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SPARC Regulates Cell Cycle Progression in Mesangial Cells via its Inhibition of IGF-Dependent Signaling

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Abstract Glomerular mesangial cells both synthesize and respond to insulin-like growth factor-1 (IGF-1). Increased activity of the IGF signaling pathway has been implicated as a major contributor to renal enlargement and subsequent development of diabetic nephropathy. Secreted protein acidic and rich in cysteine (SPARC), a matricellular protein, has been shown to modulate the interaction of cells with growth factors and extracellular matrix. We have reported that primary glomerular mesangial cells derived from SPARC-null mice exhibit an accelerated rate of proliferation and produce substantially decreased levels of transforming growth factor β1 (TGF-β1) in comparison to their wild-type counterparts (Francki et al. [1999] J. Biol. Chem. 274: 32145–32152). Herein we present evidence that SPARC modulates IGF-dependent signaling in glomerular mesangial cells. SPARC-null mesangial cells produce increased amounts of IGF-1 and -2, as well as IGF-1 receptor (IGF-1R) in comparison to wild-type cells. Addition of recombinant SPARC to SPARC-null cells inhibited IGF-1-stimulated mitogen activated protein kinase (MAPK) activation and DNA synthesis. We also show that the observed accelerated rate of basal and IGF-1-stimulated proliferation in mesangial cells derived from SPARC-null animals is due, at least in part, to markedly diminished levels of cyclin D1 and the cyclin-dependent kinase (cdk) inhibitors p21 and p27. Since expression of SPARC in the glomerulus is especially prominent during renal injury, our findings substantiate previous claims that SPARC is involved in glomerular remodeling and repair, a process commonly associated with mesangioproliferative glomerulonephritis and diabetic nephropathy. J. Cell. Biochem. 88: 802–811, 2003.

Key words: SPARC; matricellular; mesangial cells; IGF-system; proliferation; cell cycle; cyclin D1; p21; p27

Diabetic nephropathy is one of the major complications of diabetes mellitus and is a primary cause of terminal kidney failure. After an initial proliferation of glomerular cells, especially mesangial cells, an early phase of non-proliferative glomerular hypertrophy occurs [Shankland and Wolf, 2000], prior to the development of glomerulosclerosis and tubulointerstitial fibrosis that contribute to end-stage renal disease [Wolf and Ziyadeh, 1999]. These features of diabetic nephropathy occur in patients with diabetes mellitus type I (insulin-dependent), as well as diabetes type II (insulin-independent) [Dalla Vestra et al., 2000].

Proteins that affect mesangial cell proliferation and synthesis of extracellular matrix (ECM) are important targets for the regulation of the glomerular expansion that occurs during early diabetic nephropathy [Wolf and Ziyadeh, 1999]. Secreted protein acidic and rich in cysteine (SPARC, also known as BM-40 and osteonectin), a matricellular protein that inhibits mesangial cell proliferation and increases collagen production, is expressed at high levels in glomerulosclerosis and renal interstitial fibrosis [Pichler et al., 1996a,b]. Modulation of cell-ECM interactions by SPARC has been

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attributed to one or more of its described functions: (i) binding of certain growth factors and ECM proteins [Raines et al., 1992; Sasaki et al., 1998], (ii) prevention of cell spreading, disruption of focal adhesions, and inhibition of the cell cycle in vitro, [Sage et al., 1989; Funk and Sage, 1991; Murphy-Ullrich et al., 1995], and (iii) regulation of the expression of a number of ECM proteins [Lane et al., 1992; Sasaki et al., 1998] and matrix metalloproteinases (MMP) [Tremble et al., 1993]. High levels of SPARC have been reported at sites of wound repair [Reed et al., 1993], and in remodeling tissue [Shankavaram et al., 1997]. Furthermore, SPARC is associated with various renal diseases such as passive Heymann nephritis [Floege et al., 1993], mesangioproliferative glomerulonephritis [Pichler et al., 1996b], and in diabetic nephropathy [Gilbert et al., 1995], with the data indicating that SPARC is involved in glomerular remodeling and repair. However, little is known about the role of SPARC during the progression of diabetes, other than its contextual association with the disease.

Insulin-like growth factor (IGF), a progression factor for mesangial cells, also regulates proliferation [Feld et al., 1995] and ECM production [Schreiber et al., 1995], and has been implicated in the early onset of diabetic glomerulopathy [Bach, 1992]. IGF-1 and -2 are potent mitogens for a variety of cell types [Mathews et al., 1988] and play important roles in development [Baker et al., 1993], tissue regeneration [Hammerman, 1999], and wound healing [Koshizuka et al., 1997]. Both IGF-1 and -2 act through the IGF-1R [LeRoith et al., 1992], whereas the IGF-2 receptor (IGF-2R) seems to be involved in the degradation of IGF-2 and not in IGF-dependent signaling [Ellis et al., 1996]. The activities of the IGFs are controlled by their interactions with a group of soluble proteins, the IGF binding proteins (IGFBPs), which protect IGFs against degradation [Zapf, 1995], facilitate their transport to distinct compartments within the body [Drop et al., 1992], and mediate interactions between IGFs and their cell surface receptors [Frost et al., 1993; Chen et al., 1994]. Interestingly, the kidney is a prominent site for synthesis of proteins comprising the IGF family [Bach, 1992].

The objective of this study was to identify the mechanism whereby glomerular mesangial cells lacking SPARC exhibit accelerated proliferation. We found that mesangial cells derived from SPARC-null mice express increased levels of IGF-1, IGF-2, and IGF-RI, but not other growth factors, in comparison to wild-type cells. Addition of recombinant human (rh) SPARC to wild-type and SPARC-null cells inhibited the expression of IGF-1, IGF-2, and IGF-1R mRNA. Furthermore, addition of rhSPARC to wild-type mesangial cells inhibited IGF-1-stimulated proliferation and mitogen activated protein kinase (MAPK) activation. SPARC-null mesangial cells produced markedly diminished levels of the cell cycle inhibitors p21 and p27; moreover, the suppression of p27 levels by IGF-1 was abrogated by rhSPARC. Our results demonstrate a new function of SPARC in the regulation of proteins of the IGF signaling pathway and cell cycle in mesangial cells in vitro. SPARC is therefore likely to contribute to the progression of renal diseases such as mesangioproliferative glomerulonephritis and/or diabetic nephropathy.

