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Origin of Interstitial Fibroblasts in an Accelerated Model of Angiotensin II-Induced Renal Fibrosis

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To determine whether previous renal injury accelerates the progression of glomerulosclerosis and interstitial fibrosis, we examined the effect of treating rats with angiotensin II after Habu venom injury. After initiating disease, we examined the origin of interstitial myofibroblasts by locating α -smooth muscle actin (α -SMA)-positive and Na^+ , K^+ -ATPase-positive cells relative to interstitial space, tubular epithelial cells, the tubular basement membrane (TBM), and vascular structures. Tubular epithelial-mesenchymal transition was also assessed by examining TBM integrity and by using Texas Red (TR)-dextran in intravital tracking experiments. The staining of α -SMA-positive myofibroblasts dramatically increased in peritubular interstitial spaces 48 hours after Habu venom plus angiotensin II, particularly in and around perivascular and periglomerular regions, while tubular epithelial cells were α -SMA-negative. Na^+ , K^+ -ATPase-positive and TR-dextran-labeled cells were restricted to the tubular epithelium and excluded from the interstitium. By 7 and 14 days, expanded interstitial space contained only α -SMA-positive myofibroblasts without TR-dextran endocytic particles. Epithelium of atrophic tubules containing TR-dextran remained confined by surrounding interstitium and myofibroblasts. These studies indicate that early expansion of α -SMA-positive cells in the interstitium and loss of tubular area occur via encroachment of interstitial myofibroblasts from perivascular into atrophic tubular spaces rather than via epithelial-mesenchymal transition and migration of tubular cells through the TBM into the interstitium. (*Am J Pathol* 2005, 167:1193–1205)

Progressive renal diseases lead to a common histological and functional end point referred to as end-stage renal disease. Histological changes in end-stage renal disease include loss of glomerular and peritubular capillary architecture, cell proliferation, and diffuse extracellular matrix accumulation leading to tubular atrophy, and progressive fibrosis. Recent interest has focused on the renin angiotensin system, particularly angiotensin II (Ang II), as central to the progression of chronic renal disease.^{1–6} Inhibition of renin angiotensin system by preventing synthesis of Ang II or blockade of Ang II receptor (AT1) limits renal matrix expansion^{7–12} or reverses renal vascular and glomerular fibrosis¹³ in experimental models of renal disease and has beneficial effects in lessening proteinuria and alterations in glomerular filtration rate in clinical settings of progressive disease.^{14–16} Conversely, prolonged infusion of Ang II in normal rats results in a limited focal interstitial fibrosis.^{17–20} Ang II also augments pre-existing vascular injury in balloon-injured carotid arteries.^{20,21} Because underlying disease is one of the factors determining the natural history of chronic renal fibrosis in humans²² and sequential or multiple injury appears to play an important role in progression of renal lesions in experimental disease,²³ we were interested in examining if previous renal injury would accelerate the progression of Ang II-induced renal disease. In this study, we describe an accelerated rat model of glomerulosclerosis, interstitial fibrosis, and renal functional impairment that resembles end-stage renal disease, by a sustained 14-day infusion of Ang II after preinjury of the kidney by injection of Habu venom (HV) in what would otherwise be a limited proliferative glomerulonephritis or focal periarteriolar fibrosis induced by either agent alone.

A key event in interstitial fibrosis in end-stage renal disease, regardless of the primary disease, is the marked expansion of peritubular and periglomerular space filled

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with myofibroblasts and accumulated extracellular matrix.²⁴ The resultant myofibroblast encroachment and matrix accumulation in the renal parenchyma alters normal function. The cell type responsible for most of the extracellular matrix accumulation is the myofibroblasts, distinguished by acquisition of α -smooth muscle (α -SMA) phenotype and synthesis of extracellular matrix proteins. The origin of myofibroblasts is currently under extensive investigation and evidence indicates the cells may be derived from several sources, including an expansion of activated resident fibroblasts,^{17,25} perivascular adventitial cells,^{26,27} blood-borne stem cells that migrate into the glomerular mesangial or interstitial compartment,^{28–32} or by tubular epithelial-mesenchymal transition (EMT) and migration through the TBM into the peritubular interstitial space.^{32–36} Regardless of the cellular origin, the conversion of a quiescent cell type to an activated α -SMA-positive phenotype with enhanced synthesis of extracellular matrix protein, particularly fibronectin and collagens, is the end result.

In the HV + Ang II model, myofibroblasts are detected in the peritubular interstitium by the expression of α -SMA as early as 1 day after HV + Ang II infusion followed by a course of interstitial fibrosis that resembles chronic renal disease within 14 days. Such a rapid development of fibrosis provides an excellent opportunity to examine tubulointerstitial relationships during the early stages of progressive renal disease and to follow the development of myofibroblast encroachment into the interstitial space. The temporal and spatial expression of α -SMA-positive myofibroblasts in relation to renal tubules, the TBM, and vascular structures was examined by dual-label epifluorescence microscopy. Evidence for tubular EMT and migration into the interstitial space was further examined by electron microscopy and by intravital tracking of the fate of labeled-proximal tubule cells during the course of this disease.

Materials and Methods

Disease Induction

Accelerated renal fibrosis was induced in male Sprague-Dawley rats (Charles River Laboratories, Madison WI). Animal experiments were performed in accordance with National Institutes of Health guidelines, as dictated by the Institutional Animal Care and Use Committees at the Audie Murphy Veterans Hospital and the University of Texas Health Science Center at San Antonio. The rats were unilaterally nephrectomized to increase the incidence of glomerular lesions after HV as previously described.^{37–40} Twenty-four hours later the rats were divided into four groups and subjected to the following protocol:

1. Control ($n = 12$). Rats were injected intravenously with 0.02 mol/L phosphate-buffered saline (PBS), pH 7.4. One day later miniosmotic pumps were placed subcutaneously to continuously deliver Ringer's lactate buffer or sham surgery was performed.

2. HV alone ($n = 12$). Mesangioproliferative glomerulonephritis was induced by an intravenous injection of

Habu snake (*Trimeresurus flavoviridis*) venom (HV) in PBS at a dose of 3.5 mg/kg. One day later miniosmotic pumps were placed subcutaneously to continuously deliver Ringer's lactate, pH 7.4 (diluent control for Ang II experiments described below).

3. Ang II alone ($n = 16$). Rats were injected with PBS in place of HV. One day later miniosmotic pumps were placed subcutaneously to continuously deliver Ang II in Ringer's lactate at a dose of 100 ng/minute.

