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Proximal tubular cells promote fibrogenesis by TGF-β1–mediated induction of peritubular myofibroblasts

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Proximal tubular cells promote fibrogenesis by TGF-β1–mediated induction of peritubular myofibroblasts.

Background. In proteinuric nephropathies with increasingly severe defects of the glomerular filtering barrier, interstitial fibrogenesis is a major effector of scarring. An early event in this process is the peritubular accumulation of myofibroblasts that express α-smooth muscle actin (α-SMA) and contribute to abnormal matrix production. Common trigger factors are poorly understood. Enhanced protein trafficking may play a role by up-regulating inflammatory and fibrogenic genes in proximal tubular cells.

Methods. The remnant kidney model in rats was used to (*1*) analyze interactions between activated proximal tubular cells, peritubular cells expressing the myofibroblast marker, and inflammatory cells at time intervals (days 7, 14, and 30) after surgery, and (*2*) evaluate the effects of angiotensin-converting enzyme inhibitor (ACEi) on protein trafficking, fibrogenic signaling, and α-SMA expression.

Results. Abnormal uptake of ultrafiltered proteins by proximal tubular cells (IgG staining) occurred at an early stage (day 7) and was subsequently associated with macrophage and α-SMA+ cell accumulation into the peritubular interstitium. α-SMA+ cells clustered with macrophages into the interstitium. These changes were associated with appearance of transforming growth factor-β1 (TGF-β1) mRNA in proximal tubular cells and in the infiltrating cells with time. At day 30, focal α-SMA staining also was found in the tubular cells and in peritubular endothelial cells on semithin ultracryosections. ACEi prevented both proteinuria and abnormal protein accumulation in tubular cells, as well as the inflammatory and fibrogenic reaction with peritubular α-SMA expression.

Conclusions. Profibrogenic signaling from both proximal tubular cells on challenge with filtered protein and inflammatory cells is implicated as a key candidate trigger of progressive tubulointerstitial injury.

Key words: proteinuria, α-smooth muscle actin, macrophage, fibrosis, angiotensin converting enzyme inhibitor, progressive renal disease, tubulointerstitial injury.

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In chronic nephropathies with increasingly severe dysfunction of the glomerular filtering barrier, interstitial inflammatory and fibrogenic events are important components of injury leading to end-stage kidney. Proteins ultrafiltered in excessive amounts may exert renal toxicity as a consequence of virtually any kind of persistent insult or damage to the barrier [1]. Highlights on the mechanisms underlying tubulointerstitial damage have been obtained using polarized proximal tubular cells as a model to examine cellular responses to the apical challenge to proteins. Exposing the cells to delipidated albumin, immunoglobulins (IgG) or transferrin enhanced the rate of synthesis of endothelin-1 in a concentration dependent fashion [2]. Similarly, albumin and transferrin up-regulated the monocyte chemoattractant protein 1 gene (MCP-1), an event abrogated by inhibition of protein uptake with lysine [3]. RANTES (regulated upon activation, normal T cell expressed and secreted) [4] and transforming growth factor-β1 (TGF-β1) [5] also were up-regulated in proximal tubular cells on protein challenge. Mediators were released preferentially into the basolateral cell medium in a fashion that in the kidney would incite interstitial inflammatory and fibrogenic reactions [2–4]. In fact, macrophages are recruited at tubular sites of enhanced protein uptake [6] and may additionally synthesize fibrogenic stimuli [7]. However, little is known of the interactions at these sites between proximal tubular cells, fibroblasts, and other cells relevant to fibrogenic events.

One such event in progressive interstitial injury is the accumulation of fibrogenitor cells of heterogeneous origin, the myofibroblasts, induced by local stimuli to express cytoskeletal elements (α-smooth muscle actin, α-SMA) and to produce extracellular matrix [8, 9]. In anti-glomerular basement membrane (anti-GBM) disease and mesangial glomerulonephritis, selective insults to the glomerular membrane or mesangial components are followed by the accumulation of α-SMA+ cells into the peritubular interstitium as long as the barrier dysfunction and proteinuria persist [10–13]. The myofibroblast phenotype also

appears in the interstitium in the typical setting of proteinuria due to nonimmune glomerular injury, the remnant kidney of rats after 5/6 nephrectomy [14]. Remarkably, the emergence of α-SMA myofibroblasts is not confined to animal models and, like the related fibrotic lesion itself, its detection can be used as a morphological predictor of progressive disease [15–17].

Our current study tested the hypothesis that the activation of proximal tubular epithelial cells may contribute to promote a myofibroblast reaction into peritubular areas and that the filtered protein overload may act as a local stimulatory factor. To this end, remnant kidneys of rats after 5/6 nephrectomy were studied with the following specific aims: (*1*) to provide a comparative immunohistochemical analysis of ultrafiltered protein accumulation in proximal tubular cells and of α-SMA in the tubulointerstitial compartment; (*2*) to characterize the time course and location of abnormal peritubular α-SMA expression on semithin ultracryosections; (*3*) to identify relevant macrophage-myofibroblast interactions; and (*4*) to determine whether the amelioration of the filtering barrier by angiotensin-converting enzyme inhibitor (ACEi) therapy that limits both the exposure of tubular cells to ultrafiltered proteins and subsequent macrophage accumulation, also may cause reductions of abnormal peritubular α-SMA+ cells and of TGF-β1, a common inducer of myofibroblast phenotype in fibroblasts, endothelial cells, and proximal tubular epithelial cells in the kidney.

METHODS

Animals

Studies were conducted in male Sprague Dawley, CD-COBS rats (275 to 300 g initial body weight) obtained from Charles River SpA (Calco, Italy). The animals were housed in a constant temperature room with a 12-hour dark/12-hour light cycle and fed a standard diet. Animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national (*D.L. n.116*, G.U., Suppl 40, 18 Febbraio 1992, Circolare No 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; *Guide for the Care and Use of Laboratory Animals*, U.S. National Research Council, 1996).

Disease model and protocol

Five-sixths renal mass reduction (RMR) was accomplished by surgical removal of right kidney and ligation of two or three extrarenal branches of the left renal artery [18] in anesthetized rats. Age-matched rats ($N = 5$) were used as controls after sham operation, consisting of a laparotomy and manipulation of renal pedicles. Three groups of rats with renal mass reduction ($N = 7$ each group) were sacrificed at 7, 14 and 30 days after surgery, respectively; sham-operated controls were sacrificed at day 30 ($N = 7$). To assess the effects of ACEi, rats with renal mass reduction received lisinopril (25 mg/L in the drinking water) [19, 20] starting from day 1 after surgery and were sacrificed on day 30 ($N = 7$). In all groups twenty-four hour urines were collected in metabolic cages both before the time of disease induction and at sacrifice for the determination of urinary protein excretion levels. Systolic blood pressure and serum creatinine were assessed on day 30 after surgery.

