Kidney International, Vol. 62 (2002), pp. 2073–2086

Localization of SPARC in developing, mature, and chronically injured human allograft kidneys

Charles E. Alpers, Kelly L. Hudkins, Stephan Segerer, E. Helene Sage, Raimund Pichler, William G. Couser, Richard J. Johnson, and James H. Bassuk

Departments of Pathology, Biological Structure, Medicine and Urology, University of Washington School of Medicine, Seattle Washington, and The Hope Heart Institute, Seattle, Washington, USA

Localization of SPARC in developing, mature, and chronically injured human allograft kidneys.

Background. The matricellular protein SPARC (secreted protein acidic and rich in cysteine) is expressed during development, tissue remodeling and repair. It functions as an endogenous inhibitor of cell proliferation, regulates angiogenesis, regulates cell adhesion to extracellular matrix, binds cytokines such as platelet derived growth factor and stimulates transforming growth factor-β (TGF-β) production. This study describes the expression of SPARC during human renal development, in normal kidneys and during renal allograft rejection.

Methods. A total of 60 renal specimens, including normal areas from tumor nephrectomies ($N = 24$), fetal kidneys ($N = 27$) and explanted renal allografts ($N = 9$), were included in the study. SPARC protein was localized by immunohistochemistry using two different antibodies. On consecutive sections SPARC mRNA was detected by in situ hybridization.

Results. In the normal adult kidney SPARC protein was expressed by visceral and parietal epithelial cells, collecting duct epithelium (CD), urothelium, smooth muscle cells of muscular arteries and focally in interstitial cells. During renal development immature glomeruli demonstrated a polarized SPARC expression in visceral epithelial cells at their surface abutting the capillary basement membranes. In the fully differentiated glomeruli the expression pattern mirrored that of the adult kidney. Furthermore, SPARC was abundantly expressed by derivatives of the ureteric bud, and smooth muscle cells of arterial walls. During chronic allograft rejection SPARC is expressed in neointimal arterial smooth muscle cells, infiltrating inflammatory cells as well as by interstitial myofibroblasts in areas of interstitial fibrosis. SPARC mRNA synthesis detected by in situ hybridization mirrored these protein expression patterns.

Conclusion. These studies co-localize SPARC to several sites of renal injury previously shown to be sites of PDGF B-chain expression and/or activity. We speculate that SPARC could function as an accessory molecule in chronic PDGF-mediated sclerosing interstitial and vascular injury. SPARC localization to glomerular epithelial cells corresponds to similar findings in rodents, and may reflect its role in cell adhesion and /or regulation of cell shape.

Key words: SPARC, podocyte, glomeruli, interstitium, fibroblasts, smooth muscle cells, transplant rejection, fibrosis, renal development.

Received for publication November 2, 2001
and in revised form June 7, 2002
Accepted for publication July 22, 2002

SPARC (Secreted Protein Acidic and Rich in Cysteine, osteonectin, BM-40) is a glycoprotein that binds collagen. Its proposed biological functions have been ascribed to its interactions with extracellular matrix, its interactions with specific cytokines and growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), and its ability to modulate expression of transforming growth factor-β (TGF-β), its ability to inhibit cell proliferation, and its anti-adhesive properties with respect to some cell types in culture [1, 2]. Of particular interest are putative roles for SPARC in organ development [3–5] and wound repair [2, 6], as studies have demonstrated that this molecule promotes angiogenesis, as well as the deposition and organization of extracellular matrix molecules including several members of the collagen family [2, 3, 7]. The mechanisms by which these processes occur are not well understood. However, the crystal structure of two modules of the SPARC molecule has been determined and key structural domains thought to be responsible for these activities have been defined [8, 9].

Mindful of these potentially important biological functions, our group and others have presented evidence linking SPARC expression to glomerular and tubulointerstitial injury in a variety of disease models in rodents, including chronic cyclosporine nephropathy, renal ablation, passive Heymann nephritis, diabetes, and the angiotensin II infusion model [10–14]. A common finding in these models is that SPARC is expressed by the α-smooth muscle actin expressing interstitial fibroblasts ("myofibroblasts") that participate in tubulointerstitial injury, and that SPARC expression is associated with collagen I expression in such areas of injury. An important link between SPARC expression and the deposition of extracellular matrix is the induction of TGF-β, a well described key regulator of matrix deposition, by SPARC both in vitro and in vivo [7, 14].

Up-regulated SPARC expression in glomerular epithelial cells also has been linked to immunologically me-

diated injury such as passive Heymann nephritis [10]. Therefore, it is of interest to know where SPARC is expressed in the human kidney. In one broad survey of human tissues, SPARC expression was localized to several mesenchymal cell types, including fibrocytes and vascular smooth cells, but more specific localization of SPARC expression within individual organs was not detailed [15]. In the present study, we have employed immunohistochemical and in situ hybridization techniques to localize SPARC protein and mRNA in developing, mature, and chronically injured human allograft kidneys. These studies demonstrate an association between SPARC expression and fibrosing renal injury, extending the observations previously made in rodent systems to humans.

METHODS

Tissue

Normal human renal tissue ($N = 24$) was obtained from kidneys surgically excised because of the presence of a localized neoplasm. Tissues utilized for this study were obtained from macroscopically normal portions of kidney located at some distance from the neoplastic process. Allograft kidneys ($N = 9$) excised for irreversible rejection were used as an example of inflammatory and advanced fibrosis injury. Human fetal kidneys ($N = 27$) were obtained from tissue examined after therapeutic abortions.

Tissues were fixed in methyl Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) or in 10% neutral buffered formalin for at least 12 hours, processed, paraffin embedded and sectioned using conventional techniques. Some tissues from the nephrectomy specimens also were snap frozen and utilized for RNA preparation.

These studies were performed under approval from the University of Washington Human Subjects Division (Approval #01-8008-E-01) under conditions of tissue anonymization, whereby all study investigators remain masked to the specific identity of patients from whom tissue sections were obtained. Accordingly, parameters of clinical outcome related to SPARC expression could not be evaluated.

Antibodies

SPARC. Anti-SPARC (AON-5031) is a murine monoclonal antibody directed against human, rat, and bovine SPARC/osteonectin (Haematologic Technologies, Essex Junction, VT, USA). This antibody was characterized by Western immunoblotting of purified, recombinant human SPARC [16, 17]. Specific immunoreaction was localized to a 33 kD band of the non-glycoslyated recombinant protein and a 43 kD band in tissue [12]. Epitope mapping of this antibody was achieved by enzyme-linked immunosorbent assay (ELISA) and peptide dot-immunoblotting of synthetic SPARC peptides. The epitope recognized by this monoclonal IgG was found to require amino acids 5-23 of the mature protein [18]. MAB-2 is a murine monoclonal IgG1 antibody directed against human SPARC peptide 1.1 (peptide 1.1 spans amino acids 3 to 23 of SPARC) [15].

Smooth muscle cell marker. Murine monoclonal antibody 1A4 (Dako Corp., Carpinteria, CA, USA) has been characterized by tissue immunohistochemistry and Western blotting and has been previously demonstrated to recognize smooth muscle α-actin in methyl Carnoy's-fixed tissues of human kidney [19–21].

Monocyte/macrophage marker. A murine monoclonal antibody PG-M1 (Dako) directed against the CD68 epitope of human monocytes and macrophages was used in methyl Carnoy's-fixed tissues [22]. This antibody has been shown to recognize cells of monocyte/macrophage lineage in fixed tissues with no loss of sensitivity compared with frozen tissue specimens [22]. The specificity of this antibody has been established by studies of transfected and untransfected cell lines and by immunocytochemical surveys of numerous cell lines and examples of neoplastic and non-neoplastic hematopoietic tissues, as previously described [22].

PDGF A. Anti-PDGF A chain (Santa Cruz Biotechnology, Santa Cruz, CA, USA) is an affinity-purified rabbit polyclonal antibody raised against a 30 amino acid peptide corresponding to the amino terminus of the human PDGF-A chain. It has been characterized by Western blot and peptide absorption [23] and its specificity demonstrated in human kidneys [23, 24].

PDGF B. Murine monoclonal antibody PG7-007 (provided by Mochida Pharmaceutical, Tokyo, Japan) has been characterized by Western blotting of whole kidney extracts [24] and has been previously shown to specifically recognize PDGF-B chain in methyl Carnoy's fixed tissue [21, 24].