4. HV + Ang II ($n = 15$). Rats were pretreated with HV as above, followed 1 day later with continuous subcutaneous infusion of Ang II at a dose of 100 ng/minute.

The rats were subdivided and killed 48 hours and 7 and 14 days after injection of HV or PBS. At sacrifice the rats were weighed and kidneys removed. Slices of renal cortex from each rat were obtained and processed for subsequent histological and immunohistochemical evaluation, and Western blotting. Immediately before termination of the study blood pressure was taken and blood and urine collected for measurement of renal function (see below).

Renal Function and Blood Pressure

Renal function and blood pressure were assessed at 14 days after PBS or HV and respective Ang II or Ringer's infusions. Four rats in each treatment group were placed in metabolic cages for 24-hour urine collection before termination of the experiment. At the end of each experiment, a sample of blood was obtained from the vena cava and the left kidney was removed and weighed. Urine and plasma creatinine were measured by colorimetric assay (modified Jaffe; Sigma Chemical Co., St. Louis, MO) for calculation of glomerular filtration rate. Urine protein was measured by the sulfosalicylic acid turbidimetric method using bovine serum albumin as a protein standard.

Immediately before placement of the rats in the metabolic cages, blood pressure was measured in conscious, restrained rats by plethysmography, using a tail-cuff photoelectric sensor and a model 29 amplifier with computerized monitor to measure detected pulses at systole (IITC Life Science U.S.A., Woodland Hills, CA). The rats were trained to adapt to a restraining device by short 30-minute sessions daily throughout a 5- to 7-day period before blood pressure monitoring. At least five measurements were obtained from each rat, the values were averaged and differences in means between groups were compared statistically.

Routine Histology

At sacrifice kidneys were excised and cortical tissue sliced and portions fixed in neutral buffered formalin for routine paraffin embedment and subsequent tissue staining by hematoxylin and eosin (H&E), trichrome, and periodic acid-Schiff-Jones for bright-field microscopical evaluation. The remaining cortex was sliced and frozen by immersion in liquid nitrogen for subsequent immunohistochemical detection of α -SMA and extracellular ma-

trix proteins as markers for fibroblast activation, EMT, and fibrosis.

Extracellular Matrix

Renal expression of extracellular matrix was assessed by indirect or direct immunofluorescence or immunoperoxidase microscopy as described previously^{37,39,40} using a mouse monoclonal antibody specific for the alternatively spliced EIIIA domain of fibronectin (Fn-EIIIA, clone IST-9, Serotec; Harlan Bioproducts for Science, Indianapolis, IN) and rabbit polyclonal antibodies to collagen type I and type IV (Chemicon Int., Temecula, CA). These antibodies are highly specific to their respective types with less than 0.1% cross reactivity with other collagens. Control sections were incubated with nonimmune IgG of the same species as the primary antibody or PBS in place of primary antibody. The sections were then stained with fluorescein isothiocyanate (FITC)- or biotin-labeled second antibody for immunofluorescence or immunoperoxidase, respectively. The area of Fn-EIIIA staining (diaminobenzidine reaction product) was measured using a personal computer-based morphometric analysis system with Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD).

Origin of Interstitial Myofibroblasts

In normal kidney resting fibroblasts and mesangial cells do not express α -SMA but acquire this protein during disease.^{41,42} Moreover, the extent of α -SMA expression and myofibroblast activation is related to the degree of interstitial fibrosis in renal injury.^{42–45} The progression of activated myofibroblasts and mesangial cells in all four groups was examined throughout the course of the study by Western blotting and immunofluorescence microscopy.

Western Immunoblotting

Renal cortical tissue from kidneys of each group of rats was homogenized using a Dounce tissue grinder in 0.5 ml of RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EGTA, 140 mmol/L NaCl, 1.0% Nonidet P-40); containing 1 μ g/ml leupeptin and aprotinin, 1 mmol/L sodium fluoride, 0.1 mmol/L sodium orthovanadate, and 1.0 mmol/L phenylmethyl sulfonyl fluoride. Protein concentrations of all tissue extracts were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein lysates were boiled in sample buffer for 5 minutes then equal amounts of sample were loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred to a polyvinylidene difluoride membrane. Anti- α -SMA antibody (clone 1A4, Sigma Chemical Co.) was used at a dilution of 1:1000 followed by horseradish peroxidase-labeled rabbit second antibody (1:20,000) to detect specific protein bands at 42 kd relative to rainbow markers, using standard ECL techniques as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

Tubulointerstitial Relationships

The location of interstitial myofibroblast was examined by assessing the temporal and spatial relationships of interstitial and cortical tubular cells relative to the TBM and the renal vasculature using dual-label immunofluorescence, electron microscopy, and by intravital tracking the fate of fluorescence-labeled proximal tubular cells loaded with Texas Red-dextran (TR-dextran) as a tracer as outlined below.

Fibroblast-myofibroblast conversion and tubular epithelial-mesenchymal transition was assessed by the immunofluorescence detection of myofibroblast marker α -SMA. Dual-label immunofluorescence was used to assess the expression of the individual marker proteins in peritubular myofibroblasts and tubular epithelial cells relative to the tubular basement membrane (TBM) using laminin as an indicator of tubular boundaries and using fluorescence techniques similar to those previously described.^{40,46} To detect myofibroblasts relative to the TBM, a Cy3-labeled mouse anti- α -SMA antibody (clone 1A4, Sigma) was used at a 1:100 dilution then the sections were incubated with rabbit anti-laminin IgG, 20 μ g/ml (Lab Vision Corporation, Fremont, CA), followed by incubation with FITC-labeled mouse anti-rabbit IgG to detect TBM in green. To examine epithelial cell location relative to the TBM and interstitium, a monoclonal antibody to Na^+, K^+ -ATPase (clone α 5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was used followed by staining for laminin as above. In addition, dual staining of sections with α -SMA and Na^+, K^+ -ATPase was performed to examine co-localization of these phenotypic markers. Second antibodies, developed for dual-label purposes by adsorption against IgG of multiple species other than primary, were obtained from Chemicon Int. Sections were examined by epifluorescence using excitation and band-pass filters optimal for either FITC or Cy3. Paired digital images representing each fluorochrome were taken of random fields using a Magnafire digital camera (Optronics, Inc., Goleta, CA) attached to an AX70 research microscope (Olympus, Melville, NY). The images were merged and colors balanced using Image-Pro software.