MATERIALS AND METHODS

Reagents

rhSPARC was prepared in SF9 insect cells by the use of the baculovirus protein expression system and was collected in serum-free medium as described [Bradshaw et al., 2000]. The rhSPARC had activity similar to that of rhSPARC expressed in *E. coli*, as measured by inhibition of DNA synthesis [Bassuk et al., 1996]. rhIGF-1 and antibodies against IGF-1 and SPARC were purchased from R&D Systems, Inc. (Minneapolis, MN). Antibodies against the phospho-specific form of MAPK and β-tubulin were purchased from Cell Signaling Technology, Inc. (Beverly, MA) and Sigma (St. Louis, MO), respectively. Antibodies against IGF-1R, insulin receptor, insulin-receptor substrate-1 (IRS-1), cyclin D1, cyclin-dependent kinase (cdk) 4, cdk6, p15, p16, p21, p27, and p57 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were either specific for, or cross-reactive with, the corresponding mouse protein.

Isolation and Characterization of Murine Glomerular Mesangial Cells

Primary mesangial cells from wild-type and SPARC-null mice (129/SvJ × C57BL/6J) were isolated by a partial collagenase digestion of isolated glomeruli and were characterized by

immunocytochemistry as described [Francki et al., 1999]. All experiments were conducted with low-passage (3–9) mesangial cells maintained in growth medium: Dulbecco's Modified Eagle's Medium (55%), F-12 Nutrient Mixture (20%) (Gibco, Grand Island, NY), fetal bovine serum (FBS) (20%) (Gibco), trace elements (1%) (Biofluids, Inc., Rockville, MD), L-glutamine (2 mM), transferrin (5 μg/ml), insulin (125 U/ml), penicillin G, (500 U/ml), streptomycin sulfate (500 U/ml), and amphotericin B (2 μg/ml) (Sigma).

Measurement of DNA Synthesis

[^{3}H]-Thymidine incorporation was measured as previously described for endothelial cells [Motamed and Sage, 1998]. Wild-type and SPARC-null mesangial cells in growth medium were plated at equal subconfluent densities (5–10×10^4 cells/well) in 24-well plates and were allowed to adhere overnight. Plating efficiency was determined by an initial cell count 2 h after plating. The cells were synchronized in resting medium (growth medium without serum and insulin) for 48 h, and were stimulated with IGF-1 (20 ng/ml) for 18 h in the presence or absence of rhSPARC (0.9 μM). The cells were subsequently pulse-labeled with 2 μCi/ml [^{3}H]-thymidine (6.7 Ci/mmol; Amersham, Arlington Heights, IL) for 4 h, and were finally fixed in ice-cold 10% trichloroacetic acid (TCA) (Sigma). TCA-insoluble material was solubilized in 0.2 N NaOH and incorporated cpm were measured in a liquid scintillation counter.

Preparation of RNA and Analytical Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were cultured under resting conditions (24–48 h after serum removal), prior to addition of reagents. Total cellular RNA was prepared from mesangial cells by a modified method [Francki et al., 1999] that includes the commercial TRI-reagent™ (Molecular Research Center, Cincinnati, OH). RT-PCRs were performed with the Access RT-PCR System™ (Promega, Madison, WI) with oligonucleotide primers complementary to the murine cDNAs for ribosomal protein (rp)S6, SPARC, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), TGF-β1, IGF-1, IGF-2, IGF-1R, and the insulin receptor. The primer pairs were tested for "cross-annealing" with the Amplify 1.2 program©, such that up to three primer pairs could be used together in one PCR reaction [Francki et al., 1999]. For an internal standard, we amplified rpS6 mRNA, an ubiquitously-expressed gene. Values obtained from scanning densitometry of the cDNA bands generated from the respective mRNAs were normalized to the corresponding rpS6.

Protein Extraction and Western Blot Analysis

Primary mesangial cells from wild-type and SPARC-null mice were grown to 80% confluence. The insoluble (ECM proteins and membranes) and soluble cellular protein fractions were prepared by dissolution of the cells in NP-40 lysis buffer: 50 mM Tris-HCl, pH 7.5; 0.5% NP-40; 150 mM NaCl; 1 mM ethylene diamine tetraacetic acid (EDTA); 1 mM NaF; 0.5 mM sodium orthovanadate (Sigma), 10% glycerol; complete protease-inhibitor cocktail™ (Boehringer Mannheim, Indianapolis, IN). Protein concentrations were determined by the BCA assay (Pierce Biotechnology, Inc., Rockford IL), according to the manufacturer's recommended protocol. Equal amounts of protein (30–50 μg) were boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer [Laemmli, 1970] under reducing conditions for 5 min, centrifuged for 2 min at 10,000*g*, resolved by SDS–PAGE, and electrotransferred onto nitrocellulose membranes (BioRad). The blots were subsequently blocked in 1% Tween-20 in PBS and were incubated with the primary antibodies. Immunoreactivity was visualized by incubation of the blot with the respective secondary IgG coupled to horseradish peroxidase, followed by enhanced chemiluminescence. An antibody against β-tubulin was used to control for equal protein loading.

For MAPK activation and cell cycle studies, mesangial cells were cultured in growth medium, prior to deprivation of serum and insulin for 48 h, and were subsequently incubated with IGF-1 (20 ng/ml) alone or with IGF-1 and SPARC (0.9 μM) for 5–30 min (MAPK activation), or for 24 h (cell cycle proteins). Cellular protein lysates were prepared as described above, and equal amounts of protein were subjected to immunoblot analyses with antibodies against phospho-specific MAPK, as well as the cell cycle proteins cyclin D1, p21, p27, and p57.

