Tubular Phenotypic Change in Progressive Tubulointerstitial Fibrosis in Human Glomerulonephritis

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• **There is much debate over the origins of fibroblast-type cells that accumulate in interstitial fibrosis. A controversial hypothesis, supported by data from animal and cell-culture studies, is that fibroblast-type cells can derive from tubular epithelial cells by a process of epithelial-mesenchymal transdifferentiation. However, to date, no evidence supports this postulate in human glomerulonephritis. This study sought to provide evidence that tubular epithelial cells can undergo phenotypic change toward a fibroblast-like cell in human glomerulonephritis. One hundred twenty-seven open renal biopsy specimens from patients with minimal change disease (MCD), immunoglobulin A (IgA) nephropathy, and rapidly progressive glomerulonephritis (RPGN) were examined for tubular phenotypic change by two-color immunohistochemistry using the criteria of de novo expression of α-smooth muscle actin (α-SMA), a myofibroblast marker; loss of the epithelial marker cytokeratin; and collagen production. In normal human kidney and MCD, tubular epithelial cells expressed cytokeratin with no evidence of α-SMA staining. However, in 36 of 90 cases of IgA nephropathy and 9 of 18 cases of RPGN, small numbers of tubular epithelial cells in areas of fibrosis showed de novo α-SMA expression, accounting for 0.4% ± 0.2% (IgA nephropathy) and 3.8% ± 1.5% (RPGN) of cortical tubules. An intermediate stage of phenotypic change was observed in some cuboidal epithelial cells that expressed both cytokeratin and α-SMA. Tubules containing α-SMA–positive (α-SMA$^+$) cells also stained for collagen types I and III, suggesting that tubular cells undergoing phenotypic change have an active role in the fibrotic process. There also was a marked increase in transforming growth factor-β1 (TGF-β1) tubular expression in areas with interstitial fibrosis, including tubules with phenotypic change. There was a highly significant correlation between tubular α-SMA expression and interstitial fibrosis, interstitial α-SMA$^+$ myofibroblast accumulation, deposition of collagen types I and III, tubular TGF-β1 expression, and renal dysfunction. In conclusion, this study provides evidence that tubular epithelial cells can undergo phenotypic change toward a myofibroblast-like phenotype on the basis of de novo α-SMA expression, loss of cytokeratin, and de novo collagen staining. These data, although not conclusive, provide the first support for the hypothesis that transdifferentiation of tubular epithelial cells has a role in progressive renal fibrosis in human glomerulonephritis.**

INDEX WORDS: Glomerulonephritis; transdifferentiation; epithelial; myofibroblast; fibrosis; α-smooth muscle actin (α-SMA); cytokeratin; transforming growth factor-β (TGF-β).

TUBULOINTERSTITIAL fibrosis is believed to be the common final pathway leading to end-stage renal failure in glomerulonephritis.[1] Renal fibroblasts are a heterogeneous population that is difficult to define in situ because of the lack of a specific panfibroblast antigen. Most studies of renal fibrosis have focused on a subset of fibroblasts called myofibroblasts, defined by their expression of α-smooth muscle actin (α-SMA).[1,2] Myofibroblasts are a major site of synthesis of such matrix molecules as collagen types I and III in fibrotic lesions in the kidney and fibrotic changes in other tissues,[2-6] although it should be remembered that not all matrix-producing cells are myofibroblasts. There is excellent correlation between the appearance of interstitial α-SMA–positive (α-SMA$^+$) myofibroblasts and the development of interstitial fibrosis in human and experimental glomerulonephritis, and interstitial α-SMA immunostaining is the best prognostic indicator of disease progression.[3,4,7-17]

A crucial issue for our understanding of how interstitial fibrosis develops is knowing the origin of interstitial myofibroblasts. The conventional view is that these fibroblast-type cells originate by differentiation of resident renal fibro-

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blasts or migration of pericytes or smooth muscle cells into the interstitium.[18] However, another possibility exists: the controversial hypothesis that tubular epithelial cells transdifferentiate into myofibroblasts. Epithelial-mesenchymal transition occurs in fetal development and some pathological states,[19-22] but there is relatively little evidence to support such a mechanism in renal fibrosis. Phenotypic and morphological evidence that tubular epithelial cells can transdifferentiate into fibroblast-like cells comes from cell-culture studies[23-25] and studies of mouse and rat models of kidney disease.[26-29] However, to date, no data support the hypothesis that tubular epithelial-myofibroblast transdifferentiation has a role in interstitial fibrosis in human glomerulonephritis. To this end, we sought evidence that tubular epithelial cells can undergo phenotypic change toward a myofibroblast-type cell in biopsy tissue from patients with glomerulonephritis.