Dual-Label Assessment of Myofibroblast and Tubular Epithelial Cell Location in Early Lesions

Structural compartments in early 48-hour lesions remained clearly defined and allowed assessment of location of myofibroblasts and epithelial cell markers relative to the vasculature (arteries and arterioles), glomeruli, tubules, and the TBM. Lesions are accelerated in this model so that at later time points the boundaries among the compartments progressively became obscure and determination of the relative location of cells was not always permissible by dual-label immunofluorescence. Preliminary observations also indicated that α -SMA-positive myofibroblasts in early lesions were exclusively in the interstitium particularly in perivascular areas and not observed within the tubular compartment. To verify the location of myofibroblasts in early lesions, specific struc-

Table 1. Renal Function and Blood Pressure at 2 Weeks

	Plasma Cr (mg/dl)	Kidney weight (g)	GFR (ml/minute)	GFR/kidney weight (ml/minute/g)	Urine protein (mg/24 hours)	Blood pressure (mmHg)
Control	0.50 ± 0.01	1.6 ± 0.1	1.26 ± 0.06	0.79 ± 0.06	8.9 ± 2.6	122 ± 1
HV-alone	0.45 ± 0.03	2.1 ± 0.3	1.51 ± 0.12	0.74 ± 0.06	5.8 ± 1.3	119 ± 2
All-alone	0.45 ± 0.08	1.7 ± 0.2	0.97 ± 0.07	0.59 ± 0.05	17.2 ± 4.9*	199 ± 9*
HV + All	0.84 ± 0.03*	2.4 ± 0.3	0.38 ± 0.04*	0.17 ± 0.03*	101.6 ± 30*	197 ± 8*

*Value is statistically significant versus control, $P < 0.05$.

tures associated with α -SMA-positive cells were quantified. Twenty random $\times 20$ fields showing positive α -SMA staining or expanded interstitial spaces were examined using a FITC/Cy3 multiband filter set in combination with an AX-REXBA-2 excitation balancer for simultaneous viewing of multiple fluorochromes. Interstitial or tubular staining for α -SMA-positive myofibroblasts or Na^+, K^+ -ATPase-positive tubular cells relative to the TBM were quantified in 100 tubular profiles that were directly associated with myofibroblast staining or an expanded peritubular interstitium.

The distribution of interstitial myofibroblast staining was evaluated by counting specific compartments directly associated with α -SMA staining in 20 random fields. The following interstitial compartments were assessed: 1) perivascular tree (arteries and arterioles), 2) periglomerular interstitium, and 3) the peritubular interstitium without a visible arterial or glomerular vasculature.

Intravital Tracking of Fluorescence-Labeled Proximal Tubular Cells

Evidence for epithelial-mesenchymal transition was also explored by the use of Texas Red (TR)-dextran (3000 kd; Molecular Probes, Eugene, OR), as a functional tracer to track proximal tubular cell location within renal lesions at 48 hours and 7 and 14 days after HV + Ang II. Epithelial-mesenchymal transition may lead to the loss of specific markers precluding the ability to specifically identify them as the cell of origin of peritubular myofibroblasts. The specific functional characteristic of proximal tubular cells to endocytose small molecular weight fluorescent dextrans was exploited as a means to load the cells with a tracer⁴⁷ that can be expected to remain in the cells through their transition into mesenchymal cells during the course of renal fibrosis. These experiments enabled the tracking of the location of tagged dextran-laden proximal tubule cells within renal lesions and determine whether these cells were the source of myofibroblasts located in the peritubular interstitium early in the course of HV + Ang II-induced renal fibrosis.

Proximal tubular cells were loaded with a small molecular weight TR-dextran, by endocytotic uptake of the tracking dye after a bolus injection of 1 mg in 0.5 ml of PBS via the tail vein in anesthetized rats (three rats/group) and the spatial relationship of TR-dextran-labeled proximal tubular cells in contrast to the interstitial space and peritubular myofibroblasts was examined by immunofluorescence as described above. In additional experiments, sheep antibody to extracellular matrix (principally

laminin) from Engelbreth-Holm-Swarm tumor was labeled with 7-amino-4-methylcoumarin (AMCA) according to the manufacturer's instructions (Pierce, Rockford, IL) and used in triple immunofluorescence experiments to identify the TBM. The AMCA-sheep anti-ECM was used to stain the TBM blue; FITC-anti α -SMA was used to stain myofibroblasts green and TR-dextran in proximal tubules stained red. Sections were photographed, and images merged as above.

Electron Microscopy

Tissue obtained from kidney cortex of four rats at each time point was fixed overnight in cold 4% formaldehyde and 1% glutaraldehyde in phosphate buffer, and then embedded in epon 812 resin by routine methods. Plastic sections (0.5 μm) were cut and stained with toluidine blue for identification of representative areas and subsequent ultramicrotomy. Ultrathin sections were stained with uranyl acetate and myofibroblast ultrastructure in the HV + Ang II-treated rats was evaluated and photographed using a Philips 208 S electron microscope. In addition, semithin plastic sections of early lesions of HV + Ang II rats were evaluated by light microscopy for epithelial *trans*-migration through the TBM. One hundred tubular profiles clearly associated with interstitial myofibroblast were assessed with a high-power objective ($\times 100$) and evidence of cells crossing tubular-interstitial compartments was evaluated.

Results

HV + Ang II Accelerates Renal Fibrosis

The results of this study indicate that combination of HV + Ang II leads to a progressive renal fibrosis that by 14 days resembles chronic renal failure with marked renal functional impairment and histopathological alterations substantially more severe than when either agent was used alone.

Renal Function

Renal function and blood pressure measurements are summarized in Table 1. The results showed a loss in renal function (glomerular filtration rate) in animals administered HV + Ang II, corresponding to the histological alterations observed at 14 days described below. Rats receiving Ang II alone also showed modest reductions in

Figure 1. Histopathology of rat kidneys 14 days after treatment with HV alone (**a**), Ang II infusion alone (**b**), or HV + Ang II infusion (**c–f**). **a:** Lesions 14 days after HV are characterized by resolving mesangial proliferative nodules (**arrow**) and no noticeable interstitial disease. **b:** Ang II infusion alone results in small foci of interstitial fibrosis adjacent to vessels (**arrows**) with minimal glomerular histological changes. **c:** Combined treatment with HV + Ang II results in dilated and atrophic renal tubules, glomerulosclerosis, and interstitial fibrosis. **d–f:** Higher magnification of HV + Ang II-induced nephropathy illustrates mesangioproliferative glomerulonephritis and crescents (**d**), a small artery with a thickened media and expanded adventitia (**e**), and tubulointerstitial hypercellularity and fibrosis (**f**). H&E stain. Scale bars: 100 μ m (**a–d, f**); 50 μ m (**e**).

glomerular filtration rate, most likely related to focal perivascular fibrosis observed at this time. Glomerular filtration rate in rats receiving HV alone was not statistically different from sham controls. HV + Ang II rats showed marked proteinuria compared to the other groups and Ang II alone showed a modest but not significant urine protein compared to control.

