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SPARC Is Expressed by Mesangial Cells in Experimental Mesangial Proliferative Nephritis and Inhibits Platelet-Derived-Growth-Factor-Mediated Mesangial Cell Proliferation *in Vitro*

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Mesangial cell proliferation is a characteristic feature of many glomerular diseases and often precedes extracellular matrix expansion and glomerulosclerosis. This study provides the first evidence that SPARC (secreted protein acidic and rich in cysteine) could be an endogenous factor mediating resolution of experimental mesangial proliferative nephritis in the rat. SPARC is a platelet-derived-growth-factor-binding glycoprotein that inhibits proliferation of endothelial cells and fibroblasts. We now show that SPARC is synthesized by mesangial cells in culture and that SPARC mRNA levels are increased by platelet-derived growth factor and basic fibroblast growth factor. Recombinant SPARC or the synthetic SPARC peptide 2.1 inhibited platelet-derived-growth-factor-induced mesangial cell DNA synthesis in vitro. In a model of experimental mesangioproliferative glomerulonephritis, SPARC mRNA was increased 5-fold by day 7 and was identified in the mesangium by in situ hybridization. Similarly, SPARC was increased in glomerular mesangial cells and visceral epithelial cells by day 5 and reached maximal expression levels by day 7. Mesangial cell proliferation increased by 36-fold on day 5 and decreased abruptly on day 7. Maximal expression of SPARC was correlated with the resolution of mesangial cell proliferation. We propose that SPARC functions in part as an endogenous inhibitor of platelet-derived-growth-factor-mediated mesangial cell proliferation in glomerulonephritis and that it could account for the resolution of cellular proliferation in this disease. (Am J Pathol 1996, 148:1153–1167)

Mesangial cell proliferation is a common feature of many different glomerular diseases, including IgA nephropathy, membranoproliferative glomerulonephritis, lupus nephritis, and others.[1] Previous studies by our group and by others have demonstrated that mesangial cell proliferation precedes and is tightly linked to expansion of extracellular matrix and the development of segmental or global glomerular sclerosis.[2–5] As measures that reduce proliferation also reduce matrix expansion, understanding the mechanisms that regulate proliferation is of paramount interest.[6,7]

We studied the regulation of mesangial cell proliferation in the Thy-1 model, in which the intravenous injection of an anti-Thy-1 antibody into rats induces complement-dependent lysis of mesangial cells. Subsequently, proliferation of the mesangial cells (days 2 to 7) and expansion of the extracellular matrix occur with histological similarities to acute mesangial proliferative glomerulonephritis in man. Our group has previously shown that proliferation in this model is primarily dependent on platelet-derived

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growth factor (PDGF)[7] and basic fibroblast growth factor (bFGF).[8,9] During the proliferative response to injury, an autocrine/paracrine cycle develops in which glomerular mesangial cells produce increased amounts of PDGF and also augment their PDGF receptors, events that could theoretically contribute to a potentially endless cycle of proliferation. However, an interesting feature of our model is that proliferation resolves spontaneously and mesangial cellularity returns to normal levels within several weeks after induction of the disease, in part via apoptosis.[10] Endogenous mechanisms must therefore exist that lead to resolution of mesangial cell proliferation and hypercellularity.

In this paper we have investigated whether SPARC (secreted protein acidic and rich in cysteine) might be involved in the resolution of mesangial proliferative nephritis. SPARC, also known as osteonectin, BM-40, or 43 K protein, is a glycoprotein produced by a variety of cells and is expressed at sites of tissue remodeling.[11,12] Studies *in vitro* have demonstrated that SPARC can inhibit proliferation of various cell types, including endothelial cells, fibroblasts, and smooth muscle cells.[13–16] SPARC specifically binds PDGF B-chain and blocks the interaction of PDGF with its receptors on fibroblasts.[17] SPARC also diminishes the proliferation of bovine aortic endothelial cells stimulated by bFGF,[13,16] although this inhibition does not involve the binding of SPARC to the cytokine.[13,16]

This study provides evidence that SPARC affects the proliferation of mesangial cells. Recombinant SPARC inhibits mesangial cell proliferation in response to PDGF *in vitro*. SPARC mRNA and protein are expressed by mesangial cells in experimental glomerulonephritis, and the time course of the expression is concordant with the resolution of proliferation. SPARC might therefore function as an endogenous regulator of PDGF-mediated mesangial cell proliferation *in vivo*.

Materials and Methods

Rat Glomerular Mesangial Cell Cultures

Studies were performed on primary cultures of rat glomerular mesangial cells that were originally isolated from kidneys of six male Sprague-Dawley rats weighing 75 to 100 g.[18] Mesangial cells were grown in RPMI medium (Irvine Scientific, Santa Ana, CA) that contained 15% fetal bovine serum, 15 mmol/L HEPES (Sigma Chemical Co., St. Louis, MO), 89 μg/ml sodium pyruvate (Sigma), 200 μmol/L L-glutamine (Sigma), 81 μg/ml penicillin G (Irvine Scientific), 81 μg/ml streptomycin sulfate (Irvine Scientific), and 0.66 U/ml insulin (GIBCO BRL, Grand Island, NY). The pH of this medium was adjusted to pH 7.3 with 7.5% sodium bicarbonate. Cells were passaged every 72 to 96 hours by treatment with trypsin. We demonstrated that cells maintained in this manner exhibit many features of differentiated glomerular mesangial cells. The cells displayed a stellate morphology and were stained for cytoskeletal filament proteins desmin, vimentin, and α-actin and for the antigens Thy-1 and thrombospondin-1. Cultures failed to bind antibody directed against the rat endothelial cell antigen-1, OX-1 (common leukocyte antigen), and von Willebrand factor. Epithelial cell contamination was excluded by visual examination.

Preparation of Kidney Glomeruli for RNA and Protein Analysis

Sprague-Dawley male rats (100 to 150 g) were sacrificed under ether anesthesia. The kidneys were removed and perfused *in situ* with 50 ml of ice-cold phosphate-buffered saline (10 mmol/L $NaPO_4$, 50 mmol/L NaCl, pH 7.5) containing 1 mmol/L each of the following proteinase inhibitors: Pefabloc (Center Chemicals, Stanford, CT), pepstatin (Sigma), leupeptin (Sigma), and antipain (Sigma). After removal of the cortex, the glomeruli were isolated by differential sieving in the presence of proteinase inhibitors. Isolated glomeruli from normal and anti-Thy-1-treated animals at days 2, 5, and 7 were counted and were subsequently dissolved in either RNAzol B (Cinna/Biotecx Laboratories, Friendswood, TX) for RNA isolations or in 1 μl of 2X protein sample buffer (4% sodium dodecyl sulfate (SDS), 0.125 mol/L Tris-HCl, pH 6.8, 10% glycerol, 0.05% bromphenol blue) per 20 glomeruli. Protein samples were stored at −20°C.

Western Immunoblot Analysis of Mesangial Cell Proteins

Media from subconfluent rat dermal fibroblasts or from subconfluent glomerular mesangial cells were collected in the absence of serum for 16 to 24 hours, made 0.1% in SDS, dialyzed against 0.0625 mol/L Tris-HCl (pH 6.8), lyophilized, and dissolved in 1X protein sample buffer (2X buffer defined above). Cellular protein solutions were incubated for 5 minutes with 5% β-mercaptoethanol or 0.05 mol/L dithiothreithol for 5 minutes at 95°C and were resolved by electrophoresis through polyacrylamide gels that contained 0.1% SDS. Fractionated proteins were

electrotransferred onto nitrocellulose filters and were reacted with a monoclonal antibody against human SPARC (Haematologic Technologies, Essex Junction, VT). Bound SPARC-IgG complexes were visualized by either anti-mouse IgG conjugated to alkaline phosphatase (Promega, Madison, WI) and subsequent colorometric development with nitro blue tetrazolium/5-chromo-4-chloro-3-indolyl phosphate (Sigma) or rabbit anti-mouse IgG (Promega) and subsequent incubation with ^{125}I-labeled protein A (Amersham, Arlington Heights, IL).