Tissue preparation

At the end of the study, the animals were anesthetized with sodium pentobarbital IP (0.1 mL/100 g body wt of a 65 mg/mL solution in saline) and the kidneys were fixed by perfusion via abdominal aorta [21]. Kidneys were perfused with Hanks' solution for five minutes and then fixed with periodate-lysine paraformaldehyde (PLP) fixative [22] for 10 minutes, followed by overnight fixation in the same fixative at 4°C. The tissue fragments from remnant kidneys were taken from the center of non-infarcted areas. Fixed tissue specimens were extensively washed with phosphate-buffered saline (PBS; 0.9% NaCl in 10 mmol/L sodium phosphate buffer, pH 7.4) and stored in the same buffer. Selected specimens were embedded in Epon and processed for electron microscopy using standard protocol. For in situ hybridization studies, fragments were fixed in Dubosq-Brazil solution, dehydrated, and embedded in paraffin [23].

Immunohistochemistry

The tissue specimens were immersed in 30% sucrose/PBS for at least one hour at room temperature, embedded in OCT medium, and frozen in liquid nitrogen. Tissue sections were cut at 5 μm thickness using a Mikrom 500 O cryostat (Mikrom, Walldorf, Germany) and either stained immediately or stored at −20°C until further processing. Non-specific binding of antibodies was blocked with PBS/1% BSA for 15 minutes at room temperature. Sections were incubated for direct immunofluorescence with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (30 μg/mL in PBS; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for one hour at room temperature. After washes in PBS, the slides were mounted using 100 mmol/L Tris-HCl:glycerol 50:50, 2% N-propyl gallate, pH 8. Sections were examined with a Leika DM-R microscope equipped with epifluorescence and appropriate filters.

Mouse monoclonal antibodies were used for the detection of α-smooth muscle actin (1A4; Sigma Co., St. Louis, MO, USA) and rat macrophages (ED1; Serotec, Oxford, UK). Tissue sections were blocked with PBS/1% bovine serum albumin (BSA) and incubated overnight (18 hours) at 4°C with the primary antibody diluted in PBS (1A4, ascites fluid, 1:400; ED1, 10 μg/mL). After washes in PBS, they were incubated with Cy-3-conjugated don-

key anti-mouse IgG antibodies (5 μg/mL in PBS; Jackson ImmunoResearch) for one hour at room temperature. Double labeling was performed by overnight incubation with anti α-SMA antibody followed by Cy-3-anti-mouse IgG, and then with FITC-anti-rat IgG as described above.

In other experiments, sections were double-stained in sequence for α-SMA and, in the second cycle of staining, either RECA-1 or ED1 antigen. Cy3-conjugated and FITC-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch, 20 μg/mL) were used as secondary reagents, respectively. In control experiments primary antibodies were either omitted or substituted with non-immune mouse serum.

Evaluation of tubular staining, ED1 macrophages, and α-smooth muscle actin expression

For the evaluation of immunofluorescence staining, randomly selected, consecutive fields of view (at least 20 in each section, ×400) were examined by an investigator without knowledge of the experimental group. Total numbers of proximal tubuli and of tubuli positive for intracellular IgG staining were counted for assessment of percentage of positive profiles. ED1-positive cells also were counted and the mean values were calculated for each specimen [20]. The staining for α-SMA was assessed by a semiquantitative evaluation assigning a score for each field as follows: 0, no staining; 1+, few isolated α-SMA+ cells in peritubular areas; 2+, moderate α-SMA peritubular staining; 3+, marked accumulation of α-SMA cells surrounding more than 50% of the tubuli, associated or not with expression in tubular epithelial cells. The mean values were calculated for each specimen and used for statistical analysis.

Preparation and staining of semithin ultracryosections

Fragments of PLP-fixed renal cortex were infiltrated with 2.3 mol/L sucrose for at least one hour, and sectioned at about −100°C (approximate thickness, 0.5 μm) using a Reichert FCS ultracryomicrotome. Only tissues with well preserved morphology were selected for further analysis, and the slides were stored at −20°C until use.

The following sequence of incubation was used for double immunofluorescence labeling: anti-α-SMA antibody, Cy3-conjugated anti-mouse IgG antibody, and mouse monoclonal anti-rat endothelial cell antigen-1 (RECA-1) antibody (HIS52; Serotec, Kidlington, UK) revealed with FITC-conjugated anti-mouse IgG (33 μg/mL). In control experiments primary antibodies were substituted with non-immune mouse serum. The adequacy of the double staining technique was assessed by preliminary experiments on control rat kidneys showing sharp separation of colors between α-SMA+ cells (yellow staining due to recognition by both the CY3- and FITC-conjugated secondary antibodies) and RECA-1+ cells (green staining produced by the second cycle of the incubation). The ultracryosections were prepared from three rats per group, and at least five fields in each specimen (three specimens for each rat) were analyzed using a 100× objective.

In situ hybridization

The rat TGF-β1 antisense and sense RNA probes were prepared and labeled by in vitro transcription using digoxigenin-labeled uridine triphosphate (Roche, Milan, Italy) [23]. A 700 bp rat TGFβ cDNA was cloned into the EcoRI/Hin*dIII* sites of the pBluescript vector between T7 and T3 promoters. Fragments of renal cortex were fixed in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Sections were cut at 4 μm and processed as described. Briefly, after permeabilization with proteinase K (40 μg/mL; Sigma), the sections were hybridized with the RNA probes at the final concentration of 0.2 ng/μL in 2× standard sodium citrate (SSC), 10% dextran sulfate, 1× Denhardt's solution, 20 mmol/L Vanadyl Ribonucleoside Complex (Invitrogen, Life Technologies, Milan, Italy), 0.1 mol/L sodium phosphate and incubated overnight in a moist chamber at 45°C. After being washed in 0.2× SSC and blocked with a buffer blocking solution (50 mg/mL skimmed dried milk, 150 mmol/L NaCl in 100 mmol/L Tris-HCl, pH 7.8) at room temperature for 30 minutes, the sections were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche) at the dilution of 1:1000 for 45 minutes at 37°C. Colorimetric detection with nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Roche) was then performed, and the sections were mounted in 60% glycerol and examined by light microscopy. The negative control included hybridization step with the sense probe.

Systolic blood pressure

Systolic blood pressure was recorded by tail plethysmography in conscious rats [24].

Analytic methods

Proteinuria was determined by the modified Coomassie blue G dye-binding assay for proteins with BSA as standard [25]. Serum creatinine was measured by the alkaline picrate method [26].

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Data were analyzed using the non-parametric Kruskal-Wallis test. Statistical significance was defined as $P < 0.05$. Analysis of correlations between parameters was performed using the nonparametric Spearman rho rank-order correlation coefficient.