Systolic blood pressure taken near termination of the experiment in control and HV-alone rats were in the normotensive range showing values of 122 ± 1 mmHg and 119 ± 4 mmHg, respectively. Both Ang II-alone and HV + Ang II rats were hypertensive at this time with mean systolic pressures of 199 ± 9 mmHg and 197 ± 8 mmHg, respectively. Differences in mean systolic pressures of Ang II and HV + Ang II groups versus controls were statistically significant at $P < 0.05$.

Histology

Kidneys of normal control rats were free of pathological abnormalities. Kidneys of rats given HV alone showed typical progression of mesangioproliferative glomerular characterized by microaneurysms in early lesions followed by micronodules as previously described.^{37–40} The lesions begin to resolve and decrease in size by 14 days after HV (Figure 1a). Glomerular lesions were observed with an incidence of $10.2 \pm 1.7\%$, $11.8 \pm 3.1\%$, and $5.8 \pm 0.7\%$ at 48 hours and 7 and 14 days after HV, respectively. Interstitial or vascular abnormalities were not observed in kidneys of rats treated with HV alone.

Kidney sections of rats infused with Ang II alone showed no lesions at 48 hours and 7 days; however at 14 days after Ang II infusion, areas of fibrosis (Figure 1b)

Figure 2. a–d: ECM accumulation illustrated by immunoperoxidase expression of Fn-EIIIA protein in kidney sections from rats 14 days after administration of HV alone (**a**), Ang II alone (**b**), and HV + Ang II (**c**, **d**). **e** and **f**: Accumulation of collagens type I (**e**) and type IV (**f**). Matrix proteins are abundant HV + Ang II relative to HV or Ang II treatments alone. Sections lightly counterstained with hematoxylin. Scale bars: 100 μ m (**a–c**); 50 μ m (**d–f**).

were observed in small perivascular foci near arteries and veins with an average incidence of 7.0 ± 2.4 foci per kidney cross-section (Figure 1b). Most of the glomeruli and the renal parenchyma appeared normal.

Kidneys of rats given a combination of HV + Ang II had an accelerated diffuse and progressive nephropathy. At 48 hours, 93% of glomeruli had lesions characterized as microaneurysms or micronodules. Tubular casts and increased interstitial cellularity was observed throughout the kidney. At 7 days, glomeruli, tubules, and interstitium were hypercellular and interstitial space was expanded. Lesions at 14 days were characteristic of progressive renal disease (Figure 1; c to f) showing mesangioproliferative glomerulonephritis, and glomerular crescents (Figure 1d), arterial medial thickening and adventitial widening (Figure 1e), dilated and atrophied tubules, and expanded interstitial space and fibrosis (Figure 1f). Periodic acid-Schiff-Jones and trichrome staining at 2 weeks after treatment was markedly enhanced in HV + Ang II-treated rats compared to other groups (data not shown), indicating increased matrix accumulation as defined below.

Matrix Accumulation

Normal control kidneys at all time points showed only weak staining for Fn-EIIIA in the glomerular mesangium.^{39,40} Kidneys of rats receiving HV alone showed a course of Fn-EIIIA expression similar to that previously described^{37,39} peaking at 7 days and diminishing at 14 days (Figure 2a). Fn-EIIIA expression in kidneys of rats administered Ang II alone was increased in the glomerular mesangium at all time points examined. Similarly, foci of increased expression of Fn-EIIIA were observed in the peritubular interstitium, particularly near arteries, increasing in intensity and area throughout time (Figure 2b). However, most of the peritubular interstitium stained faintly or did not stain for Fn-EIIIA (Figure 2b). On the other hand, kidneys of rats treated with HV + Ang II demonstrated an accelerated and progressive increase in the expression of Fn-EIIIA throughout the course of the disease. The area and intensity of Fn-EIIIA protein progressively increased, showing large expanses of staining by 14 days (Figure 2, c and d). Kidneys also stained with a similar distribution for collagen types I (Figure 2e) and

Extracellular Matrix Expansion (2wks) Fibronectin EIIIA

Figure 3. Quantitative image analysis of matrix accumulation at 2 weeks based on the total area of the kidney occupied by Fn-EIIIA protein by immunohistochemistry. Area occupied by Fn-EIIIA in sections from HV + AI was 42% versus HV alone (7%), AI alone (11%), or controls (2%).

IV (Figure 2f) in renal lesions. Fibrillary collagen was observed by electron microscopy and dimpling of the kidney subcapsular surface was apparent at 7 and 14 days, indicating progression of the disease toward a more advanced fibrotic process. Quantitative image analysis of matrix expansion at 14 days measured a substantial increase in area occupied by Fn-EIIIA from HV + Ang II ($42 \pm 3\%$) compared to HV alone ($7 \pm 2\%$), Ang II alone ($11 \pm 1\%$), or controls ($2 \pm 0.5\%$), $P < 0.05$ HV + Ang II versus control, HV and Ang II alone (Figure 3).

Myofibroblast Activation (Expression of α -SMA)

Western blot analysis of protein extracts also showed a progressive increase in α -SMA expression throughout the course of HV + Ang II-induced fibrosis (Figure 4a). At 14 days, when α -SMA protein was maximally expressed in kidney lysates from rats administered HV + Ang II, only faint expression was observed in lysates from control, HV alone, and Ang II alone, reflecting the large difference in fibrosis in glomeruli and the interstitium in HV + Ang II histological sections relative to the other groups (Figure 4b).

Figure 4. Western blot analysis of α -SMA expression in renal cortical lysates throughout the course of HV + Ang II-induced renal fibrosis, showing progressive increments in protein expression (a) and in comparison with controls, HV alone, and Ang II infusion alone at 14 days (b).

Temporal-Spatial Relationships of Interstitial Fibroblasts with Tubules and the Microvasculature

Location of Myofibroblast

Expression of α -SMA in renal lesions paralleled expression of Fn-EIIIA, an observation in agreement with previous studies from this laboratory.⁴⁰ As previously described, α -SMA in normal control kidneys was seen exclusively in arteries and arterioles and not the glomerular mesangium or interstitium.⁴⁰ In rats receiving HV alone, staining for α -SMA was present in activated mesangial cells in glomerular lesions at 48 hours (Figure 5a), peaking at 7 days (Figure 5b), and subsiding by 14 days (Figure 5c). Staining in the periglomerular and peritubular interstitium was weak to negligible reflecting the sparse myofibroblast population in these areas. Tubules did not stain for α -SMA.

