SPARC is expressed in renal interstitial fibrosis and in renal vascular injury

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SPARC is expressed in renal interstitial fibrosis and in renal vascular injury. Tubulointerstitial inflammation and fibrosis are critical determinants for renal function and prognosis in a variety of human nephropathies. Yet, the pathophysiology of the injury remains obscure. We investigated the expression of SPARC (secreted protein acidic and rich in cysteine) by immunohistochemistry and *in situ* hybridization in experimental models characterized by tubulointerstitial fibrosis and matrix expansion in rats. SPARC is a secreted glycoprotein that has been demonstrated to affect cellular interaction with matrix proteins, modulate cell proliferation, bind to and/or inhibit growth factors such as PDGF and bFGF, and regulate angiogenesis. Interstitial expression of SPARC was most prominent in passive Heyman nephritis (PHN), chronic cyclosporine A (CsA) nephropathy, and the remnant kidney model and, to a lesser extent, in angiotensin II (Ang II)-infused animals. SPARC protein and mRNA were substantially increased at sites of tubulointerstitial fibrosis/matrix expansion. In the PHN model, SPARC protein was expressed by interstitial fibroblasts that also produced α-smooth muscle actin ("myofibroblasts") and correlated both temporally ($r = 0.97$) and spatially with sites of type I collagen deposition. Interstitial cell proliferation preceded the development of interstitial fibrosis, and maximal SPARC expression (d15) coincided with the initial decline in interstitial proliferation. In the Ang II-infusion model, which is characterized by arteriolopathy and tubulointerstitial injury, an increase in SPARC protein and mRNA was also seen in injured blood vessels. SPARC was shown to be expressed by vascular smooth muscle cells and also by cells in the adventitia of hypertrophied arteries. In summary, SPARC was transiently expressed by interstitial fibroblasts at sites of tubulointerstitial injury and fibrosis, and by smooth muscle cells and cells in the adventitia of injured arteries in the Ang II-model. In addition to its proposed role in extracellular matrix deposition, the antiproliferative properties of SPARC might contribute to the resolution of interstitial fibroblast proliferation in the PHN model.

Tubulointerstitial inflammation and fibrosis are typical of virtually all progressive renal diseases and are characterized by an alteration of the normal tubulointerstitial architecture due to the accumulation of extracellular matrix proteins within the interstitial space. Numerous studies have demonstrated that the extent of chronic tubulointerstitial damage has been the best histologic correlate of the decline in renal function and the best predictor of

long-term renal outcome in a broad variety of renal diseases including mesangial proliferative glomerulonephritis and focal segmental glomerulosclerosis [1, 2], lupus nephritis [3, 4], membranous nephropathy [2, 5] and chronic interstitial nephritis of all types [6, 7].

In this study we investigated the role of SPARC in the development of interstitial fibrosis in a variety of models of renal disease that are characterized by interstitial fibrosis. SPARC (secreted protein acidic and rich in cysteine), also known as osteonectin and BM-40, is an extracellular matrix-associated glycoprotein, synthesized by a variety of cells and expressed at sites of tissue remodeling [8, 9]. SPARC has been shown to bind to collagen types I, III, IV, V and VIII and has been implicated in extracellular matrix deposition [8]. In addition to affecting cell shape [10], SPARC has been shown to inhibit proliferation of various cell types, including endothelial cells, fibroblasts, and smooth muscle cells [11–14]. Specifically, in aortic endothelial cells, the cell cycle was arrested in the mid-G1 phase [14]. SPARC also binds to the B-chain of PDGF (platelet-derived growth factor), a mitogen that stimulates the proliferation of vascular smooth muscle cells, fibroblasts, and mesangial cells [15, 16]. In addition, SPARC was shown to augment the production of plasminogen activator inhibitor-1 (PAI-1) and was associated with a decrease in the synthesis of fibronectin [17, 18]. SPARC can induce the expression of metalloproteases such as the 92 kDa gelatinase, stromelysin, and collagenase in synovial fibroblasts indicating a potential role in the regulation of extracellular matrix turnover [19]. SPARC expression has been shown to be increased by a variety of cytokines and growth factors, including transforming growth factor (TGF)-β1, IL-1, CSF-1, progesterone, and glucocorticoids [8].

This study demonstrates that SPARC is expressed by interstitial α-smooth muscle actin-positive fibroblasts ("myofibroblasts") in the tubulointerstitium, and that it colocalizes with cells expressing type I collagen in experimental models in which interstitial fibrosis occurs. When individual animals were compared, a close correlation ($r = 0.97$) could be shown between SPARC and collagen type I expression in the first 15 days of the PHN model, an experimental model of membranous nephropathy. Interstitial expression of SPARC correlated with the resolution of interstitial cell proliferation at day 15 of the PHN model. We also show that SPARC was

expressed by vascular smooth muscle cells and adventitial cells in injured arteries of angiotensin II (Ang II)-infused animals.

Methods

Experimental protocols

Passive Heyman nephritis (PHN), an experimental model of membranous nephropathy [20], was induced in male Sprague-Dawley rats (190 to 210 grams body wt; Tyler Laboratories, Bellevue, WA, USA) by a single i.v. injection of a sheep anti-Fx1A antibody ($N = 6$). The antibody was prepared as described elsewhere [21]. Controls included six Sprague-Dawley rats injected with sterile 0.9% NaCl solution and six rats injected with anti-Fx1A but depleted of complement with cobra venom factor (CVF; Diamedix, Miami, FL, USA) before disease induction [22]. Twenty-four-hour urinary protein excretion was determined in all rats before sacrifice to assure significant disease. Rats were sacrificed at days 5, 10, 15, 21 and 30, and renal biopsies were obtained.

Experimental cyclosporine (CsA) *nephropathy* was induced in male Sprague-Dawley rats ($N = 8$; Charles River, Wilmington, MA, USA) weighing 225 to 250 grams [23]. Rats were housed in individual cages in a temperature- and light-controlled environment. All rats received a low salt diet (0.05% sodium, Teklad, Madison, WI, USA). CsA, provided by Sandoz Research Institute (East Hanover, NJ, USA), was diluted in olive oil to a final concentration of 15 mg/ml. Rats were treated with CsA 15 mg/kg s.c. (CsA, $N = 8$) or vehicle olive oil 1 ml/kg s.c. (VH, $N = 8$) for 35 days.

Remnant kidney model. We studied male Sprague-Dawley rats (Simonsen, Gilroy, CA, USA) weighing 140 to 160 grams at the start of the experiment [24]. In the remnant kidney group ($N = 8$), a right subcapsular nephrectomy and infarction of approximately two-thirds of the left kidney was accomplished by ligation of the posterior and 1 or 2 anterior extrarenal branches of the main renal artery. In the control group ($N = 8$), we performed a sham operation consisting of laparotomy and manipulation of the renal pedicles but without destruction of renal tissue. Rats in each group underwent renal biopsy by flank incision under ether anesthesia at four weeks and were subsequently killed by an overdose of sodium pentobarbital.

Angiotensin II infusion model. Male Sprague-Dawley rats (400 to 500 g; $N = 6$; Zivic Miller, Allison Park, PA, USA) received Angiotensin II infusion (200 ng/min dissolved in Ringer's lactate; Sigma Chemical Co., St. Louis, MO, USA) continuously by subcutaneous osmotic minipumps (Alzet model 2002, Alza Corp., Palo Alto, CA, USA) [25]. Control rats ($N = 6$) received Ringer's lactate by minipump. Rats in each group underwent renal biopsy by flank incision under ether anesthesia at 7 and 14 days and were subsequently killed by an overdose of sodium pentobarbital.

Renal morphology and immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid) and were embedded in paraffin [26]. Four-micron sections were stained with periodic acid and Schiff's reagent (PAS) and were counterstained with hematoxylin. Indirect immunoperoxidase staining and immunofluorescence were performed on 4 μm sections as described previously [27–29], with the following primary antibodies: A murine monoclonal antibody (*AON-5031*) directed against human, rat, and bovine SPARC/osteonectin (Haematologic Technologies Inc., Essex Jct., VT, USA) [30, 31]; a murine monoclonal IgM antibody against the proliferating cell nuclear antigen (PCNA) (*19A2*; Coulter Immunology, Hialeah, FL, USA) [32]; a murine monoclonal IgG$_2$ antibody against α-smooth muscle actin (Sigma Chemical Co.) [32]; *ED-I*, a monoclonal antibody used as a marker for macrophages/monocytes and dendritic cells [32], and a polyclonal guinea pig antibody directed against rat collagen I [9].