RESULTS

IgG staining in proximal tubuli and expression of α-smooth muscle actin after 5/6 nephrectomy

In the kidneys of sham-operated rats IgG staining was undetectable in proximal tubular cells except that for a weak intracellular granular reactivity with focal distribution. Intracellular IgG staining was found in increasingly higher numbers of proximal tubular profiles at different time points starting from day 7 (day 7, 21.22 ± 8.66%; day 14, 58.17 ± 9.16%; day 30, 66.71 ± 10.67%; sham control, 3.68 ± 1.51% staining). This change was associated with increasingly higher levels of urinary protein excretion during time after surgery (day 7, 45.2 ± 18.87; day 14, 116.18 ± 34.21; day 30, 223.65 ± 94.70; sham control, 24.36 ± 4.31 mg/24 h; IgG mean score and proteinuria, $P < 0.05$ day 7 vs control, $P < 0.01$ day 14 vs. day 7, and $P < 0.01$ day 30 vs. day 14).

No differences were visualized in the staining for α-SMA in the tubulointerstitial compartment of remnant kidneys at day 7 as compared to sham control kidneys (Fig. 1 A, B; mean score for remnant kidney at day 7, 0.07, and control, 0.03). The staining was confined to vascular smooth muscle cells of arteries, arterioles, and their branches splitting into capillaries. In contrast, in remnant kidneys at 14 days after surgery, α-SMA+ cells became detectable in the peritubular areas, either isolated or in clusters. In most fields the abnormal staining appeared to concentrate in the interstitium (Fig. 1C) in cells with round or elongated fibroblast-like morphology. Abnormal α-SMA expression was more evident and diffuse in the sections of 30-day remnant kidneys. At this time point, it was focally detectable in tubular epithelial cells in addition to the α-SMA+ cells in adjacent peritubular areas (Fig. 1D). The mean scores for α-SMA staining were 0.71 at day 14 and 1.11 at day 30 ($P < 0.01$ vs. day 7 and vs. day 14, respectively).

Comparison of the distribution of IgG and α-smooth muscle actin staining

By double staining of the sections for IgG and α-SMA, abnormal IgG staining was visualized in remnant kidneys at day 7 in proximal tubular cells, in the absence of α-SMA staining in surrounding areas (Fig. 2B, compare to sham control in Fig. 2A) as predictable from results of single immunofluorescence staining. The initial expression of abnormal α-SMA at 14 days was revealed in the peritubular areas in the vicinity of proximal tubuli that stained positive for IgG as a consequence of abnormally high cellular uptake of ultrafiltered protein (Fig. 2C). This association was maintained at 30 days (Fig. 2D). Additionally, α-SMA staining was visualized in remnant kidneys at 30 days in tubular epithelial cells of IgG-positive proximal tubuli, although not confined exclusively to these segments. Some of the α-SMA+ cells showed less or no IgG staining in respect to other IgG-positive epithelial cells within individual profiles of proximal tubule, most presumably reflecting impaired protein absorption following the induction of abnormal cell phenotype. Correlation analysis revealed significant positive correlations both between proteinuria and proximal tubular IgG staining and between the latter and peritubular α-SMA staining (Spearman rho: +0.80 and +0.88, respectively, $P < 0.001$).

TGF-β1 mRNA expression in the tubulointerstitium in remnant kidneys

By in situ hybridization, in kidneys of sham operated rats signal for TGF-β1 mRNA was found in tubular epithelial cells in distal segments, and in occasional interstitial cells or peritubular capillary endothelial cells. Stain of proximal tubular cells was absent (Fig. 3A). In remnant kidneys at 7 days after surgery, despite a similar distribution of TGF-β1 mRNA, a focal and weak staining of epithelial cells was revealed in proximal tubuli (Fig. 3B). A focal increase in the extension and intensity of staining was also visualized in proximal tubuli in 14-day remnant kidneys. In addition, abnormal signal was present in peritubular interstitial and capillary areas, part of which was associated with infiltrating cells (Fig. 3C). In 30-day remnant kidneys the increase in TGF-β1 mRNA hybridization signal in proximal tubuli also was prominent and diffuse in parallel with marked TGF-β1 mRNA expression in peritubular areas (Fig. 3D).

Time course of ED1 macrophage accumulation and comparison with α-smooth muscle actin in remnant kidneys

We and others have documented that ED1 monocyte/macrophages and major histocompatibility complex-II (MHC-II) positive cells initially accumulate into the interstitium of remnant kidneys during the early proteinuric stages of progressive disease, with significant increases at 14 days after surgery. The time course of ED1 macrophage accumulation was confirmed here, and is illustrated in Figure 4 (ED1, green color). Mean counts of ED1+ cells (number/high power field) were 6.2 ± 2.2 at day 7, 16.6 ± 3.5 at day 14, and 28.1 ± 5.7 at day 30 ($P < 0.01$ days 14 and 30 vs. control, 5.3 ± 1.0). Additionally, infiltrating macrophages were identified in areas surrounding proximal tubuli in which protein traffic was enhanced [20], thus in a distribution remarkably similar to that of peritubular α-SMA+ cells in remnant kidneys as described above in the present study. Consistent with such distribution, the results of double staining experiments revealed a close association of ED1+ cells and α-SMA+ cells in peritubular areas in the sections of 14 day remnant kidneys (Fig. 4C), reflecting the interaction between the recruited macrophages and α-SMA+ cells at those sites. ED1 macrophages and α-SMA+ cells