PDGF α-receptor. Anti-PDGF α-receptor (A951; Santa Cruz Biotechnology) is an affinity-purified rabbit polyclonal antibody. This antibody has been characterized by Western blotting [25] and has been shown to specifically immunolabel PDGF α-receptor in methyl Carnoy's fixed tissue [25, 26].

Western blotting

Western blotting was performed using lysates from whole human kidneys or recombinant SPARC protein. Lysates were fractionated on a 12% polyacrylamide electrophoretic gel that contained 0.1% SDS and then transferred onto nitrocellulose membranes by electroblotting. The blots were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour at 37°C and then incubated with 0.2 to 2 μg/mL of SPARC (AON-5031) antibody or an equal concentration of normal rabbit IgG diluted in PBS containing 0.1% BSA for

two hours at room temperature. After washing, the blots were incubated with alkaline phosphatase conjugated horse-anti-mouse IgG antibody (Vector Labs, Burlingame, CA, USA) for one hour. The blots were then visualized with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium (BCIP/NBT).

Immunohistochemistry

Immunohistochemistry was performed on methyl Carnoy's fixed, paraffin-embedded tissues following a standard avidin-biotin complex (ABC) method, as previously described in detail [20, 27]. Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide and non-specific binding was blocked by incubation in 10% normal goat serum. The sections were then incubated for one hour with the primary antibody. Following washes in PBS, the sections were sequentially incubated with biotinylated second antibodies (Vector Labs), the ABC-Elite reagent (Vector Labs) and finally 3,3′-diaminobenzidine (with nickel chloride enhancement) was used as the chromogen. The sections were counterstained with methyl green, dehydrated and cover slipped.

Specificity controls for the labeling procedures included substitution of irrelevant non-immune mouse IgG or rabbit sera for the primary antibodies, and pre-absorption of the antibody with recombinant protein (for AON-5031), followed by the otherwise identical immunocytochemical procedures.

Double label immunohistochemistry

Slides were first immunostained as described above using MAB-2 or CD68 and DAB without nickel chloride enhancement to visualize either SPARC or monocytes/macrophages, respectively, with a brown reaction product. Following a PBS rinse and further block with 3% hydrogen peroxide, the slides were incubated sequentially with 1A4 or MAB-2 (to localize alpha smooth muscle actin or SPARC, respectively) overnight at 4°C, HRP conjugated anti-mouse antibody (Dako, Carpinteria, CA, USA) and then developed with the True Blue substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) to give a blue color reaction.

In situ hybridization

Primer design. Two oligonucleotide primers were designed to be used for the amplification of human SPARC. The 3′ primer (5′-GGAGAGGTACCCGTCAATGG-3′) spans the eighth intron/exon junction (bases 374-395) of the human SPARC cDNA and the 5′ primer (5′-CGA GTTTGAGAAGGTGTGCAGC-3′) spans the fifth intron/exon junction (bases782-801). The primers were synthesized by Bio-Synthesis, Inc. (Lewisville, TX, USA).

RNA preparation, reverse transcription (RT) and polymerase chain reaction (PCR). Total RNA was extracted from snap frozen, normal human kidney tissue with TRI Reagent (MRC, Inc., Cincinnati, OH, USA), followed by precipitation with isopropanol. First strand cDNA was synthesized from RNA by Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Gibco BRL, Gaithersburg, MD, USA) using either random hexamer primers or the SPARC 3′ primer. The reaction mixture consisted of 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 10 mmol/L dithiothreitol (DTT), 3 mmol/L $MgCl_2$, 1 U RNase inhibitor, 1 μg RNA, 1 mmol/L 4dNTP, 200 ng primer and 20 units of M-MLV RT in a total volume of 20 μL. The reactions were allowed to proceed for 45 minutes at 37°C and then the enzyme was inactivated by incubation at 65°C for five minutes.

The synthesized first strand cDNA from the RT reaction was amplified by PCR. The reaction mixture contained 60 pmol of each primer, 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 500 mmol/L KCl, and 200 μmol/L 4dNTP. The PCR reactions were performed using a PTC 100 (MJ Research, Inc., Watertown, MA, USA) temperature cycler. After a five minute incubation at 85°C, 2.5 U Taq DNA polymerase (Boehringer Mannheim, Indianapolis IN, USA) were added to each tube, followed by a denaturation step at 94°C for one minute, an annealing step at 64°C for one minute and an extension step at 72°C for one minute. The reaction tubes were then cycled 35 times for thirty seconds at 94°C, followed by one minute at 64°C and one minute at 72°C. After a final extension step at 72°C for ten minutes, the tubes were held at 4°C. A 10 μL aliquot of the PCR products was fractionated on a 1% agarose gel, stained with ethidium bromide and visualized with an ultraviolet transilluminator. The expected 427 bp product was obtained from two different RNA preparations using both the random hexamer primer and the 3′ SPARC primer.

Probe preparation. The product from the PCR reaction was cloned using the pGEM-T Vector System (Promega, Madison, WI, USA). The ligation reaction mixture contained 2 Weiss Units of T4 DNA Ligase, 50 ng pGEM-T Vector DNA, 30 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MgCl_2$, 10 mmol/L DTT, 1 mmol/L adenosine 5′-triphosphate (ATP) and 25 ng SPARC PCR product DNA. The reaction was incubated for two hours at 15°C and then transformed into Maximum Efficiency DH5α competent *E. coli* cells (Gibco BRL). After plating on LB/amp/IPTG/X-gal plates, white colonies were selected and used in a standard plasmid miniprep procedure. After restriction enzyme mapping, one clone was selected as it contained a single insert of the SPARC cDNA. The presence of the insert was confirmed by dideoxy sequencing of both strands from the T7 and SP6 promoters of the pGEM-T vector. This construct was linearized and transcribed into a riboprobe using reagents from Promega (Madison, WI, USA), except

[^{35}S]-uridine triphosphate (UTP), which was obtained from New England Nuclear (Boston, MA, USA). The transcription reaction mixture contained 1 μg of SPARC cDNA (sense or anti-sense), 250 μCi of [^{35}S]-UTP, 500 μmol/L each of ATP, cytidine 5′-triphosphate (CTP) and guanosine 5′-triphosphate (GTP), 40 U of RNAsin, 10 mmol/L DTT, 40 mmol/L Tris-HCl and 10 U of T7 polymerase. After 75 minutes at 37°C, the template DNA was digested by adding 1 U of DNAse (Promega) and incubating at 37°C for an additional 15 minutes. Free nucleotides were separated with a Sephadex G-50 column. Probes were used within 48 hours.

Sequencing of probe and verification of identity. One nanogram of the insert DNA cDNA fragment of the human SPARC/pGEM-T plasmid was characterized by Taq polymerase dye terminator cycle sequencing (Perkin Elmer Corporation, Foster City, CA, USA). Annealing of template to oligonucleotide primers was achieved through the use of SP6 and T7 primers specific for the PGEM-T plasmid. Both strands of the insert were completely sequenced. This SPARC DNA cDNA fragment, obtained from the uninvolved kidney tissue of a single patient with a localized neoplasm, was found to be 427 bp in length. The fragment was 99% identical (423/427 bp) with the coding region of human SPARC cDNA (accession #Y00755 and #J03040). The lack of complete identity was due to differences at the position of the wobble base of 4 codons. These differences were found in exon 8 and correspond to the following amino acids: #183, CAT (His) to CAC (His); #190, GAG (Glu) to GAC (Glu); #212, TTC (Phe) to TTT (Phe); and #213, CCT (Pro) to CCC (Pro). The insert of this recombinant plasmid corresponds to amino acids 85 through 226 of the human SPARC protein sequence.