Ang II-infusion induced an increase in expression of α -SMA in the glomerular mesangium at all time points examined (Figure 5; d to f). Peritubular staining for α -SMA was negligible at 48 hours (Figure 5d), but increased throughout time particularly near arteries and limited to a fine peritubular interstitial myofibroblast staining after 7 and 14 days of Ang II infusion (Figure 5, e and f). The tubular epithelium remained negative for α -SMA with intact TBMs.

Kidneys of rats treated with HV + Ang II showed an accelerated and progressive increase in α -SMA staining throughout the course of the disease (Figure 5; g to i) consistent with development of an expanded myofibroblast population in interstitial spaces and fibrosis and glomerulosclerosis. α -SMA-positive myofibroblasts were first observed, at 48 hours, around arteries, arterioles, and glomeruli (Figure 5g, arrows), with progressively larger expanses of fibroblast in these areas and peritubular interstitium by 7 and 14 days. At 48 hours after HV + Ang II, tubules showed no evidence of α -SMA expression and TBM were intact by linear staining of laminin. Tubular interstitial space and α -SMA staining progressively increased throughout time. At 7 days after HV + Ang II areas of increased peritubular α -SMA expression widened. The majority of tubules in these areas were α -SMA-negative. Occasionally, weakly stained material was detected in small punctuate spots within the center of the lumen within the confines of the proximal tubule. In large areas of expanded fibrosis, many tubules were atrophied, but retained an intact TBM assessed by laminin staining. However, the TBM of some atrophied tubules stained weakly for laminin and α -SMA-positive myofibroblast were observed throughout the area of fibrosis. Fibrotic lesions with α -SMA staining were more expansive by 14 days after HV + Ang II (Figure 5i).

Evaluation of the distribution of myofibroblast markers in tubulointerstitial compartments during early lesions revealed that α -SMA-positive cells were located exclusively within interstitium and not within the tubules (Table 2). Examination of staining in individual interstitial compartments showed that the predominant location of α -SMA-positive cells occurred in perivascular (periarterial, arte-

Figure 5. Dual-label immunofluorescence micrographs of kidneys stained for α -SMA (red) relative to the TBM (laminin, green) in rats treated with HV (**a–c**), Ang II (**d–f**), and HV + Ang II (**g–i**) at 48 hours (**a, d, g**), and 7 (**b, e, h**) and 14 (**c, f, i**) days. α -SMA in glomerular and interstitial lesions after HV diminishes by 14 days. In rats given Ang II alone, mesangial and interstitial expression of α -SMA increases throughout time, but without substantial histopathological lesions by H&E. Combination of HV + Ang II results in an abundant expression of α -SMA-positive myofibroblasts in glomeruli and the interstitium reflecting the progressive fibrosis observed by H&E in Figure 1. Scale bars, 100 μ m.

riolar, and periglomerular) regions, comprising a total of 87% of the staining. The remaining 13% of area with α -SMA-positive staining was observed within peritubular interstitial areas that could not be directly associated with vascular profiles (Table 2).

Position of Tubular Epithelial Cells

Immunohistochemistry for Na^+, K^+ -ATPase showed epithelial staining of proximal and distal convoluted tubules, thick ascending limb of Henle, and collecting

Table 2. Tubulo/Interstitial Distribution of Myofibroblast and Tubular Markers

Marker/cell type		Peritubular interstitium*	Tubular epithelium*
α -SMA/myofibroblast		100	0
Na^+, K^+ -ATPase/proximal and distal tubules		0	100
TR-dextran/proximal tubules		0	100
Location of Myofibroblasts in Interstitial Compartments [†]			
Marker	Peritubular with no visible vasculature [‡]	Vascular tree	Glomeruli
α -SMA	2.7/ 13%	10.2/ 51%	7.2/ 36%

*Distribution of staining for cell markers was evaluated in 100 tubular profiles adjacent to myofibroblast encroachment or expanded interstitial spaces.

[†]Location of myofibroblast staining directly connected to listed interstitial compartments.

[‡]Arteries, arterioles, or glomeruli.

ducts as reported in the literature.⁴⁸ Dual-label staining of this epithelial marker with laminin showed staining in cells exclusively within confines of proximal and distal tubules. Staining was not detected in the interstitial compartment. Similarly, dual-label immunofluorescence examination of Na^+, K^+ -ATPase relative to α -SMA-positive myofibroblast verified these findings where each marker stained exclusively in their respective compartments, with no co-expression determined by direct observation (Table 2) or in merged photographs (Figure 6, a to c).

Intravital Tracking Experiments

Injection of TR-dextran in control rats resulted in a uniform pattern of proximal tubular endocytotic uptake of the red fluorescence dextran, identifying the cells by fluorescence of numerous punctate endosomes. The dye was detected in proximal tubular cells of controls up to 7 days after injection; however, only trace amounts remained in controls at 14 days. Staining was not detected in other cell types including interstitial cells, mesangial, endothelial, or circulating leukocytes. Studies in HV + Ang II rats indicated that TR-dextran-laden endosomes accumulated and remained in proximal tubules for a longer duration and greater extent and intensity compared to controls, most likely reflecting retarded metabolic degradation of the tracer. Therefore, tracking experiments could easily be performed up to the 14-day time point when proximal tubular content of TR-dextran was detected and used for multiple staining experiments.

Careful examination of tubular-interstitial compartments using TR-dextran as a functional marker for labeled proximal tubular cells relative to FITC-labeled TBM revealed no evidence for EMT and cell migration through the basement membrane into the interstitial space in early lesions (Table 2) or throughout the course of HV + Ang II-induced fibrosis. TR-dextran-labeled-proximal tubule cells remained within the confines of intact TBM and the interstitium was negative for the tubular cell marker at all time points (Figure 6, d and e). Likewise, interstitial myofibroblasts stained with FITC anti- α -SMA were not observed to contain TR-dextran in tricolor merged images using AMCA anti-ECM antibody (Figure 6, f and g; Table 2) as a structural marker for the TBM.