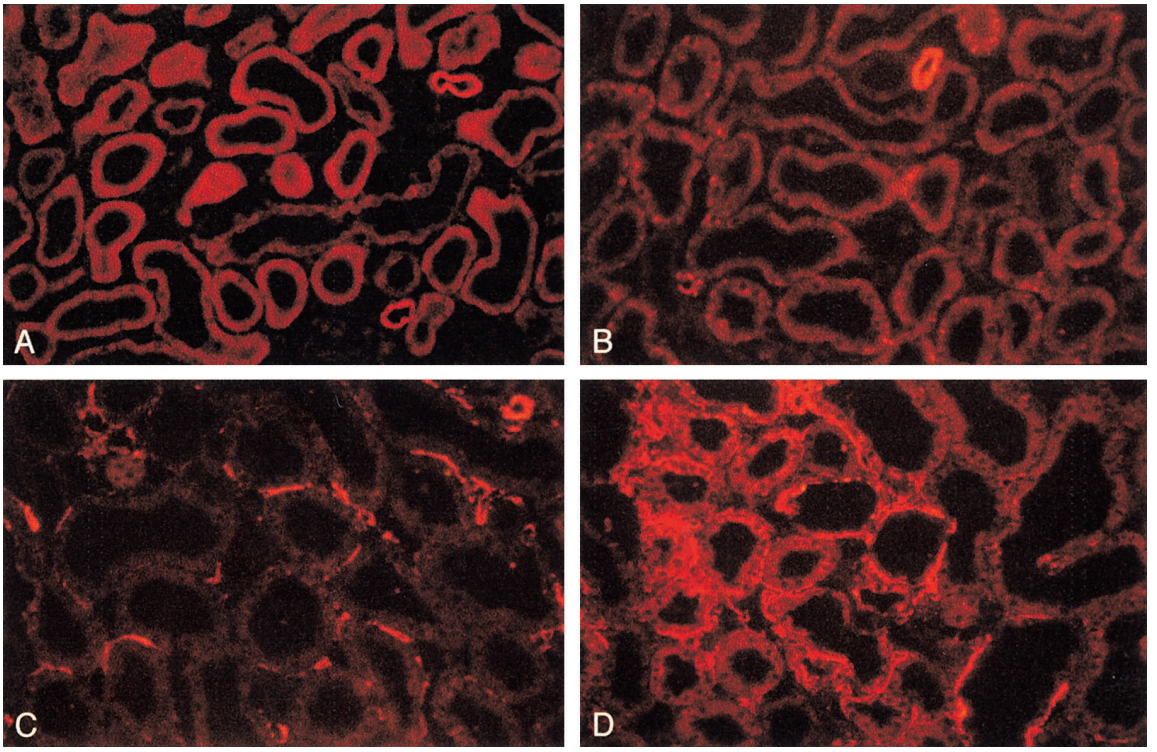

Fig. 1. Immunofluorescence staining of α-smooth muscle actin (α-SMA) on cryostat sections of kidney from sham operated rat and of remnant kidneys after 5/6 renal mass ablation. In contrast to the sham control (*A*) and to a remnant kidney at day 7 after surgery (*B*), in which the specific staining is confined to blood vessels, abnormal α-SMA expression in peritubular areas is visible in remnant kidney at day 14 (*C*). A further increase in the peritubular α-SMA expression and focal staining in the tubuli was observed in remnant kidneys at day 30 (*D*). (Fixation by perfusion with paraformaldehyde-lysine-metaperiodate fixative; CY3-conjugated secondary antibody. Magnification ×250.) (Reproduction of this figure in color was made possible by the generous sponsorship of Wyeth Ledorle, Aprilia, Italy.)

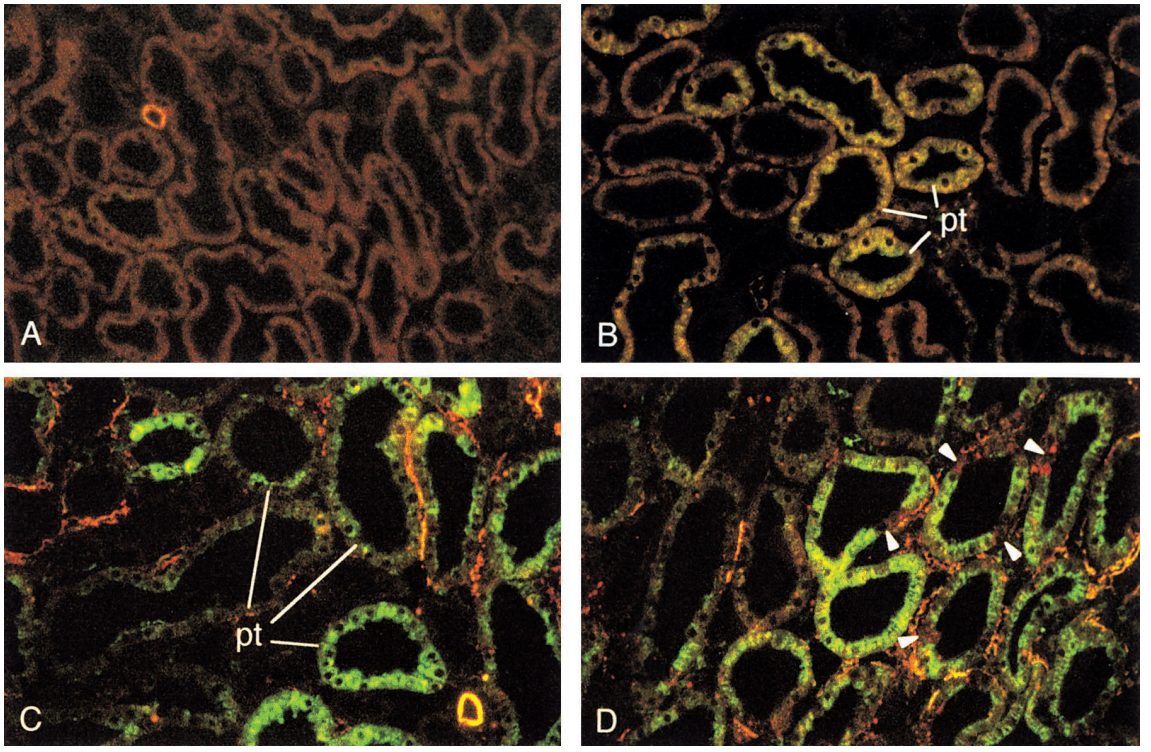

Fig. 2. Comparison of IgG staining in tubular epithelial cells and peritubular α-SMA staining. In contrast to sham operated kidney (*A*), intracellular IgG (green) is present in proximal tubular epithelial cells (pt) in remnant kidney at day 7 after surgery, in the absence of α-SMA expression in peritubular areas (*B*). The latter (red) is visible in remnant kidney at day 14 (*C*) and more pronounced at day 30 (*D*) in the areas surrounding proximal tubuli in which the ultrafiltered protein uptake is enhanced. At day 30 at these sites some cells in IgG-positive proximal tubuli appear to be in a stage of reduced protein reabsorption (arrowheads). (IgG detected by FITC-conjugated anti-rat IgG antibody; α-SMA revealed by indirect technique using CY3-conjugated secondary antibody. Magnification ×250.) (Reproduction of this figure in color was made possible by the generous sponsorship of Wyeth Ledorle, Aprilia, Italy.)