METHODS

Patients

Open renal biopsy specimens from 127 patients from the Department of Nephrology, Tokai University School of Medicine (Isehara, Japan), were examined on this study. The study was approved by the University Review Board. Disease category was based on histological examination of biopsy specimens. The cohort consisted of 19 cases of minimal change disease (MCD), 90 cases of immunoglobulin A (IgA) nephropathy, and 18 cases of rapidly progressive glomerulonephritis (RPGN; Table 1). Patients with IgA nephropathy were graded according to the histological classification of Clarkson et al[30] and divided into two groups. Group 1 included grades I (minimal alteration) and II (pure mesangial proliferation). Group 2 included grades III (focal and/or diffuse mesangial proliferation and <50% crescents) and IV (focal and/or diffuse mesangial proliferation and 50% to 70% crescents). Data for serum creatinine level, creatinine clearance, and total urinary protein excretion were obtained at the time of biopsy (Table 1). Normal human kidney was obtained from the uninvolved pole of carcinoma nephrectomies (4 cases) and unmatched donor organs (2 cases) from the Department of Nephrology, Monash Medical Centre (Clayton, Australia).

Histological Evaluation

Biopsy tissue was fixed in 10% neutral buffered formalin and sections (2 μm) were stained with hematoxylin and eosin or periodic acid–Schiff (PAS). The percentage of cortical tubulointerstitium showing fibrosis was scored on PAS-stained sections by point-counting on coded slides.[27]

Antibodies

The following antibodies were used in this study: mouse monoclonal antibody (mAb) 1A4 that specifically recognized the smooth muscle α-actin isoform (Sigma Chemical Co, St Louis, MO), pan cytokeratin mouse mAb AE1/AE3 (Dako Corp, Carpenteria, CA), mouse antihuman collagen type I mAb I-8H5 (Fuji Chemical Industries, Toyama, Japan), mouse antihuman collagen type III mAb III-53 (Fuji Chemical Industries), and rabbit antihuman transforming growth factor-β1 (TGF-β1; Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls used isotype-matched mouse mAb or normal rabbit IgG. In addition, peroxidase-conjugated goat antimouse immunoglobulin, mouse peroxidase-conjugated antiperoxidase complexes (PAPs), alkaline phosphatase–conjugated goat antimouse and goat antirabbit immunoglobulins, and mouse and rabbit alkaline phosphatase–conjugated antialkaline phosphatase complexes (APAAPs) were purchased from Dakopatts (Glostrup, Denmark).

Immunohistochemistry

Immunohistochemistry staining for α-SMA and collagen types I and III used a three-layer PAP method, as previously described.[27,31] Paraffin sections (4 μm) were dewaxed. For collagen staining, sections were treated with 10 minutes of microwave oven heating in 400 mL of 0.01 mol/L of sodium citrate, pH 6.0, at 2,450 MHz and 800 W, followed by incubation with 0.05% trypsin for 30 minutes at 37°C. Sections then were preincubated for 20 minutes in 10% fetal calf serum (FCS) and 10% normal goat serum to block nonspecific binding, incubated with a mouse mAb for 60

Table 1. Patient Renal Function Data

	Serum Creatinine (mg/mL)	Creatinine Clearance (mL/min)	Urinary Protein (g/day)
MCD (n = 19)	0.72 ± 0.31	90.7 ± 28.2	4.0 ± 4.0
IgA nephropathy group 1 (n = 40)	0.79 ± 0.18	89.3 ± 23.4	0.31 ± 0.33*
IgA nephropathy group 2 (n = 50)	1.59 ± 1.39†	57.0 ± 28.3*	1.55 ± 1.48*†
RPGN (n = 18)	4.32 ± 2.86‡§‖	23.4 ± 22.5*§‖	1.88 ± 1.98†‡

NOTE. Data expressed as mean ± SD.
*$P < 0.001$ versus MCD.
†$P < 0.05$ versus IgA nephropathy group 1.
‡$P < 0.01$ versus MCD by analysis of variance.
§$P < 0.001$ versus IgA nephropathy group 1 by analysis of variance.
‖$P < 0.001$ versus IgA nephropathy group 2 by analysis of variance.

minutes, and washed three times in phosphate-buffered saline. After inactivating endogenous peroxidase in 0.3% hydrogen peroxide in methanol, sections were labeled sequentially with peroxidase-conjugated goat antimouse IgG and mouse PAP, followed by development with diaminobenzidine to produce a brown color. Sections were then counterstained with PAS and mounted.