Electron Microscopy

Renal cortex of controls showed normal architecture with tubules and capillaries directly adjacent to each other, with limited interstitial space and few fibroblasts in toluidine blue-stained plastic sections by bright-field and electron microscopy. In early lesions of HV + Ang II, spaces around arteries, arterioles, and tubules were increased and occupied by interstitial cells (Figure 7a). These cells had characteristics of fibroblasts and showed no vestigial or remnants of differentiated characteristics of proximal tubules such as brush border, elaborate endocytotic or dense lysosomal apparatus, elongated mitochondria, and so forth (Figure 7b). Tubules frequently appeared normal, but the basement membrane was thickened. Some tubules showed signs of atrophy by cellular separation from the TBM, increased vacuolization, and diminution in size of the cells and nuclear condensation consistent with apoptosis. Breaks in the TBM or cells traversing the epithelial-interstitial margin were not observed. By 7 days, the interstitial space around vessels (Figure 7d) and between TBM and peritubular capillary endothelium was greatly expanded and filled with cells similar in morphology as described at 48 hours. Tubules were frequently smaller in size with thickened basement membrane. Atrophied tubules were frequently comprised of collapsing, involuting epithelia, increased vesicles, and loss of tubular lumens (Figure 7c). Perivascular and peritubular interstitial compartments were greatly expanded and atrophic tubules were smaller at 14 days. Fibrillary collagen was frequently observed in interstitial spaces at 7 and 14 days. Light microscopical evaluation of 0.5- μm semithin sections at areas showing varying degrees of peritubular interstitial fibroblast encroachment showed no cellular profiles crossing from the TBM from the epithelium or vice versa.

Discussion

In this study we describe an accelerated model of renal fibrosis induced by Ang II infusion superimposed on mesangial and interstitial cell injury. Similar to the balloon-injury model in carotid arteries, Ang II infusion augments renal vascular injury²¹ showing increased medial thickness. In addition, combination of preinjury and Ang II

Figure 6. Expression of Na⁺,K⁺-ATPase (**a**) and α-SMA (**b**), and a merged image (**c**) by dual-label immunofluorescence at 48 hours after HV + Ang II. Na⁺,K⁺-ATPase is present exclusively in epithelia of proximal (P) and distal (D) tubular compartments, but not in the interstitium or glomeruli (G). α-SMA is present exclusively within the interstitium as indicated in their respective monochrome and in merged (**c**) photomicrographs. TR-dextran experiments show staining in proximal tubules, but not in interstitial cells in two-color merged images using laminin as a marker for the TBM (FITC-second antibody) at 48 hours (**d**) and 7 days (**e**) after HV + Ang II. Triple-dye immunofluorescence at 48 hours (**f**) and 7 days (**g**) after HV + Ang II shows myofibroblasts (FITC-mouse anti-α-SMA-positive cells) in interstitial spaces without co-localization of TR-dextran. The TBM stains blue with AMCA sheep anti-BM in tri-colored merged images. Scale bars, 100 μm.

infusion lead to severe interstitial expansion, accumulation of fibronectin and collagens I and IV, periarteriolar thickening, glomerulosclerosis, tubular atrophy and/or dilation, all of which are features of chronic renal disease similar to the renal ablation model^{49,50} and end-stage renal disease in humans.⁵¹ HV alone induces a self-limiting mesangial proliferative glomerulonephritis with lesions that by 14 days after injection begin to decrease in size and lose expression of α-SMA, and extracellular matrix synthesis, findings that are consistent with previ-

ous studies from this laboratory.^{37–40} Infusion of Ang II alone also resulted in a limited, focal perivascular interstitial fibrosis similar to, but less extensive than, that described in studies by Johnson and colleagues¹⁷ and Giachelli and colleagues¹⁹ who used a continuous infusion of a higher dose of Ang II (200 ng/min) throughout the same time period. Thus, the combination of preinjury and Ang II infusion results in acceleration of renal lesions far greater in extent and severity than either of the treatments alone.

Figure 7. Electron micrographs showing myofibroblasts (arrows) surrounding proximal tubules (PT) at 48 hours (a, b) and 7 days (c) after HV + Ang II. The perivascular and interstitial space is expanded and filled with numerous myofibroblasts. c: Epithelial cells show evidence of apoptosis including vacuolization, loss of brush border, nuclear abnormalities, and reduction in size. d: Expanded perivascular space shows numerous adventitial cells/myofibroblasts (arrows). En, Endothelium. Scale bars: 10 μm (a, d); 2 μm (b, c).

Because of the accelerated development of interstitial fibrosis, this model provided an ideal opportunity to examine the intrarenal origins of myofibroblasts that rapidly encroach in the peritubular interstitium. Evidence indicates the myofibroblasts may be derived from activated resident fibroblasts,^{17,25} perivascular adventitial cells,^{26,27} blood-borne stem cells,^{28–31} or by tubular epithelial-mesenchymal transition and migration through the TBM into the peritubular interstitial space.^{32–35} This study shows that myofibroblasts rapidly populate the peritubular interstitium within 1 day after infusion of Ang II in the HV model. α -SMA-positive myofibroblasts were first detected in perivascular regions and glomeruli in the absence of tubular epithelial staining for this mesenchymal marker suggesting that these cells primary origin is from a vascular source.

Unexpectedly, evidence for epithelial-mesenchymal transmigration into the interstitium was not observed ei-

ther by dual-labeling experiments examining the location of Na^+, K^+ -ATPase-positive epithelial cells and α -SMA-positive cells relative to the TBM or ultrastructural examination of tubulointerstitial relationships. No Na^+, K^+ -ATPase-positive cells were identified in the interstitial compartment 48 hours after HV + Ang II. Conversely, tubular epithelial cells did not stain for α -SMA at this time; however, some weak sporadic staining could be detected in a few tubules at 1 and 2 weeks. Epithelial cell tracking with TR-dextran and ultrastructural studies indicated that primary source of myofibroblast in the peritubular interstitium was not the result of proximal tubular cell transmigration across the TBM into the interstitium. Proximal tubules are the most prevalent tubular type in the cortex and are the cell type most implicated in EMT during renal fibrosis. If the primary source of myofibroblast was a result of epithelial-mesenchymal transition and transmigration through the TBM, TR-dextran-laden

myofibroblasts or Na⁺,K⁺-ATPase-positive cells in transition from the proximal tubule to the interstitial space would have been detected, particularly early, within 1 day in the disease process when tubular cells retained the TR-dextran and peritubular myofibroblasts are abundant. Moreover, the large expanses of interstitial space occupied by myofibroblasts at 7 and 14 days did not contain TR-dextran-positive cells at a time when numerous proximal tubules retained staining for fluorescence-laden granular endosomes.