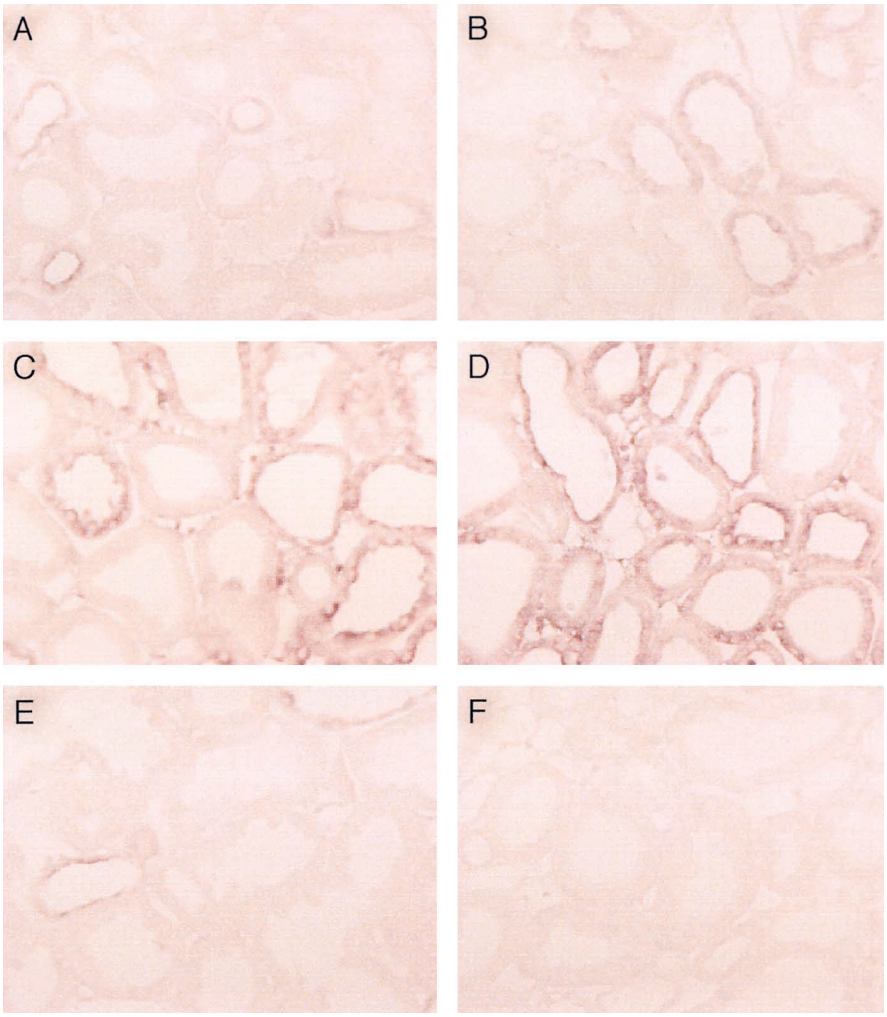

Fig. 3. Photomicrographs showing TGF-β1 mRNA expression by in situ hybridization in kidneys from a sham operated rat (*A*) and from remnant kidneys at 7 (*B*), 14 (*C*), or 30 days (*D*) after surgery, and the effect of ACEi on TGF-β1 expression (*E*). Compared with control kidneys showing hybridization signal in distal segments, remnant kidneys at day 7 also revealed a focal and weak TGF-β1 expression in occasional proximal tubular cells. Marked proximal tubular TGF-β1 up-regulation was detected in kidneys at 14 and 30 days after surgery, associated with signal in peritubular infiltrating cells. Similar to control kidney, TGF-β1 mRNA was not detectable in proximal tubular cells in remnant kidneys of ACEi treated rats. (*F*) Negative control using TGF-β1 sense probe in a section of remnant kidney at day 30. (Magnification ×300.) (Reproduction of this figure in color was made possible by the generous sponsorship of Wyeth Ledorle, Aprilia, Italy.)

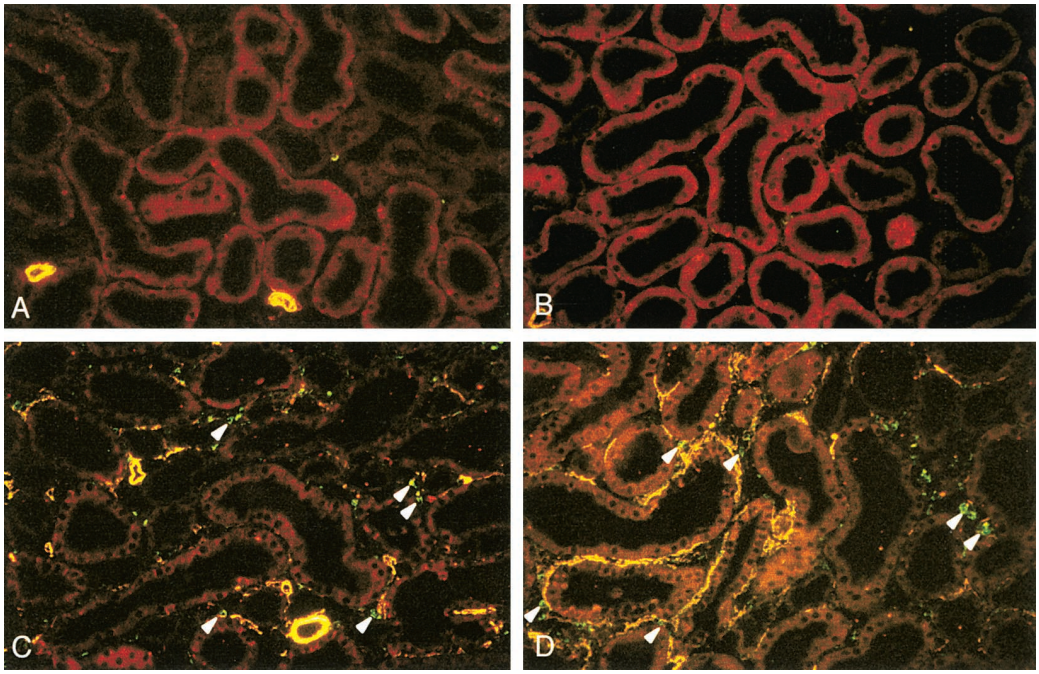

Fig. 4. Immunofluorescence localization of ED1 macrophages (green) and α-SMA+ cells (yellow). In contrast to sham control (*A*) and remnant kidneys at day 7 (*B*), positive cells (arrowheads) are found in many areas in the peritubular interstitium in remnant kidneys at days 14 (*C*) and 30 (*D*). There is evidence of interaction in most of the areas of initial injury. In remnant kidney at day 30, ED1 macrophages are also present in areas in which very few or no α-SMA+ cells are yet detectable. (Magnification ×250.) (Reproduction of this figure in color was made possible by the generous sponsorship of Wyeth Ledorle, Aprilia, Italy.)