Biotinylation of rhSPARC and Binding Studies for IGF-1 and SPARC

Biotinamidocaproate NHS-ester (Sigma) was mixed with rhSPARC at a 20:1 molar ratio in Hank's Balanced Salt Solution (HBSS) (Gibco), and the solution was rotated in the dark for 2 h at room temperature. The reaction was stopped by addition of Tris-HCl/glycine (pH 7.6) to a concentration of 10 mM. Biotinylated rhSPARC was purified on a PD-10 column (Sephadex G-25) (Amersham).

rhIGF-1 and biotinylated rhSPARC were mixed at 1:1 and 3:1 molar ratios in binding buffer (HBSS containing 0.1% Tween-20 (Sigma) and 0.5% Tryptone (Sigma)) and were rotated for 4 h at 4°C. Competition experiments with unlabeled rhSPARC used at a 100-fold molar excess were performed to ascertain specific binding. Subsequently, 50 µl immobilized UltraLink NeutraAvidin Beads (Pierce) were added and the reactions were rotated for an additional 4 h at 4°C. The reactions were centrifuged for 5 min at 10,000*g* and the pellets were washed four times with washing buffer (binding buffer containing 20% NP-40 lysis buffer). The pellets were boiled in SDS–PAGE sample buffer [Laemmli, 1970] for 5 min and were centrifuged for 5 min at 10,000*g*. The immunoprecipitates were resolved by SDS–PAGE under reducing conditions and were electrotransferred onto nitrocellulose membranes. The blots were subsequently blocked (PBS containing 1% Tween-20) and were incubated with antibodies against IGF-1 and rhSPARC. Immunoreactivity was visualized by incubation of the blot with the appropriate secondary IgG coupled to horseradish peroxidase, followed by enhanced chemiluminescence. An antibody against β-tubulin was used to control for equal protein loading.

Statistical Analysis

Autoradiograms, immunoblots, and agarose gels were photographed and converted to digital computer files with a flatbed scanner and Adobe Photoshop™ software. Files were analyzed quantitatively with NIH Image™ [Francki et al., 1999]. The data presented in Figures 1–6 were derived from five independent preparations of mesangial cells isolated from pools of eight kidneys each. Data are reported as means ± SEM.

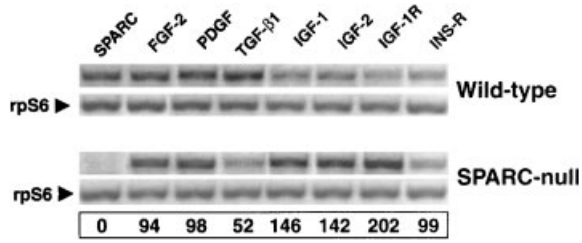

Fig. 1. Altered levels of IGF-1, IGF-2, IGF-1R, and TGF-β1 mRNAs in SPARC-null mesangial cells. Total RNA extracted from wild-type and SPARC-null cells was reverse-transcribed and was amplified in the presence of specific primers for the mRNAs shown, as well as for rpS6 (loading control). The SPARC-null cells showed increases in the levels of IGF-1 (146%), IGF-2 (142%), and IGF-1R (202%) mRNA, and a decrease in the level of TGF-β1 (52%) mRNA, in comparison to the corresponding levels in wild-type cells. The steady-state levels of FGF-2, PDGF, and insulin receptor (INS-R) mRNA were similar between wild-type and SPARC-null cells. Numbers under each lane denote percent change in levels of respective mRNAs, relative to wild-type control (set at 100%). Results shown are from one experiment that was representative of 8 independent experiments. Variability among experiments was less than 11% for each mRNA.

RESULTS

SPARC Influences the Expression of IGF-1 and IGF-2

A potential mechanism by which SPARC controls proliferation, cell shape, and ECM production is the regulation of the activity of pleiotropic growth factors. In a previous study, we demonstrated that SPARC-null mesangial cells exhibited significantly decreased steady-state levels of TGF-β1 mRNA and secreted protein [Francki et al., 1999]. In Figure 1 we

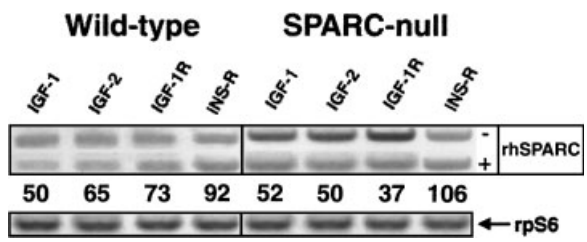

Fig. 2. rhSPARC decreases the steady-state levels of IGF-1, IGF-2, and IGF-1R mRNA. Total RNA extracted from wild-type and SPARC-null cells (cultured ± rhSPARC) was reverse-transcribed and amplified in the presence of the primers indicated. Incubation with rhSPARC (0.9 µM) for 6 h reduced the levels of IGF-1, IGF-2, and IGF-1R mRNA in SPARC-null cells and wild-type cells, but had virtually no effect on the levels of the insulin receptor (INS-R) mRNA. Numbers under each lane denote percent change in the levels of respective mRNAs, relative to untreated (-rhSPARC) cells (set at 100%). Results shown are from one experiment that was representative of 8 independent experiments. Variability among experiments was less than 8% for each mRNA.

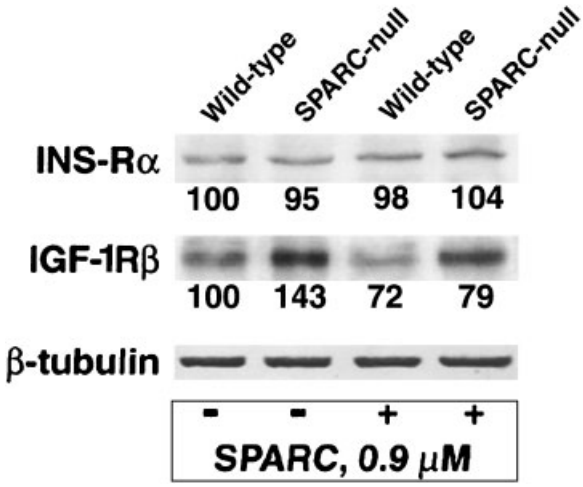

Fig. 3. Elevated levels of IGF-1R protein in SPARC-null mesangial cells. Analysis of cellular protein derived from wild-type and SPARC-null cells by immunoblotting with antibodies specific for the β-subunit of the IGF-1R (IGF-1Rβ) and the α-subunit of the insulin receptor (INS-Rα). The amount of IGF-1Rβ protein in SPARC-null cells was increased by 43% in comparison to that observed in wild-type cells. Incubation with rhSPARC (0.9 μM) for 24 h decreased the levels of IGF-1Rβ protein, but had no effect on the levels of INS-Rα protein. An antibody against β-tubulin was used as an internal control. Numbers under each lane denote percent change in levels of respective proteins, relative to wild-type control (set at 100%). Results shown are from one experiment that was representative of 5 independent experiments. Variability among experiments was less than 10% for each protein.