Immunostaining for cytokeratin and TGF-β1 used a three-layer APAAP method. This was performed alone or after a three-layer PAP stain, described previously. Briefly, sections were microwaved for 10 minutes. Sections then were preincubated in block solution as described previously and incubated with a mouse or rabbit antibody for 60 minutes, followed by alkaline phosphatase–conjugated goat antimouse or goat antirabbit IgG and mouse or rabbit APAAP. Finally, sections were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Roche Diagnostics Australia Pty Ltd, Castle Hill, NSW, Australia) to produce a dark blue/purple color. Sections were counterstained with PAS without hematoxylin and coverslipped in an aqueous mounting medium. Negative controls included the use of irrelevant isotype-matched antibodies in the first or second round of staining. In some cases, serial sections were stained with individual antibodies and compared with double staining.

Quantitation of Immunohistochemistry

Tubules were identified by morphological characteristics on the basis of PAS staining of the tubular basement membrane. Tubular expression of α-SMA was defined as containing one or more α-SMA$^+$ cells and expressed as a percentage of total cortical tubules (500 to 1,000 cells) examined. Interstitial α-SMA$^+$ myofibroblasts were counted in high-power fields (original magnification $\times$400) covering the entire cortical interstitium using a 0.02-mm^2 graticule fit in the eyepiece of the microscope, and data are expressed as α-SMA$^+$ cells per square millimeter. The area of positive staining for collagen types I and III in the interstitium was determined by point counting and expressed as a percentage of interstitial involvement. Tubular staining of TGF-β1 was assessed in a semiquantitative fashion as follows: 0, no staining; 1, less than 10% of tubules stained; 2, 10% to 25% of tubules stained; 3, 25% to 50% of tubules stained, and 4, greater than 50% of tubules stained. All scoring was performed on coded slides.

Statistical Analysis

Data from quantitation of immunohistochemistry staining was compared by one-way analysis of variance using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Correlation analysis for parametric data used Pearson's single correlation coefficient, whereas analysis of nonparametric data used Spearman's correlation coefficient.

RESULTS

Normal Human Kidney

Immunohistochemistry staining identified the presence of α-SMA protein in smooth muscle cells of blood vessels in normal human kidney (Fig 1A). No staining of tubular epithelial cells or glomeruli was apparent in normal human kidney.

Tubular Phenotypic Change in Human Glomerulonephritis

Tubular phenotypic change was examined on the basis of de novo expression of myofibroblast marker, α-SMA. No tubular expression of α-SMA was detected in the 19 cases of MCD (Fig 2B). However, de novo expression of α-SMA protein was identified in small numbers of tubules in 36 of 90 cases of IgA nephropathy and 9 of 18 cases of RPGN. Tubular α-SMA expression was restricted to areas of tubular damage and fibrosis. Both intact and damaged tubules showed α-SMA staining. Figure 1(B) shows α-SMA staining of large cuboidal tubular epithelial cells in an area of tubulointerstitial damage with mild fibrosis. This α-SMA expression in tubules clearly is caused by large cuboidal epithelial cells, rather than the possibility of interstitial α-SMA$^+$ myofibroblasts infiltrating into the tubule (Fig 1B). Tubular α-SMA expression was an infrequent event. Quantitation of the percentage of tubules expressing α-SMA is shown in Fig 2 and compared with the degree of interstitial fibrosis and number of interstitial α-SMA$^+$ myofibroblasts.

A second way to examine tubular phenotypic change is to look for the loss of a specific epithelial marker, such as cytokeratin. Figure 1C shows that cytokeratin is expressed by all tubules in normal human kidney. Double immunostaining provided clear evidence of tubular phenotypic change. Figure 1D through F shows the presence of tubular cells that express both cytokeratin and α-SMA and tubular cells that express α-SMA but have lost cytokeratin expression. The presence of cuboidal epithelial cells expressing both markers represents an intermediate stage in the process of phenotypic change. Such double staining was usually present in epithelial cells that maintained a cuboidal appearance with relatively normal tubular morphological characteristics (Fig 1D). Both cuboidal epithelial cells and flattened epithelial cells that expressed α-SMA but lacked cytokeratin were seen (Fig 1D through F). The flattened rim of elongated cells in damaged tubules that express α-SMA, but not cytokeratin, suggests a change to a mesenchymal phenotype (Fig 1D).

