsies all antigens of tubular proliferation, phenotype change and ECM production remained associated with serum creatinine levels, in our opinion suggesting the value of these markers in early or at least more precise detection of disease severity.

The absence of association with urinary proteins indirectly supports the concept that quality and not quantity of proteins is important for progression of the disease [61] and our laboratory's results demonstrate a good correlation between tubular positivity for mesenchymal antigens and the excretion of proteins of a very low molecular weight, which might indicate a less preserved tubular reabsorption due to damage of the tubular epithelial cells (manuscript in preparation).

In conclusion, our study confirms previous data and indicates that phenotype variations in humans characterize tubular epithelial cells during disease, independently of the etiology. Many questions remain, especially about the timing of these changes and their possible reversibility [8, 38, 62]. Since they seem to eventually enable tubular cells to produce extracellular matrix proteins directly, they give tubular cells further centrality in the progression of renal damage.

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