SPARC and type I collagen expression by interstitial cells was graded by calculation of the percentage of tubules surrounded by SPARC or collagen I-positive interstitial cells per field (0.25 mm^2) at a $\times 200$ magnification with an ocular grid. At least 12 fields measuring 0.25 mm^2 were counted. For each time point, biopsies of at least six different animals were assessed in a blinded fashion.

Interstitial cell proliferation was assessed by determination of the number of interstitial PCNA-positive cells per 0.0625 mm^2 at a $\times 400$ magnification. At least 12 fields, each measuring 0.0625 mm^2, were counted. Interstitial cells were defined as cells that were located in between tubules and did not consist of arterial, arteriolar, or glomerular cells.

Interstitial fibrosis was scored semiquantitatively by a blinded observer, who examined cortical tubulointerstitial fields on Masson's Trichrome-stained renal biopsies with the $\times 20$ objective. A minimum of 30 fields was assessed in each biopsy. The following semiquantitative score was used: score 0 = normal interstitium and tubules; score 1 = mild fibrosis with minimal interstitial thickening between the tubules; score 2 = moderate fibrosis with moderate interstitial thickening between the tubules; score 3 = severe fibrosis with severe interstitial thickening between the tubules.

Immunohistochemical double labeling. Double immunolabeling for SPARC and α-smooth muscle actin was performed to determine the cell types expressing SPARC in our disease models. Tissue was fixed with Methyl-Carnoy's solution, embedded in paraffin, and stained by an indirect immunofluorescence technique for SPARC and for α-smooth muscle actin, a marker for myofibroblasts [32] and vascular smooth muscle cells [33]. Tissue sections were first incubated with a murine monoclonal IgG1 antibody directed against SPARC (*AON-5031*) (Haematologic Technologies Inc.) overnight at 4°C, followed sequentially by a 60 minute incubation with a biotinylated rabbit anti-mouse IgG1 antibody (Zymed, San Francisco, CA, USA) at room temperature (RT), a 60 minute incubation with a biotinylated goat anti-rabbit antibody (Vector) at RT, and a 60 minute incubation with Texas Red-labeled Avidin D (Vector) at RT in the dark. The next step consisted of an overnight incubation with a murine monoclonal IgG2a antibody directed against α-smooth muscle actin (Sigma), followed by a 60 minute incubation with an FITC (fluorescein isothiocyanate)-labeled anti-mouse IgG2a antibody (Cappel, West Chester, PA, USA). Cross reactivity between the FITC-conjugated anti-mouse IgG2a antibody used to detect α-smooth muscle actin and the IgG1 (SPARC antibody) was ruled out by omission the α-smooth muscle actin antibody. FITC staining was not observed under these circumstances.

Double immunolabeling for SPARC and type I collagen was performed to determine whether collagen I was expressed by the same cells that produce SPARC and whether the two proteins
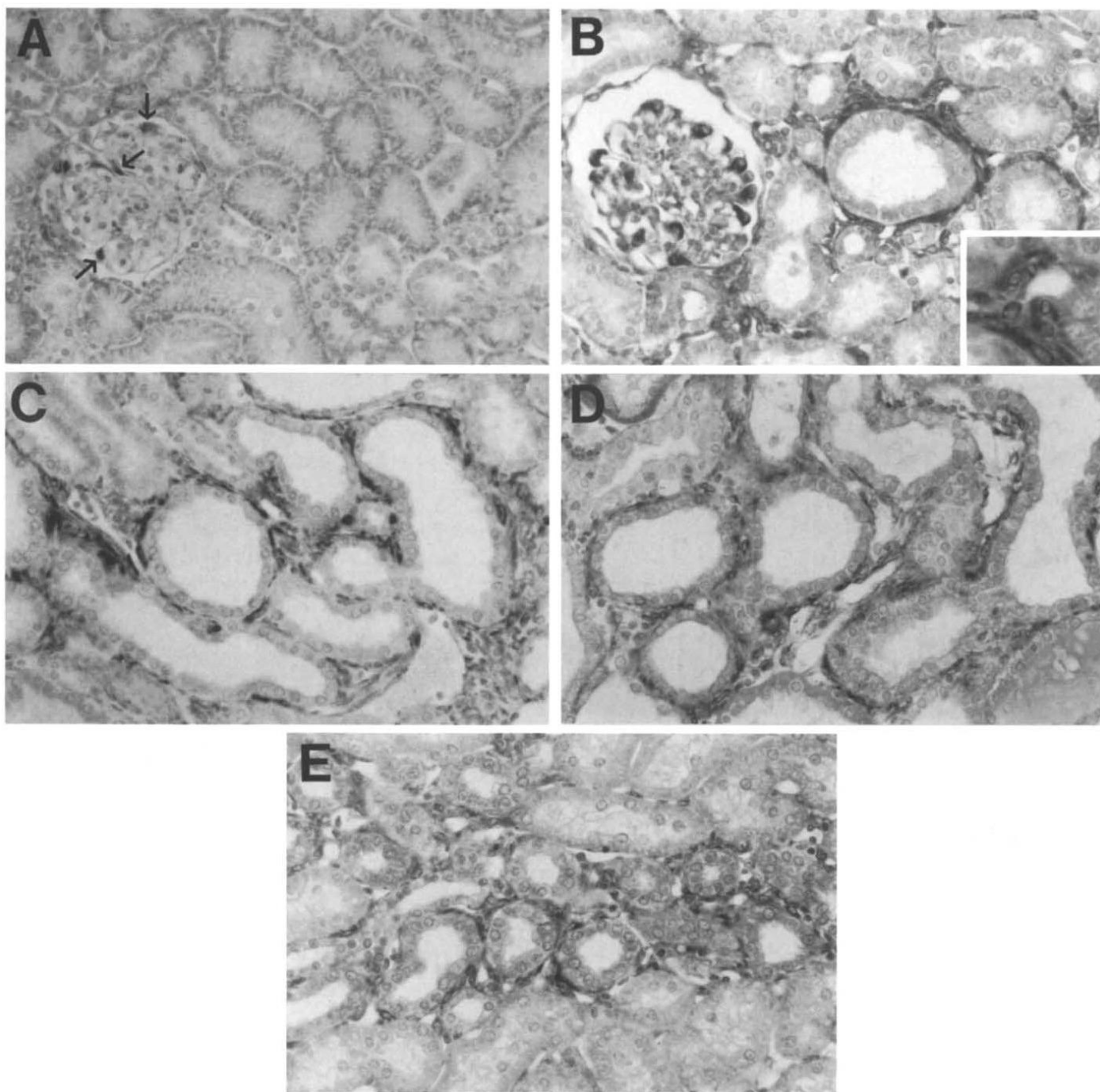
Fig. 1. *SPARC protein is expressed in areas of tubulointerstitial fibrosis.* In normal rats (**A**), SPARC was expressed by visceral glomerular epithelial cells (arrows) in the glomeruli but was absent from the tubulointerstitium. SPARC was increased in glomerular epithelial cells and in interstitial fibroblasts in passive Heyman nephritis (PHN) (**B**) at day 10. In experimental CsA (cyclosporine) nephropathy (**C**), SPARC was expressed by interstitial cells around dilated and atrophic tubules at day 35. In the remnant kidney model (**D**) (4 weeks), SPARC was also expressed in the tubulointerstitium, in large part by interstitial fibroblasts. In the angiotensin II-infusion model (day 14) (**E**), SPARC was also present in the tubulointerstitium.

were colocalized. For SPARC, the same staining protocol as in the SPARC/α-smooth muscle actin double labeling was used. After these steps were completed, tissue sections were treated with an Avidin-Biotin blocking kit (Vector). The next step consisted of an overnight incubation with a guinea-pig anti-type I collagen antibody at 4°C, followed by a 60 minute incubation with a biotinyl-

ated goat anti-guinea-pig antibody (Vector) subsequently detected by FITC-conjugated streptavidin D (Vector).