Preparation of Mesangial Cell RNA and Northern Analysis

Total RNA was extracted from cultured glomerular mesangial cells with RNAzol B followed by precipitation with LiCl. Ten μg of RNA per lane was resolved by electrophoresis through a 1% agarose gel that contained 3% formaldehyde and 0.2 mol/L morphilinopropanesulfonic acid (pH 7.0), and was transferred to a Hybond N^+ nylon membrane (Amersham) by capillary blotting. A 930-bp *Sma*I-*Xba*I rat SPARC cDNA fragment was radiolabeled with [^{32}P]dCTP by random primer extension. Membranes were prehybridized for 20 minutes in Quickhyb solution (Stratagene, La Jolla, CA), hybridized with 2×10^6 cpm probe/ml for 1 to 2 hours at 68°C in Quickhyb solution, and washed twice for 30 minutes at 65°C with 18 mmol/L NaCl, 10 mmol/L $NaPO_4$ (pH 7.7), 0.1 mmol/L EDTA, 0.1% SDS. The signal intensity of the autoradiograms was determined by Phospho-Imager analysis (Molecular Dynamics, Sunnyvale, CA) and was corrected for 28S ribosomal RNA from a reprobing of the blot with a 280-bp cDNA probe for 28S RNA (gift of Dr. L. Iruela-Arispe). All Northern blots were repeated at least twice with RNA from different tissue culture experiments.

Rat glomerular mesangial cells were growth arrested as described below. The medium was replaced with RPMI medium with or without 5 ng/ml PDGF-BB or 10 ng/ml bFGF for 3, 6, and 24 hours, respectively. Total RNA was extracted from cells as described above.

Assay of DNA Synthesis

Cultures of rat mesangial cells were seeded in 24-well plastic tissue culture plates at a density of 10,000 cells/dish and were grown in RPMI media that contained 15% fetal calf serum and 0.025 mg/ml (0.66 U/ml) insulin until the culture reached 50 to 60% confluence. After the media were removed, cells were rinsed and growth arrested for 72 hours in RPMI/0.5% serum/insulin, or in Dulbecco's modified Eagle's medium/insulin. Growth was subsequently stimulated by the addition of serum or PDGF with or without insulin. [^{3}H]thymidine was added to the cultures at a final concentration of 2 μCi/ml. After 16 to 18 hours at 37°C, the medium was removed and was used subsequently for cell viability assays (below). A 1-ml volume of wash medium was added to the cells in each well and was removed by gentle aspiration. The cells were permeabilized with cold methanol (twice for 5 minutes), followed by a fixation and precipitation with 10% trichloroacetic acid. After a wash with H_2O, cells were solubilized in 0.3 N NaOH (0.3 ml) by incubation at 50°C for 10 minutes. Constant aliquots (0.2 ml) were removed for radioactive scintillation counting for determination of the incorporation of [^{3}H]thymidine into the acid-insoluble cellular DNA fraction.

Lactate dehydrogenase activity was measured by a modification of the procedure of Wroblewski and LaDue.[19] Constant volumes of conditioned media (0.04 ml) were transferred to a 96-well plate, followed by 0.04 ml of sodium phosphate (pH 7.0), 0.04 ml of nicotinamide adenine dinucleotide, and 0.02 ml of sodium pyruvate. After 3 minutes, the reaction was stopped by addition of 0.1 vol of glacial acetic acid, and the absorbance at 340 nm was measured with a plate reader (Molecular Devices UV-MAX, Palo Alto, CA).

Total DNA content was determined from constant volumes (0.07 ml) of mesangial cell DNA that had been dissolved in 0.3 N NaOH. DNA samples were diluted into a 100 mmol/L Tris-HCl (pH 8.0) buffer solution that contained 10 mmol/L EDTA, 100 mmol/L NaCl, and 1.5 mg/ml 4,6-diamino-2-phenylindole. The fluorescence of each sample was measured with a Perkin-Elmer LS50B fluorimeter set at the following parameters: resolution slit widths of 5 and 6 nm for excitation and emission, respectively, an excitation wavelength of 340 nm, and an emission wavelength of 400 nm.

Recombinant human SPARC (rSPARC) was isolated as described.[20] Briefly, *Escherichia coli* cultures that expressed human endothelial rSPARC were grown in 1.3-L fermentations. Soluble rSPARC was purified from the bacterial lysate by sequential chromatography on ion-exchange and nickel-chelate affinity resins. rSPARC was isolated at a concentration of 0.2 mg/ml and was exchanged into RPMI media by sterile, disposable gel filtration columns. The levels of contaminating bacterial endotoxin in preparations of rSPARC were determined by the *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD).

After correcting for the contribution of endotoxin by media and buffers, we calculated that preparations of rSPARC contained 0.014 to 0.018 ng endotoxin/mg rSPARC. This ratio is below the threshold *in vitro* that affects protein synthesis and that causes detachment of bovine aortic endothelial cells.[21]

SPARC synthetic peptide 2.1 (NH_2-CQNHHCKHGKVCELDESNT-COOH) was purified after synthesis by preparative reverse-phase high pressure liquid chromatography and was verified by mass spectrometry to be 19 residues corresponding to the correct molecular weight. The peptide was dissolved in 50 mmol/L NaOH and was used immediately after addition to RPMI medium.

Experimental Protocol

Experimental mesangial proliferative glomerulonephritis (anti-Thy-1.1 nephritis) was induced in male Wistar rats (180 to 220 g; Simonsen Laboratories, Gilroy, CA) by intravenous injection of goat anti-rat thymocyte plasma (0.4 ml/100 g body weight). Rats were sacrificed at days 2, 5, 7, and 14 (n = 6 per group). Six additional rats were also complement depleted for 5 days with cobra venom factor (Diamedics Corp., Miami, FL). In these rats, C3-complement levels were measured by radial immunodiffusion, and values of <10% were maintained throughout the study period. Six untreated rats served as normal controls.

Renal Morphology and Immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution and were embedded in paraffin.[22] Four-micron sections were stained with periodic acid and Schiff's reagent and were counterstained with hematoxylin. Indirect immunoperoxidase staining and immunofluorescence were performed on four-micron sections as described previously[22] with the following primary antibodies: ascites 2, a murine monoclonal IgG_1 antibody directed against SPARC peptide 1.1 (peptide 1.1 spans amino acids 3 to 23 of SPARC[23]); a rabbit polyclonal antibody (5944A) directed against native SPARC[24]; a murine monoclonal antibody (AON-5031) directed against human, rat, and bovine SPARC/osteonectin (Haematologic Technologies); a murine monoclonal IgM antibody against the proliferating cell nuclear antigen (PCNA; 19A2; Coulter Immunology, Hialeah, FL); a murine monoclonal IgG_2 antibody against α-smooth muscle actin (Sigma); ED-I, a monoclonal antibody used as a marker for macrophages/monocytes and dendritic cells; and a murine monoclonal antibody PGF-007 (Mochida Pharmaceutical, Tokyo, Japan) against a 25-amino-acid peptide located near the carboxy terminus of the human PDGF B-chain. All antibodies directed against SPARC produced the same staining pattern.

