SPARC Regulates the Expression of Collagen Type I and Transforming Growth Factor-β1 in Mesangial Cells*

(Received for publication, March 22, 1999, and in revised form, July 20, 1999)

Aleksandar Francki‡, Amy D. Bradshaw‡‡, James A. Bassuk¶, Chin C. Howe¶, William G. Couter**, and E. Helene Sage‡‡‡‡

From the ‡Department of Biological Structure, University of Washington, Seattle, Washington 98195-7420, the †Department of Urology, University of Washington, Seattle, Washington 98195, the ¶Division of Nephrology, University of Washington, Seattle, Washington 98195

The matricellular protein SPARC is expressed at high levels in cells that participate in tissue remodeling and is thought to regulate mesangial cell proliferation and extracellular matrix production in the kidney glomerulus in a rat model of glomerulonephritis (Pichler, R. H., Bassuk, J. A., Hugo, C., Reed, M. J., Eng, E., Gordon, K. L., Pippin, J., Alpers, C. E., Couser, W. G., Sage, E. H., and Johnson, R. J. (1997) Am. J. Pathol. 148, 1153–1167). A potential mechanism by which SPARC controls both cell cycle and matrix production has been attributed to its regulation of a pleiotropic growth factor. In this study we used primary mesangial cell cultures from wild-type mice and from mice with a targeted disruption of the SPARC gene. SPARC-null cells displayed diminished expression of collagen type I mRNA and protein, relative to wild-type cells, by the criteria of immunocytochemistry, immunoblotting, and the reverse transcription-polymerase chain reaction. The SPARC-null cells also showed significantly decreased steady-state levels of transforming growth factor-β1 (TGF-β1) mRNA and secreted TGF-β1 protein. Addition of recombinant SPARC to SPARC-null cells restored the expression of collagen type I mRNA to 70% and TGF-β1 mRNA to 100% of wild-type levels. We conclude that SPARC regulates the expression of collagen type I and TGF-β1 in kidney mesangial cells. Since increased mitosis and matrix deposition by mesangial cells are characteristics of glomerulopathies, we propose that SPARC is one of the factors that maintains the balance between cell proliferation and matrix production in the glomerulus.

SPARC (secreted protein acidic and rich in cysteine), a matricellular glycoprotein also known as BM-40, osteonectin, or 43-kDa protein, modulates the interaction of cells with the extracellular matrix through its regulation of cell adhesion and binding of growth factors (1, 2). It has been shown to inhibit proliferation, disrupt focal adhesions, and prevent cell spreading in vitro (3). In addition, SPARC is known to bind to certain growth factors, for example platelet-derived growth factor (PDGF) (4), and to bind extracellular matrix proteins such as collagen type I (5). SPARC regulates the expression of a number of secreted proteins (6) as well as matrix metalloproteinases (7) in certain cell types and is thought to modulate the interactions between cells and the surrounding extracellular matrix at least partially through this activity. In vivo, it is expressed during development (8) and is produced at sites of wound repair (9) and tissue remodeling (10). Furthermore, the production of SPARC mRNA is increased in certain types of carcinoma (11), in sclerosis (12), atherosclerotic lesions (4), passive Heymann nephritis (13), and mesangioproliferative glomerulonephritis (14). For example, SPARC has been shown to be involved in the resolution of mesangioproliferative glomerulonephritis in the Thy 1.1 model in the rat and to inhibit PDGF-induced proliferation of mesangial cells in vitro (14).

Transforming growth factor-β1 (TGF-β1) is also produced by mesangial cells during mesangioproliferative glomerulonephritis (15). A multifunctional growth factor that belongs to a family of proteins, TGF-β1 functions in various physiological processes such as growth, differentiation, proliferation, tissue remodeling, and wound healing (16). Although specific receptors have been found on nearly all mammalian cells, the effects of TGF-β1 differ according to cell type, growth conditions, and concentration of growth factor (17). TGF-β1 has been implicated in development and in the remodeling of tissues that takes place during adult life (18), although its effects on proliferation and differentiation can be stimulatory or inhibitory (19). TGF-β1 mediates the formation of extracellular matrix via its stimulation of the synthesis of components such as collagen type I. Moreover, it inhibits the degradation of extracellular matrix by suppression of matrix metalloproteinases and induction of tissue inhibitors of these enzymes (20). A number of publications have identified TGF-β1 as a critical factor in kidney diseases such as glomerulosclerosis (21) and mesangioproliferative glomerulonephritis (22). Furthermore, it has been shown that TGF-β1 augments the accumulation of glomerular matrix through its induction of collagen type I (23).