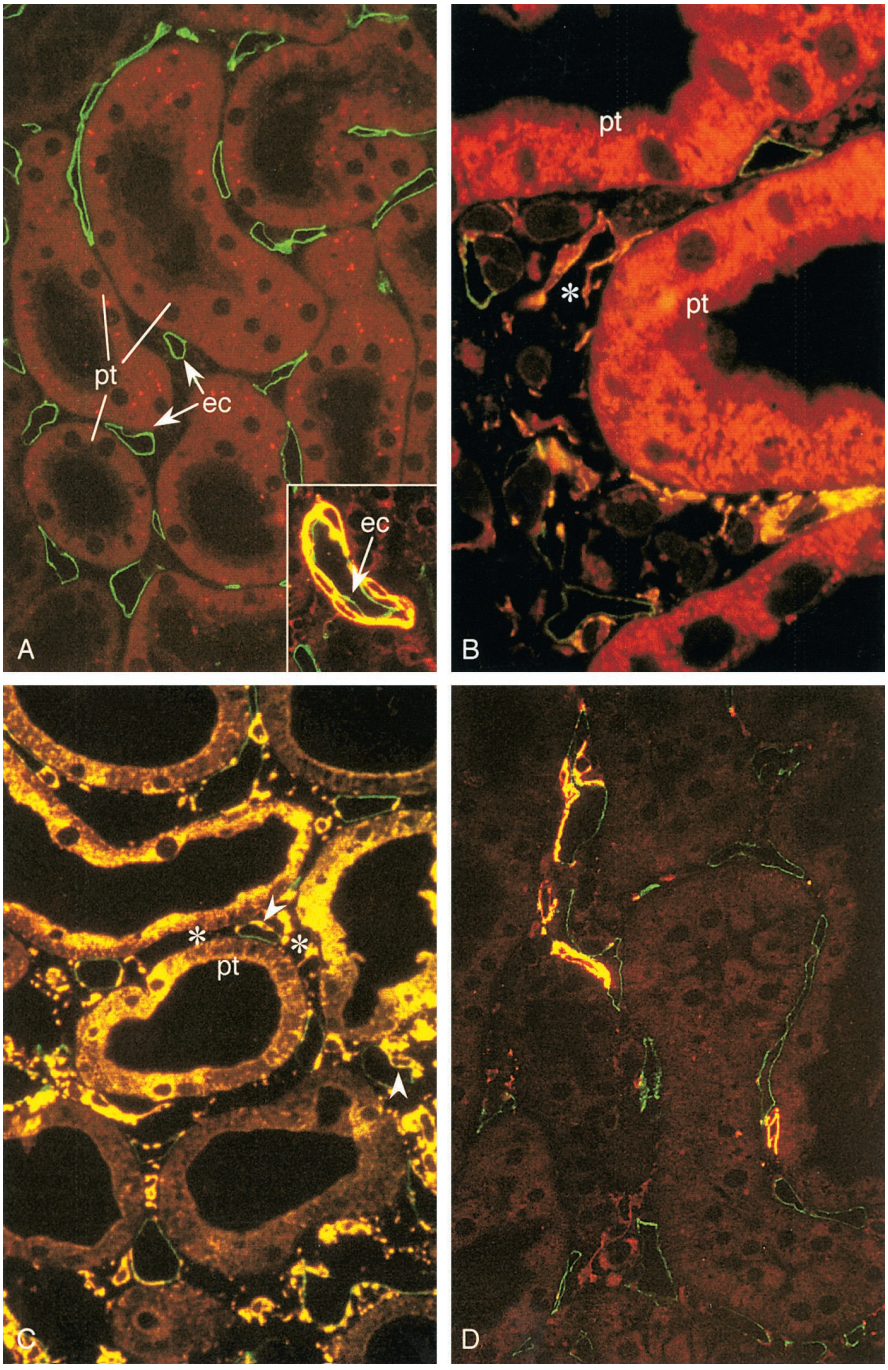

Fig. 5. Identification of α-SMA in tubulointerstitial areas on semithin ultracryosections of remnant kidneys, and effect of lisinopril on abnormal α-SMA expression. No α-SMA staining was localized in peritubular areas in remnant kidney at day 7 (*A*). Endothelial cells (ec), stained in green for rat endothelial cell antigen -1, are sharply distinguishable from α-SMA+ smooth muscle cells (in yellow, inset). In a remnant kidney at day 14 (*B*), abnormal peritubular α-SMA staining can be seen in myofibroblasts in areas (asterisk) between proximal tubuli (pt) and peritubular capillaries. In a remnant kidney at day 30 (*C*), α-SMA is additionally expressed by tubular epithelial cells and appears to be associated with cells showing both endothelial location and morphology in focal areas (arrowheads). (*D*) Remnant kidney of lisinopril-treated rat. (Magnification ×400 in A, C, D, and inset; ×1000 in B.) (Reproduction of this figure in color was made possible by the generous sponsorship of Wyeth Ledorle, Aprilia, Italy.)

also were found to colocalize in peritubular areas in kidneys at 30 days (Fig. 4D), despite more irregular and diffuse distribution of macrophages during the persistent inflammatory wave.

Localization of α-smooth muscle actin positive cells on ultracryosections

To gain information on the cell type(s) initially induced to express α-SMA, and to extend the results from the above analysis, we performed a detailed study using semithin ultracryosections, which provide much higher level of resolution and precise delineation of peritubular structures. The sections were analyzed for detection of α-SMA and the endothelial cell marker, RECA-1. Results were unequivocal by inspection and are illustrated in Figures 5 and 6. Both in sham kidneys and in remnant kidneys at day 7 (Fig. 5A), endothelial cells were distinguishable by RECA-1 staining in green color from