Glomerular expression of SPARC was graded semiquantitatively by the following scoring system: 1, normal distribution of SPARC, ie, staining in glomerular epithelial cells but no mesangial staining; 2, <30% of the cells in mesangial areas staining for SPARC; 3, 30 to 60% of cells in mesangial areas staining for SPARC; 4, >60% of cells in mesangial areas staining for SPARC.

Glomerular expression of PDGF was graded semiquantitatively by the following score: 0, very weak or no staining for PDGF-β; 1, weak staining with <25% of glomerular tuft showing focally increased staining; 2, 25 to 49% of glomerular tuft exhibiting staining; 3, 50 to 75% of the glomerular tuft demonstrating increased staining; 4, >75% of the glomerular tuft exhibiting staining.

Expression of PCNA was quantified according to the number of PCNA-positive cells per glomerular cross section. For each variable, a minimum of 35 glomeruli per biopsy was assessed from which an individual mean score was determined. An overall score per group (mean $\pm$ SD) was subsequently calculated from the individual mean scores from each biopsy.

Immunohistochemistry of cultured mesangial cells was performed on cells plated onto chamber slides (Nunc, Naperville, IL). Cells were fixed in 50% methanol/50% acetone solution for 1 minute at room temperature and were subsequenly incubated with a murine monoclonal antibody (AON-5031) directed against human, rat, and bovine SPARC/osteonectin for 90 minutes at room temperature. After a 40-minute incubation with a biotinylated rabbit anti-mouse IgG_1 antibody (Zymed Laboratories, San Francisco, CA), mesangial cells were incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) and finally with fluorescein-isothiocyanate-conjugated streptavidin (Amersham). Control experiments included the replacement of primary antibody with an irrelevant monoclonal antibody. Under no circumstances did controls exhibit staining.

Immunohistochemical double labeling was performed to determine the cell types expressing SPARC in our disease model. Tissue was fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid), embedded in paraffin,

and stained by an indirect immunofluorescence technique for SPARC and for α-smooth muscle actin, a marker for activated mesangial cells.[1,25] Both primary antibodies (SPARC ascites 2, an IgG_1, and α-smooth muscle actin, an IgG_2) were incubated together overnight at 4°C. Detection was accomplished by sequential incubation of the sections with a biotinylated rabbit anti-mouse IgG_1 antibody, Texas-red-conjugated avidin D (Vector), and a fluorescein-isothiocyanate-labeled anti-mouse IgG_2 antibody (Cappel Laboratories, West Chester, PA).

Immunoblotting of SPARC

Kidneys were perfused *in situ* with 50 ml of ice-cold 0.9% sterile saline containing 1 mmol/L each of the following protease inhibitors: Pefabloc, leupeptin, pepstatin, and antipain. The kidneys were removed, the cortex was separated from the medulla, and glomeruli were isolated by differential sieving in the presence of protease inhibitors. Isolated glomeruli of normal control animals and of rats with anti-Thy-1 disease (days 0, 2, 5, and 7) were counted (three aliquots each) and were lysed in 2X protein sample buffer at room temperature (1 μl of buffer per 20 glomeruli). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA). The samples were incubated in 5% β-mercaptoethanol (Sigma), boiled for 5 minutes, and centrifuged for 5 minutes at 11,600 $\times$ *g*. Each lane contained the protein extract from 160 glomeruli. Murine PYS-SPARC served as a positive control. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 5% stacking gel and a 15% separating gel.[26] Proteins were subsequently electrotransferred to polyvinylidine fluoride membranes (Millipore, Bedford, MA) and reacted with a monoclonal antibody against human SPARC (AON-5031). Bound SPARC-IgG complexes were visualized by anti-mouse IgG conjugated to alkaline phosphatase and subsequent colorometric development with nitroblue tetrazolium/5-chromo-4-chloro-3-indolyl phosphate.

Preparation of Glomerular RNA and Northern Analysis

Total RNA was extracted from three separate isolated glomerular preparations (>90% purity) with RNAzol B followed by precipitation in LiCl.[27] Fifteen micrograms of denatured glomerular RNA per lane was resolved on a 1% agarose gel containing 3% paraformaldehyde and was subsequently transferred to a nylon membrane (Hybond N^+, Amersham) as previously described.[25] An isolated 557-bp fragment of murine SPARC cDNA was radiolabeled with [^{32}P]dCTP (10 μCi/ml; New England Nuclear, Boston, MA) by random primer extension. Membranes were prehybridized for 20 minutes, hybridized with 2×10^6 cpm/ml for 60 minutes at 68°C in Quickhyb solution (Stratagene), and washed with 0.1X standard saline phosphate-EDTA containing 0.1% SDS twice for 30 minutes at 65°C.[25] Signal intensities of Northern blots were determined by analysis of hybridized membranes with a PhosphoImager and were normalized to a signal corresponding to 28S ribosomal RNA.[25,28] Northern blots were performed in triplicate with RNA from different sets of animals.

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 Holland et al[29] with ^{35}S-labeled antisense and sense RNA probes for SPARC, as previously described.[11]

Statistical Analysis

All values are expressed as mean $\pm$ SD. Statistical significance was defined as $P < 0.05$ and was evaluated by analysis of variance followed by the Fisher's protected least significant difference procedure.

Results

Studies in Vitro

Mesangial Cells in Culture Synthesize and Secrete SPARC

We cultured mesangial cells from isolated rat glomeruli and examined them for the presence of SPARC by immunohistochemistry (Figure 1A) and by immunoblotting (Figure 1B). When cultured mesangial cells were incubated with a specific anti-SPARC IgG, staining could be detected in a perinuclear granular pattern, which is consistent with that of a secreted protein (Figure 1A). Immunoreaction with an irrelevant monoclonal antibody or with secondary antibody alone was negative (data not shown).

We found that SPARC was detected readily in the media from cells grown in 0.5% serum (Figure 1B, lane 2). The observed signal from cells grown in 0.5% serum was dependent on protein synthesis and secretion by cultured mesangial cells, because