Double immunolabeling for SPARC and ED-I positive macrophages was performed to determine whether SPARC was expressed by ED-I-positive macrophages in the adventitia of injured blood vessels. The same staining protocol described above in the

Table 1. Interstitial expression of SPARC[a] and type 1 collagen[b], interstitial cell proliferation[c], and interstitial fibrosis score[d] in animals with experimental membranous nephropathy (PHN model)

	Normal control	PHN day 5	PHN day 10	PHN day 15	PHN day 21	PHN day 30
SPARC express %[a]	0	0.7 ± 0.2	53.3 ± 2.2^e	62.5 ± 4.4^e	23.1 ± 6.6^e	6.9 ± 3.8
Collagen expression %[b]	0	0.7 ± 0.1^e	46.6 ± 2.0^e	61.2 ± 2.3^e	45.7 ± 4.8^e	35.1 ± 7.0^e
Interstitial cell proliferation[c]	6.4 ± 0.64	51.2 ± 9.6^e	168 ± 49.6^e	65.6 ± 8^f	33.6 ± 4.8	11.2 ± 1.6
Interstitial fibrosis score (0–3)[d]	0	0	0.5 ± 0.1	0.6 ± 0.2	2.1 ± 0.4^e	2.3 ± 0.6^e

For an explanation of scoring, see **Methods** section. Values are expressed as mean $\pm$ SE
[a] Percent of tubules surrounded by cells staining for SPARC
[b] Percent of tubules surrounded by cells staining for type I collagen
[c] Number of PCNA-positive interstitial cells per mm^2
[d] Described in **Methods** section
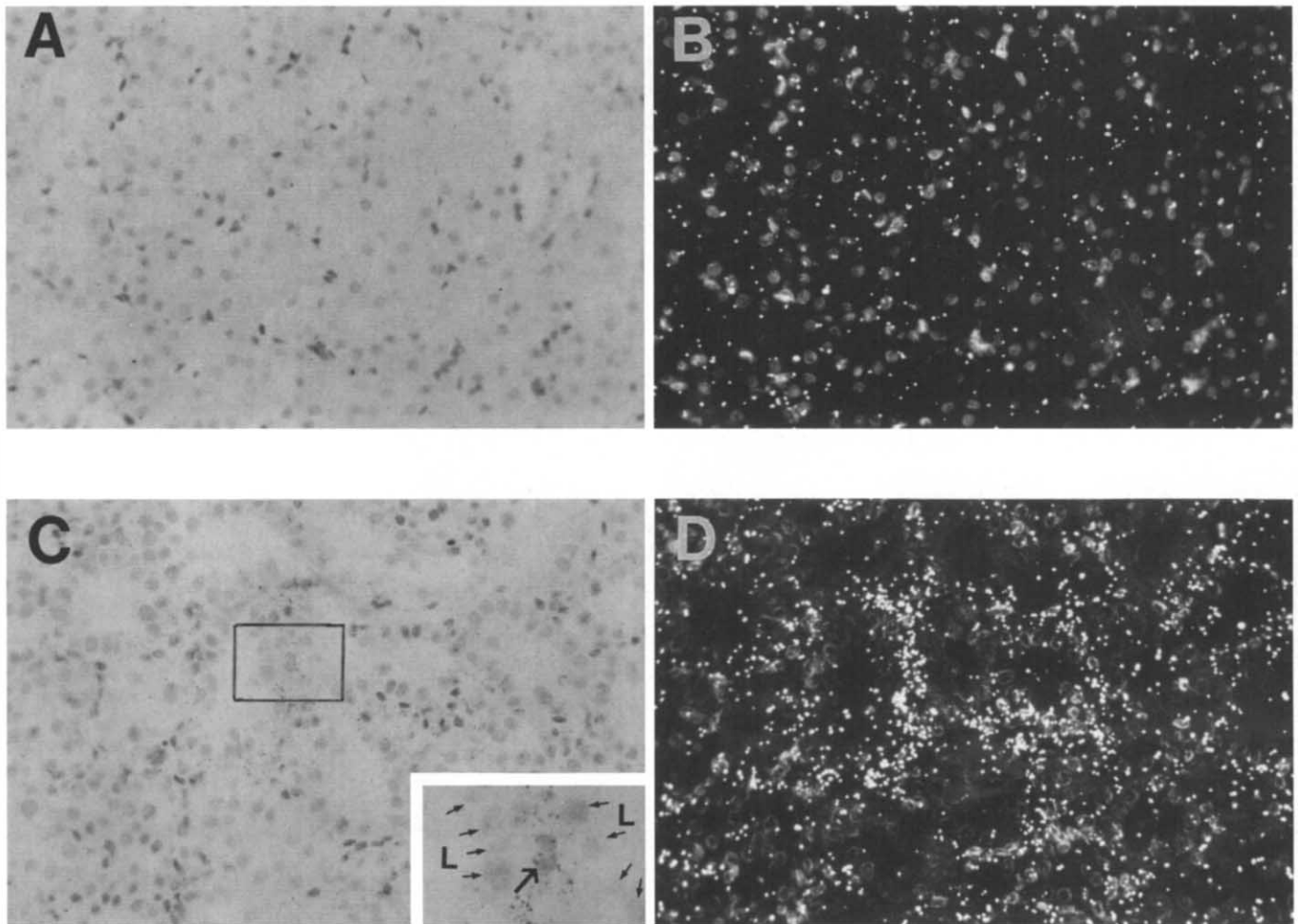[e] $P < 0.005$ vs. control
[f] $P < 0.05$ vs. control.

Fig. 2. *SPARC mRNA is expressed by interstitial cells in areas of tubulointerstitial injury.* In normal control rats the levels of SPARC were low, as illustrated by the presence of very few grains [black in the brightfield (**A**) and white in the darkfield (**B**)]. In contrast, in a PHN animal at day 10, an increase in the number of grains could be observed in interstitial areas [black in the brightfield (**C**) and white in the darkfield (**D**)]. In C, the high power inset ($\times$400) shows that grains primarily localized to interstitial cells (large arrow), and not to tubular epithelial cells (small arrows) or the tubular lumen (L).