The most abundant fibrillar collagen expressed by a variety of cell types, collagen type I maintains the structural integrity of tissues such as bone, skin, organ capsules, and blood vessels (24). A number of factors modulate expression of the collagen

* This work was supported in part by National Institutes of Health Grants GM 40711, HL 18445, DK 47549, and GM 18705 (to A. D. B.), by the University of Washington Royalty Research Fund, by the Seattle Diabetes Research Council, by National Institutes of Health Training Grant DK 07467 (to A. D. B.), and by Fr 1223/1-1 from the Deutsche Forschungsgemeinschaft (to A. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Vascular Biology, The Hope Heart Institute, 528 18th Ave., Seattle, WA 98122. Tel.: 206-903-2025; Fax: 206-903-2044.

‡‡ To whom correspondence should be addressed: Dept. of Vascular Biology, The Hope Heart Institute, 528 18th Ave., Seattle, WA 98122. Tel.: 206-903-2025; Fax: 206-903-2044.

1 The abbreviations used are: PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-β1; rh, recombinant human; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse-transcribed polymerase chain reaction; rp, ribosomal protein; FGF, fibroblast growth factor; EtBr, ethidium bromide; VEGF, vascular endothelial growth factor.
genes during development (24), wound healing (25), inflammation (26), cancer (27), and glomerulonephritis (20). Numerous studies have shown that collagen synthesis and deposition are regulated by TGF-β1 (28) and by alterations in cell-extracellular matrix interactions that are accompanied by reorganization of the cytoskeletal network (29). Under pathological conditions, changes in regulatory pathways occur that can lead to the elevated expression of collagen type I (30), with eventual fibrosis or sclerosis and impaired organ function. Thus, it is critical to understand the different factors involved in the regulation of this predominant collagen.

To investigate the function of SPARC in the regulation of collagen type I and TGF-β1, we chose a model in which we could study interactions among SPARC, collagen type I, and TGF-β1 in primary mesangial cell cultures from wild-type and SPARC-null mice. We present evidence that SPARC regulates the expression of both collagen type I and TGF-β1 in mouse mesangial cells. SPARC-null cells exhibited a significantly diminished expression of collagen type I and TGF-β1. After treatment of these cells with recombinant human (rh) SPARC, the levels of collagen type I and TGF-β1 were restored to 70% and 100%, respectively, of those produced by wild-type cells. Furthermore, we show that SPARC exhibits some of its effects on collagen type I expression via a TGF-β1-dependent pathway. Since TGF-β1 can induce the expression of SPARC under certain conditions (26), it is likely that SPARC and TGF-β1 participate in a reciprocal, positive autocrine feedback loop that is especially prominent in mesangial cells.

**EXPERIMENTAL PROCEDURES**

**Preparation and Characterization of Mouse Glomerular Mesangial Cells—**129SvJ × C57BL/6J wild-type and SPARC-null mice (31) were maintained in a specific pathogen-free facility. Mice were euthanized at 3–6 months of age, and the kidneys were removed. Mesangial cells were detached in a solution of trypsin/EDTA (0.125%/0.010%, w/v) (Life Technologies, Inc.) and were replated at a density of 1.3. The cells were seeded between passages 3 and 8. For the TGF-β1 assays, wild-type and SPARC-null cells were grown to 80% confluence in growth medium, as described above. The cells were washed 2 times with PBS and were changed into fresh growth medium for 96 h. To measure the amounts of TGF-β1 protein in the conditioned culture media of wild-type and SPARC-null cells, we used an enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems Inc., Minneapolis, MN) according to the manufacturer’s instructions. The assay was repeated five times.