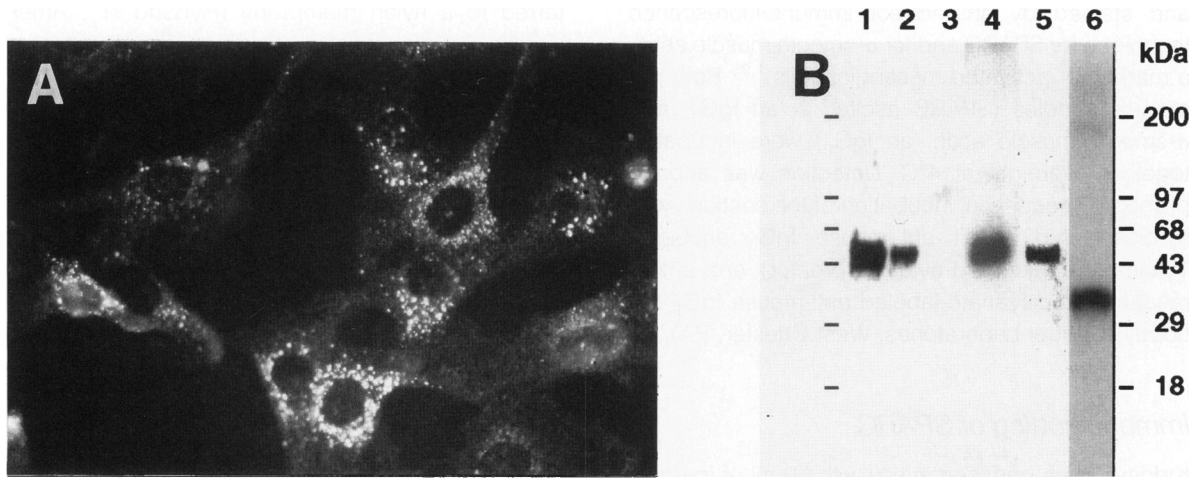

Figure 1. *SPARC is expressed and secreted by mesangial cells in culture.* A: *Cultured rat mesangial cells that exhibit the typical staining pattern for SPARC, a granular, perinuclear distribution that presumably delimits the Golgi apparatus. Magnification, ×630.* B: *Soluble proteins were collected from the media of subconfluent rat mesangial cells, resolved on a 10 to 20% gradient SDS-polyacrylamide gel, and electrotransferred to a nitrocellulose filter. Proteins bound to the filter were exposed to an anti-SPARC-specific monoclonal antibody. Immunocomplexes were detected with* ^{125}*I-labeled protein A.* Lane 1, *conditioned medium from rat lung fibroblasts in 0% serum;* lane 2, *conditioned medium from rat glomerular mesangial cells in 0.5% serum;* lane 3, *RPMI medium/10% serum;* lane 4, *SPARC purified from murine teratocarcinoma cells;* lane 5, *NP-40 extract of adult rat glomeruli;* lane 6, *recombinant human SPARC produced in* E. coli.

an equivalent volume of 10% serum in RPMI media yielded no signal (Figure 1B, lane 3). Also shown for comparison is the positive signal from the conditioned media of rat lung fibroblasts (Figure 1B, lane 1). The anti-SPARC monoclonal antibody also recognized SPARC from a detergent extract of adult rat glomeruli (Figure 1B, lane 5) and recombinant human SPARC (lane 6).

Rat SPARC displayed an electrophoretic mobility consistent with a molecular mass of 43 kd, a value 10 kd greater than the molecular mass predicted from its DNA sequence. This discrepancy in apparent molecular weight is due to the post-translational addition of carbohydrate, as well as the low pI of the protein, characteristics known to retard the mobility of many globular proteins on SDS-polyacrylamide gel electrophoresis.

SPARC Mesangial Cell mRNA Is Increased by PDGF and bFGF in Vitro

SPARC mRNA was detected readily in mesangial cells grown *in vitro*. Steady-state levels of SPARC mRNA were lower in mesangial cells grown in maintenance medium before growth arrest. The signal detected at 2.2 kb was consistent with the previously reported value from total glomerular RNA[24]; however, we detected no other molecular species with our SPARC cDNA probe. When subconfluent cultures were treated with PDGF-BB or bFGF, a 1.5-fold increase in SPARC steady-state mRNA levels could be observed by Northern blot analysis at 3 and 6 hours, after correction for loading errors by normalization of signals to that for 28S rRNA. Figure 2 shows quantification of the levels of SPARC mRNA at 3, 6, and 24 hours after treatment with either PDGF or bFGF; the level of SPARC mRNA at time 0 (growth-arrested mesangial cells before treatment) was defined as 1.

SPARC Inhibits Mesangial Cell DNA Synthesis in Vitro

We asked whether SPARC could have a modulatory effect on PDGF-mediated mesangial cell proliferation. Human rSPARC was added to PDGF-stimulated

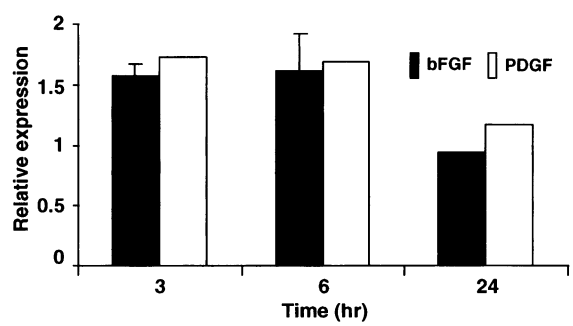

Figure 2. *Increased expression of rat SPARC mesangial mRNA by bFGF and PDGF. Relative expression of SPARC mRNA by mesangial cells that were cultured for 3, 6, and 24 hours with either 5 ng/ml PDGF-BB or 10 ng/ml bFGF (compared with growth-arrested mesangial cells) from three different Northern blots as determined by densitometric scanning, when corrected for equal loading by probing for 28S RNA.*