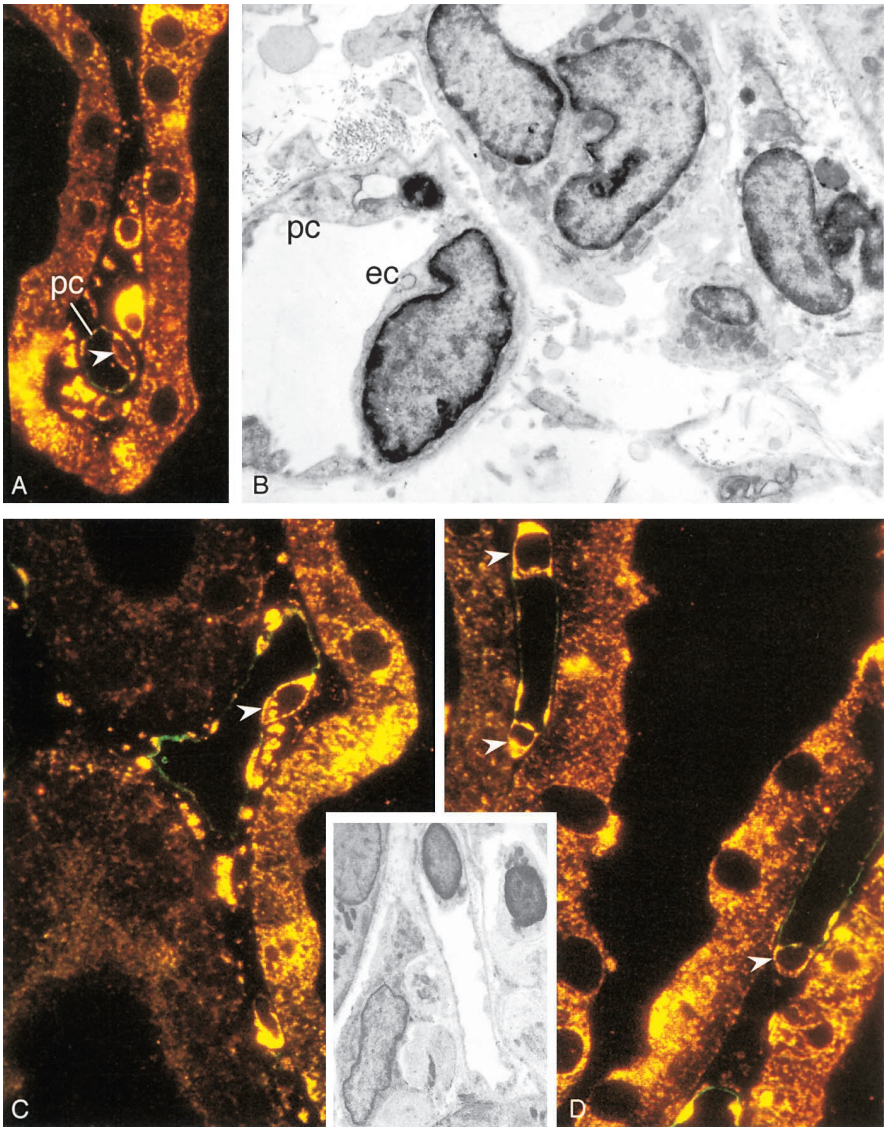

Fig. 6. Localization of α-SMA+ cells in semithin cryosections of remnant kidney at day 30. In addition to brightly stained cells in the interstitium, α-SMA is detected (in yellow) in a peritubular endothelial cell (arrow) of a capillary (pc) labeled in green for rat endothelial cell antigen-1 (*A*). The electron micrograph shows a peritubular interstitial area containing a similar capillary structure (pc) for comparison (*B*, ×3000). Other examples are shown in *C* and *D* (compare with the peritubular capillary endothelium in the electron micrograph, inset). (Magnification ×600 in A; ×800 in C, D; ×3000 in B; ×1000 in inset.) (Reproduction of this figure in color was made possible by the generous sponsorship of Wyeth Ledorle, Aprilia, Italy.)

α-SMA+ cells that stained yellow up to arteriolar branches. No abnormal α-SMA staining could be found in remnant kidneys at day 7. By contrast, in remnant kidneys at 14 days, in the initial stages of myofibroblast formation the α-SMA+ cells were detectable in peritubular areas (yellow color in Fig. 5B), they had round or elongated shape and concentrated exclusively in the interstitium between tubuli and peritubular capillaries in all the sections. In remnant kidneys at day 30, α-SMA expression also became evident in tubular epithelial cells (Fig. 5C). A remarkable finding at 30 days at this resolution was α-SMA+ cells with endothelial morphology in peritubular capillaries in some areas. At higher magnification, α-SMA+ endothelial cells, showing a low intensity of RECA-1 staining at this time, were recognized to line the wall of peritubular capillaries adjacent to α-SMA+ cells in the peritubular interstitium (Fig. 6). Glomerular endothelial cells did not stain for the myofibroblast marker in contrast to the presence of abnormal expression in mesangial cells (data not shown).

Effects of lisinopril on proteinuria and proximal tubular traffic of proteins, TGF-β1 mRNA, macrophage accumulation into the interstitium, and abnormal peritubular α-smooth muscle actin expression

Rats with a remnant kidney given lisinopril since day 1 after surgery were protected against proteinuria and IgG accumulation in proximal tubular cells (Table 1). These effects at day 30 were associated with reduced systolic blood pressure (RMR, 160 ± 20; RMR+ACEi, 92 ± 13; control, 119 ± 8 mm Hg) and serum creatinine levels (RMR, 1.41 ± 0.42; RMR+ACEi, 0.96 ± 0.17; control, 0.63 ± 0.03 mg/dL). Lisinopril also prevented

Table 1. Effect of ACE inhibitor (ACEi) on proteinuria, IgG accumulation in proximal tubular cells, ED1 macrophages, and peritubular α-SMA expression

Groups	Proteinuria *mg/day*	Proximal tubular IgG staining %	ED1 *N of cells/HPF*	α-SMA *staining score*
30-day RMR	224 ± 95[a]	66.7 ± 10.6[a]	28.1 ± 5.6[a]	1.10 ± 0.13[a]
30-day RMR + ACEi	27 ± 9[b]	3.9 ± 0.9[b]	6.3 ± 1.7[b]	0.05 ± 0.06[b]
30-day Control	24 ± 4	3.7 ± 1.5	5.3 ± 1.0	0.03 ± 0.05

Values are mean ± SD. Abbreviations are: ACE, angiotensin-converting enzyme; α-SMA, α-smooth muscle actin; RMR, renal mass reduction
[a] $P < 0.01$ vs. control
[b] $P < 0.01$ vs. 30-day RMR

both the increases in TGF-β1 expression thus abrogating the prominent up-regulation in proximal tubular epithelial cells (Fig. 3E), and the interstitial accumulation of macrophages in remnant kidneys at 30 days (Table 1). Finally, the ACEi prevented the abnormal accumulation of α-SMA+ cells in peritubular areas and the tubular α-SMA expression (Table 1), despite persistent expression of α-SMA in vascular walls and arteriolar branches. The effect of lisinopril on induction of myofibroblasts was confirmed by examination of ultrathin cryosections double-stained for α-SMA and the endothelial specific marker revealing absence of abnormal α-SMA staining (Fig. 5D).

DISCUSSION

Interstitial fibrogenesis is recognized as a multifactorial process leading to architectural disruption and renal scarring. Our current study focused on the proximal tubular epithelial cell, specifically in relation to the inflammatory and profibrogenic phenotype that is activated after a nonimmune insult to the glomerular barrier [1, 27]. Our first aim was to assess by morphological techniques whether an association may exist between excess tubular protein load and the peritubular accumulation of myofibroblasts. The role of myofibroblasts in the fibrogenic process is indicated by studies showing that α-SMA+ cells become detectable in the interstitium, followed by collagen deposition in the areas of accumulation [28–30], and these events can be antagonized by renoprotective intervention [31]. We found that in remnant kidneys of rats with renal mass ablation, after the onset of abnormally high protein trafficking, α-SMA+ cells appear in areas adjacent to proximal tubular cells actively engaged in enhanced protein uptake. Positive correlations also were found between proteinuria and tubular protein overload, and between the latter and peritubular α-SMA staining. In experimental nephropathy, a relationship between protein overload and fibrogenesis was suggested by studies in aging rats [32] and in rats with proteinuria induced by intraperitoneal injections of albumin that caused macrophage infiltration as well as up-regulation and deposition of interstitial collagen [7, 33]. The accumulation of α-SMA+ cells has been described more recently as a typical event in renal fibrogenesis. However, in remnant kidneys, which also are characterized by mononuclear infiltrates and subsequent interstitial collagen deposition [14, 34], the link with a pathophysiologic mechanism could not be documented.