**Preparation of RNA and Cellular Protein—**For the mRNA preparta, wild-type and SPARC-null cells were grown to 80% confluence in growth medium. Total cellular RNA was prepared from mesangial cells by a modified method (33) that incorporated TRI-reagent (Molecular Research Center Inc., Cincinnati, OH). To increase the purity of the RNA samples, we added an additional step with 4 x 1 L of LiCl to eliminate residual contaminating polysaccharides, a DNase digestion step to eliminate DNA, and an additional precipitation of the RNA with ethanol. For preparation of cellular protein, wild-type and SPARC-null cells were grown to 80% confluence in growth medium. The insoluble (extracellular matrix proteins and membranes) and soluble cellular protein fractions were prepared either with the TRI-reagent or by dissolution of the cells in 1% SDS. The protein concentrations of the cell fractions were determined by the Bradford protein assay (34).

**Preparation of rhSPARC—**rhSPARC was prepared in SF9 cells by the use of the baculovirus protein expression system (35) and was collected in serum-free medium. rhSPARC was isolated by anion-exchange chromatography and was identified by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with a specific monoclonal anti-SPARC antibody (Haemotological Technologies, Essex Junction, VT). The rhSPARC had activity similar to that of recombinant SPARC expressed in *Escherichia coli* (36) and to SPARC synthesized by cultured mammalian cells (37), as measured by inhibition of proliferation and spreading (35).

**Analytical Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—**RT-PCR reactions containing 1 μg of total RNA were performed with the Access RT-PCR System™ (Promega) with oligonucleotide primers complementary to mouse β-tubulin, mouse ribosomal protein (rp) S6, mouse SPARC, mouse collagen α(I) and α(II), mouse collagen α(I) (III), mouse collagen α(IV) and α(IV), mouse collagen α(VIII) and α(VIII), mouse fibroblast growth factor 1 (FGF-1), fibroblast growth factor 2 (FGF-2), PDGF-A and -B chain, and TGF-β1. The primers were designed according to the Entrez nucleotide query program to retrieve the appropriate cDNAs from GenBank™ and the Primer selection TM oligonucleotide search program. Furthermore, with the Amplify 1.2 program, the primer pairs were tested for “cross-anneling” such that up to three primer pairs could be used together in one PCR reaction (38).

To establish conditions that allow comparison of the amounts of cDNA produced by RT-PCR, we varied the number of cycles from 24 to 40. For an internal standard, we either amplified β-tubulin mRNA or rpS6 mRNA, two ubiquitously expressed genes. After electrophoresis of 1/10 of the PCR reaction (5 μl), the bands (stained with ethidium bromide (EtBr), 0.5 μg/ml) became visible after 22 PCR cycles, and the staining reached saturation after 28 cycles. Therefore, a cycle number of 24 was chosen to compare the different levels of expression of the various mRNAs and to avoid saturation of the PCR DNA product and EtBr staining. The amounts of β-tubulin or rpS6 appeared to be unchanged between wild-type and SPARC-null cells. For quantification, values obtained from scanning densitometry of the cDNA bands generated from the respective mRNAs were normalized to the β-tubulin or rpS6 band. Since the β-tubulin/rpS6 and the other cDNAs were synthesized in the same tube, a direct comparison of the levels of expression is reasonable. Amplification of the newly synthesized first strand cDNA was performed in a Thermolyne Temptronic Thermal Cycler™. Equivalent aliquots of each amplification reaction were separated on a 1.2% agarose gel containing 0.5 μg/ml EtBr in 0.14 μl Tris acetate, 0.001 μl EDTA, 0.0007 μl MgCl₂, and 0.0001 μl HEPES. The gels were subjected to electrophoresis for 3 h at 100 V and were subsequently photographed.

**Western Blot Analysis and Metabolic Labeling—**Primary mesangial cell cultures from wild-type and SPARC-null mice were grown to 80% confluence in growth medium, and the kidneys were removed. Mesangial cells were detached in a solution of trypsin/EDTA (0.125%/0.010%, w/v) (Life Technologies, Inc.) and were replated at a density of 1.3. The cells were seeded between passages 3 and 8. For the TGF-β1 assays, wild-type and SPARC-null cells were grown to 80% confluence in growth medium, as described above. The cells were washed 2 times with PBS and were changed into fresh growth medium for 96 h. To measure the amounts of TGF-β1 protein in the conditioned culture media of wild-type and SPARC-null cells, we used an enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems Inc., Minneapolis, MN) according to the manufacturer’s instructions. The assay was repeated five times.