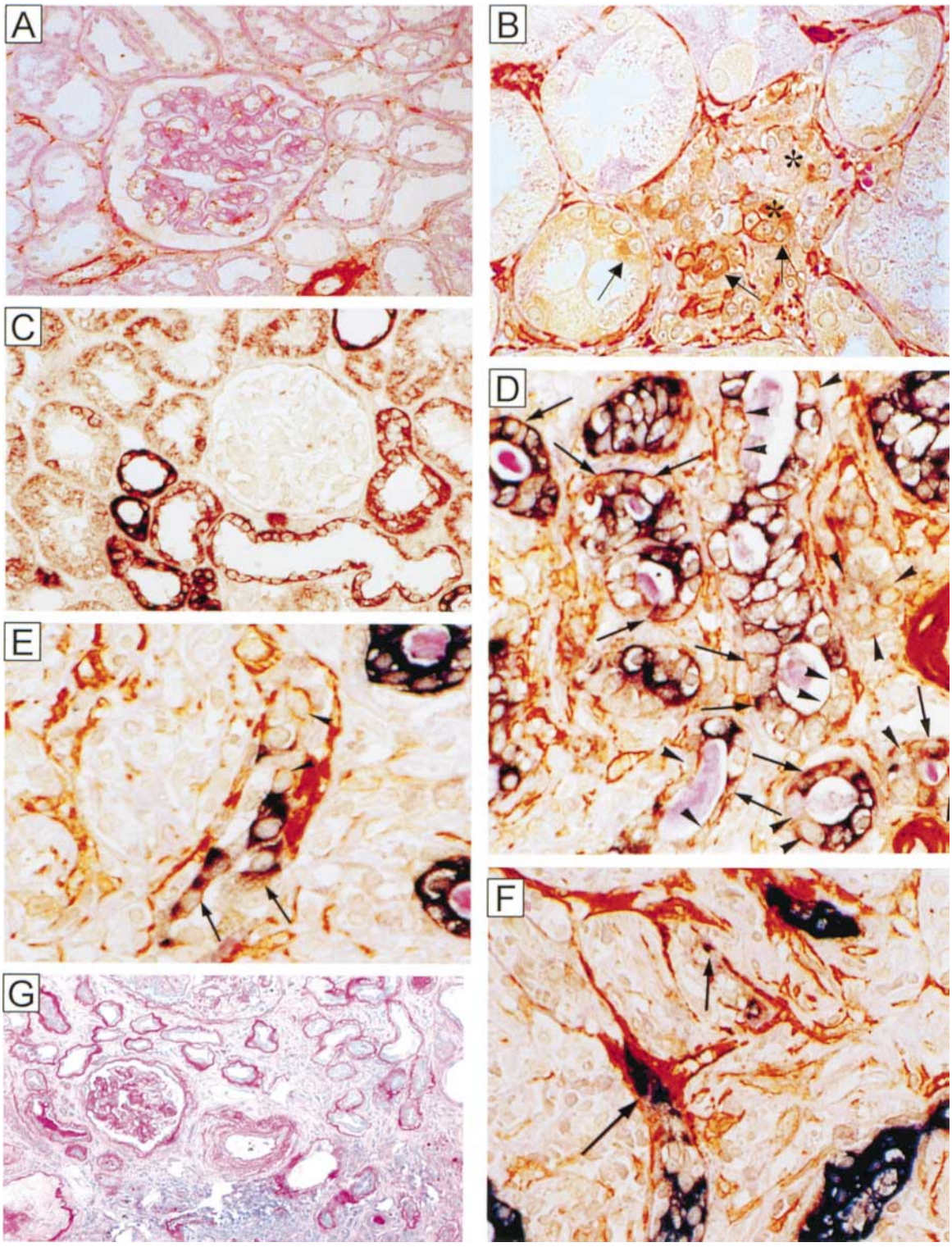

Fig 1.

Collagen Production by Tubular Epithelial Cells in Human Glomerulonephritis

A functional characteristic of myofibroblasts is the production of extracellular matrix proteins, particularly collagen types I and III.[3-5] Weak immunohistochemistry staining of collagen types I and III was seen in the interstitium in normal human kidney, but this was completely absent from tubular epithelial cells (not shown). An increase in interstitial deposition of collagen types I and III was evident in IgA nephropathy and RPGN (Fig 2). In addition, some tubules in areas with marked interstitial collagen deposition contained small numbers of epithelial cells that showed strong staining for collagen types I and III. Figure 3A shows a tubule in an area of fibrosis in which several α-SMA$^+$ cells are present. Sections from the same tubule show the presence of cells positively stained for collagen type I and type III (Fig 3B and C).