show that SPARC-null mesangial cells displayed elevated steady-state levels of IGF-1 mRNA (46%) and IGF-2 mRNA (42%), relative to wild-type cells. The levels of bFGF and PDGF mRNAs remained essentially the same in wild-type and SPARC-null cells, whereas, consistent with our previous report [Francki et al., 1999], the levels of TGF-β1 mRNA were diminished (by 48%) in SPARC-null mesangial cells. The addition of rhSPARC to SPARC-null and wild-type cells diminished the levels of IGF-1 mRNA (by 50% and 48%, respectively), but did not alter those of bFGF and PDGF (Fig. 2). There was also a reduction in steady-state levels of IGF-2 mRNA induced by rhSPARC (35% and 50% in wild-type and SPARC-null cells, respectively) (Fig. 2).

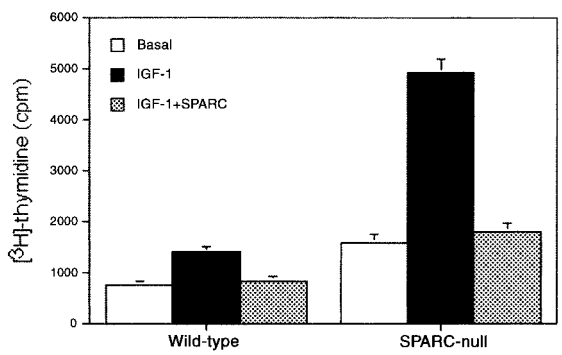

Fig. 4. SPARC inhibits IGF-1-induced DNA synthesis. Wild-type and SPARC-null mesangial cells were synchronized under serum-free conditions for 48 h, prior to stimulation with IGF-1 (20 ng/ml) or resting medium (basal) for 18 h in the presence or absence of rhSPARC (0.9 μM). Cells were subsequently pulsed with [^{3}H]-thymidine for 4h, and incorporation of radiolabel was measured in a liquid scintillation counter. Results shown are from one experiment that was representative of 3 separate experiments. Variability among experiments was less than 12%.

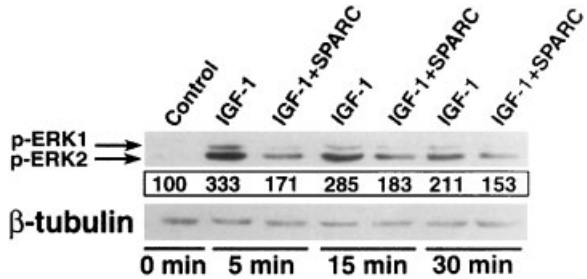

Fig. 5. SPARC suppresses IGF-1-stimulated MAPK activation. Wild-type mesangial cells were synchronized under serum-free conditions for 48 h and were pretreated with PBS (control) or rhSPARC (0.9 μM) for 2 h prior to stimulation with IGF-1 (20 ng/ml) or resting medium (control) for 5 – 30 min. Cell extracts were immunoblotted with a phospho-specific MAPK antibody, and with an antibody against β-tubulin as an internal control. Numbers under each lane denote percent change in levels of respective proteins, relative to untreated cells (set at 100%). Results shown are from one experiment that was representative of 3 separate experiments. Variability among experiments was less than 9% for each protein.

SPARC Influences the Expression of IGF-1R

To determine whether the expression of downstream effectors associated with the IGF pathway is also dependent on SPARC, we examined the mRNA and protein levels of IGF-RI and the insulin receptor in SPARC-null and wild-type mesangial cells. The SPARC-null cells produced increased amounts (~100% relative to wild-type values) of IGF-1R mRNA, whereas the levels of insulin receptor mRNA were equivalent (Fig. 1). Similarly, levels of IGF-1R protein were enhanced in SPARC-null cells (by 43% relative to wild-type cells), whereas the levels of insulin receptor protein (Fig. 3) as well as the levels of IRS-1 protein (data not shown) were unchanged. The mRNA levels (Fig. 1) and the protein levels (Fig. 3) of IGF-1R in SPARC-null cells were reverted nearly to wild-type values by the addition of rhSPARC, whereas the levels of insulin receptor remained

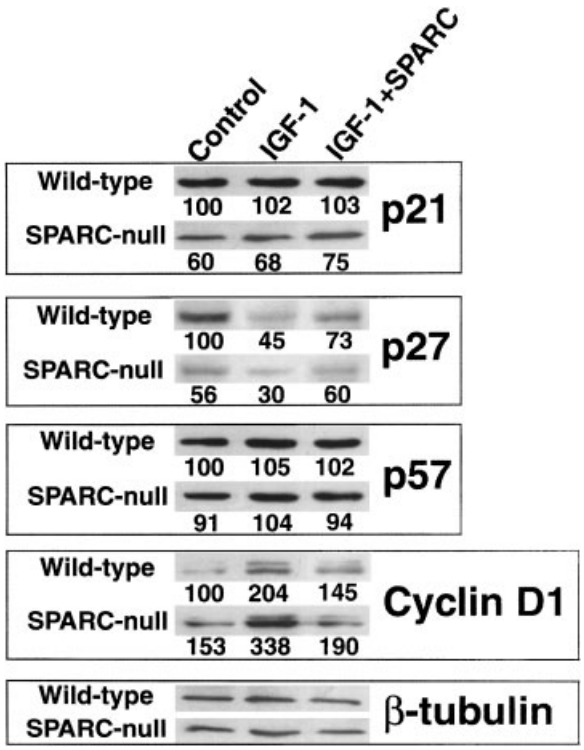

Fig. 6. SPARC inhibits IGF-1-stimulated mesangial cell cycle progression through regulation of the levels of cyclin D1 and the cdk inhibitor p27. Wild-type and SPARC-null mesangial cells were synchronized under serum-free conditions for 48 h, prior to stimulation with IGF-1 (20 ng/ml) or resting medium (control) for 24 h in the absence (control) or presence of rhSPARC (0.9 μM). Equal amounts of cell lysates were separated by SDS–PAGE and were analyzed for levels of cyclin D1, p21, p27, and p57 by immunoblotting. An antibody against β-tubulin was used as an internal control for equal loading. Numbers under each lane denote percent change in levels of respective proteins, relative to wild-type control (set at 100%). Results shown are from one experiment that was representative of 4 independent experiments. Variability among experiments was less than 8% for each protein.