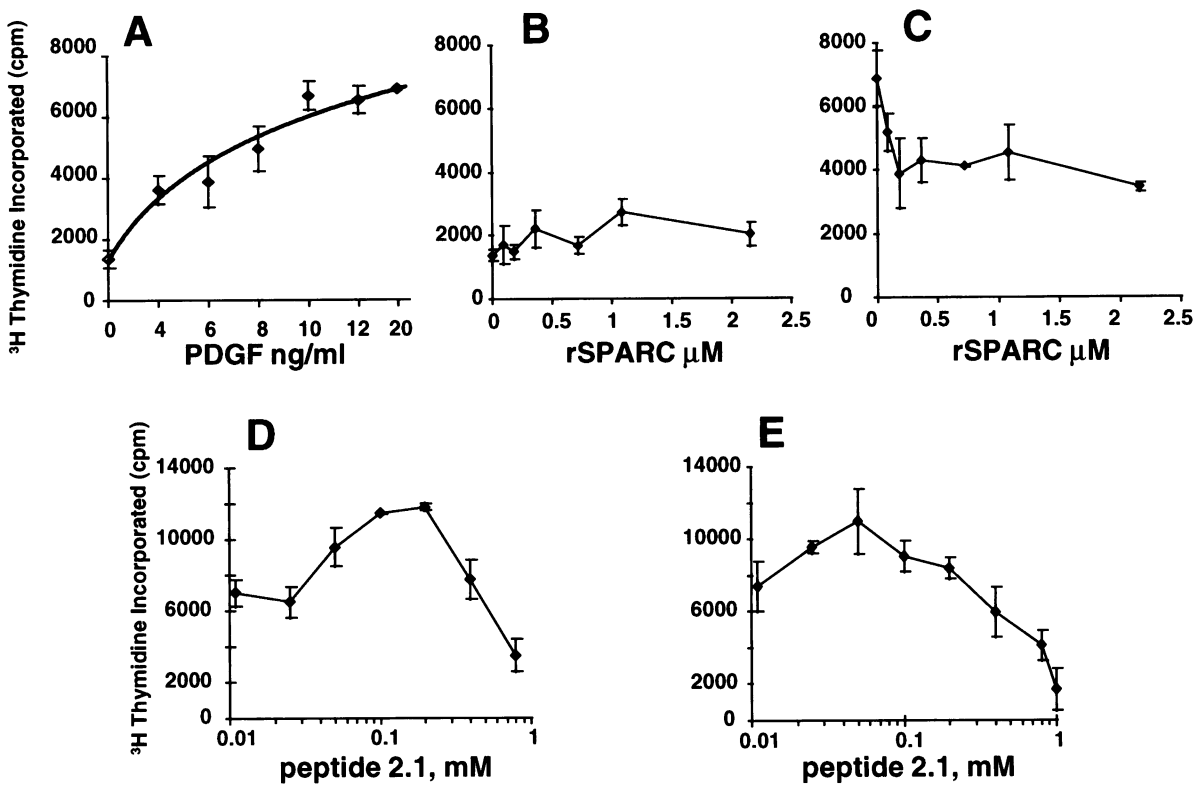

Figure 3. *Inhibition of glomerular mesangial cell DNA synthesis by rSPARC* (A *to* C) *and the SPARC peptide 2.1* (D *and* E). *Cultures of rat glomerular mesangial cells were seeded in 24-well plastic tissue culture plates at a density of 10,000 cells/dish and were grown in RPMI media that contained 15% fetal calf serum and 0.025 mg/ml (0.66 U/ml) insulin until the culture reached 50 to 60% confluence. After the medium was removed, cells were rinsed and growth arrested for 72 hours in 0.5% serum/RPMI/insulin. Growth was restimulated by the addition of PDGF* (A *to* C *and* E) *or 15% serum* (D). *DNA synthesis was monitored by the incorporation of [^{3}H]thymidine into acid-precipitable cpm. Each data point (mean ± SD) represents three experiments.* A: *Growth curve with varying concentrations of PDGF-BB.* B: *No PDGF; rSPARC used at 0, 3, 6, 12, 24, 36, and 72 μg/ml.* C: *PDGF used at 10 ng/ml; rSPARC used at 0, 3, 6, 12, 24, 36, and 72 μg/ml.* D: *15% serum/insulin/RPMI; peptide 2.1 used at 0.01, 0.1, and 1 mmol/L.* E: *10ng/ml PDGF; peptide 2.1 used at 0.01, 0.1, and 1 mmol/L.*

mesangial cells that had been growth arrested for 72 hours. When compared with the amino acid sequence of rat SPARC (J. A. Bassuk and R. Meek, unpublished observations), the sequence of rSPARC was 96.1% identical. We therefore felt that it was reasonable to use human rSPARC on rat mesangial cells. The rSPARC was not contaminated with growth factors or cytokines and contained <0.014 ng endotoxin/μg rSPARC.

Figure 3A shows that growth-arrested mesangial cells could be stimulated to synthesize DNA by the addition of PDGF. This stimulation was concentration dependent and achieved a fivefold increase over the range measured at PDGF concentrations of 0 to 2.1 μmol/L. In contrast, the addition of increasing concentrations of rSPARC to growth-arrested mesangial cells had no influence on the incorporation of [^{3}H]thymidine into mesangial cell DNA; rSPARC therefore appears to be nonmitogenic for these cells (Figure 3B). When growth-arrested mesangial cells were stimulated with 10 ng/ml PDGF, we found that increasing amounts of rSPARC inhibited the PDGF-dependent synthesis of cellular DNA by a maximum of 50% (Figure 3C). This effect was found to be similar to that of heparin (Table 1).

SPARC Peptide 2.1 Inhibits Mesangial Cell DNA Synthesis

Peptide 2.1 is a peptide of 19 amino acid residues that has previously been shown to mimic the effect of SPARC on DNA synthesis by endothelial cells and fibroblasts.[13,16] This sequence, termed peptide 2.1, was tested for biological activity in assays of [^{3}H]thymidine incorporation into mesangial cell DNA. Figure 3 shows that this peptide inhibited the incorporation of [^{3}H]thymidine in the presence of serum (Figure 3D) or PDGF (Figure 3E). It is of interest that peptide 2.1 was associated with a biphasic response of [^{3}H]thymidine incorporation into mesangial cell DNA, an observation similar to that made for human and

Table 1. *DNA Synthesis, Total DNA, and LDH Activity of Glomerular Mesangial Cells*

	[^{3}H]Thymidine incorporation, % control	DAPI fluorescence, % control	LDH activity, % control
Peptide 2.1 (mmol/L)			
0	100	100	100
0.1	126.0 ± 16.1	92.8 ± 5.0	93.4 ± 2.7
1.0	3.4 ± 1.4	48.0 ± 7.3	91.6 ± 2.7
Heparin (μg/ml)			
0	100	100	100
10	80.6 ± 5.4	65.9 ± 16.8	101.8 ± 0.6
100	61.6 ± 5.0	54.5 ± 4.5	98.7
1000	16.4 ± 7.2	43.2 ± 19.3	92.9
rSPARC (μmol/L)			
0	100	100	100
0.3	109.6	102.4	98.9
1.5	55.6	53.1	97.7
3	29.8	37.5	94.7

All procedures are described in Materials and Methods. Values are expressed as mean ± SD. Values without SD represent averages of experiments performed only in duplicate; in these instances, the differences between the two determinations did not exceed 14%. DAPI, 4,6-diamino-2-phenylindole; LDH, lactate dehydrogenase.

bovine fibroblasts.[13] In the presence of serum (Figure 3D), a stimulation of [^{3}H]thymidine incorporation was observed at 0.05 to 0.3 mmol/L. At higher concentrations (0.8 mmol/L), peptide 2.1 reduced [^{3}H]thymidine incorporation by approximately 40%. In the presence of PDGF (Figure 3E), a stimulatory effect of peptide 2.1 was observed between 0.025 and 0.2 mmol/L, and a reduction of >90% was seen at a concentration of 1 mmol/L.

Table 1 displays three measurable parameters of our mesangial cell culture assay. Over a range of concentration of peptide 2.1 (0.1 and 1.0 mmol/L), we calculated that, at concentrations of peptide greater than 1 mmol/L, [^{3}H]thymidine incorporation was essentially zero in comparison with the control. This reduction in deoxyribonucleotide incorporation represents a real decline in DNA content, as revealed by 4,6-diamino-2-phenylindole fluorescence. At concentrations of 0.1 mmol/L peptide 2.1, the total DNA content of our culture system was reduced by approximately 7%, whereas a reduction of nearly 50% was noted at a concentration of 1 mmol/L. Lactate dehydrogenase activity, a measure for cell viability, was within normal limits at concentrations of peptide 2.1 of ≤1 mmol/L. Peptide 2.1 therefore appeared to have no effect on cell viability at these concentrations.

Glomerular Cell Proliferation in Experimental Mesangial Proliferative Nephritis (Thy-1 model) Is Transient

Glomerular cell proliferation was detected at day 2 after induction of the disease, was maximal at day 5, and was resolved by day 14. The proliferation at day 5 was 36 times greater than that observed in normal animals (11 *versus* 0.3 PCNA-positive cells/glomerular cross section for anti-Thy-1 (day 5) *versus* normal, respectively; Table 2). Previously we demonstrated by double immunolabeling that more than 85% of the proliferating cells at day 5 were mesangial cells (ie, these cells expressed Thy-1 or α-smooth muscle actin).[22]

Immunohistochemical Staining Corresponding to PDGF-B Is Increased in Mesangial Areas and Correlates with the Initial Increase in Mesangial Cell Proliferation

Glomerular immunostaining with an anti-PDGF antibody increased as early as day 2, was maximal at days 5 and 7, and declined by day 14 (Figure 4, Table 2). The initial increase in glomerular cell proliferation correlated closely with the increase in glomerular immunoreactivity for PDGF-B until day 5 of the Thy-1 model ($r = 0.83$, $P < 0.0001$). Subsequently, glomerular immunoreactivity with anti-PDGF antibody remained at maximal levels until day 7, whereas glomerular cell proliferation declined by more than 30%.