**Preparation of RNA and Cellular Protein—**For the mRNA preparta, wild-type and SPARC-null cells were grown to 80% confluence in growth medium. Total cellular RNA was prepared from mesangial cells by a modified method (33) that incorporated TRI-reagent (Molecular Research Center Inc., Cincinnati, OH). To increase the purity of the RNA samples, we added an additional step with 4 x 1 L of LiCl to eliminate residual contaminating polysaccharides, a DNase digestion step to eliminate DNA, and an additional precipitation of the RNA with ethanol. For preparation of cellular protein, wild-type and SPARC-null cells were grown to 80% confluence in growth medium. The insoluble (extracellular matrix proteins and membranes) and soluble cellular protein fractions were prepared either with the TRI-reagent or by dissolution of the cells in 1% SDS. The protein concentrations of the cell fractions were determined by the Bradford protein assay (34).

**Preparation of rhSPARC—**rhSPARC was prepared in SF9 cells by the use of the baculovirus protein expression system (35) and was collected in serum-free medium. rhSPARC was isolated by anion-exchange chromatography and was identified by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with a specific monoclonal anti-SPARC antibody (Haemotological Technologies, Essex Junction, VT). The rhSPARC had activity similar to that of recombinant SPARC expressed in *Escherichia coli* (36) and to SPARC synthesized by cultured mammalian cells (37), as measured by inhibition of proliferation and spreading (35).

**Analytical Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—**RT-PCR reactions containing 1 μg of total RNA were performed with the Access RT-PCR System™ (Promega) with oligonucleotide primers complementary to mouse β-tubulin, mouse ribosomal protein (rp) S6, mouse SPARC, mouse collagen α(I) and α(II), mouse collagen α(I) (III), mouse collagen α(IV) and α(IV), mouse collagen α(VIII) and α(VIII), mouse fibroblast growth factor 1 (FGF-1), fibroblast growth factor 2 (FGF-2), PDGF-A and -B chain, and TGF-β1. The primers were designed according to the Entrez nucleotide query program to retrieve the appropriate cDNAs from GenBank™ and the Primer selection TM oligonucleotide search program. Furthermore, with the Amplify 1.2 program, the primer pairs were tested for “cross-anneling” such that up to three primer pairs could be used together in one PCR reaction (38).

To establish conditions that allow comparison of the amounts of cDNA produced by RT-PCR, we varied the number of cycles from 24 to 40. For an internal standard, we either amplified β-tubulin mRNA or rpS6 mRNA, two ubiquitously expressed genes. After electrophoresis of 1/10 of the PCR reaction (5 μl), the bands (stained with ethidium bromide (EtBr), 0.5 μg/ml) became visible after 22 PCR cycles, and the staining reached saturation after 28 cycles. Therefore, a cycle number of 24 was chosen to compare the different levels of expression of the various mRNAs and to avoid saturation of the PCR DNA product and EtBr staining. The amounts of β-tubulin or rpS6 appeared to be unchanged between wild-type and SPARC-null cells. For quantification, values obtained from scanning densitometry of the cDNA bands generated from the respective mRNAs were normalized to the β-tubulin or rpS6 band. Since the β-tubulin/rpS6 and the other cDNAs were synthesized in the same tube, a direct comparison of the levels of expression is reasonable. Amplification of the newly synthesized first strand cDNA was performed in a Thermolyne Temptronic Thermal Cycler™. Equivalent aliquots of each amplification reaction were separated on a 1.2% agarose gel containing 0.5 μg/ml EtBr in 0.14 μl Tris acetate, 0.001 μl EDTA, 0.0007 μl MgCl₂, and 0.0001 μl HEPES. The gels were subjected to electrophoresis for 3 h at 100 V and were subsequently photographed.
confluence in the presence of 50 μg/ml sodium ascorbate for the final 24 h, and protein was prepared as described above. Equal amounts of protein per lane were resolved by SDS-PAGE (7% gels) under reducing conditions and were electrotransferred onto nitrocellulose membranes, which were subsequently blocked for 1 h with 5% nonfat dry milk and 0.05% Tween 20 (Sigma) in PBS. The blots were incubated with antibodies against collagen I (guinea pig anti-rat collagen I that cross-reacts with mouse collagen type I) (24) for 1 h. Immunoreactivity was detected by incubation of the blot with goat anti-guinea pig IgG coupled to horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA), followed by enhanced chemiluminescence (Amersham Pharmacia Biotech). For assessment of differences in protein loading, the blots were incubated with rabbit anti-human a-enolase IgG (gift from Dr. E. Plow, Cleveland Clinic, Cleveland, OH) that cross-reacts with mouse a-enolase, followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase.