Tubular TGF-β Production

The growth factor TGF-β1 has been implicated in the process of interstitial fibrosis.[32,33] TGF-β1 was detected in a small number of tubules in MCD and was upregulated significantly in IgA nephropathy and RPGN (Fig 2F). Areas of severe fibrosis showed strong tubular TGF-β1 expression. Strong TGF-β1 staining of a tubule that contains α-SMA$^+$ epithelial cells is shown in Fig 3A and D.

Correlation of Tubular α-SMA Expression With Renal Function and Histological Damage

There was a highly significant correlation between the percentage of cortical tubules showing de novo α-SMA expression and renal dysfunction and interstitial fibrosis, including interstitial α-SMA$^+$ myofibroblasts, tubular expression of TGF-β1, and interstitial deposition of collagen types I and III (Table 2). A highly significant correlation was also evident between the number of interstitial α-SMA$^+$ myofibroblasts, interstitial collagen deposition, renal dysfunction, and interstitial fibrosis (Table 2).

DISCUSSION

This study has identified phenotypic change in tubular epithelial cells in areas of tubular damage and fibrosis in human glomerulonephritis. This is based on immunohistochemistry staining showing de novo expression of the mesenchymal marker α-SMA and loss of the epithelial marker cytokeratin by cuboidal tubular epithelial cells. In addition, these phenotypic changes were associated with tubular collagen production.

Although transdifferentiation of epithelial cells into mesenchymal cells has been described in fetal development and a number of pathological conditions,[19-22] there is little evidence to support the occurrence of tubular epithelial cell transdifferentiation into fibroblast-like cells in kidney disease. This is a difficult hypothesis to prove for several reasons. First, should tubular epithelial cells transdifferentiate into fibroblast-like cells and then migrate into the interstitium, it is no longer possible to identify such cells as having a tubular origin. Second, areas of interstitial fibrosis contain many fibroblasts in very close proximity to tubular epithelial cells. Therefore, it is difficult to distinguish between interstitial fibroblasts that have entered damaged tubules from

Fig 1. Immunohistochemistry staining showing tubular phenotypic changes in human glomerulonephritis. (A) Normal human kidney showing α-SMA expression (red/brown) in vascular smooth-muscle cells of an artery, but no staining is seen in tubules or glomeruli. (B) IgA nephropathy group 1 showing a focal area of tubulointerstitial fibrosis. Note the presence of α-SMA$^+$ cells within the damaged area (*) that have cuboidal epithelial-like morphological characteristics (arrows), representing an early stage of tubular phenotypic change. (C-F) Sections were double stained for the myofibroblast marker α-SMA (red/brown) and the epithelial marker cytokeratin (blue/purple). (C) Normal human kidney showing cytokeratin staining in all tubular epithelial cells, whereas glomeruli are negative. (D) IgA nephropathy group 2 showing a focal area of tubulointerstitial fibrosis. An intermediate stage of phenotypic change can be seen in tubular epithelial cells coexpressing cytokeratin and α-SMA (arrows). Tubular epithelial cells that have lost cytokeratin expression and gained α-SMA expression are shown by arrowheads. This includes tubular epithelial cells with a cuboidal appearance and tubules with a flattened appearance suggestive of epithelial differentiation into a myofibroblast-like phenotype. (E, F) RPGN showing areas of severe tubular fibrosis. Several damaged tubules show colocalization of cytokeratin and α-SMA staining (arrows), indicating an early stage in phenotypic change, whereas other α-SMA$^+$ tubular epithelial cells can be seen that have lost cytokeratin staining (arrowheads). (G) No staining is seen in an area of tubular damage using an isotype-matched negative control antibody. Sections were counterstained with PAS (minus hematoxylin). (Original magnification [A, C, G] ×100, [B, D, E] ×250, [F] ×400.)