SPARC/α-smooth muscle actin double labeling was used to detect SPARC. After completion of these steps, tissue sections were incubated with goat anti-mouse Fab1 fragments (Vector) for 45 minutes at room temperature as a blocking step, followed by incubation with the ED-I antibody for 60 minutes at room temperature. The last step consisted of an incubation with an
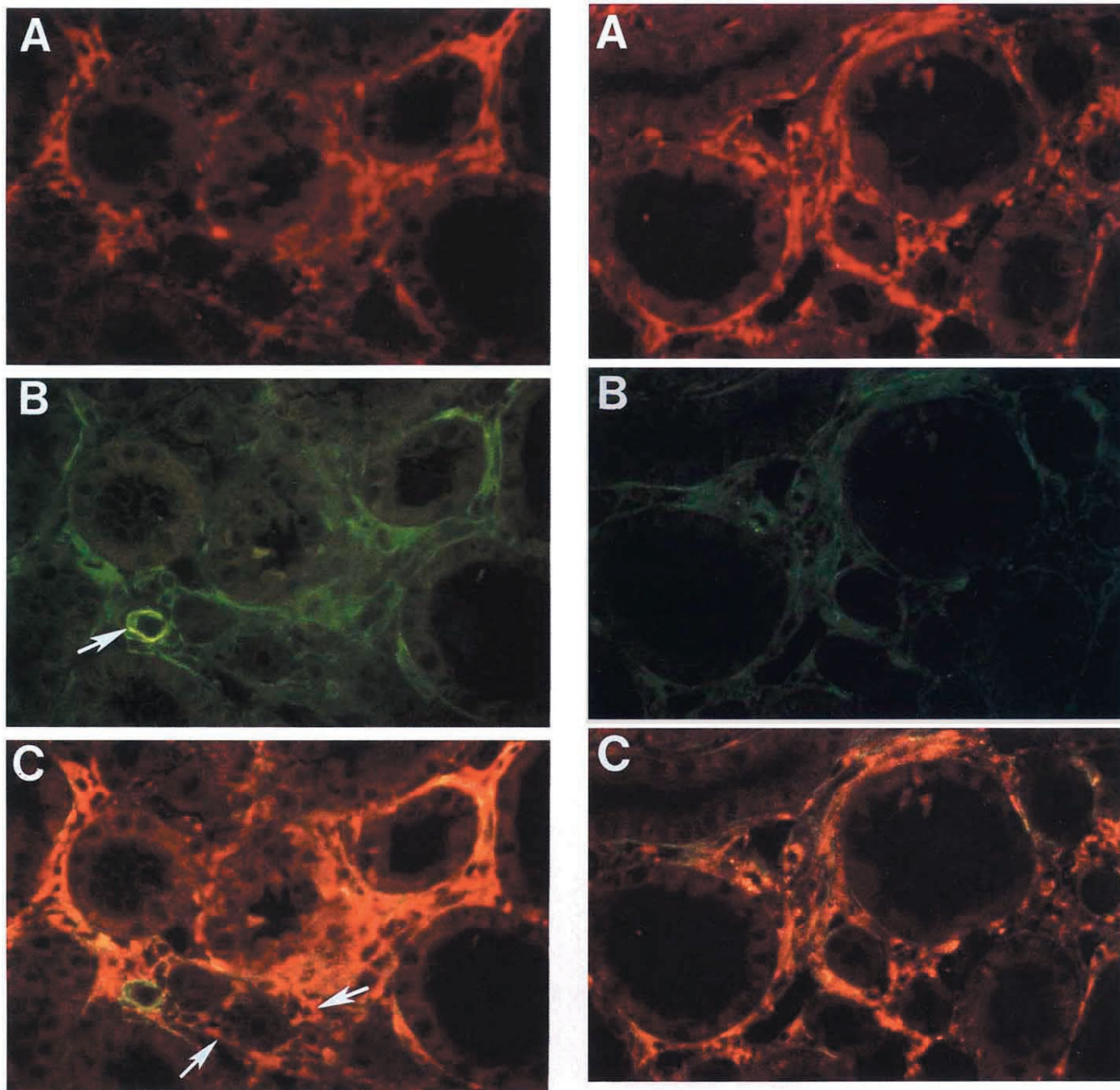
Fig. 3. *SPARC is expressed by α-smooth muscle actin positive "myofibroblasts."* Immunohistochemistry for SPARC (red) revealed that SPARC was expressed by interstitial cells (**A**). α-Smooth muscle actin expression (green) showed a similar distribution on the same tissue section (**B**). In contrast to SPARC, however, α-smooth muscle actin also strongly stained a smooth muscle cell in a small artery (arrow). The double exposure revealed that the majority of SPARC-positive cells also stained for α-smooth muscle actin (orange, **C**). The vascular smooth muscle cell was labeled only with the antibody directed against α-smooth muscle actin and therefore appears green (**C**), whereas very few cells in the interstitium (possibly macrophages) contained SPARC alone (arrows) (PHN, day 10).

FITC-conjugated donkey anti-mouse-IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Controls for the double-immunostaining included replacement of each primary antibody with an irrelevant antibody of the same species or omission of each secondary or tertiary antibody. Under no circumstances did controls exhibit staining.

In situ hybridization for SPARC mRNA

SPARC mRNA was detected by *in situ* hybridization on formalin-fixed tissue according to a modification of the method of Reed et al [9], Pichler et al [34] and Holland et al [35]. Riboprobes were transcribed with an RNA transcription kit (Promega; Madison,
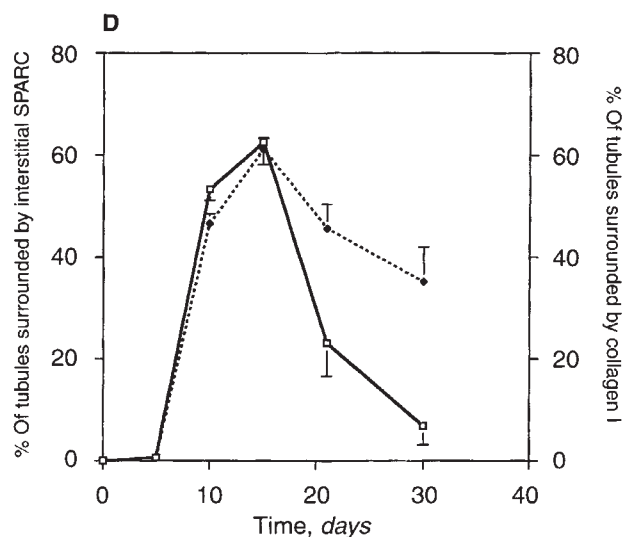
D

Fig. 5. *Interstitial SPARC expression colocalizes and correlates with interstitial type I collagen deposition.* When immunohistochemistry for SPARC was performed on a section of a PHN animal at day 10 (**A**, opposite page), positive staining was observed in interstitial areas (red). Staining for type I collagen on the same tissue section (**B**, opposite page) showed a similar distribution (green). The double exposure (**C**, opposite page) revealed that the majority of interstitial cells expressed both SPARC and type I collagen (orange). However, type I collagen seemed to predominate in the extracellular space and exhibited a reticular staining pattern. When quantification was performed for interstitial SPARC and type I collagen staining in the PHN model, the time course of both molecules was similar through day 15 (**D**, above). Comparison of individual animals (day 0 until day 15) revealed a close correlation ($r = 0.97$). After day 15, however, expression of SPARC decreased more rapidly than that of type I collagen. Symbols are: (□) interstitial SPARC; (◆) interstitial collagen I.
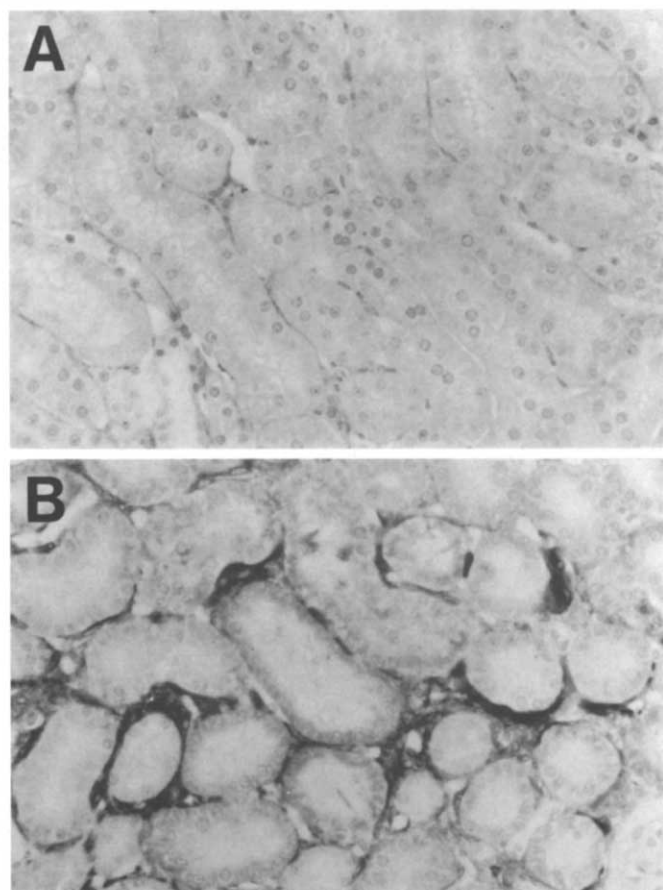
Fig. 4. *Type I collagen expression is increased in areas of tubulointerstitial fibrosis.* When immunohistochemistry for type I collagen was performed on kidney sections of normal control animals (**A**), only faint staining could be observed in interstitial areas. In an animal with interstitial matrix expansion, however (PHN, day 10), a significant increase in type I collagen staining was observed (**B**).