unchanged (Figs. 1 and 3). Addition of rhSPARC to wild-type cells diminished the levels of IGF-1R mRNA and protein by approximately 20%, whereas the levels of insulin receptor were not changed (Figs. 1 and 3). These data suggest that SPARC specifically modulates the levels of proteins of the IGF-system, but not proteins of the insulin-dependent pathway, in mesangial cells.

SPARC Modulates IGF-1-Induced Proliferation

We have established previously that mesangial cells derived from SPARC-null animals proliferate at a higher rate than their wild-type counterparts, and that this property was due, at least in part, to the diminished expression of TGF-β1 [Francki et al., 1999]. It also has been shown that IGF-1 stimulates proliferation in mesangial cells [Feld et al., 1995]. We were therefore interested in the effect of SPARC on IGF-1-regulated proliferation in mesangial cells. As shown in Figure 4, stimulation of serum-deprived, wild-type mesangial cells by IGF-1 resulted maximally in a 350% increase in DNA synthesis, and the addition of rhSPARC inhibited this increase by more than 75%. This result indicates that SPARC diminishes the mitogenic effect IGF-1 in mesangial cells.

SPARC Inhibits IGF-1-Induced MAPK Activation

IGF-1 has been shown to induce activation of MAPK and cell proliferation in various types of cells [Ge and Rudikoff, 2000; Kurihara et al., 2000; Boney et al., 2001; Xu et al., 2001]. SPARC is known to inhibit vascular endothelial growth factor (VEGF)-stimulated MAPK activation in human endothelial cells [Kupprion et al., 1998]. Given that SPARC modulates the activity of several growth factors, we asked whether SPARC could affect the activation of MAPK stimulated by IGF-1 in mesangial cells. A brief (2 h) pretreatment with SPARC diminished IGF-1-stimulated MAPK activation in wild-type mesangial cells by $>300\%$ after 5 min (Fig. 5). These data indicate that IGF-1 activates MAPK in mesangial cells and that this activation can be suppressed significantly by SPARC.

SPARC Modulates IGF-1-Dependent Cell Cycle Progression

In a previous study, we showed that mesangial cells from SPARC-null animals exhibited an accelerated rate of serum-stimulated DNA synthesis, relative to their wild-type counterparts [Bradshaw et al., 1999]. This augmented rate of DNA synthesis appeared to be mediated by an increase in the levels of the cell cycle regulatory protein cyclin A [Bradshaw et al., 1999]. To elucidate the inhibitory effect of SPARC on mesangial cell cycle progression, we compared levels of various G1-phase cyclins and cdk inhibitors (CKI) in serum-deprived cells from wild-type and SPARC-null mice, after stimulation with IGF-1. Levels of cyclin D1, an early G1 cyclin, were higher in serum-starved (53%) and IGF-1-stimulated (238%) SPARC-null mesangial cells, relative to wild-type controls (Fig. 6). Moreover, diminished steady-state levels of the G1 phase CKIs p21 and p27

(40% and 44%, respectively), but not p57, were detected in serum-starved SPARC-null cells (Fig. 6). Interestingly, stimulation with IGF-1 resulted in a significant diminution of p27 levels in wild-type (55%) and SPARC-null cells (54%), but not p21 or p57, which was rescued almost completely by addition of rhSPARC (Fig. 6). A significant difference in steady-state or IGF-1-induced levels of other G1-specific cyclin-dependent kinases (cdk4 and cdk6) or CKIs (p15 and p16) was not detected in SPARC-null and wild-type cells (data not shown). These data indicate that SPARC modulates IGF-1-driven cell cycle progression in mesangial cells, at least in part, via regulation of p27 levels.

SPARC Does not Bind Directly to IGF-1

Because it has been shown that SPARC suppresses the proliferative activity of PDGF and VEGF by direct binding to these growth factors [Raines et al., 1992; Kupprion et al., 1998], we performed binding studies with biotinylated rhSPARC and rhIGF-1 at various molar ratios to determine whether SPARC interacts directly with IGF-1. We were unable to show any specific binding between IGF-1 and SPARC under a variety of assay conditions, as described in the experimental procedures (data not shown). This result is suggestive that SPARC modulates the activity of IGF-1 through a novel pathway different from that described for other growth factors.

DISCUSSION

Mesangial cells are specialized, smooth muscle-like cells in the kidney glomerulus that maintain glomerular integrity, control filtration rate, and clear immune complexes. These functions are accomplished in part by the regulation of the production of growth factors and ECM proteins [Floege et al., 1991; Poncelet and Schnaper, 1998]. Furthermore, mesangial cells are associated with the remodeling that occurs after kidney injury or during diabetes. Typically, the mesangial cell response to injury is hyperproliferation that is coincident with an elevated production of various growth factors and ECM constituents [Francki and Sage, 2001].

Major factors during the glomerular response to injury are the proteins of the IGF system, which regulate mesangial proliferation as well as the expression of ECM proteins and glucose levels in the plasma, all of which have been implicated in glomerular enlargement during diabetic nephropathy [Lehmann and Schleicher, 2000]. For example, IGF-1 regulates its own level [LeRoith et al., 1992] and that of its receptors [Hernandez-Sanchez et al., 1997] in an autocrine fashion, and modulates the expression of ECM proteins such as collagen type I [Feld et al., 1995]. We have reported previously that primary glomerular mesangial cells isolated from SPARC-null animals show higher rates of proliferation and substantially decreased levels of collagen type I and TGF-β1, and that SPARC regulates the expression of collagen type I and TGF-β1 in these cells via a TGF-β1-dependent pathway [Francki et al., 1999]. SPARC has been shown to inhibit the proliferation of cultured mesenchymal cells stimulated with PDGF, VEGF, bFGF, and serum [Raines et al., 1992; for review see Brekken and Sage, 2000]. However, essentially nothing is known about the potential regulation of either the levels and/or the activities of the IGF family of proteins by SPARC.