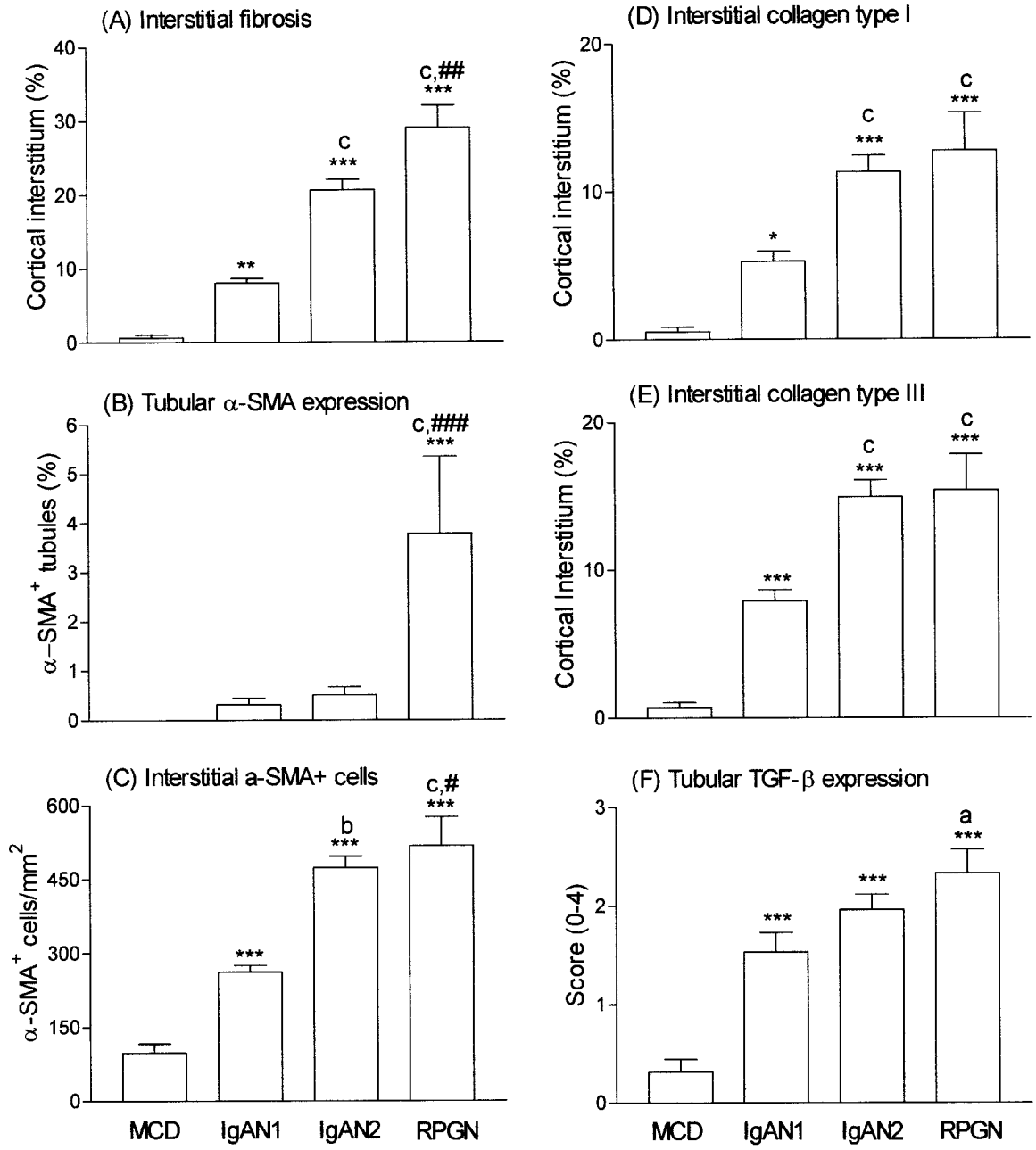

Fig 2. Quantitation of tubular α-SMA expression and interstitial fibrosis in human glomerulonephritis. (A) Cortical interstitial fibrosis. (B) Percentage of tubules with α-SMA staining. (C) Interstitial α-SMA+ myofibroblasts. (D) Interstitial deposition of collagen type I. (E) Interstitial deposition of collagen type III. (F) Semiquantitative score of tubular TGF-β1 expression. Data expressed as mean ± SEM. Abbreviations: IgAN1, IgA nephropathy group 1; IgAN2, IgA nephropathy group 2. *$P < 0.05$ versus MCD. **$P < 0.01$ versus MCD. ***$P < 0.001$ versus MCD. [a]$P < 0.05$ versus IgA nephropathy group 1. [b]$P < 0.01$ versus IgA nephropathy group 1. [c]$P < 0.001$ versus IgA nephropathy group 1. [#]$P < 0.05$ versus IgA nephropathy group 2. [##]$P < 0.01$ versus IgA nephropathy group 2. [###]$P < 0.001$ versus IgA nephropathy group 2. *P* by analysis of variance with (A-E) Tukey's multiple comparison test for parametric data and (F) Kruskal-Wallis test with Dunn's multiple comparison for nonparametric data.