These intravital dye studies do not rule out possible EMT and transmigration of epithelial cells from distal tubules or collecting ducts, because these cell types do not endocytose TR-dextran. However, the contribution of distal tubular epithelial cells is small relative to proximal tubules. Moreover, the distal nephron stains intensely for Na⁺,K⁺-ATPase and there was no indication that these cells gained access to the interstitium. Also, no evidence of cells traversing the TBM was observed in high-resolution plastic sections or by electron microscopy. The increase in interstitial area was accompanied by tubular atrophy, most likely due to apoptosis and drop-out of tubular epithelial cells beginning at 48 hours and continuing throughout the course of HV + Ang II-induced disease, suggesting that the myofibroblast encroach and crowd out a shrinking tubular mass.

Expression of α -SMA by interstitial myofibroblasts after HV + Ang II treatment was found exclusively in the perivascular and peritubular interstitium similar to that described in the laboratories of Johnson and colleagues^{17,20} except with a more rapid and severe course. With regard to the origin of myofibroblasts, our studies are in agreement with Wiggins and colleagues²⁶ and Lloyd and colleagues²⁷ indicating the myofibroblasts are derived from perivascular and periglomerular regions in anti-GBM disease in the rabbit and mouse, respectively. Both groups used *in situ* hybridization to detect myofibroblast expression of type I collagen RNA during the course of the disease. Expression of collagen I RNA was first revealed within a pool of cells in the periarterial, periarteriolar, and periglomerular interstitium. The authors concluded that the perivascular adventitial cells are among the first cells to respond to glomerular inflammation, migrating into the interstitial space leading to fibrosis.²⁶ Similarly, in our study collagen type I was strongest in perivascular and periglomerular locations and fibronectin was detected in perivascular and peritubular interstitial spaces correlating with myofibroblast α -SMA expression as described in the anti-GBM and HV model in previous studies.⁴⁰ Our studies show that more than 87% of interstitial myofibroblast staining was directly associated with vascular (arterial and glomerular) structures. Thirteen percent of interstitial staining was not directly linked to vascular structures. Because no evidence of EMT was observed, it is likely that these cells represent areas of staining near vascular structures outside the plane of the section.

A perivascular source of myofibroblasts could also reflect infiltration of circulating stem cells. Circulating fibrocytes have been shown to migrate to wound sites in the skin.⁵³ A stem cell origin of myofibroblasts has been

reported to be a small component in a mouse model of renal fibrosis.³² Such a source of myofibroblasts cannot be ruled out in our experiments and remains an area for further investigation. The mechanisms of the rapid progression of glomerular and interstitial fibrosis with features of chronic renal failure in this model also remain to be determined. This accelerated model of fibrosis provides a new means to study early cellular mechanisms of tubulointerstitial pathology.

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References

1. Border WA, Noble NA: Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. *Hypertension* 1998, 31:181–188
2. Klahr S, Morrissey JJ: Comparative study of ACE inhibitors and angiotensin II receptor antagonists in interstitial scarring. *Kidney Int* 1997, 52:S111–S114
3. Wolf G, Neilson EG: Angiotensin II as a renal growth factor. *J Am Soc Nephrol* 1993, 3:1531–1540
4. Klahr S, Morrissey JJ: The role of vasoactive compounds, growth factors and cytokines in the progression of renal disease. *Kidney Int* 2000, 57:S7–S14
5. Matsusaka T, Hymes J, Ichikawa I: Angiotensin in progressive renal diseases: theory and practice. *J Am Soc Nephrol* 1996, 7:2025–2043
6. Fogo AB: The role of angiotensin II and plasminogen activator inhibitor-1 in progressive glomerulosclerosis. *Am J Kidney Dis* 2000, 35:179–188
7. Peters H, Border WA, Noble NA: Targeting TGF- β overexpression in renal disease: maximizing the antifibrotic action of angiotensin II blockade. *Kidney Int* 1998, 54:1570–1580
8. Shihab FS, Bennett WM, Tanner AM, Andoh TF: Angiotensin II blockade decreases TGF- β 1 matrix proteins in cyclosporine nephropathy. *Kidney Int* 1997, 52:660–673
9. Peters H, Border WA, Noble NA: Angiotensin II blockade and low-protein diet produce additive therapeutic effects in experimental glomerulonephritis. *Kidney Int* 2000, 57:1493–1501
10. Tanaka R, Kon V, Yoshioka T, Ichikawa I, Fogo A: Angiotensin converting enzyme inhibitor modulates glomerular function and structure by distinct mechanisms. *Kidney Int* 1994, 45:537–543
11. Oikawa T, Freeman M, Lo W, Vaughan DE, Fogo A: Modulation of plasminogen activator inhibitor-1 in vivo: a new mechanism for the anti-fibrotic effect of renin-angiotensin inhibition. *Kidney Int* 1997, 51:164–172
12. Moulder JE, Fish BL, Cohen EP, Bonsib SM: Angiotensin II receptor antagonists in the prevention of radiation nephropathy. *Radiat Res* 1996, 146:106–110
13. Boffa J-J, Lu Y, Placier S, Stefanski A, Dussault J-C, Chatziantoniou C: Regression of renal vascular and glomerular fibrosis: role of angiotensin II receptor antagonism and matrix metalloproteinases. *J Am Soc Nephrol* 2003, 14:1132–1144
14. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. *N Engl J Med* 1993, 329:1456–1462
15. Cattran DC, Greenwood C, Ritchie S: Long-term benefits of angiotensin-converting enzyme inhibitor therapy in patients with severe immunoglobulin A nephropathy: a comparison to patients receiving treatment with other antihypertensive agents and to patients receiving no therapy. *Am J Kidney Dis* 1994, 23:247–254
16. Taal MW, Brenner BM: Renoprotective benefits of RAS inhibition: from ACEI to angiotensin II antagonists. *Kidney Int* 2000, 57:1803–1817
17. Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, Schwartz SM: Renal injury from angiotensin II-mediated hypertension. *Hypertension* 1992, 19:464–474