In situ hybridization. Fetal and adult kidney tissue that had been fixed in 10% formalin and embedded in paraffin was deparaffinized following standard protocol. The sections were washed with 0.5× standard saline citrate (SSC; 1× SSC = 150 mmol/L NaCl, 15 mmol/L Na citrate, pH 7.0) and digested with proteinase K (1 μg/mL; Sigma Chemical Co., St. Louis, MO, USA) in Tris-Cl buffer (500 mmol/L NaCl, 10 mmol/L Tris, pH 8.0) for 40 minutes at 37°C. Several 0.5× SSC washes were followed by prehybridization for two hours in 50 μL of prehybridization buffer (0.3 mol/L NaCl, 20 mmol/L Tris pH 8.0, 5 mmol/L EDTA, 1× Denhardt's solution, 10% dextran sulfate, 10 mmol/L DTT). The hybridizations were started by adding 500,000 cpm of ^{35}S-labeled riboprobe in 50 μL of prehybridization buffer and allowed to proceed overnight at 50°C. After hybridization, sections were washed with 0.5× SSC, treated with RNase A (20 μg/mL, 30 min at room temperature), washed in 2× SSC (2 × 2 min), followed by three high-stringency washes in 0.1× SSC/0.1% Tween 20 (Sigma) at 50°C, and several 2× SSC washes. After the tissue was dehydrated and air dried, it was dipped in NTB2 nuclear emulsion (Eastman Kodak, Rochester, NY, USA) and exposed in the dark at 4°C for two weeks. After developing, the sections were counterstained with hematoxylin and eosin, dehydrated, mounted and viewed. Positive cellular labeling was defined as five or more silver grains concentrated over a single cell.

In addition, non-radioactive in situ hybridization was performed. The slides were prepared as above and the hybridization was carried out using a digoxigenen-labeled riboprobe (Boehringer Mannheim, Indianapolis, IN, USA). The slides were washed as above and the probe was detected using horseradish peroxidase labeled anti-digoxigenin antibody (Boehringer Mannheim), followed by amplification using the TSA-Indirect Kit (NEN) and then DAB with nickel enhancement to give a black reaction product. The slides were then counterstained with methyl green and cover slipped.

RESULTS

Histologic characterization of renal tissues

Developing kidneys were obtained from fetuses of estimated 57 to 120 days gestational age, as previously described. These kidneys demonstrated age appropriate features of glomerulogenesis and tubulogenesis, and uniformly lacked features of maldevelopment such as dysplasia, scarring, or cyst development. Mature renal tissues obtained from nephrectomy specimens demonstrated focal changes typically associated with aging, including focal global glomerulosclerosis involving less than 10% of glomeruli sampled, mild patchy interstitial fibrosis and associated tubular atrophy, and focal mild intimal sclerosis involving muscular arteries. Pathologic features characteristic of other specific glomerular, tubulointerstitial, or vascular injuries were not identified in this sample. Kidneys with chronic allograft nephropathy demonstrated more extensive global glomerulosclerosis, and some demonstrated variable, focal, and generally mild features of transplant glomerulopathy as detected by histological examination. Features of transplant glomerulopathy included increased glomerular lobularity, thickening and splitting of peripheral capillary walls, and mesangial expansion. These changes were clearly present in four out of nine allograft nephrectomies, and were equivocally present in three additional kidneys in this group. Allograft kidneys demonstrated somewhat diffuse fibrosis of the cortical interstitium, associated with frequent and focally prominent infiltrates of mononuclear leukocytes in the interstitium. Muscular arteries demonstrated marked, typically concentric thickening of the intima by accumulations of matrix and spindle shaped cells. In several cases, there were additional features of superimposed acute rejection characterized by infiltration of the subendothelial space by mononuclear

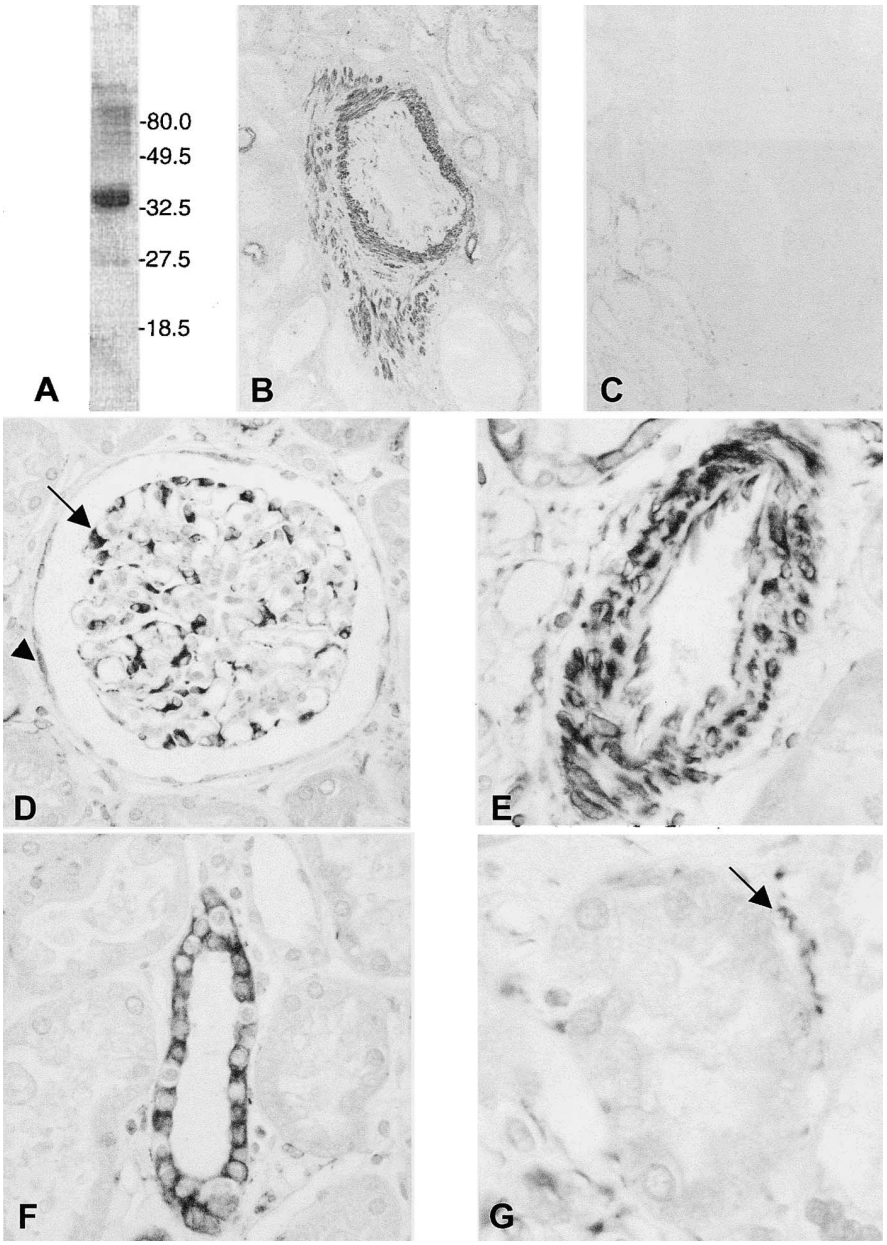

Fig. 1. Characterization of the anti SPARC antibody and SPARC expression in normal renal tissue (*A*) Western blot of recombinant SPARC protein using the monoclonal antibody AON-5031. (*B*) Immunohistochemistry with the anti-SPARC antibody AON-5031 shows staining of myocytes of the arterial wall. (*C*) This staining is abolished after preincubation with recombinant SPARC protein (original magnification ×200) (B, C, *D*, *E*) Immunohistochemistry for SPARC using the monoclonal antibody MAB-2 on mature kidney tissue as part of a tumor nephrectomy specimen (B, C, ×40; D ×400; E-G, ×600 original magnifications). (D) In the normal adult glomerulus, SPARC expression was usually restricted to visceral (arrow) and parietal epithelium (arrowhead). Myocytes of the arterial wall, as well as endothelial cells, constitutively express SPARC as do periarterial adventitial fibroblasts (E). (*F*) Illustrates SPARC expression in collecting ducts. (*G*) Focal SPARC expression was detected in interstitial cells (arrow).

leukocytes, presumably due to diminished immunosuppression prior to anticipated allograft nephrectomy.