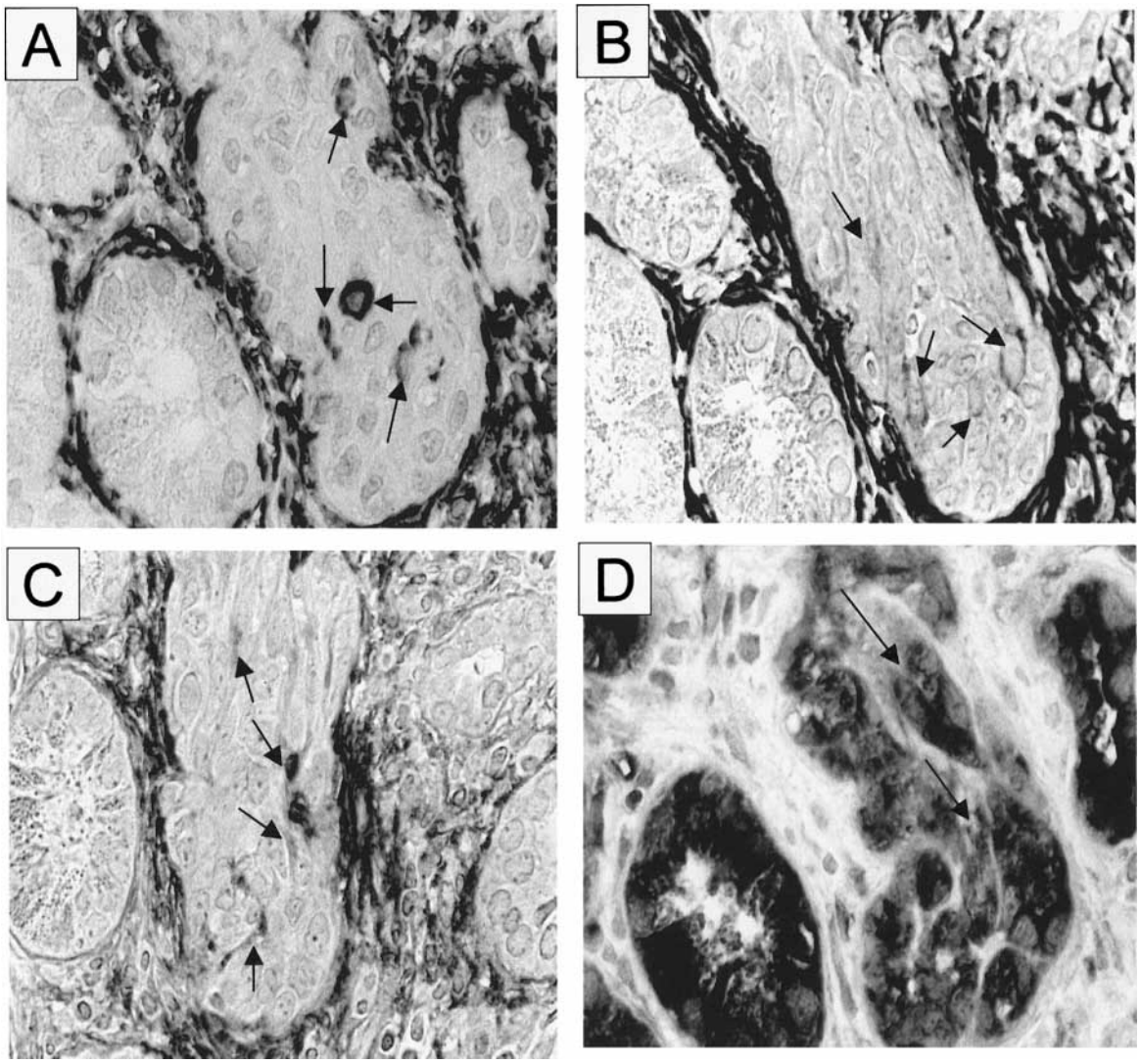

Fig 3. Immunostaining of serial sections showing colocalization of tubular α-SMA, collagen, and TGF-β1 in an area of fibrosis in RPGN. (A) De novo α-SMA expression by tubular epithelial cells indicating phenotypic change (arrows). Production of collagen types I (B) and III (C) by cells with elongated morphological characteristics (arrows) in close association with α-SMA$^+$ cells seen in (A). (D) TGF-β1 staining of tubular epithelial cells in this area of fibrosis. Note that some tubular epithelial cells show elongated morphological characteristics (arrows). (Original magnification $\times$400.)