Herein, we report an enhanced activity of the IGF-1 system in SPARC-null mesangial cells. Comparison of the steady-state mRNA levels of pertinent growth factors and cognate receptors revealed that, relative to wild-type controls, SPARC-null mesangial cells expressed significantly higher levels of IGF-1, IGF-2, and IGF-1R, similar levels of insulin receptor, PDGF, and FGF-2, and lower levels of TGF-β1 (Fig. 1). Consistent with these findings, addition of rhSPARC to wild-type and SPARC-null mesangial cells resulted in a significant suppression of the levels of IGF-1, IGF-2, and IGF-1R mRNA, but not that of the insulin receptor (Fig. 2). The inhibitory effect of rhSPARC on IGF-1R mRNA, but not insulin receptor mRNA, was also observed at the protein level (Fig. 3).

It has been shown that the mitogenic action of IGF-1 is mediated through activation of the MAPK pathway [Ge and Rudikoff, 2000; Kurihara et al., 2000; Boney et al., 2001; Xu et al., 2001]. A brief (1–2 h) exposure of cells to SPARC, prior to the addition of VEGF and bFGF, has been shown to be required for maximum diminution of ligand-mediated MAPK activity [Kupprion et al., 1998; Motamed et al., 1999]. We have previously reported that the direct molecular interaction between SPARC and VEGF can account for approx. 50% inhibition of the ligand-induced MAPK activity and proliferation in endothelial cells [Kupprion

et al., 1998]. SPARC has also been shown to inhibit bFGF-stimulated proliferation of endothelial cells, in the absence of a direct molecular interaction with bFGF [Hasselaar and Sage, 1992; Motamed et al., 1999]. Our results indicate that similar pretreatment with SPARC is also required for inhibition of the IGF-1-mediated MAPK activity in mouse mesangial cells (Fig. 4). It is therefore conceivable that the inhibition of ligand-induced MAPK activity following pretreatment of cells with SPARC could be mediated by the regulation of effector molecules downstream from ligand-induced receptor phosphorylation. In support of this idea, SPARC has been found associated with the nuclear matrix [Gooden et al., 1999], an observation suggesting that it might also modulate intracellular and nuclear processes. Consistent with the inhibitory effect of SPARC on IGF-1-stimulated MAPK activity, we showed that SPARC-null mesangial cells had a higher rate of basal (>85%) and IGF-1-stimulated (>350%) DNA synthesis, relative to wild-type controls (Fig. 4), and addition of rhSPARC to both cell types inhibited IGF-1-stimulated DNA synthesis almost completely (Fig. 4).

Expression of cyclin A and activation of its associated kinase (cyclin-dependent kinase (cdk)-2) in late G1 has been implicated as the rate-limiting step for entrance into S phase [Resnitzky et al., 1995]. Moreover, inhibition of monomeric collagen-mediated proliferation of human smooth muscle cells by SPARC has recently been shown to be the result of a significant decrease in the levels of cyclin A and in the activity of its associated kinase cdk2 [Motamed et al., 2002]. To elucidate how SPARC transduces its antiproliferative effect on glomerular mesangial cells, we investigated whether SPARC modulates the expression of regulators of G1 to S. Cell cycle progression through the G1 restriction point requires appropriate association of cyclins (D, E, and A) with specific cdks (4/6 and 2) to form active enzymatic complexes that are capable of phosphorylating ligands such as retinoblastoma protein, a known regulator of transcription factor action and G_1/S cell cycle traverse [for review see Morgan, 1995; Kaelin, 1999; Ekholm and Reed, 2000]. Inhibitors of cdk activity [for review see Peter and Herskovitz, 1994; Sherr and Roberts, 1995] provide a counter balance in the regulation of cell cycle progression and are believed to be critical determinants for the onset and magnitude of renal cell proliferation [Shankland and Wolf, 2000]. Stimulation of the proliferation of cultured mesangial cells by different mitogens has been shown to be associated with changes in the levels or activity of specific cyclins, cdks, and cdk inhibitors [Shankland et al., 1997; Lang et al., 2000]. Expression of cyclin D1 in early to mid-G1 appears to be required for mesangial cell proliferation in vitro and in vivo [Lang et al., 2000], and stimulation of growth-arrested rat mesangial cells in culture with serum or PDGF has been shown to result in rapid increases in the expression of cyclin D1 protein [Lang et al., 2000]. Our results show that IGF-1 efficiently stimulates cyclin D1 protein expression in mouse mesangial cells and that SPARC abrogates this function almost completely (Fig. 6). We also observed substantially lower steady-state levels of p21 and p27, but not p57, in SPARC-null mesangial cells in comparison to wild-type cells. Furthermore, a 24 h stimulation of SPARC-null and wild-type mesangial cells with IGF-1 resulted in a significant decrease in the levels of p27, but did not affect the levels of p21 or p57 (Fig. 6). Importantly, the addition of exogenous SPARC inhibited this effect of IGF-1 by augmentation of p27 levels to those of unstimulated controls (Fig. 6). These results are consistent with reports showing that the onset of mesangial cell proliferation in vitro and in vivo is correlated with a diminution in levels of p27 [Shankland et al., 1996].

In conclusion, our data indicate that SPARC controls the proliferation of mesangial cells by modulating the levels and/or activity of IGF-1R and its ligands. The results also indicate that SPARC influences the levels and the activity of certain cell cycle proteins that are critical for the early onset of the glomerular hypertrophy and subsequent enlargement observed during diabetic nephropathy. Since increased mitosis and matrix deposition by mesangial cells are characteristics of glomerulopathies and diabetic nephropathy, we propose that SPARC is one of the factors that maintain the balance between cell proliferation and matrix production in the glomerulus.