For metabolic labeling, the mesangial cells were grown to 80% confluence and were incubated with sodium ascorbate (50 μg/ml) for 24 h. Cultures were subsequently incubated in fresh growth medium containing 50 μCi/ml L-[2,3,4,5-3H]proline (100 Ci/mmol, NEN Life Science Products). After 18 h, media containing radiolabeled proteins were collected, and the cells were washed twice with PBS. The supernatants were centrifuged to remove cell debris, and the media were counted using a scintillation counter. Incorporation of [3H]proline was measured in a scintillation counter. Protein content was determined by densitometric analysis of Coomassie Brilliant Blue-stained gels and used to normalize the data. The amount of procollagen type I secreted by the SPARC-null cells was lower than that secreted by wild-type cells.

**RESULTS**

**Diminished Expression of Collagen Type I in SPARC-Null Cells**—SPARC has been implicated as a modulator of interactions between cells and the extracellular matrix. It is known to bind to growth factors and to matrix proteins as well as to matrix proteins. Alterations in cell-matrix interactions usually occur during wound healing, tissue remodeling, and fibrosis (29). Since SPARC (14), collagen type I (20), and TGF-β1 (22) were among the proteins shown to be augmented during mesangiolamellar nephritis, we examined the effect of SPARC on the expression of collagen type I and TGF-β1 in mesangial cells cultured from wild-type and SPARC-null mice. The following results were observed in five independent preparations of mesangial cells isolated from pools of eight kidneys each. The experiments were performed four times with all five preparations. Means ± S.D. were calculated for all experiments.

One of the first observations we made was that SPARC-null mesangial cells proliferated faster and exhibited a more rounded, cobblestone-like cell shape in comparison to their wild-type counterparts (35). Confluent monolayers of SPARC-null mesangial cells displayed very few of the hillocks (localized accumulations of extracellular matrix) that typify mesangial cell cultures. Mesangial cells also produced diminished levels of collagen type I, as shown in Fig. 1. Wild-type cells incubated with anti-collagen type I antibody exhibited a typical granular staining pattern throughout the cytoplasm (A), whereas SPARC-null cells showed significantly less staining for collagen type I (B). Immunoreactivity with an irrelevant antibody or the secondary antibody alone was negative (data not shown).

The amount of procollagen type I secreted by the SPARC-null cells was also considerably diminished, relative to levels produced by wild-type cells (Fig. 2, lanes 1 and 3). Digestion of the secreted protein with collagenase (Fig. 2, lanes 2 and 4) prior to SDS-PAGE confirmed the identity of these bands as procollagen and its processed α chains. Under the conditions used for SDS-PAGE, the α1(I) and the α2(I) chains comigrated in our metabolic labeling and immunoblotting experiments.