WI, USA) in the presence of α-[^{35}S]-dUTP (Amersham, Arlington Heights, IL, USA). The specific activity of the probe was approximately 3 to 4 × 10^7 cpm/μg. The SPARC riboprobe was transcribed from a 1147 bp *Bam*HI/*Hind*III mouse cDNA fragment, cloned in an antisense orientation relative to the SP6 promoter in a pGEM-1 vector. Before use the SPARC riboprobe was reduced to less than 150 bp fragments by alkaline hydrolysis. Sections were mounted overnight on Probe-On slides (Fisher, Pittsburgh, PA, USA), hydrated with a graded series of ethanol, rinsed in PBS, post-fixed with 4% PFA for 20 minutes, rinsed in PBS, and digested for 2 to 3 minutes with proteinase K (2.5 μg/ml in 50 mM Tris-HCl with 5 mM EDTA) (BRL, Gaithersburg, MD, USA). Samples were rinsed with PBS, fixed with 4% PFA for five minutes, dipped in water treated with diethylpyrocabonate (DEPC-water), exposed to 0.1 M triethanolamine (pH 8) with 0.05% (vol/vol) acetic anhydride for 10 minutes, rinsed in PBS for five minutes, dipped in DEPC-water, dehydrated in graded solutions of ethanol (70 to 100%), and air-dried. Sections were treated ovenight at 50°C in prehybridization solution [0.6 M NaCl, 0.12 M Tris-HCl (pH 8], 8 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.1% Pentex BSA (Sigma), 500 μg/ml salmon sperm DNA, 600 μg/ml yeast total RNA, and 50% deionized formamide]. The samples were hybridized at 50°C overnight in prehybridization solution that contained 10% dextran sulfate and 0.1 μg/ml of the SPARC anti-sense and sense riboprobes. Samples were washed for 30 minutes at 50°C in formamide buffer (50%

formamide, 5% 20 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M Na-citate, pH 7.5, 1% 1 M DTT (dithiotreitol) in DEPC-water], for 30 minutes at room temperature in 0.5 × SSC, and for 30 minutes at 37°C in 3.5 × SSC with 40 μg/ml RNAse A (Sigma) and 50 μl RNAse T1 (Sigma). Slides were incublated sequentially in 3.5 × SSC for 10 minutes at room temperature and 0.1 × SSC for 90 minutes at 65°C. Sections were dehydrated with ethanol (solutions of 50, 70, 95, and 100%) containing 0.3 M sodium acetate and were subsequently air-dried. We performed autoradiogaphy with NTB 3 emulsion (Kodak, Rochester, NY, USA) diluted to 40% with a solution of 4% Dreft detergent; slides exposed for 5 to 7 days at 4°C were developed in Kodak D-19 developer and fixed in GBX fixer (Kodak). Samples were counterstained with toluidine blue, dehydrated with ethanol (solutions of 70 to 100%), cleared in xylene, and mounted with Permount.

Statistical analysis

Statistical significance was defined as $P < 0.05$ and was evaluated by ANOVA (analysis of variance) followed by the Fisher's PLSD (protected least significant difference) procedure. Statistical values are expressed as mean ± SEM.
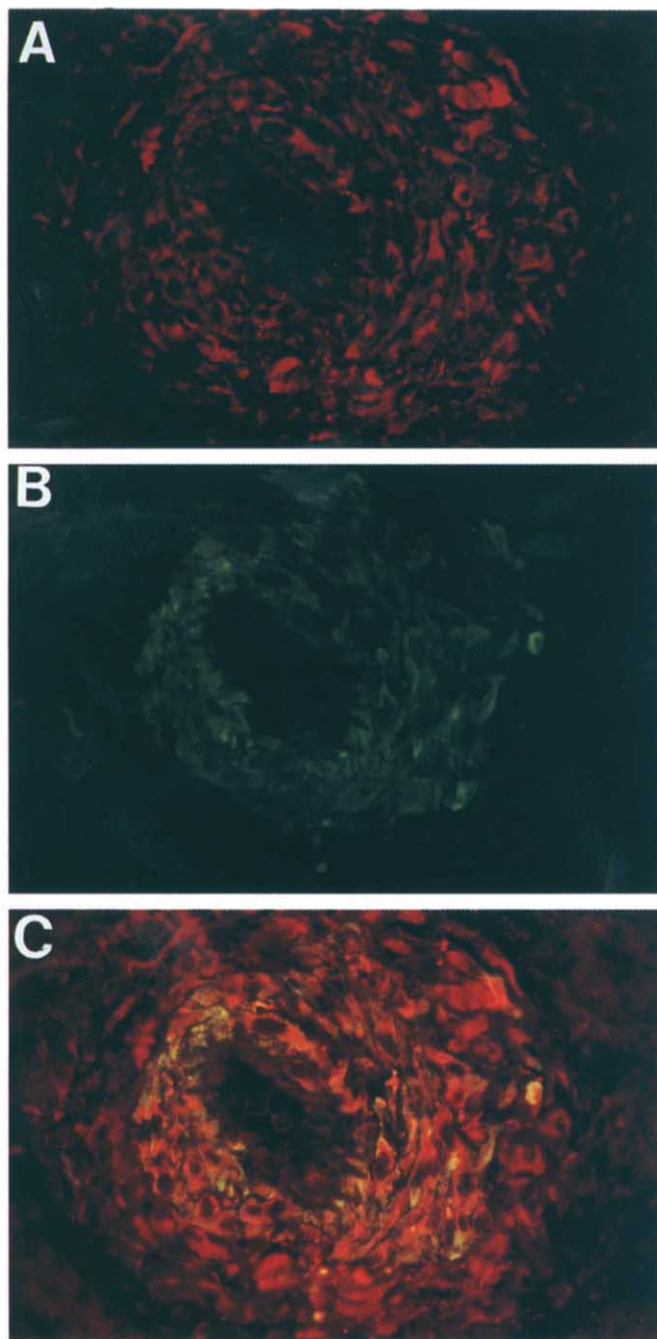
Fig. 6. *Proliferation of interstitial α-smooth muscle actin positive "myofibroblasts" in the PHN model precedes interstitial fibrosis.* We performed double immunolabeling for PCNA and α-smooth muscle actin in the PHN model to identify the proliferating interstitial cells. This technique revealed that the majority of interstitial PCNA-positive cells was α-smooth muscle actin-positive (**A**). PCNA (nuclear staining; small arrow) is shown in dark gray, and SPARC (cytoplasmic staining; large arrow) is shown in brown. Several PCNA-positive cells are also present in the tubular lumen. **B.** The proliferative response clearly preceded the development of interstitial fibrosis. Symbols are: (□) interstitial PCNA; (♦) interstitial fibrosis. Maximal interstitial SPARC-expression levels correlate with the resolution of interstitial cell proliferation. **C.** The relationship between interstitial SPARC expression and interstitial cell proliferation. Maximal expression levels of SPARC coincide with the initial decline in interstitial cell proliferation. Symbols are: (□) interstitial PCNA; (♦) interstitial SPARC.

Fig. 8. *Vascular smooth muscle cells are a major source of SPARC in injured blood vessels of angiotensin II-infused animals.* Double immunolabeling for SPARC and α-smooth muscle actin was performed to determine the cell type that expressed SPARC. Immunohistochemistry for SPARC (red) showed expression in the media and adventitia of the blood vessel (**A**). Immunostaining for α-smooth muscle actin (green) on the same tissue section outlined the vascular smooth muscle cells (**B**). The double exposure demonstrated that vascular smooth muscle cells are a major source of SPARC (orange; **C**), however, SPARC was also expressed by cells in the adventitia.

Results

SPARC protein and mRNA are expressed by interstitial cells in areas of tubulointerstitial fibrosis in experimental models of renal disease

We performed immunohistochemistry for SPARC in several models of renal disease which are characterized by the develop-
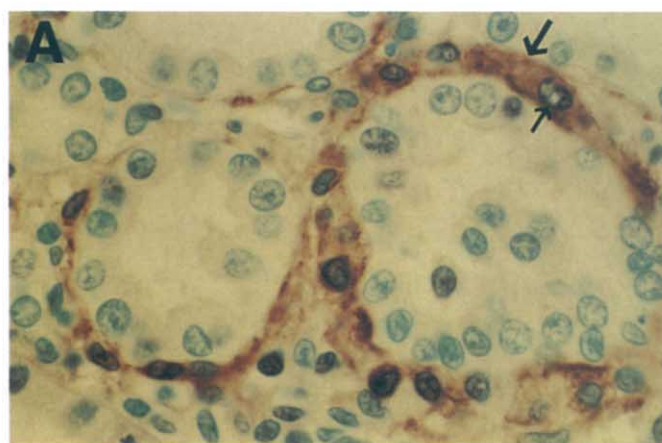
ment of interstitial fibrosis. In normal rats SPARC was primarily expressed by visceral glomerular epithelial cells but was absent from the interstitium (Fig. 1A). In passive Heymann nephritis (PHN), an experimental model of membranous nephropathy, SPARC was not only expressed in increased amounts by glomerular epithelial cells, as reported previously [36], but was also expressed by spindle-shaped interstitial cells in areas of extracellular matrix expansion (Fig. 1B). In the PHN model, interstitial SPARC expression was minimally elevated at day 5, when only 0.6% of the tubules were surrounded by interstitial cells staining for SPARC. SPARC expression was significantly elevated at day 10 (53.3%), was maximal at day 15 (62.5%), and decreased thereafter (Table 1). Complement depletion with CVF prevented the development of disease, and no increase in SPARC expression was observed.