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REFERENCES

Bach LA. 1992. IGF-I and IGF binding proteins in diabetes-related kidney growth. Growth Regul 2:30–39.

Baker J, Liu JP, Robertson EJ, Efstratiadis A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73–82.

Bassuk JA, Baneyx F, Vernon RB, Funk SE, Sage EH. 1996. Expression of biologically active human SPARC in Escherichia coli. Arch Biochem Biophys 325:8–19.

Boney CM, Sekimoto H, Gruppuso PA, Frackelton AR. 2001. Src family tyrosine kinases participate in insulin-like growth factor I mitogenic signaling in 3T3-L1 cells. Cell Growth Differ 12:379–386.

Bradshaw AD, Francki A, Motamed K, Howe CC, Sage EH. 1999. Primary mesenchymal cells isolated from SPARC-null mice exhibit altered morphology and rates of proliferation. Mol Biol Cell 10:1569–1579.

Bradshaw AD, Bassuk J, Francki A, Sage EH. 2000. Expression and purification of ecombinant human SPARC produced by baculovirus. Mol Cell Biol Res Comm 3:345–351.

Brekken RA, Sage EH. 2000. SPARC, a matricellular protein: At the crossroads of cell-matrix communication. Matrix Biol 19:816–827.

Chen JC, Shao ZM, Sheikh MS, Hussain A, LeRoith D, Roberts CT, Fontana JA. 1994. Insulin-like growth factor-binding protein enhancement of insulin-like growth factor-I (IGF-I)-mediated DNA synthesis and IGF-I binding in a human breast carcinoma cell line. J Cell Physiol 158:69–78.

Dalla Vestra M, Saller A, Bortoloso E, Mauer M, Fioretto P. 2000. Structural involvement in type 1 and type 2 diabetic nephropathy. Diabetes Metab 26(Suppl 4):8–14.

Drop SL, Schuller AG, Lindenbergh-Kortleve DJ, Groffen C, Brinkman A, Zwarthoff EC. 1992. Structural aspects of the IGFBP family. Growth Regul 2:69–79.

Ekholm SV, Reed SI. 2000. Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr Opin Cell Biol 6:676–684.

Ellis MJ, Leav BA, Yang Z, Rasmussen A, Pearce A, Zweibel JA, Lippman ME, Cullen KJ. 1996. Affinity for the insulin-like growth factor-II (IGF-II) receptor inhibits autocrine IGF-II activity in MCF-7 breast cancer cells. Mol Endocrinol 10:286–297.

Feld SM, Hirschberg R, Artishevsky A, Nast C, Adler SG. 1995. Insulin-like growth factor 1 induces mesangial proliferation and increases mRNA and secretion of collagen. Kidney Int 48:45–51.

Floege J, Topley N, Resch K. 1991. Regulation of mesangial cell proliferation. Am J Kidney Dis 17:673–676.

Floege J, Johnson RJ, Alpers CE, Fatemi-Nainie S, Richardson CA, Gordon K, Couser WG. 1993. Visceral glomerular epithelial cells can proliferate in vivo and synthesize platelet-derived growth factor B-chain. Am J Pathol 142:637–650.

Francki A, Sage EH. 2001. SPARC and the kidney glomerulus: Matricellular proteins exhibit diverse functions under normal and pathological conditions. Trends Cardiovasc Med 11:32–37.

Francki A, Bradshaw AD, Bassuk JA, Howe CC, Couser WG, Sage EH. 1999. SPARC regulates the expression of collagen type I and transforming growth factor-1 in mesangial cells. J Biol Chem 274:32145–32152.

Frost RA, Mazella J, Tseng L. 1993. Insulin-like growth factor binding protein-1 inhibits the mitogenic effect of insulin-like growth factors and progestins in human endometrial stromal cells. Biol Reprod 49:104–111.

Funk SE, Sage EH. 1991. The Ca^{2+}-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. Proc Natl Acad Sci USA 88:2648–2652.

Ge NL, Rudikoff S. 2000. Insulin-like growth factor I is a dual effector of multiple myeloma cell growth. Blood 96: 2856–2861.

Gilbert RE, McNally PG, Cox A, Dziadek M, Rumble J, Cooper ME, Jerums G. 1995. SPARC gene expression is reduced in early diabetes-related kidney growth. Kidney Int 48:1216–1225.

Gooden MD, Vernon RB, Bassuk JA, Sage EH. 1999. Cell cycle-dependent nuclear location of the matricellular protein SPARC: Association with the nuclear matrix. J Cell Biochem 74:152–167.

Hammerman MR. 1999. The growth hormone-insulin-like growth factor axis in kidney re-revisited. Nephrol Dial Transplant 14:1853–1860.

Hasselaar P, Sage EH. 1992. SPARC antagonizes the effect of basic fibroblast growth factor on the migration of bovine aortic endothelial cells. J Cell Biochem 49:272–283.

Hernandez-Sanchez C, Werner H, Roberts CT, Woo EJ, Hum DW, Rosenthal SM, LeRoith D. 1997. Differential regulation of insulin-like growth factor-I (IGF-1) receptor gene expression by IGF-1 and basic fibroblastic growth factor. J Biol Chem 272:4663–4670.

Kaelin WG, Jr. 1999. Functions of the retinoblastoma protein. BioEssays 21:950–958.

Koshizuka S, Kanazawa K, Kobayashi N, Takazawa I, Waki Y, Shibusawa H, Shumiya S. 1997. The beneficial effects of recombinant human insulin-like growth factor-I (IGF-I) on wound healing in severely wounded senescent mice. Surg Today 27:946–952.

Kupprion C, Motamed K, Sage EH. 1998. SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. J Biol Chem 273:29635–29640.

Kurihara S, Hakuno F, Takahashi S. 2000. Insulin-like growth factor-1-dependent signal transduction pathways leading to the induction of cell growth and differentiation of human neuroblastoma cell line SH-SY5Y: The roles of MAPK kinase pathway and PI 3-kinase pathway. Endocr J 47:739–751.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lane TF, Iruela-Arispe ML, Sage EH. 1992. Regulation of gene expression by SPARC during angiogenesis in vitro. Changes in fibronectin, thrombospondin-1, and plasminogen activator inhibitor-1. J Biol Chem 267:16736–16745.