18. Ruiz-Ortega M, Lorenzo O, Ruperez M, Blanco J, Egido J: Systemic infusion of angiotensin II into normal rats activates nuclear factor- κ B and AP-1 in the kidney. Role of AT1 and AT2 receptors. *Am J Pathol* 2001, 158:1743–1756
19. Giachelli CM, Pichler R, Lombardi D, Denhardt DT, Alpers CE, Schwartz SM, Johnson RJ: Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. *Kidney Int* 1994, 45:515–524
20. Lombardi DM, Viswanathan M, Vio CP, Saavedra JM, Schwartz SM, Johnson RJ: Renal and vascular injury induced by exogenous angiotensin II is AT1 receptor-dependent. *Nephron* 2001, 87:66–74
21. Daemen MJAP, Lombardi DM, Bosman FT, Schwartz SM: Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ Res* 1991, 68:450–456
22. Locatelli F, Del Vecchio L, Andrulli S, Marai P, Tentori F: The role of underlying nephropathy in the progression of renal disease. *Kidney Int* 2000, 57:S49–S55
23. Yamamoto T, Noble NA, Miller DE, Border WA: Sustained expression of TGF- β 1 underlies development of progressive kidney fibrosis. *Kidney Int* 1994, 45:916–927
24. Desmouliere A, Darby I, Gabbiani G: Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest* 2003, 83:1689–1707
25. Tang WW, Ulich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, Van GY, Tarpley JE, Yee JS: Platelet-derived growth factor-BB induces renal tubulointerstitial myofibroblast formation and tubulointerstitial fibrosis. *Am J Pathol* 1996, 148:1169–1180
26. Wiggins R, Goyal M, Merritt S, Killen PD: Vascular adventitial cell expression of collagen I messenger ribonucleic acid in anti-glomerular basement membrane antibody-induced crescentic nephritis in the rabbit. A cellular source for interstitial collagen synthesis in inflammatory renal disease. *Lab Invest* 1993, 68:557–565
27. Lloyd CM, Minto AW, Dorf ME, Proudfoot A, Wells TN, Salant DJ, Gutierrez-Ramos JC: RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J Exp Med* 1997, 185:1371–1380
28. Al-Awqati Q, Oliver JA: Stem cells in the kidney. *Kidney Int* 2002, 61:387–395
29. Cornacchia F, Fornoni A, Plati AR, Thomas A, Wang Y, Inveradi L, Striker LJ, Striker GE: Glomerulosclerosis is transmitted by bone marrow-derived mesangial cell progenitors. *J Clin Invest* 2001, 108:1649–1656
30. Forbes SJ, Poulosom R, Wright NA: Hepatic and renal differentiation from blood-borne stem cells. *Gene Therapy* 2002, 9:625–630
31. Ito T, Suzuki A, Imai E, Okabe M, Hori M: Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling. *J Am Soc Nephrol* 2001, 12:2625–2635
32. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002, 110:341–350
33. Strutz F, Muller GA, Neilson EG: Transdifferentiation: a new angle on renal fibrosis. *Exp Nephrol* 1996, 4:267–270
34. Okada H, Danoff TM, Kalluri R, Neilson EG: Early role of Fsp1 in epithelial-mesenchymal transformation. *Am J Physiol* 1997, 273:F563–F574
35. Ng YY, Huang TP, Yang WC, Chen ZP, Yang AH, Mu W, Nikolic-Paterson DJ, Atkins GB, Lan HY: Tubular epithelial-myofibroblast transdifferentiation in progressive tubulointerstitial fibrosis in 5/6 nephrectomized rats. *Kidney Int* 1998, 54:864–876
36. Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003, 112:1776–1784
37. Barnes JL, Hastings RR, De La Garza MA: Sequential expression of cellular fibronectin by platelets, macrophages, and mesangial cells in proliferative glomerulonephritis. *Am J Pathol* 1994, 145:585–597
38. Barnes JL, Abboud HE: Temporal expression of autocrine growth factors corresponds to morphological features of mesangial proliferation in Habu snake venom-induced glomerulonephritis. *Am J Pathol* 1993, 143:1366–1376
39. Barnes JL, Torres ES, Mitchell RJ, Peters JH: Expression of alternatively spliced fibronectin variants during remodeling in proliferative glomerulonephritis. *Am J Pathol* 1995, 147:1361–1371
40. Barnes VL, Musa J, Mitchell RJ, Barnes JL: Expression of embryonic fibronectin isoform EIIIA parallels α -smooth muscle actin in maturing and diseased kidney. *J Histochem Cytochem* 1999, 47:787–797
41. Johnson RJ, Floege J, Yoshimura A, Iida H, Couser WG, Alpers CE: The activated mesangial cell: a glomerular "myofibroblast"? *J Am Soc Nephrol* 1992, 2:S190–S197
42. Alpers CE, Pichler R, Johnson RJ: Phenotypic features of cortical interstitial cells potentially important in fibrosis. *Kidney Int* 1996, 49:S28–S31
43. Alpers CE, Hudkins KL, Gown AM, Johnson RJ: Enhanced expression of "muscle-specific" actin in glomerulonephritis. *Kidney Int* 1992, 41:1134–1142
44. Alpers CE, Hudkins KL, Floege J, Johnson RJ: Human renal cortical interstitial cells with some features of smooth muscle cells participate in tubulointerstitial and crescentic glomerular injury. *J Am Soc Nephrol* 1994, 5:201–209
45. Boukhalfa G, Desmouliere A, Rondeau E, Gabbiani G, Sraer JD: Relationship between alpha-smooth muscle actin expression and fibrotic changes in human kidney. *Exp Nephrol* 1996, 4:241–247
46. Barnes JL, Mitchell RJ, Torres ES: Expression of plasminogen activator-inhibitor-1 (PAI-1) during cellular remodeling in proliferative glomerulonephritis in the rat. *J Histochem Cytochem* 1995, 43:895–905
47. Dunn KW, Sandoval RM, Kelly KJ, Dagher PC, Tanner GA, Atkinson SJ, Bacallao RL, Molitoris BA: Functional studies of the kidney of living animals using multicolor two-photon microscopy. *Am J Physiol* 2002, 283:C905–C916
48. Kashgarian M, Biemesderfer D, Caplan M, Forbush BE: Monoclonal antibody to Na,K-ATPase: immunocytochemical localization along nephron segments. *Kidney Int* 1985, 28:899–913
49. Shimamura T, Morrison AB: A progressive glomerulosclerosis occurring in partial five-sixths nephrectomized rats. *Am J Pathol* 1975, 79:95–106
50. Hostetter T, Olson JL, Rennke HG, Venkatachalam MA, Brenner BM: Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation. *Am J Physiol* 1981, 241:F85–F93
51. Hughson MD: End-stage renal disease. *Heptinstall's Pathology of the Kidney*. Edited by Jennette JC, Olson JL, Schwartz MM, Silva FG. Philadelphia, Lippincott-Raven Publishers, 1998, pp 1371–1408
52. Stouffer GA, Owens GK: Angiotensin II-induced mitogenesis of spontaneously hypertensive rat-derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-beta. *Circ Res* 1992, 70:820–828
53. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN: Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 2001, 166:7556–7562