In all other models that we studied, SPARC was also increased in the interstitium in association with interstitial fibrosis. In experimental cyclosporine A (CsA) nephropathy, SPARC was expressed by interstitial cells in areas of interstitial fibrosis at day 35. Staining for SPARC was particularly prominent around dilated and atrophic tubules (Fig. 1C). In the remnant kidney model, SPARC was expressed by interstitial cells in areas of fibrosis/matrix expansion (Fig. 1D). In the angiotensin II (Ang II)-infusion model, SPARC was also expressed by spindle-shaped interstitial cells in areas of interstitial fibrosis (Fig. 1E), as well as in injured blood vessels (see below).

When *in situ* hybridization for SPARC on tissue sections of normal control animals was performed, only low levels of SPARC mRNA could be detected in the tubulointerstitium (Fig. 2A). A significant increase in interstitial SPARC mRNA, however, could
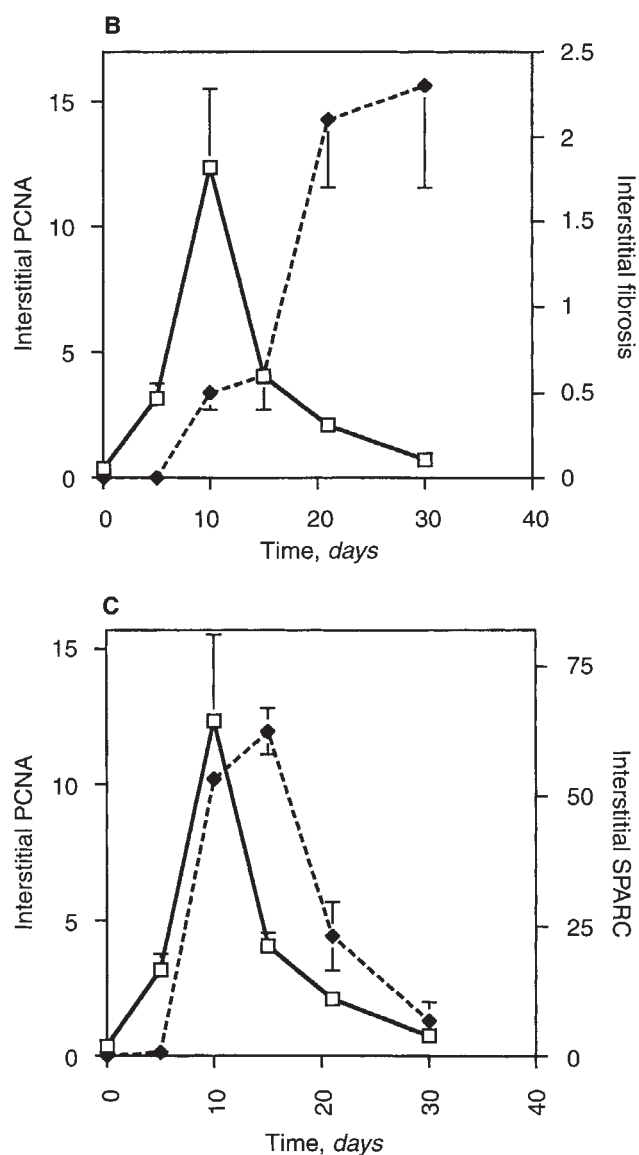
Fig. 6 *(continued)*

be observed in areas of interstitial fibrosis for all the disease models, including the PHN model (Fig. 2B).

Interstitial cells expressing SPARC are α-smooth muscle actin-positive (myofibroblasts)

To identify the cell type expressing SPARC in the interstitium, we performed double-immunolabeling for SPARC and α-smooth muscle actin, since the latter is expressed by activated interstitial fibroblasts (myofibroblasts) [32]. This technique revealed that the majority (> 90%) of interstitial cells expressing SPARC also expressed α-smooth muscle actin and therefore corresponded to "myofibroblasts" (Fig. 3).

Collagen type I is also expressed in interstitial areas of tubulointerstitial fibrosis

In normal rats type I collagen was expressed at low levels by some interstitial cells (Fig. 4A), presumably fibroblasts [37], around collecting ducts, in the media and adventitia of blood vessels, and in the renal capsule. In rats with interstitial fibrosis,

however, such as in the PHN model, a substantial increase in interstitial collagen I was observed by immunohistochemical staining (Fig. 4B). Similar to SPARC, the type I collagen was localized largely to spindle-shaped interstitial cells. In the PHN model, expression of type I collagen was only minimally elevated at day 5, when 0.6% of the tubules were surrounded by interstitial cells staining for this collagen. The percentage of tubules surrounded by type I collagen-positive cells was substantially increased at day 10 (46.6%), reached maximal levels at day 15 (61.2%), and decreased thereafter to 35.1% on day 30 (Table 1).

Interstitial SPARC expression colocalizes and correlates with interstitial collagen type I deposition in the PHN model

Since immunohistochemistry for type I collagen showed a highly similar distribution compared to SPARC in areas of tubulointerstitial injury/fibrosis, we performed double-immunolabeling for SPARC and type I collagen in the PHN model to determine whether they were in fact colocalized. The majority of SPARC-expressing cells also expressed type I collagen (Fig. 5A-C). Whereas immunohistochemistry for SPARC was limited to the cells (cytoplasm and cell surface), immunostaining for collagen type I also included extracellular reticular fibers (Fig. 5C).

When the timecourse of interstitial SPARC expression was compared with that of interstitial type I collagen in the PHN model, a significant correlation ($r = 0.97$) for the expression of both molecules could be shown for the time between the induction of the disease (day 0) and day 15 of the PHN model. Expression of both SPARC and of type I collagen reached maximal levels at day 15 in the PHN model, when 62.5 and 61.2% of the tubules were surrounded by SPARC or type I collagen-positive interstitial cells, respectively (Table 1; Fig. 5D). Thereafter, interstitial SPARC decreased more rapidly than interstitial type I collagen (Table 1).

Interstitial proliferation of α-smooth muscle actin positive cells precedes the development of interstitial fibrosis in the PHN model

Interstitial cell proliferation was assessed in the PHN model by the counting of PCNA (proliferating cell nuclear antigen, a marker for cell proliferation)-positive interstitial cells. A mild increase in proliferation ($P = 0.83$, not statistically significant) was noted at day 5, with 51.2 PCNA positive cells per mm^2 versus 6.4 proliferating cells in the control animals (Table 1). Interstitial cell proliferation reached maximal levels at day 10 with 168 PCNA positive cells per mm^2 ($P < 0.0001$) and started to resolve at day 15 with 65.6 PCNA positive cells per mm^2 ($P < 0.04$). At days 21 and 30, no statistically significant increase in PCNA-positive cells could be observed (Table 1).

To identify the PCNA-positive interstitial cells, we performed double immunolabeling for PCNA and α-smooth muscle actin. This technique revealed that the majority of interstitial proliferating cells (> 75%) stained for α-smooth muscle actin (Fig. 6A).

Interstitial cell proliferation preceded the development of interstitial fibrosis. At day 5, no fibrosis was detected despite modest interstitial cell proliferation. (Fig. 6B). Proliferation had also subsided by day 21, despite a progressive increase in fibrosis, which was maximal at day 30 (Fig. 6B and Table 1).

Interstitial SPARC expression correlates with the resolution of interstitial cell proliferation in the PHN model

Interstitial cell proliferation in passive Heyman nephritis was maximal at day 10. By day 15 a rapid and substantial decrease in proliferating cells of more than 60% was observed. The initial decline of interstitial cell proliferation coincided with the maximal levels of SPARC on day 15 (Fig. 6C).