Lang S, Hartner A, Sterzel RB, Schocklmann HO. 2000. Requirement of cyclin D1 in mesangial cell mitogenesis. J Am Soc Nephrol 11:1398–1408.

Lehmann R, Schleicher ED. 2000. Molecular mechanism of diabetic nephropathy. Clin Chim Acta 297:135–144.

LeRoith D, McGuinness M, Shemer J, Stannard B, Lanau F, Faria TN, Kato H, Werner H, Adam M, Roberts CT. 1992. Insulin-like growth factors. Biol Signals 4:173–181.

Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL, Palmiter RD. 1988. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 123:2827–2833.

Morgan DO. 1995. Principles of CDK regulation. Nature 374:131–134.

Motamed K, Sage EH. 1998. SPARC inhibits endothelial cell adhesion but not proliferation through a tyrosine phosphorylation-dependent pathway. J Cell Biochem 70:543–552.

Motamed K, Angello J, Hauschka S, Sage EH. 1999. SPARC inhibits the mitogenic activity of basic fibroblast growth factor on microvascular endothelial cells by blocking the phosphorylation of FGFR-1. FASEB J 13(5):A704 (Abstract).

Motamed K, Funk SE, Koyama H, Ross R, Raines ER, Sage EH. 2002. Inhibition of PDGF-stimulated and matrix-mediated proliferation of human vascular smooth muscle cells by SPARC is independent of changes in cell shape or cyclin-dependent kinase inhibitors. J Cell Biochem 84: 759–771.

Murphy-Ullrich JE, Lane TF, Pallero MA, Sage EH. 1995. SPARC mediates focal adhesion disassembly in endothelial cells through a follistatin-like region and the Ca^{2+}-binding EF-hand. J Cell Biochem 57:341–350.

Peter M, Herskovitz I. 1994. Joining the complex: Cyclin-dependent kinase inhibitory proteins and the cell cycle. Cell 79:181–184.

Pichler RH, Hugo C, Shankland SJ, Reed MJ, Bassuk JA, Andoh TF, Lombardi DM, Schwartz SM, Bennett WM, Alpers CE, Sage EH, Johnson RJ, Couser WG. 1996a. SPARC is expressed in renal interstitial fibrosis and in renal vascular injury. Kidney Int 50:1978–1989.

Pichler RH, Bassuk JA, Hugo C, Reed MJ, Eng E, Gordon KL, Pippin J, Alpers CE, Couser WG, Sage EH, Johnson RJ. 1996b. SPARC is expressed by mesangial cells in experimental mesangial proliferative nephritis and inhibits platelet-derived-growth-factor-mediated mesangial cell proliferation in vitro. Am J Pathol 148:1153–1167.

Poncelet AC, Schnaper HW. 1998. Regulation of human mesangial cell collagen expression by transforming growth factor-beta1. Am J Physiol 275:458–466.

Raines EW, Lane TF, Iruela-Arispe ML, Ross R, Sage EH. 1992. The extracellular glycoprotein SPARC interacts with platelet-derived growth factor (PDGF)-AB and -BB and inhibits the binding of PDGF to its receptors. Proc Natl Acad Sci USA 89:1281–1285.

Reed MJ, Puolakkainen P, Lane TF, Dickerson D, Bornstein P, Sage EH. 1993. Differential expression of SPARC and thrombospondin 1 in wound repair: Immunolocalization and in situ hybridization. J Histochem Cytochem 41:1467–1477.

Resnitzky D, Hengst L, Reed SI. 1995. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27Kip1. Mol Cell Biol 15:4347–4352.

Sage EH, Vernon RB, Funk SE, Everitt EA, Angello J. 1989. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits Ca^{+2}-dependent binding to the extracellular matrix. J Cell Biol 109:341–356.

Sasaki T, Hohenester E, Göhring W, Timpl R. 1998. Crystal structure and mapping by site-directed mutagenesis of the collagen-binding epitope of an activated form of BM-40/SPARC/osteonectin. EMBO J 17:1625–1634.

Schreiber BD, Hughes ML, Groggel GC. 1995. Insulin-like growth factor-1 stimulates production of mesangial cell matrix components. Clin Nephrol 43:368–374.

Shankavaram UT, DeWitt DL, Funk SE, Sage EH, Wahl LM. 1997. Regulation of human monocyte matrix metalloproteinases by SPARC. J Cell Physiol 173:327–334.

Shankland SJ, Wolf G. 2000. Cell cycle regulatory proteins in renal disease: Role in hypertrophy, proliferation, and apoptosis. Am J Physiol Renal Physiol 278: F515–529.

Shankland SJ, Hugo C, Coats SR, Nangaku M, Pichler RH, Gordon KL, Pippin J, Roberts JM, Couser WG, Johnson RJ. 1996. Changes in cell-cycle protein expression during experimental mesangial proliferative glomerulonephritis. Kidney Int 50:1230–1239.

Shankland SJ, Pippin J, Flanagan M, Coats SR, Nangaku M, Gordon KL, Roberts JM, Couser WG, Johnson RJ. 1997. Mesangial cell proliferation mediated by PDGF and bFGF is determined by levels of the cyclin kinase inhibitor p27Kip1. Kidney Int 51:1088–1099.

Sherr CJ, Roberts JM. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149–1163.

Tremble PM, Lane TF, Sage EH, Werb Z. 1993. SPARC, a secreted protein associated with morphogenesis and tissue remodeling, induces expression of metalloproteinases in fibroblasts through a novel extracellular matrix-dependent pathway. J Cell Biol 121:1433–1444.

Wolf G, Ziyadeh FN. 1999. Molecular mechanisms of diabetic renal hypertrophy. Kidney Int 56:393–405.

Xu A, Suh PG, Marmy-Conus M, Pearson RB, Seok OY, Cocco L, Gilmour RS. 2001. Phosphorylation of nuclear phospholipase C beta1 by extracellular signal-regulated kinase mediates the mitogenic action of insulin-like growth factor 1. Mol Cell Biol 21:2981–2990.

Zapf J. 1995. Physiological role of the insulin-like growth factor binding proteins. Eur J Endocrinol 132: 645–654.