Characterization of the antibodies and SPARC expression in normal adult kidneys

During the establishment of SPARC immunohistochemistry we tested two antibodies against SPARC. The murine monoclonal antibody directed against human, rat, and bovine SPARC/osteonectin (AON-5031; Haematologic Technologies) antibody was characterized by Western immunoblotting of purified, recombinant human SPARC (Fig. 1A) [16, 17]. Specific immunoreaction was localized to a 33 kD band of the non-glycoslyated recombinant protein (Fig. 1A) and to a 43 kD band in tissue [12]. Incubation with recombinant protein abolished the staining by immunohistochemistry (Fig. 1 B, C). MAB-2 is a murine monoclonal IgG1 antibody directed against SPARC peptide 1.1 (acids 3 to 23 of SPARC) [28]. Both antibodies resulted in comparable staining patterns in methyl Carnoy's fixed, paraffin embedded tissue sections. The staining pattern with the monoclonal antibody MAB-2 was slightly superior to the results obtained with AON-5031. MAB-2 demonstrated a higher sensitivity and a lower background, as compared to AON-5031. These differences were especially apparent in vascular smooth muscle cells and glomerular visceral epithe-

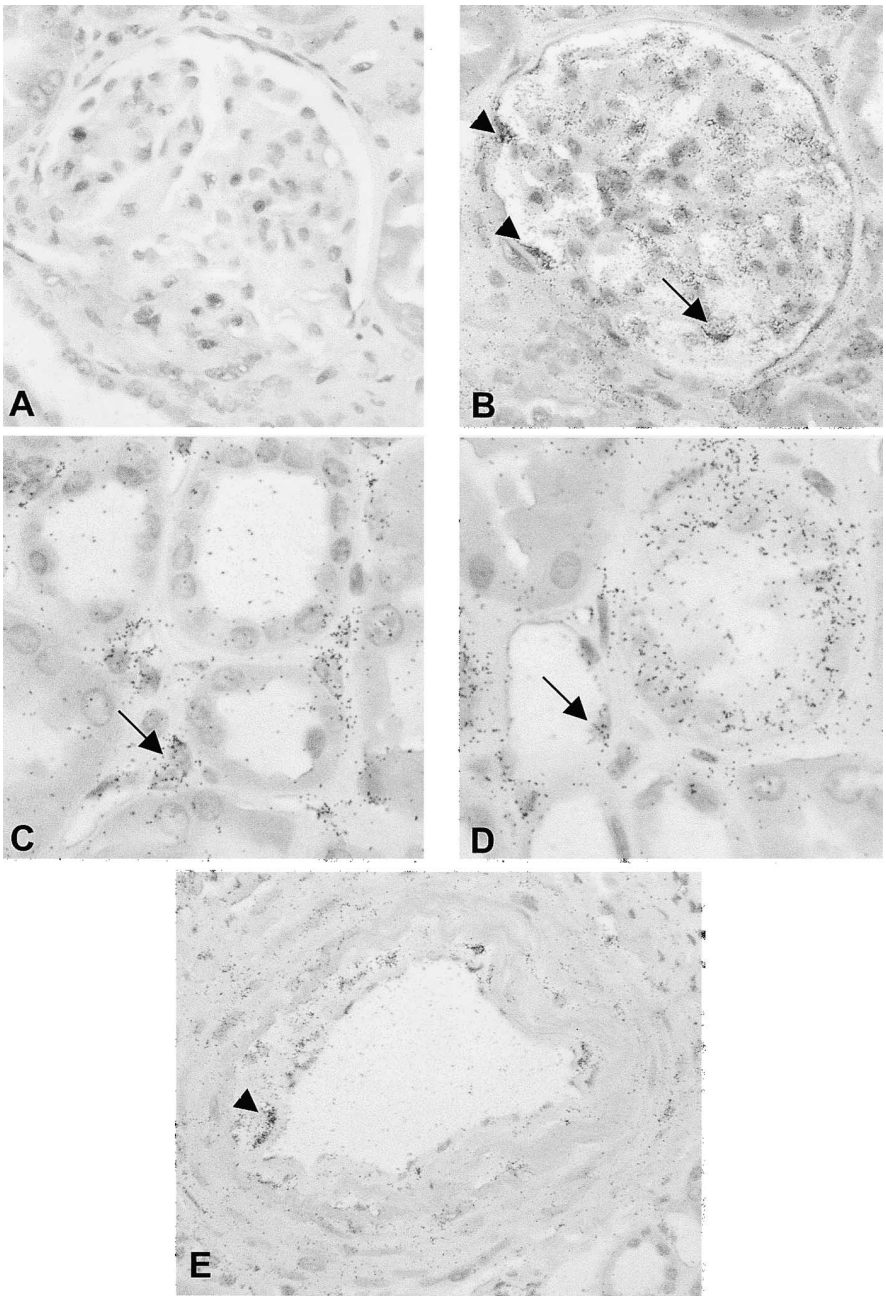

Fig. 2. SPARC mRNA expression in the normal adult kidney. In situ hybridization on mature kidney tissue as part of a tumor nephrectomy specimen, using SPARC sense (*A*) and SPARC antisense probes (*B–E*, all orig. ×400). (A) The tissue section that was hybridized with the sense riboprobe illustrates the low number of non-specifically deposited silver grains. (B) Strong SPARC mRNA expression was detectable in adult glomeruli with an accentuation of silver grains in the peripheral glomerular tuft over visceral (arrow) and parietal (arrowhead) epithelial cells. (C) Scattered interstitial cells were SPARC positive (arrow). (D) SPARC mRNA expression by tubular epithelial cells, most likely collecting duct epithelium. Note the localization of mRNA to the endothelium of a peritubular capillary (arrow). (E) Arteriosclerotic artery, which demonstrated SPARC mRNA in the neointima (arrowhead).

lial cells. The detection of SPARC expression in interstitial cells and collecting ducts was similar when either of the two antibodies was utilized. Four different lots of antibody AON-5031 were obtained from the manufacturer, and noticeable lot-to-lot differences in sensitivity in immunohistochemical studies of the same tissue were observed. Overall, the combined immunoreactive properties of AON-5031 and MAB-2 and the correlative localization of mRNA by in situ hybridization solidify the validity of these reagents as tools for localization of SPARC in human tissues. For these reasons, we relied on antibody MAB-2 for the principal findings of this study.

In the mature adult kidney (Figs. 1 and 2), SPARC is uniformly expressed by glomerular visceral epithelial cells and very commonly in parietal epithelial cells (Figs. 1D and 2B). In the vasculature, arterial smooth muscle cells (Figs. 1E and 2E), adventitial fibroblasts, and collecting duct cells demonstrated widespread SPARC expression (Figs. 1F and 2D). Focal expression of SPARC was detected in distal tubular epithelium. Neointimal smooth muscle cells, routinely encountered in the arteriosclerosis of aging human kidneys, also demonstrated expression of SPARC (Fig. 2E). SPARC-expressing endothelial cells were common in large and medium sized