tubular epithelial cells that may have become fibroblasts. The most readily identifiable step in a process of epithelial-mesenchymal transdifferentiation would be early phenotypic changes that occur while the tubular epithelial cell still maintains its distinctive cuboidal appearance and before tubular integrity is lost. This phenotypic change was identified in the current study by double immunostaining for α-SMA and cytokeratin. A key aspect of this identification was the use of PAS counterstaining to identify tubular basement membrane. This allowed a clear distinction between α-SMA staining of intact tubules from the closely associated interstitial α-SMA myofibroblasts. This is probably the reason, together with the infrequent occurrence of such cells, that tubular α-SMA staining has not been reported in previous studies of human glomerulonephritis.

Although phenotypic changes observed in the

Table 2. Correlation Analysis of Tubular α-SMA Expression and Renal Function and Histological Damage

	Serum Creatinine (mg/mL)	Creatinine Clearance (mL/min)	Urine Protein (g/d)	Interstitial Fibrosis (%)	α-SMA$^+$ tubules (%)	Interstitial α-SMA$^+$ (cell/mm^2)	Tubular TGF-β1 (0-4)	Interstitial Collagen I (%)	Interstitial Collagen III (%)
α-SMA$^+$ tubules (%)	0.419	−0.294	−0.01	0.469	—	0.317	0.262	0.45	0.41
P	<0.0001	0.0011	NS	<0.0001	—	0.0003	0.0029	<0.0001	<0.0001
Interstitial α-SMA$^+$ (cells/mm^2)	0.510	−0.519	−0.063	0.692	0.317	—	0.381	0.516	0.612
P	<0.0001	<0.0001	NS	<0.0001	0.0003	—	<0.0001	<0.0001	<0.0001

NOTES. Results shown as correlation coefficient. All analyses used Pearson's correlation coefficient, except for tubular TGF-β and FGF-2, which used Spearman's correlation coefficient. Analysis was performed on results from the entire 127 patients.

Abbreviation: NS, not significant.

current study are not de facto proof of epithelial-mesenchymal transdifferentiation in human glomerulonephritis, they support the hypothesis that transdifferentiation has a role in interstitial fibrosis in glomerulonephritis. A key functional characteristic of myofibroblasts is collagen production. The association of collagen production and phenotypic change in tubular epithelial cells is consistent with the process of transdifferentiation, although again, this does not constitute proof of transdifferentiation. However, phenotypic changes observed in this study of human glomerulonephritis are important because they validate the relevance of animal studies in which evidence of tubular epithelial-mesenchymal transdifferentiation has been presented. Studies of mouse models of antitubular basement membrane disease and polycystic kidney disease have shown phenotypic evidence of epithelial-mesenchymal transdifferentiation based on tubular epithelial cell expression of fibroblast-specific protein-1.[26,29] We observed tubular epithelial-myofibroblast transdifferentiation in the rat remnant kidney based on de novo expression of α-SMA by tubular epithelial cells and morphological characteristics with the presence of characteristic actin microfilaments and dense bodies within tubular epithelial cells with intact tight junctions.[27] These results were confirmed in a separate study of the rat remnant kidney.[28] In addition, differentiation of cultured tubular epithelial cells into fibroblast-like cells has been reported.[23-25]

What factors regulate this phenotypic change of tubular epithelial cells? TGF-β1 has been implicated in promoting renal fibrosis.[32,33] The current study showed that tubular α-SMA staining was associated with tubular TGF-β1 expression. There was a highly significant correlation between tubular TGF-β1 expression and de novo α-SMA expression in tubular epithelial cells. Although these results do not prove that TGF-β1 induces tubular phenotypic change, they are consistent with cell-culture studies showing that TGF-β1, either alone or in combination with epithelial growth factor (EGF), can induce mesenchymal transdifferentiation of tubular epithelial cell lines.[23,24]

In summary, this study provides the first evidence that tubular epithelial cells can undergo phenotypic change toward a myofibroblast-like cell during progressive renal fibrosis in human glomerulonephritis on the basis of de novo α-SMA expression, loss of cytokeratin, and de novo collagen staining. These events were restricted to areas of fibrosis. In particular, identification of cuboidal tubular epithelial cells coexpressing α-SMA and cytokeratin is believed to represent the earliest stage of phenotypic change in tubular epithelial cells. Although not conclusive, these studies support the hypothesis that tubular epithelial-myofibroblast transdifferentiation has a role in progressive interstitial fibrosis in human glomerulonephritis.

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