Our findings rather directly implicate a novel interaction between activated proximal tubular cells and cells induced to express the myofibroblast phenotype in the interstitium. The hypothesis that this type of interaction might occur has been proposed based on the evidence that proximal tubular cells, presumably by virtue of ability to synthesize PDGF-AB and TGF-β1, stimulate renal cortical fibroblasts in coculture to grow and synthesize collagen [35]. TGF-β1 does act as a common inducer of α-SMA expression and collagen and fibronectin synthesis in fibroblasts and other cell types [8, 36]. Furthermore, the phenotypic change can be related to activating effects of filtered proteins in vivo. Of major interest in this venue, high concentrations of albumin have been recently shown to up-regulate TGF-β1 mRNA expression in cultured proximal tubular cells (abstracts; Zhao J et al, *J Am Soc Nephrol* 11:523A, 2000; Mao et al, *J Am Soc Nephrol* 12:710A, 2001) [5]. Here, TGF-β1 was up-regulated in proximal tubular cells in concomitance with protein over-reabsorption at day 7 and more markedly at day 30 after surgery. These data extend observations of enhanced TGF-β1 expression in this model [34], while the stimulus or the local relationship with a functional response in the interstitium has not yet been addressed simultaneously. A unifying interpretation is that the excess protein uptake acts as a stimulus for secondary up-regulation of fibrogenic mediators such as TGF-β1 in proximal tubular cells to induce abnormal peritubular α-SMA expression. Other mediators in this response include platelet-derived growth factor [9] and endothelin-1, which also activates α-SMA gene expression [37] and is up-regulated by protein load in proximal tubular cells [2] as well as in remnant kidney and other progressive models [38, 39]. As to specific components in the ultrafiltrate, hepatocyte growth factor or TGF-β1 itself [40] may act as an additional and independent factor.

In addition to a direct tubular cell-dependent mechanism, fibrogenic events can be induced via macrophage-

mediated pathways. The codistribution of the tubular staining for IgG with peritubular α-SMA in the present study replicates the codistribution found with inflammatory cells after either immune or nonimmune injury [6]. A cellular interaction is disclosed consistently between macrophages and peritubular α-SMA+ cells at day 14 after surgery. No studies have specifically looked at this aspect in progressive disease models. The macrophage-myofibroblast interaction, however, has been recently proposed to have pathogenic significance in chronic immune tubulointerstitial injury [16]. In the remnant kidney the initial hit responsible for proteinuria and tubulointerstitial damage is nonimmune. The macrophage-myofibroblast interaction can be at least favored thereby by inflammatory mediators, such as MCP-1, RANTES, osteopontin, and complement, released or activated locally by tubular cells as a consequence of protein overreabsorption [3, 4, 7, 41]. That the macrophages further elaborate fibrogenic molecules is known by results of in vitro studies [42, 43] and by evidence here as well as in the protein overload model that peritubular infiltrating cells express TGF-β1 [7]. On the same line, macrophage-derived TGF-β1 stimulated rat kidney interstitial fibroblasts to produce collagen types I and III and fibronectin [40].

Regardless of the source of stimulation, the present report provides a clue to the origin(s) of peritubular myofibroblasts. Studies have left unanswered questions about whether the initial α-SMA expression in remnant kidney was associated with interstitial cells [14] or proximal tubular cells [44]. First, dual-staining experiments reveal that cells located in peritubular areas, most presumably albeit not exclusively the fibroblast [9, 14], are the initial targets of fibrogenic signaling. On the other hand, the finding of α-SMA in tubular cells at day 30 is in line with previous results [44], confirming that a phenotypic transformation of tubular cells occurs in a relatively late stage, as suggested originally for autoimmune interstitial nephritis [45]. The available data indicate that the phenotypic change is mediated in an autocrine fashion and/or by inflammatory cells via TGF-β1, epidermal growth factor, or other pathways [36]. Of note, the appearance of α-SMA in proximal tubular cells in some profiles was associated with no or less IgG staining as compared with cells showing more abundant reabsorbed protein, a change that could reflect reduced reabsorption by the transdifferentiating epithelium. Another new finding is the detection of α-SMA in peritubular endothelial cells in semithin ultracryosections of remnant kidneys at day 30. Certain types of endothelial cells in culture can be induced to express α-SMA [46–48], for instance after heparin depletion, and transdifferentiate into mesenchymal cells again in response to TGF-β1 [49]. Thus, it seems that the peritubular endothelium, less than the fibroblasts, is responsive to signaling from the nearby epithelium and immune cells and may participate to the fibrogenic reaction. Importantly in this perspective, antisense oligodeoxynucleotides complementary to α-SMA inhibited endothelial-mesenchymal transformation and the associated TGF-β1–dependent migration in vitro [50].

Angiotensin converting enzyme inhibitors are the treatment of choice in chronic proteinuric nephropathies. Studies on mechanisms of renoprotection have identified, among other targets, glomerular permselective dysfunction. We found previously that lisinopril prevents the excess exposure of proximal tubular cells to ultrafiltered proteins [20], which are potentially responsible for macrophage accumulation into the interstitium [20, 34]. That ACEi treatment can limit α-SMA expression and collagen accumulation also has been reported (abstract; Jiang et al, *J Am Soc Nephrol* 10:660A, 1999) [31]. In addition, in our current study lisinopril abrogated TGF-β1 up-regulation and tubular cell- and macrophage-myofibroblast interactions at sites of abnormal protein reabsorption. These effects offer further support to the possibility that proximal tubular cells become activated to promote local myofibroblast accumulation. ACEi does ameliorate the permselective function of the barrier by now emerging effects on podocyte functional molecules such as nephrin [51]. As a less likely alternative from such standpoint, ACEi may act on separate angiotensin II pathways of interstitial injury [52]. However, given the relevant effects of angiotensin II including the ability to induce proteinuria, interstitial α-SMA expression [53], and TGF-β1 expression in tubular cells [54], its interrelated role as an upstream factor certainly must be considered.

In summary, the results of this study indicate that in an experimental model of progressive nephropathy proximal tubular cells promote peritubular accumulation of α-SMA+ cells, either directly and via macrophage mediated pathways. The initial target cell lies in the interstitium, and presumably the interstitial fibroblasts are a major component. In addition to tubular cells at subsequent stages, peritubular endothelial cells may participate to the fibrogenic response as a consequence of common signaling partly mediated by TGF-β1.

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