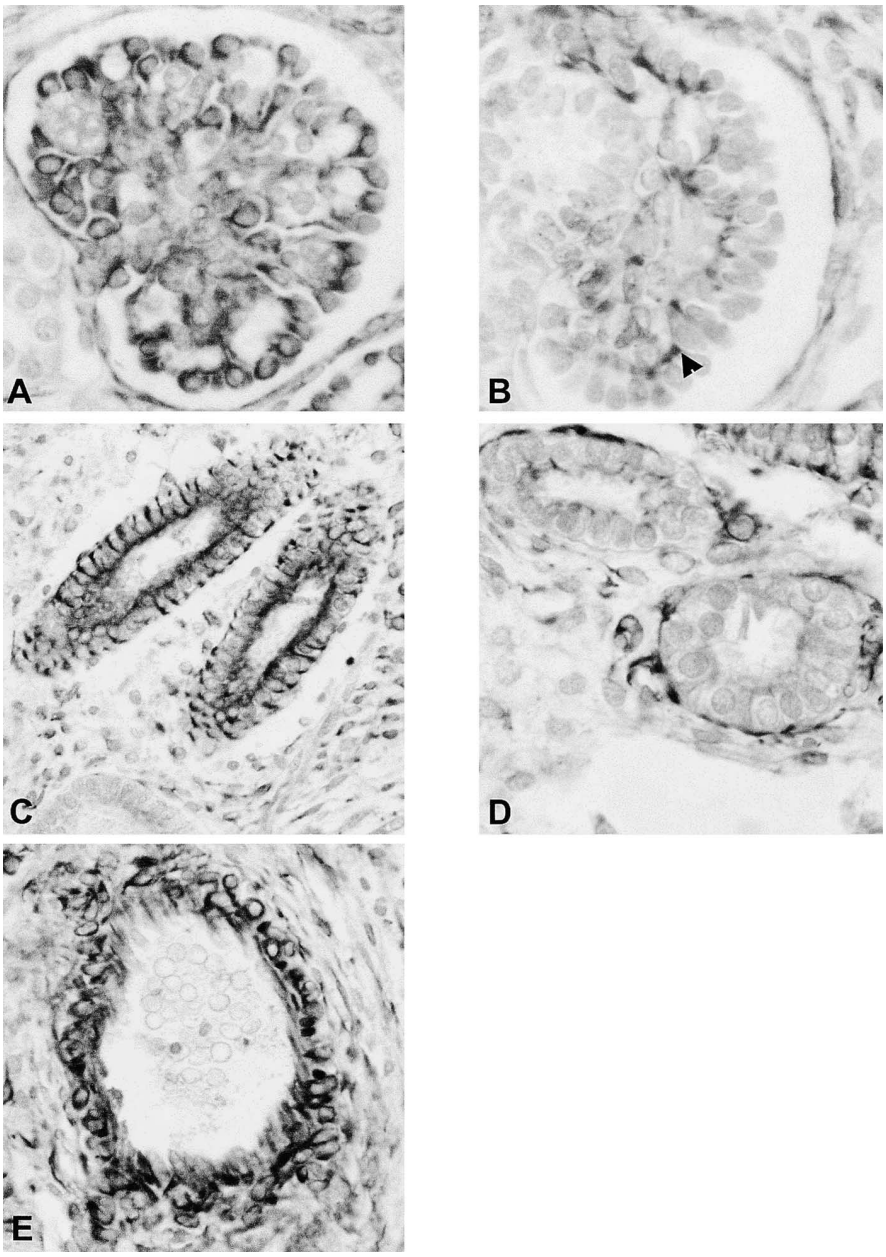

Fig. 3. SPARC protein expression in the developing kidney. Immunohistochemistry for SPARC using the monoclonal antibody MAB-2 on sections of human fetal kidneys (*A*, *B*, *C*, *E* orig. ×400; *D* orig. ×600). (A) In well differentiated glomeruli, visceral epithelial cells were uniformly SPARC positive, commonly with a diffusely stained cytoplasm around the unstained nuclei. (B) In earlier stages of glomerular development, SPARC staining became detectable at the base of the visceral epithelial cells (arrowhead). (C) Collecting ducts were uniformly SPARC positive with a prominent polarization toward the luminal surface and a weaker staining on the abluminal side. (D) Peritubular interstitial cells were commonly found to express SPARC. (E) Vascular smooth muscle cells uniformly expressed SPARC (compare to Fig. 1E).

arteries, whereas glomerular endothelium was not stained for SPARC (Fig. 1 D, E, and Fig. 2D). Interstitial fibroblasts in the uninjured kidney were usually only weakly and focally SPARC positive by our immunohistochemical techniques (Figs. 1G and 2C). There was possible focal staining of peritubular capillary endothelium, but we could not achieve sufficient resolution by light microscopy to exclude localization of SPARC in adjacent interstitial fibroblasts. The protein expression mirrored the mRNA expression detected by in situ hybridization (Figs. 1 and 2).

SPARC expression during renal development

SPARC expression in developing glomeruli was most prominent at late stages of differentiation of cells comprising the visceral and parietal epithelium of developing glomeruli (Fig. 3 A, B). In earlier glomerular structures SPARC commonly became apparent in a polarized distribution at the base of immature, columnar appearing podocytes, where they attach to the capillary basement membranes (Fig. 3B). During glomerular maturation the pattern changed toward the diffuse cytoplasmic pattern seen in adult visceral epithelial cells (Figs. 3A and 1D). Expression of SPARC by mesangial cells in differentiated glomeruli was identified in 8 of 21 kidneys. Although these structures often failed to demonstrate immunohistochemically detectable SPARC expression, SPARC also was expressed focally by differentiating epithelial

cells comprising comma- and S-shaped bodies in about half of the cases. There was no evidence of SPARC expression by structures involved in earlier stages of nephrogenesis such as metanephric blastema, tips of ureteric buds, or condensing vesicles.

SPARC was expressed in each of the extraglomerular tissue compartments in the fetal kidney (Fig. 3). Tubular expression of SPARC was generally confined to differentiated cells of the collecting duct system and pelvic urothelium (Fig. 3 C, D). In these sites, SPARC protein appeared polarized with localization primarily at the luminal surface of these cells. The basolateral cell surface at times also demonstrated localized expression of SPARC (Fig. 3C). Medial smooth muscle cells of arteries demonstrated widespread expression (Fig. 3E). Prominent expression of SPARC by the adventitial fibroblast-like cells adjacent to muscular arteries also was identified. In the interstitium, SPARC was widely expressed by mesenchymal cells likely corresponding to interstitial fibroblasts both within the cortex and medulla (Fig. 3D). As in adult kidney, sites of SPARC protein expression were coincident with sites of SPARC mRNA synthesis as detected by in situ hybridization (data not shown).

SPARC expression during human renal allograft rejection

In chronic allograft nephropathy, a prominent change in SPARC expression became apparent. Noteworthy in this set of tissues was the prominent expression of SPARC in the neointima of arteries with histologic features of chronic rejection (obliterative arteriopathy; Fig. 4 A, B). Expression of SPARC by smooth muscle cells of the medial portions of the arterial wall persisted (Fig. 4 A, B). An increased number of SPARC positive interstitial cells became detectable in areas of interstitial fibrosis (Fig. 4E). The distribution pattern in the interstitium mirrored the distribution of smooth muscle actin positive interstitial myofibroblasts (compare Fig. 4 panels E and F), which was further evidenced by double immunolabeling of these cells for actin and SPARC as illustrated in Figure 5. At sites of interstitial leukocytic infiltrates, a high number of SPARC positive cells were scattered between infiltrating inflammatory cells. However, a minority of interstitial leukocytes, expressing the monocyte/macrophage differentiation marker CD68, could be shown on replicate tissue sections to express SPARC peptide as detected by immunohistochemistry (Fig. 5).

Additionally, endothelium of peritubular capillaries commonly demonstrated SPARC expression, especially in areas of interstitial fibrosis (Fig. 4D). A small subset of inflammatory cells, typically seen in lumina of peritubular capillaries, also expressed SPARC. Glomerular epithelial cells demonstrated persistent expression of SPARC without clear change in its expression by other components of the glomerular tufts. As illustrated in Figure 4C, there were rare instances of possible SPARC expression by glomerular endothelial cells.

Figure 6 illustrates glomerular expression of SPARC and members of the PDGF ligand/receptor family in glomeruli from a renal allograft with chronic allograft nephropathy. Particularly noteworthy is the colocalization of SPARC and PDGF A chain in visceral epithelial cells; PDGF B chain expression is confined to the mesangium. Figure 7 illustrates SPARC and PDGF expression in an artery with features of acute and chronic rejection, in which SPARC expression by medial and neointimal smooth muscle cells (expressing α smooth muscle actin) is congruent with PDGF A chain expression and in which SPARC expression by endothelial cells and some infiltrating leukocytes is congruent with expression by PDGF B-chain and PDGFα receptor by these cells.

DISCUSSION

This study localized the expression of both SPARC protein and mRNA to glomeruli, collecting ducts, urothelium, interstitial cells, and arteries of developing as well as mature human kidneys. These sites of SPARC expression in humans generally, but not invariably, conform to previous observations obtained from rodent models of renal injury [10, 11, 13, 29]. Some differences with a prior published study localizing SPARC in human kidney were apparent in the present investigation. Mundlos et al described SPARC expression only in glomeruli in fetal human kidneys [30]. This stands in contrast to the findings of SPARC expression in collecting ducts, urothelium, arteries, and interstitial cells reported here. Our findings were made both on protein and mRNA level and conform to findings in other mammalian systems (for example, rat and mouse) as well as to general observations in human fibrocytes and arterial cells reported by Porter et al [15]. The reagents utilized both for in situ hybridization and immunocytochemistry in the study of Mundlos et al are distinct from those utilized in our own study, and this may account for much of the difference. It is possible their reagents lacked the sensitivity to detect extraglomerular expression.