SPARC Is Expressed in Experimental Mesangial Proliferative Nephritis

In normal rats, SPARC was expressed primarily by visceral glomerular epithelial cells (Figure 4, Table 2). Expression of SPARC, as detected by immuno-

Table 2. *Glomerular Cell Proliferation and Glomerular PDGF and SPARC Expression*

	Normal control	Thy-1, day 2	Thy-1, day 5	Thy-1, day 7	Thy-1, day 14
PCNA-positive cells per glomerulus	0.3 ± 0.05	4.6 ± 0.6*	10.8 ± 1.1*	7.5 ± 0.4*	0.7 ± 0.2
Glomerular PDGF score (0 to 4)	0.6 ± 0.08	1.0 ± 0.04†	2.5 ± 0.06*	2.5 ± 0.2*	1.4 ± 0.2*
Glomerular SPARC score (1 to 4)	1.0 ± 0.02	1.2 ± 0.05	2.1 ± 0.1*	2.4 ± 0.1*	1.9 ± 0.2*
Glomerular SPARC mRNA (relative to control)	1.0 ± 0.0	2.5 ± 0.9	4.0 ± 1.5*	5.4 ± 0.7*	2.6 ± 0.8

For explanation of scoring, see Materials and Methods. Values are expressed as mean ± SE.
*$P < 0.0001$ *versus* control.
†$P < 0.05$ *versus* control.

staining, remained at low levels on day 2 after injection of anti-Thy-1 antibody, rapidly increased at day 5, and was maximal at day 7. Thereafter, expression decreased almost to normal levels by day 14. By immunohistochemistry, the qualitative increase in SPARC was detected primarily in mesangial areas but could also be observed in visceral glomerular epithelial cells.

The increase in SPARC expression in anti-Thy-1 nephritis was confirmed by Western immunoblotting of protein lysates from equal numbers of glomeruli (Figure 5). An increase in SPARC was evident at days 5 and 7, in comparison with normal glomeruli. A positive control consisting of murine PYS-SPARC migrated with a molecular weight similar to that of the rat protein (Figure 5).

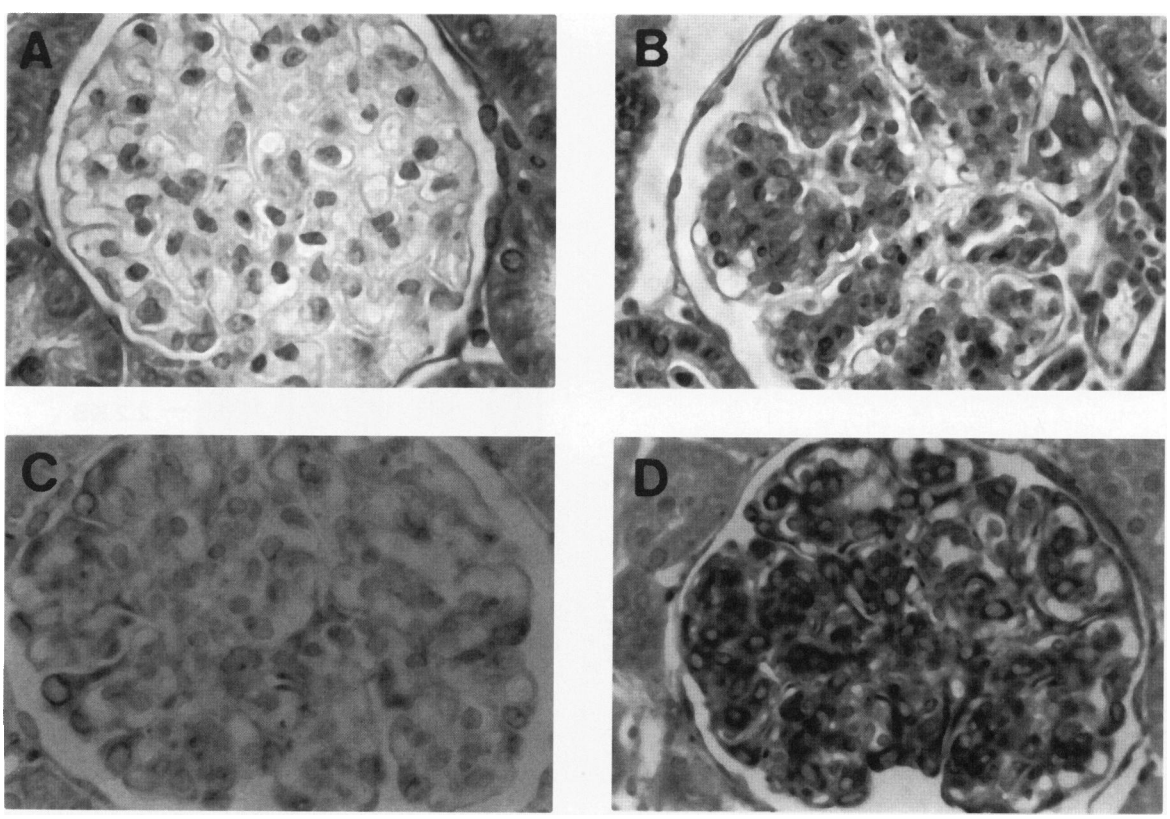

Figure 4. *PDGF B-chain and SPARC protein expression are increased in experimental mesangial proliferative nephritis. Whereas immunohistochemical staining for PDGF is minimal in a normal control animal* (**A**), *a marked increase can be observed in mesangial areas in an animal injected with anti-Thy-1 antibody* (**B**). *Magnification,* ×*630. Immunohistochemical reaction for SPARC in a normal control animal demonstrates reactivity confined almost exclusively to glomerular epithelial cells* (**C**). *An increase in mesangial reactivity was observed at day 7* (**D**), *which decreased by day 14* (*see Table 2*).

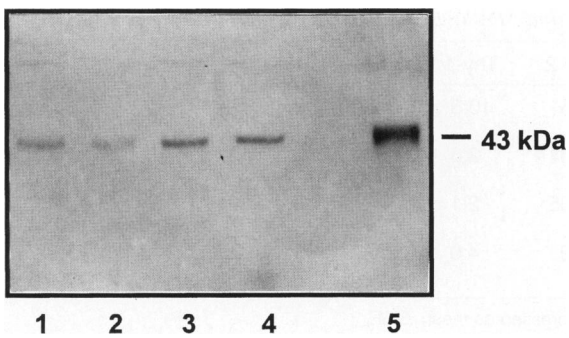

Figure 5. *Western blot of SPARC in whole glomerular protein lysates in anti-Thy-1 nephritis. In comparison to normal glomeruli* (lane 1), *a marked increase in SPARC protein was observed in glomerular extracts at days 5* (lane 3) *and 7* (lane 4) *from rats with anti-Thy-1 nephritis. The reduction in SPARC protein at day 2* (lane 2) *could result from lysis of mesangial cells. A positive control consisting of murine PYS-SPARC is shown in* lane 5. *Proteins were resolved on a 15% SDS-polyacrylamide gel.*

Positive Correlation between SPARC mRNA and Protein Expression within the Glomeruli in Experimental Mesangial Proliferative Nephritis

Expression of SPARC mRNA, as quantified by Northern blot analysis of total glomerular RNA, was coincident with that of SPARC and was increased more than fivefold over normal levels at day 7 in rats with experimental mesangial proliferative nephritis (Figure 6).

In situ hybridization for SPARC mRNA revealed a substantial increase in SPARC mRNA in glomeruli from rats with anti-Thy-1 nephritis (Figure 7). The density of silver grains was much higher in glomeruli from rats with anti-Thy-1 nephritis at days 5 and 7 in comparison with those from normal rats. Sections hybridized with a sense cRNA probe were negative.

Mesangial Cells Are a Major Source of SPARC in Anti-Thy-1 Nephritis

To determine the cell type that expressed increased amounts of SPARC in anti-Thy-1 nephritis, we performed double immunolabeling for SPARC and α-smooth muscle actin (a marker for activated mesangial cells[25]). In mesangial areas, the majority of cells overexpressing SPARC contained α-smooth muscle actin. This observation indicates that the SPARC-expressing cells were most likely glomerular mesangial cells (Figure 8). Visceral glomerular epithelial cells, which could be identified due to their location on the outer side of the basement membrane, were also positive for SPARC but were negative for α-actin (Figure 8).