SPARC protein and mRNA are expressed in injured blood vessels of angiotensin II-infused rats

In angiotensin II-infused rats, focal areas of fibrinoid necrosis involving the afferent arteriole and other small arteries were observed by light microscopy (PAS stain) by day 7. A striking finding was that SPARC protein was substantially increased in the areas of vascular injury (Fig. 7B), in comparison to SPARC expression in blood vessels of vehicle-infused animals (Fig. 7A). Within the blood vessels, high levels of SPARC protein were noted in the media, adventitia, and endothelium (Fig. 7B). Since macrophages are a potential source of SPARC [8] and since macrophages have been found in the adventitia of injured blood vessels in this model [32], immunohistochemistry for ED-1-positive macrophages was performed on serial sections (Fig. 7C). Macrophages were located primarily at the periphery of the blood vessel. Only 10 to 20% of these macrophages were also positive for SPARC by double immunolabeling (data not shown).

In situ hybridization for SPARC in control animals showed only low levels of SPARC mRNA (Fig. 7 D, E). In contrast, angiotensin II-infused animals demonstrated a substantial increase of SPARC mRNA in injured blood vessels (Fig. 7 F, G), a result consistent with the immunohistochemical data shown in Figure 7B.

SPARC is expressed by vascular smooth muscle cells and by cells in the adventitia of injured blood vessels in angiotensin II infused rats

To identify the cells expressing SPARC in angiotensin II-mediated vascular injury, we performed double-immunolabeling for SPARC and α-smooth muscle actin. Whereas immunostaining for SPARC primarily labeled the smooth muscle cells in the media of the artery (Fig. 8A), weak staining for SPARC was also observed in the media, adventitia, and endothelium. The double exposure (Fig. 8C) revealed that more than 50% of the cells that expressed SPARC were vascular smooth muscle cells in the tunica media; the remainder appeared to be distibuted among cells in the adventitia and intima.

Discussion

In this study we have examined the expression of SPARC in interstitial fibrosis and in injured blood vessels in experimental models of renal disease. SPARC was consistently expressed by interstitial cells in areas of interstitial fibrosis in experimental models of membranous nephropathy (PHN model), CsA nephropathy (CsA model), focal segmental glomerulosclerosis (remnant kidney model), and hypertensive renal disease (Ang II-model). In the PHN model, SPARC was expressed by interstitial "myofibroblasts" in areas of interstitial fibrosis, in which the degree of expression and the time course correlated closely with that of type I collagen ($r = 0.97$). Maximal interstitial SPARC in this model correlated with the resolution of interstitial cell proliferation. SPARC expression was also found to be substan-
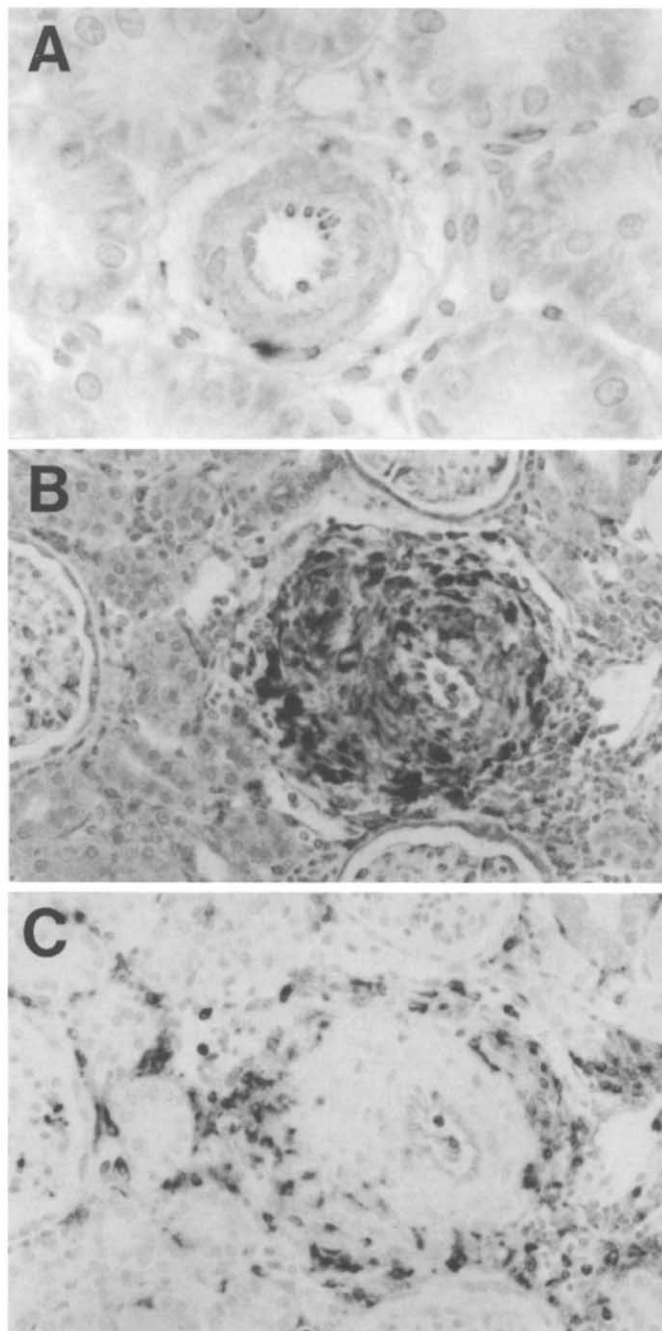

Fig. 7. *SPARC protein and mRNA are increased in injured blood vessels of angiotensin II-infused animals.* When immunohistochemistry for SPARC was performed on a normal blood vessel, only faint staining could be observed (**A**). A substantial increase in vascular SPARC was observed at day 7 in the angiotensin II-infusion model (**B**). Immunohistochemistry for ED-1-positive macrophages on a serial section showed that macrophages localized primarily to the periphery of the blood vessel (**C**). In situ hybridization for SPARC on a section of a vehicle-infused animal demonstrated low expression levels of SPARC mRNA in a blood vessel [black grains in brightfield **D**); white grains in darkfield (**E**)]. In injured blood vessels of angiotensin II-infused rats, however, a substantial increase in SPARC mRNA was observed [black grains in brightfield (**F**); white grains in darkfield (**G**)].

tially increased in vascular smooth muscle cells and in adventitial cells of proliferating, injured blood vessels of angiotensin-II infused rats.

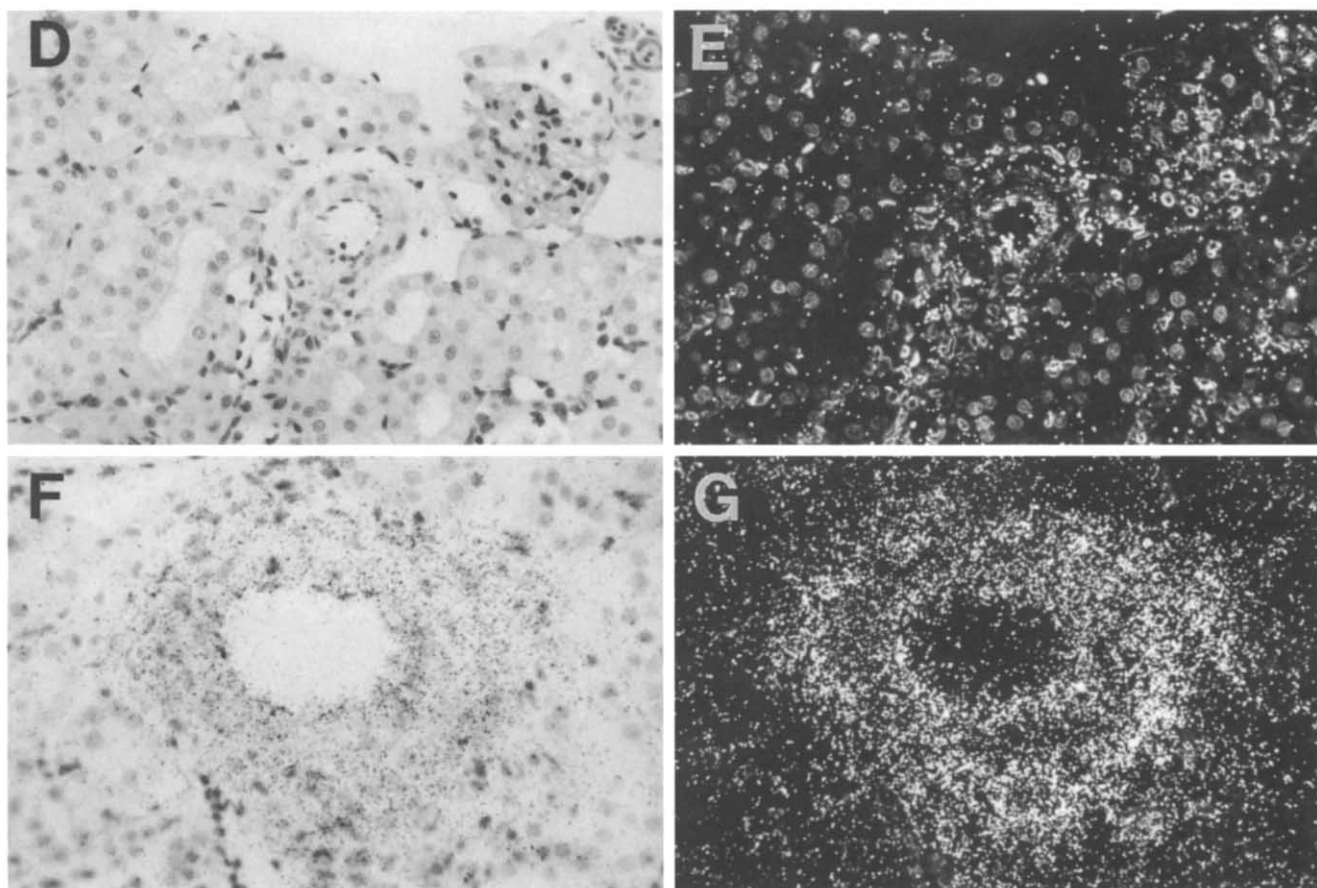Our studies provide the first documentation that SPARC is