In the human glomerulus, the localization of constitutive expression of SPARC to visceral epithelial cells is of interest in view of the anti-proliferative properties of this molecule for cells in vitro. There is increasing evidence that the glomerular visceral epithelial cell has an extremely limited capacity for replication [31, 32]. Therefore, SPARC may contribute to the resistance to proliferative stimuli in these cells. It is also of interest that we have previously localized PDGF A-chain expression to the human glomerular visceral epithelial cell [23]. A role for SPARC in mediating renal injury as well as injury in other tissues is suggested in part by its ability to bind the growth factor PDGF and hence modulate signaling

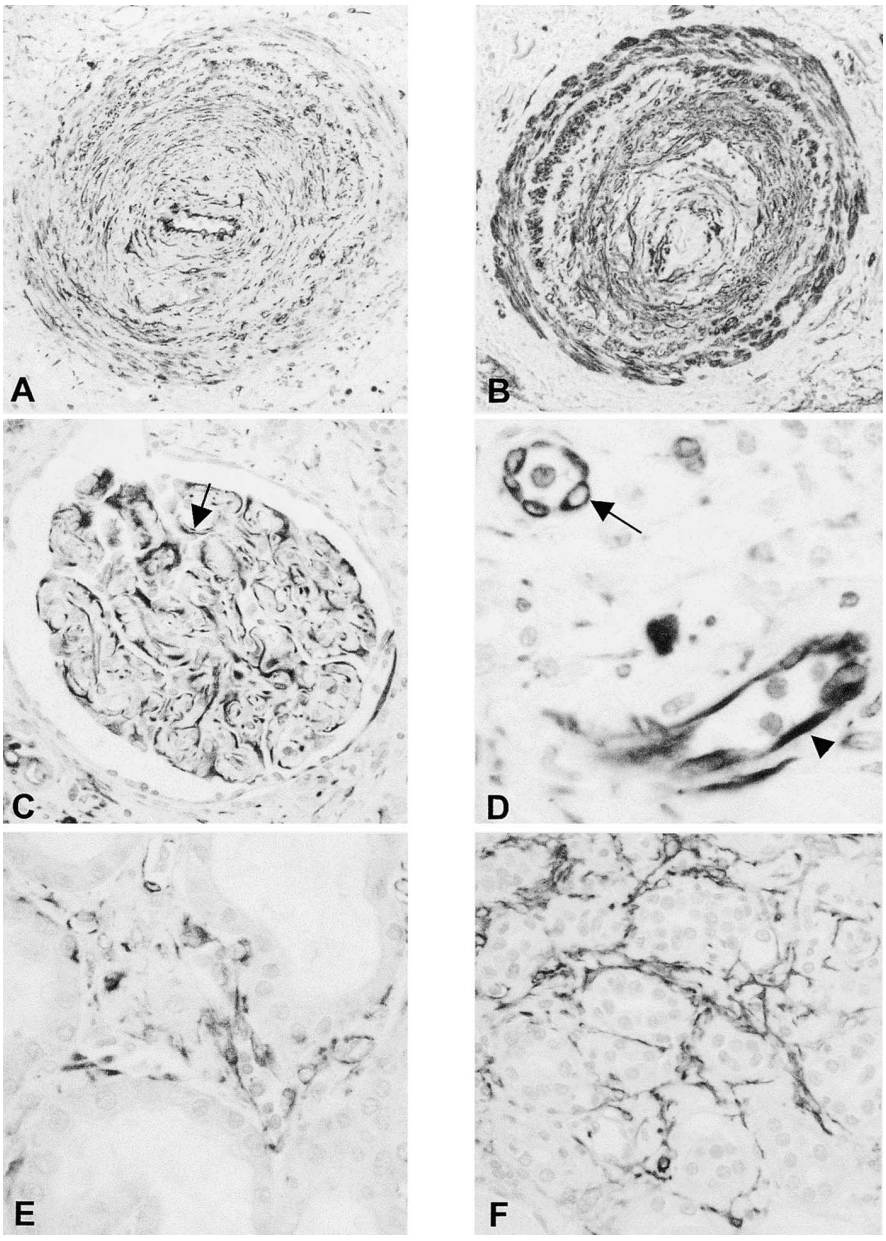

Fig. 4. SPARC expression in transplant nephrectomies. Immunohistochemistry for SPARC (*A* ×200; *C* ×400; *D*, *E* ×600 original magnification) and for smooth muscle actin (*B* ×200; *F* ×400 original magnification) performed on sections from explanted transplants. (A) Prominent neointima formation with a principal component of SPARC positive cells. (B) The distribution of smooth muscle actin in an artery from the specimen illustrated in panel A. (C) Transplant glomerulopathy, with persistent expression of SPARC by visceral epithelial cells (compare to Fig. 1D). Uncommonly, there appeared to be expression of SPARC by cells on both sides of the glomerular basement membrane (arrow), which could represent focal expression by glomerular endothelium or possibly an unusual sectioning artifact. (D) Several peritubular capillaries with SPARC expression by endothelium, here associated with the presence of intraluminal leukocytes (cross section: arrow, longitudinal section: arrowhead). (E) SPARC-expressing interstitial cells were present at sites of interstitial fibrosis. (F) Expression of smooth muscle actin by myofibroblasts in the interstitium. The pattern mirrored the expression pattern of SPARC (compare panel E from a different specimen).

pathways regulated by this factor. However, the binding of SPARC to PDGF is thought to occur via the PDGF B-chain, and thus would affect only activities of the PDGF heterodimers PDGF-BB and PDGF-AB, but not the PDGF-AA or PDGF A-chain per se [33]. Therefore, we are uncertain whether any biological interaction results from the co-localization of these two molecules.

The current study also demonstrates that SPARC is constitutively expressed by parietal epithelial cells, a cell type previously shown by us and others to be one in which constitutive expression of PDGF receptor β (PDGFRβ) is found [34]. This is of particular interest in view of the studies by Raines et al, showing that SPARC can modulate the ability of PDGF-BB and PDGF-AB to interact with this receptor by virtue of its binding to the PDGF B-chain molecule [33]. We speculate that SPARC may modulate the response of parietal epithelial cells to PDGF in glomerular injury, but a means to test this hypothesis in human cells is not currently available.

Mesangial expression of SPARC has been documented in the rodent both in vivo and in vitro. In rat mesangial cells, SPARC expression may be up-regulated by several growth factors including PDGF and FGF-2 [12]. In turn, its up-regulated expression by mesangial cells has been correlated with resolution of the acute mesangial cell proliferation that occurs in the anti-Thy-1 mesangial pro-