Time Course of SPARC Expression Correlates with the Resolution of Mesangial Cell Proliferation

Previous studies from our group have demonstrated that proliferating mesangial cells in the anti-Thy-1 model exhibit substantial increases in PDGF A- and B-chain expression and PDGF-β receptors.[30,31] Mesangial cell proliferation decreased abruptly after day 5 (when PCNA was expressed maximally), despite a sustained elevation of PDGF-B expression (Table 2). It is therefore of interest that the peak of SPARC expression at day 7 correlated with the initial decrease in cell proliferation (Figure 9), an effect that could be due to the binding of PDGF by SPARC.

Discussion

In this study we have examined a potential role for SPARC in the regulation of mesangial cell proliferation. Recombinant SPARC was found to inhibit mesangial cell proliferation *in vitro* in growth-arrested and subsequently PDGF-stimulated mesangial cells. SPARC mRNA and protein were expressed by mesangial cells in culture, and SPARC mRNA was increased by PDGF and bFGF. SPARC mRNA and protein were also expressed by mesangial cells in an experimental model of mesangial proliferative nephritis (Thy-1 model). In this model, SPARC expression correlated with the resolution of glomerular cell proliferation.

A number of mediators of mesangial cell proliferation have been demonstrated to be important, eg, platelets, PDGF, bFGF, interleukin-6, interleukin-1,

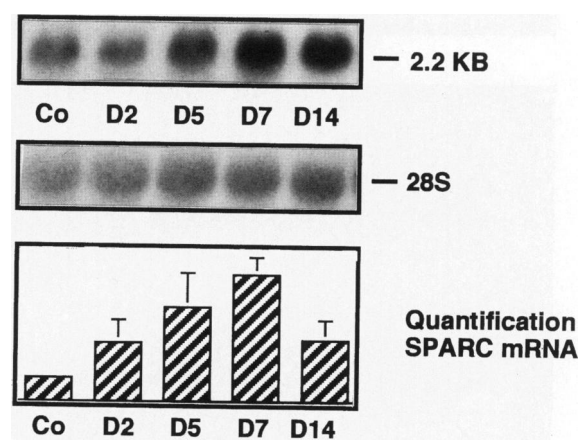

Figure 6. *Northern blot of SPARC mRNA in anti-Thy-1 nephritis. Each lane contained equal amounts of glomerular total RNA. A marked increase in SPARC mRNA expression that was maximal on day 7 was evident. This result is consistent with the temporal expression of SPARC. Quantification of signal for SPARC mRNA, which was normalized to that of 28S, is shown in the lower panel.*

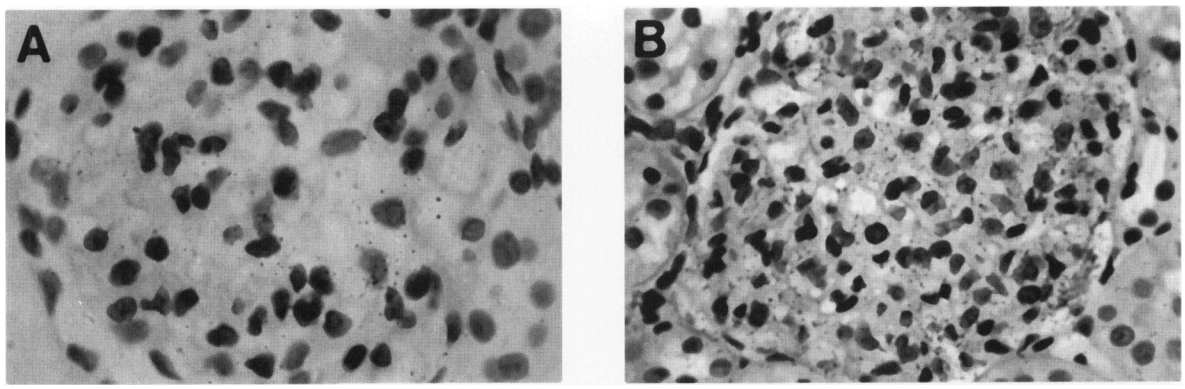

Figure 7. *SPARC mRNA is increased in Thy-1 disease as shown by* in situ *hybridization. Minimal amounts of SPARC mRNA are present in glomeruli in a normal control animal* (**A**). *In contrast, animals with mesangial proliferative nephritis exhibited a substantial increase in SPARC mRNA in the glomeruli at day 7* (**B**). *Magnification,* ×*630.*

endothelin, and insulin-like growth factor-1.[22,32] A central role has been demonstrated for PDGF, which is not only released by platelets and macrophages but is also produced by mesangial cells in the anti-Thy-1 model.[30,33] Treatment with an anti-PDGF antibody in this disease model reduced mesangial cell proliferation at day 4 by 60%.[7]

In contrast to the abundance of work on mediators of mesangial cell proliferation, only a few studies have been devoted to the identification of factors that are involved in the resolution of glomerular cell proliferation. Potential mediators include transforming growth factor-β, heparin, heparan sulfate proteoglycan, atrial natriuretic factor, nitric oxide, prostaglandin I_2, and interferon-γ.[34–38] This paper provides the first evidence for SPARC as an endogenous factor mediating the resolution of this proliferation in the Thy-1 model of mesangial proliferative nephritis.

SPARC, also known as osteonectin[39] and BM-40,[40] is widely expressed during development and remodeling and interacts with both cytokines[17] and components of the extracellular matrix.[23] From the predicted secondary structure of SPARC, the molecule was assigned four distinct domains.[41] The highly acidic domain I (amino acids 3 to 51) binds 5 to 8 Ca^{2+} molecules with a K_d of 10^{-3} to 10^{-5} mol/L and has an α-helical character.[12] Domain II (amino acids 52 to 132) contains 10 cysteines and has sequence similarity to follistatin, an inhibitor of transforming-growth-factor-β-like cytokines.[12] Domain II also is related to serpin-type protease inhibitors and contains epidermal-growth-factor-like repeats[12] as well as two Cu^{2+}-binding sites. The Cu^{2+}-binding sites have been implicated in the regulation of cellular proliferation and angiogenesis.[12,42] Domain III (amino acids 133 to 227) contains a series of α-helical segments, binds to collagen, and has an endogenous protease-sensitive site.[12] Domain IV (amino acids 228 to 285) contains a Ca^{2+}-binding EF-hand motif and has been implicated in Ca^{2+}-dependent binding to collagen[23,40] and endothelial cells.[43]

Previous studies have established that SPARC modulates cell proliferation. Specifically, both SPARC and a 19-residue synthetic peptide from SPARC domain II (termed peptide 2.1) inhibit the incorporation of [^{3}H]thymidine into newly synthesized DNA in cultured bovine aortic endothelial cells in a concentration-dependent fashion.[16] Similar effects of SPARC and peptide 2.1 on DNA synthesis could also be shown for human umbilical vein endothelial cells, for a transformed fetal bovine aortic endothelial cell line, and for bovine capillary endothelial cells.[13] Interestingly, human foreskin fibroblasts and fetal bovine ligament fibroblasts exhibited a biphasic response to peptide 2.1. Whereas concentrations of peptide 2.1 increased [^{3}H]thymidine incorporation, inhibition was observed at concentrations in excess of 0.4 mmol/L.[13] Furthermore, SPARC inhibited [^{3}H]thymidine incorporation into DNA in bovine aortic endothelial cells stimulated by bFGF.[15] SPARC, secreted by endothelial cells transformed by the polyoma middle T oncogene, also inhibited the growth of normal endothelial cells *in vitro*.[14] Relevant to our work are studies showing that SPARC also complexes with and/or inhibits cytokines known to mediate mesangial cell proliferation *in vitro* and *in vivo*.[8,15,17]