Fig. 7. *Continued.*

expressed by interstitial fibroblasts during renal interstitial fibrogenesis. Progressive fibrogenesis can be divided into three different stages: initiation of fibrogenesis, fibrotic matrix deposition, and attenuation of fibrogenesis [38]. The initiation process involves cytokines and growth factors such as transforming growth factor-β (TGF-β), interleukin-1 (IL-1), PDGF, tumor necrosis factor-α (TNF-α) and γ-interferon that stimulate motility, growth and/or proliferation of fibroblasts [38]. TGF-β and IL-1 have also been shown to be positive regulators of SPARC expression [8]. More fibroblasts are attracted into the tubulointerstitial space by chemotactic factors including cleavage products of fibronectin and type I collagen [39, 40]. Movement of fibroblasts through the extracellular matrix is facilitated by the secretion of metalloproteinases, which compromise the integrity the extracellular matrix [41]. The second stage is characterized by the deposition of extracellular matrix. After fibroblasts acquire a synthetic phenotype, increase in number, and migrate to areas of active inflammation, they begin to deposit extracellular matrix. The third and last stage consists of either alleviation or attenuation of inflammation, reduction in synthesis of fibrotic matrix, and stimulation of proteolysis of fibrotic matrix.

We were able to demonstrate that SPARC is expressed by interstitial fibroblasts in areas of tubulointerstitial injury, where it could be involved in several stages of tubulointerstitial fibrogenesis. First, SPARC could function in the deposition of extracellular matrix. We showed that SPARC colocalized and correlated with interstitial type I collagen, a finding not entirely restricted to

the tubulointerstitium. Our group has previously shown that both SPARC and collagen type I are also expressed by glomerular epithelial cells in the experimental model of membranous nephropathy [27, 36] and that SPARC and collagen type I are also expressed by mesangial cells in experimental mesangial proliferative glomerulonephritis [34, 42]. In both instances, overexpression of SPARC was associated with increased extracellular matrix deposition. Since SPARC has been shown to bind to collagen type I, it is conceivable that SPARC could act as a nucleation site for type I and other interstitial collagen fibrils in tubulointerstitial fibrosis, similar to its proposed role in bone [43] or in hepatic fibrosis [44]. The fact that immunohistochemistry for SPARC was primarily restricted to the fibroblasts themselves (and not to the extracellular matrix), despite the fact that SPARC is a secreted protein, could result from an antigen-masking effect of the interstitial collagen fibers bound to extracellular SPARC. In the PHN model, staining for SPARC declined faster than that for type I collagen (Table 1 and Fig. 5D). This observation could also be due to the shorter half-life of extracellular SPARC.

Second, SPARC could be involved in the regulation of interstitial fibroblast proliferation. Gilbert et al [45] have demonstrated that a reduction of renal cortical SPARC expression was associated with an increase in early diabetes-related kidney growth. Our group has previously shown that SPARC might be a potential inhibitor of mesangial cell proliferation in glomerulonephritis [34]. In this study we have demonstrated and confirmed previous reports from other groups that interstitial fibroblasts proliferate in

areas of tubulointerstitial injury/fibrosis. One of the growth factors involved in interstitial fibroblast proliferation appears to be PDGF. PDGF has been shown to be synthesized by macrophages, which have been invariably identified in human acute and chronic interstitial injury [46, 47], and also by tubular epithelial cells. Interstitial fibroblasts have also been shown to express PDGF receptors [48]. Recently, Tang et al have shown that infusion of PDGF into rats induces proliferation of interstitial fibroblasts [49]. PDGF mRNA is also expressed at sites of interstitial cell proliferation and fibrosis in the hypertensive injury induced by angiotensin II [32]. SPARC has been shown to bind to PDGF B-chain and thereby inhibit the interaction with its receptor [50]. SPARC has also been shown to inhibit proliferation independently of PDGF through the arrest of aortic endothelial cells, which lack PDGF receptors, in the mid-G1 phase of the cell cycle [14]. Since the maximum of interstitial SPARC expression correlated with the initial decline in interstitial cell proliferation, SPARC could be involved in the regulation of interstitial fibroblast proliferation [11].

Third, SPARC could also play a role in the regulation of fibroblast migration. SPARC has been shown to induce the expression of three different metalloproteinases in synovial fibroblasts [19], some of which also have been shown to be expressed by renal fibroblasts [51]. Therefore, SPARC could contribute to the generation of collagen cleavage products, which are chemotactic for fibroblasts [40], and also to the proteolysis of the normal extracellular matrix, to facilitate fibroblast migration [41]. Kuhn and Mason have suggested that SPARC, due to its anti-adhesive properties, could enhance fibroblast migration in the development of pulmonary fibrosis [52]. Furthermore, by inhibiting the binding of PDGF to its receptor, SPARC might interfere with the chemotactic activity of PDGF for fibroblasts [50, 53].

In the angiotensin II infusion model, SPARC was induced in blood vessels with morphological changes consistent with fibrinoid necrosis and vascular hypertrophy. Expression of SPARC was primarily restricted to vascular smooth muscle cells and cells in the adventitia. Many studies have demonstrated that the principal effect of angiotensin II-mediated hypertension on the peripheral vasculature involves both a hypertrophic and proliferative response [25, 54]. Studies *in vitro* [55] and *in vivo* [54] indicate that this hypertrophic response might be distinct from the effects of angiotensin II on hypertension and that the hypertrophic response could involve the increased production of the contractile protein, α-smooth muscle actin [55]. Our group has also previously shown that angiotensin II-mediated hypertension can also induce vascular smooth muscle cell proliferation *in vivo* [25, 32]. A component of the proliferative response to angiotensin II might occur independently of hypertension. Angiotensin II, for example, induces vascular smooth muscle cells to express PDGF [56]. Since SPARC binds to and inhibits the action of the PDGF dimers AB and BB, it could function in the regulation of PDGF-mediated smooth muscle cell proliferation.

In summary, we have shown that SPARC is expressed by interstitial, α-smooth muscle actin-positive fibroblasts in several models of renal disease in which interstitial fibrosis occurs, and that SPARC colocalizes and correlates temporally and spatially with interstitial type I collagen in the PHN model. Maximal levels of SPARC in the interstitium correlated with the initial resolution of interstitial cell proliferation in this model. SPARC is also expressed in injured blood vessels in rats with Ang II-mediated

hypertension. These studies indicate a role for SPARC in the pathogenesis of tubulointerstitial injury. Specifically, SPARC might modulate the proliferation of interstitial fibroblasts or vascular smooth muscle cells and/or the deposition of type I collagen fibers.

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