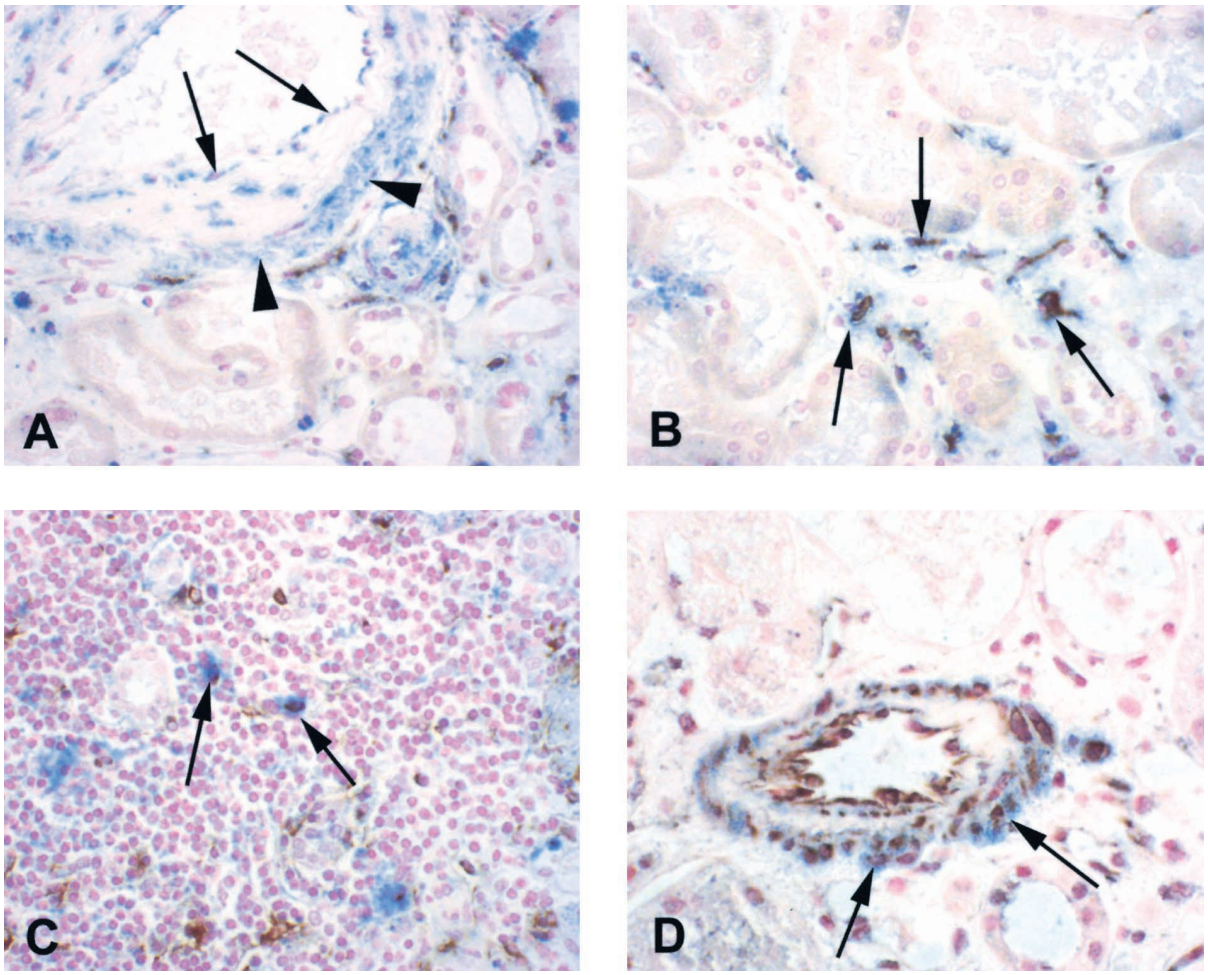

Fig. 5. Double immunolabeling of cortex from an allograft nephrectomy for expression of SPARC and the monocyte/macrophage marker CD68 in *A*, *B* and *C* and SPARC and α-smooth muscle actin in *D*. (A) SPARC (blue label) is expressed by arterial endothelial cells (arrows), smooth muscle cells of the vessel wall (arrowheads) and scattered cells in the tubulointerstitium, some of which also may express CD68 (brown label), although this is equivocal. There is no CD68 expression by vascular or tubular cells, but expression is confined to interstitial leukocytes. (B) Same case and labeling as panel A. High power view demonstrates expression of SPARC (blue) by some CD68 positive interstitial monocytes/macrophages (arrows). (C) Area of marked inflammation in the interstitium in which there are scattered CD68 positive monocytes/macrophages, a minority of which also express SPARC (blue) (arrows). (D) SPARC (brown) is expressed by arterial endothelium and medial smooth muscle cells (arrows). These smooth muscle cells also express α-smooth muscle actin (blue).

liferative glomerulonephritis model [12]. This is consistent with the observation that cultured mesenchymal cells, including mesangial cells, from SPARC deficient mice exhibit increased rates of cell proliferation [35]. Again, it has been proposed that the association between SPARC expression and resolution of mesangial cell proliferation in the anti-Thy-1 model may reflect possible interruption of binding of PDGF B-chain to its receptors, an event that mediates earlier phases of injury in this model. This study demonstrates that SPARC is occasionally expressed by human mesangial cells, most notably in the fetal kidney. Our work and that of others has previously identified several molecules such as α-actin, PDGFRβ, PDGF B-chain, and p75 nerve growth factor receptor, that are expressed by mesangial cells at the same stages of development identified for SPARC [21, 36]. Expression of each of these molecules typically is down-regulated in the healthy mature kidney, but may be re-expressed in the course of mesangial proliferative injury and repair. This has suggested a paradigm where the biologic events and the molecular signals that direct these events occurring in the repair of glomerular injury represent a recapitulation of renal development [37]. The expression of SPARC has not yet been tested in human glomerulonephritis to evaluate whether this paradigm holds for this molecule as well. The evidence concerning SPARC expression obtained from the anti-Thy-1 mesangial proliferative nephritis model indicates that the paradigm is true at least in the rat. We show in Figure 7 that, at least in chronic allograft nephropathy, mesangial expression of PDGF B-chain may be up-regulated while glomerular SPARC expression remains confined to visceral epithe-

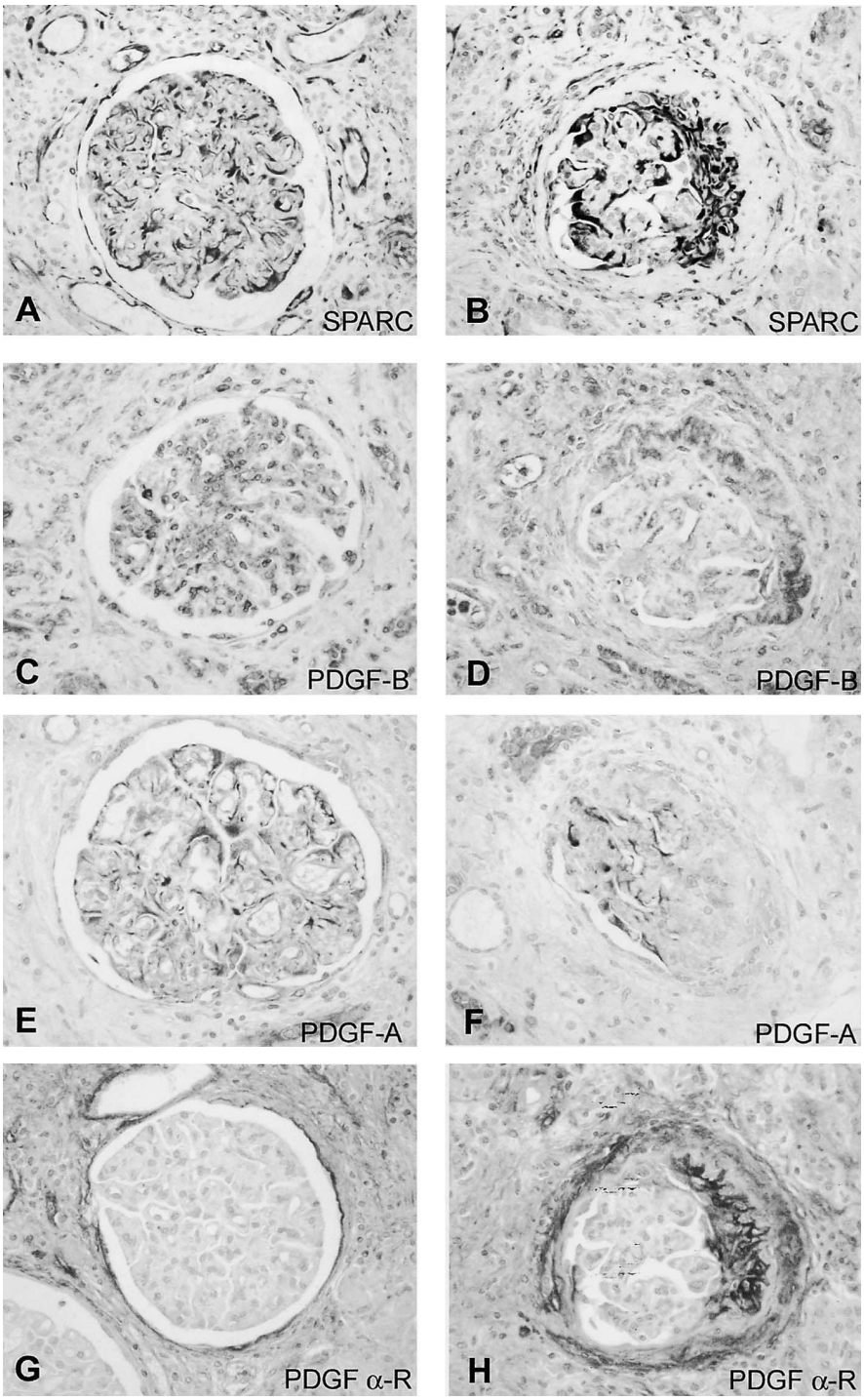

Fig. 6. Expression of SPARC (*A*, *B*), PDGF-B chain (*C*, *D*), PDGF A-chain (*E*, *F*) and PDGF α-receptor (*G*, *H*) in glomeruli in serial tissue sections from an allograft nephrectomy. A, C, E and G are from glomeruli with some features of transplant glomerulopathy (thickened capillary walls, increased lobularity), and B, D, F and H are from a glomerulus undergoing sclerosis. There is persistent expression of SPARC and PDGF A chain in visceral epithelial cells, a feature of normal mature kidneys. PDGF B is expressed in the mesangium and in tubulointerstitial cells. There is no expression of PDGF α-receptor by intrinsic cells of the glomerular tuft. Immunohistochemical analysis of PDGF β-receptor could not be accomplished for technical reasons.