To examine the potential role of SPARC as an endogenous regulator of mesangial cell proliferation, we first showed that mesangial cells transcribed SPARC mRNA as well as synthesized and secreted SPARC protein. Second, we found that both PDGF and bFGF increased the expression of mesangial

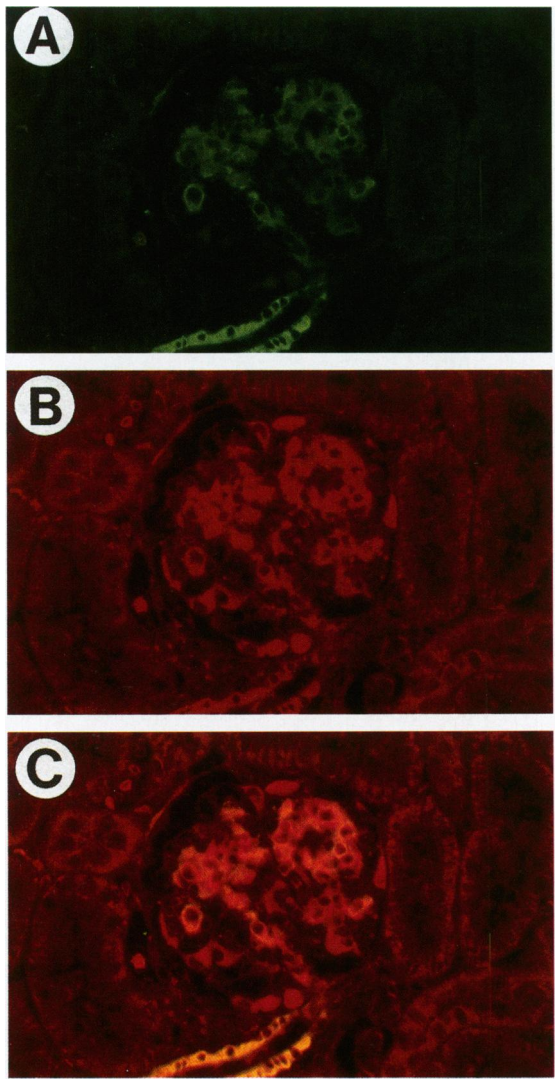

Figure 8. *Mesangial cells are a major source of SPARC in anti-Thy-1 nephritis. Immunohistochemical staining for α-smooth muscle actin (a mesangial cell marker; A) shows that glomerular mesangial cells and smooth muscle cells of the afferent arteriole are positive (green). SPARC (B) is expressed by many glomerular cells and also smooth muscle cells of the afferent arteriole (red). Upon double exposure, it is evident that many α-actin-positive cells express SPARC (orange in C).*

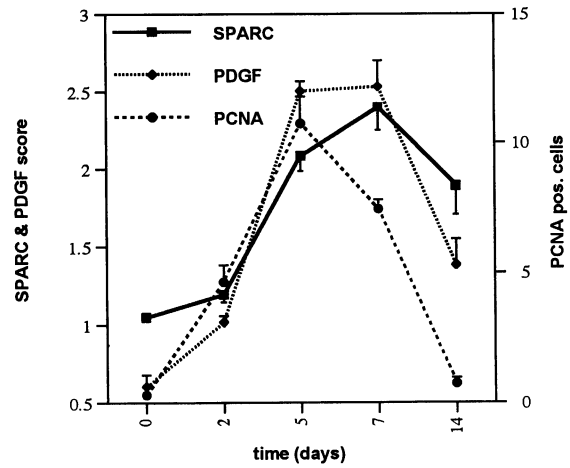

Figure 9. *Expression of SPARC correlates with the resolution of mesangial cell proliferation. Mesangial cell proliferation decreased abruptly after the peak at day 5, despite a sustained elevation of PDGF expression. Maximal SPARC expression at day 7 correlated with the initial decrease in cell proliferation.*

SPARC mRNA. This result is relevant because both growth factors are expressed in our experimental model of mesangial proliferative nephritis before the expression of mesangial SPARC; therefore,bFGF and PDGF are candidates for the regulation of SPARC expression *in vivo*.[27,33] Third, we have shown that SPARC inhibits mesangial cell proliferation *in vitro*. Human rSPARC inhibited [^{3}H]thymidine incorporation into mesangial cell DNA by 50% at a concentration of 0.3 to 2.1 μmol/L, an effect that is similar to that observed with heparin, a known potent inhibitor of mesangial cell proliferation.[6] Peptide 2.1 also inhibited mesangial cell proliferation at concentrations of 0.8 mmol/L, although a stimulatory effect was seen at lower concentrations, a result similar to that observed with fibroblasts.[13]

We next examined the expression of SPARC in the Thy-1 model in which mesangial cell proliferation is rapid and prominent but also self-limited. Glomerular SPARC mRNA was elevated twofold at day 5, was maximal at day 7, and continued to be elevated until day 14, as shown by *in situ* hybridization and Northern analysis. Increased SPARC was also confirmed by immunohistochemistry and immunoblotting. Although in normal glomeruli SPARC was expressed primarily by visceral glomerular epithelial cells,[44] double immunolabeling for SPARC and the mesangial marker α-smooth muscle actin revealed that SPARC was expressed *de novo* by mesangial cells in our model. Although platelets and macrophages express SPARC and are functionally important in our model, these cells did not seem to be a major source of SPARC in glomeruli.

Expression of PDGF in our study reached maximal levels at days 5 and 7 and remained elevated by day 14. It is of interest that glomerular expression of PDGF, one of the main mediators of mesangial cell proliferation, is tightly linked to glomerular cell proliferation until day 5. Although PDGF remained elevated after day 5, proliferation abruptly decreased (Figure 9). The findings that SPARC binds to PDGF, inhibits the interaction of PDGF with its receptor,[17] and is maximal at day 7 in our model, are consistent with our hypothesis that SPARC mediates the rapid decrease in mesangial cell proliferation and leads to resolution of the disease.

In addition to its effect on cell proliferation, SPARC might have other consequences in disease, especially in regard to regulation of cell shape and attachment. SPARC has been shown to inhibit cell spreading on collagen and other substrates and to induce cell rounding in cultured endothelial cells and fibroblasts.[45] It is also known that SPARC can disrupt focal adhesions[46] and alter the distribution of cytoskeletal elements as well as the permeability of endothelial monolayers.[47] On the other hand, SPARC might also affect matrix deposition or assembly. Endothelial cells, for example, change the synthesis of several extracellular matrix proteins after exposure to exogenous SPARC.[21,48]

These studies provide the first evidence that mesangial cells can express SPARC *de novo* in an experimental model of renal disease. Previous studies by our group have reported that visceral glomerular epithelial cells constitutively express SPARC and that the expression is increased in complement-dependent models of glomerular epithelial cell injury such as passive Heyman nephritis.[24] Thus, SPARC is produced by mesangial cells both *in vitro* and in certain pathologies *in vivo* and is a potent regulator of mesangial cell proliferation *in vitro*. SPARC is also markedly augmented in experimental mesangial proliferative nephritis, and its expression is consistent with its proposed function as an endogenous regulator of mesangial cell proliferation.

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