lial cells, similar to the expression pattern of PDGF A-chain. Moreover, the autocrine loop effected by SPARC on cultured mesangial cells, via its induction of TGF-β1 and collagen type I, is likely to prove significant in fibroproliferative disorders of the glomerulus [6, 7].

The second major finding of this study is the localization of SPARC to interstitial myofibroblast-like cells and some infiltrating inflammatory cells in areas of chronic fibrosing injury occurring as part of chronic allograft nephropathy. SPARC expression by interstitial cells is only focally and weakly detectable in the mature adult kidney, but in the setting of chronic tubulointerstitial injury, this expression is dramatically up-regulated. Serial labeling studies and double immunolabeling studies

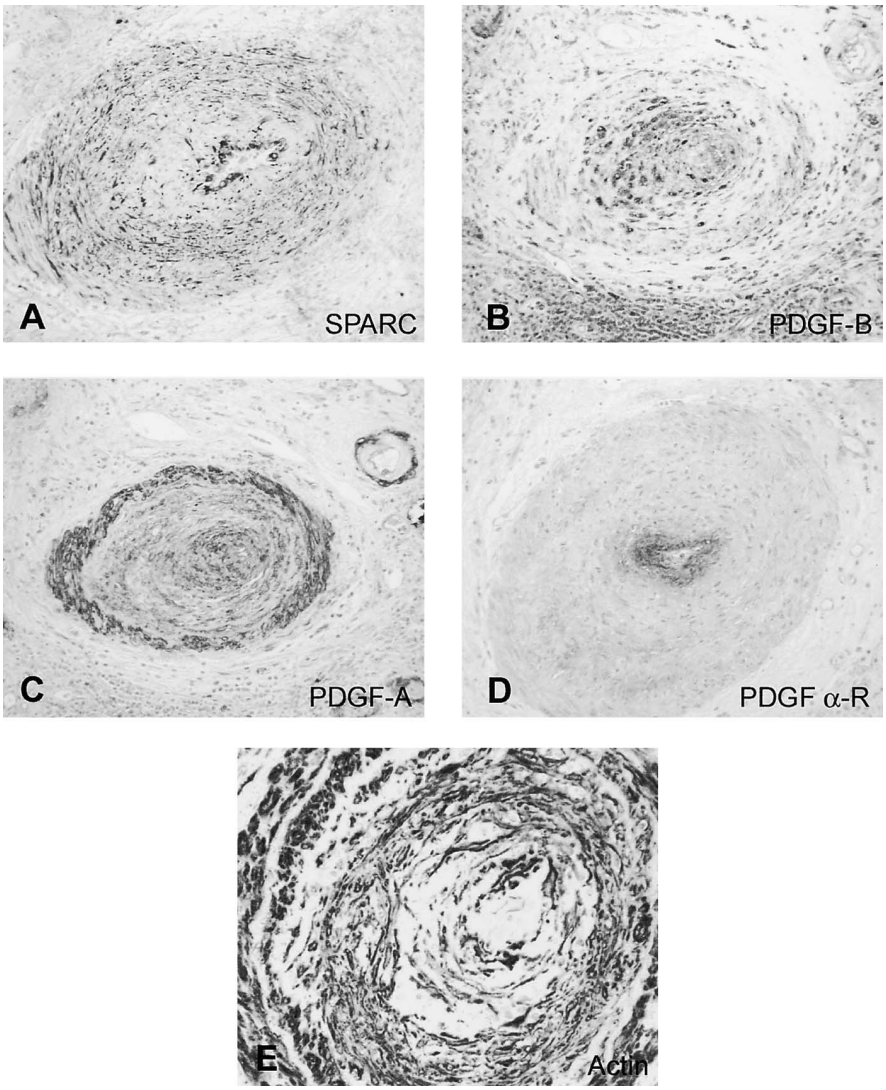

Fig. 7. Serial sections of a typical injured artery in a case of chronic allograft nephropathy. SPARC (*A*) and PDGF A-chain (*C*) are uniformly expressed by smooth muscle cells of the medial layer of the vessel wall and in the neointima. SPARC also is expressed by endothelial cells and likely some infiltrating leukocytes in the subendothelial lining, similar to expression patterns of PDGF B-chain (illustrated in *B*) and PDGF α-receptor (illustrated in *D*). Expression of α-smooth muscle actin by smooth muscle cells of the medial compartment of the artery wall and neointima (illustrated in *E*) is identical to that of the majority of SPARC expressing cells in this vessel.

demonstrated that most SPARC-expressing interstitial cells also express α-smooth muscle actin. This last feature has led to consideration of these interstitial cells as myofibroblasts [38–41]. Accumulation of these cells has been clearly identified at sites of human and experimental tubulointerstitial injury, and there is recent evidence that their enumeration may be a particularly accurate prognostic factor in studies of human diseases such as diabetes mellitus [41, 42] and membranous nephropathy [43]. It is also of note that these myofibroblast-like interstitial cells in the human express both PDGF α and β receptors [25, 44], making them likely to be responsive to stimulation by heterodimers containing the PDGF B-chain. Indeed, Tang et al have demonstrated direct stimulation of proliferation and collagen production by myofibroblast-like renal interstitial cells in rats in vivo exposed to infusions of PDGF B-chain [45]. In this site, it seems likely that up-regulated SPARC production by interstitial cells in chronic injury states may modulate the effects of continued exposure to PDGF by these cells, either through direct anti-proliferative properties of SPARC or by the direct interference with the binding of PDGF to its receptors. The induction of TGF-β by SPARC might be another important pathway involved in the regulation of tubulointerstitial fibrosis [14].

SPARC was localized also to a minority of the infiltrating mononuclear leukocytes typically present in areas of chronic tubulointerstitial injury occurring as part of chronic allograft nephropathy. SPARC previously has been identified in monocytes/macrophages in wound healing in experimental systems. However, the specific activities of leukocyte SPARC are not currently understood. SPARC has been shown to mediate some responses of human monocytes to injury, such as the production of matrix metalloproteinases [46]. This suggests the possibility of an autocrine stimulatory pathway for these ef-

fector cells, which are commonly present in various renal diseases.

Studies of SPARC deficient mice have shown that expression of SPARC is not a requirement for normal development of murine kidneys [47], but the putative biologic activities of this molecule have not been adequately tested in renal disease settings in these mice. Our current study suggests roles for SPARC in human renal development and fibrosing renal tubulointerstitial and vascular injuries. Our findings are consistent with a hypothesis that the effects exerted by SPARC in these injury processes are at least in part the result of modification of signaling through PDGF ligand/receptor pathways, but the findings in humans and experimental injury systems have not yet provided a proof of this possibility.

ACKNOWLEDGMENTS

This work was supported by grant GM 40711, and a George O'Brien Kidney Research Center Grant DK47659 from the National Institutes of Health. J.A.B. was supported by a University of Washington Royalty Research Award. The authors thank the Central Laboratory for Human Embryology at the University of Washington (supported by grant HD00836 from the National Institutes of Health) for assistance in providing fetal tissue, and Ms. Judy Renando for secretarial assistance.

Reprint requests to Dr. Charles E. Alpers, University of Washington Medical Center, Department of Pathology, Box 356100, Seattle, Washington 98195,USA.
E-mail: calp@u.washington.edu

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