Collagen type I mRNA was detectable in mesangial cells by RT-PCR. The initial amounts of reverse-transcribed mRNAs for α1(I) were significantly lower in the SPARC-null cells (lane 2) in comparison with those in wild-type cells (lane 1) (Fig. 3A). By scanning densitometry with β-tubulin or rpS6 as an internal control, we found consistently diminished levels of α1(I)
SPARC Regulates Collagen I and TGF-β1 Expression

Expression of collagen type I mRNA in wild-type and SPARC-null mesangial cells

| Addition | Changes in α1(I) mRNA expression |
|----------|----------------------------------|
| 2. rhSPARC (0.9 μg/ml) | 1.3-Fold* increase |
| 3. rhTGF-β1 (5 ng/ml) | 1.4-Fold increase |
| 4. Anti-TGF-β1 antibodies (30 μg/ml) | 1.3-Fold decrease |
| 5. rhSPARC + anti-TGF-β1 antibodies | 1.0-Fold increase |
| 6. rhTGF-β1 + anti-TGF-β1 antibodies | 1.1-Fold increase |

* Level of α1(I) mRNA in treated cells relative to that of untreated cells (untreated wild-type cells are set at 100%, in bold). In comparison to wild-type cells, SPARC-null cells expressed 44% collagen type I mRNA (2.2-fold decrease, Row 1). Treatment with rhSPARC or rhTGF-β1 increased the expression of collagen type I mRNA by 1.3- and 1.4-fold, respectively (Rows 2 and 3) in wild-type cells, whereas in SPARC-null cells the expression increased 1.6- and 1.9-fold, respectively (Rows 2 and 3). Treatment with anti-TGF-β1 antibodies alone decreased the endogenous production of collagen type I mRNA 1.3-fold in the wild-type cells and 1.6-fold in the SPARC-null cells (Row 4). The stimulatory effect of rhSPARC or rhTGF-β1 on the expression of collagen type I mRNA was reduced to nearly control levels by anti-TGF-β1 antibodies in wild-type cells (Rows 5 and 6). In SPARC-null cells, however, the stimulatory effect of rhSPARC or rhTGF-β1 was not blocked completely by anti-TGF-β1 antibodies (i.e. it was decreased from 1.6- to 1.2-fold, or 67% for rhSPARC, and from 1.9- to 1.3-fold, or 67% for rhTGF-β1) (Rows 5 and 6). The numbers represent means ± S.D. For all values, the standard deviation did not exceed 11%. Results are averaged from five independent mesangial cell preparations, and experiments were repeated four times on each preparation (n = 20).

The diminished expression of collagen type I mRNA shown in Fig. 3a was consistent with the amounts of protein synthesized by the mesangial cell strains. The SPARC-null cells expressed significantly less collagen type I relative to wild-type cells (Fig. 3B). By scanning densitometry with α-enolase as an internal control, we found the level of expression of collagen type I protein in SPARC-null cells to be diminished 1.4-fold (70 ± 5%), relative to wild-type cells. By RT-PCR, levels of other collagen types that are known to be expressed in the kidney, e.g. types III, IV, and VIII, were not altered significantly in SPARC-null cells, in comparison to wild-type cells (data not shown).

SPARC Induces Collagen Type I in Mesangial Cells—Since the data above were derived from SPARC-null cells, we asked whether exogenous SPARC would rescue the expression of collagen type I to levels typical of wild-type cells. Consequently, we added rhSPARC to a final concentration of 30 μg/ml (0.9 μM) to wild-type and SPARC-null cells grown to 80% confluence. The levels of α1(I) mRNA were increased 1.3-fold (25%) in wild-type cells and 1.6-fold (60%) in SPARC-null cells, relative to those of unstimulated cells, after exposure to rhSPARC for 6 h (Fig. 4A). We were not able to restore completely the expression of collagen type I in SPARC-null cells to levels typical of wild-type cells. It is therefore possible that SPARC might not regulate the expression of collagen type I directly but might exert its effect via an additional factor. The differences in mRNA expression were calculated as described above and are shown in Table I. These results were confirmed at the protein level by immunoblotting for collagen type I (Fig. 4B); the addition of rhSPARC increased collagen type I by 40 ± 4% (1.4-fold) in wild-type cells and by 34 ± 2% (1.3-fold) in SPARC-null cells. Interestingly, the addition of rhSPARC was also associated

---

**Fig. 2. Reduced secretion of collagen type I by SPARC-null mesangial cells.** Mesangial cells were incubated for 18 h in the absence of [3H]proline; equal cpm of radiolabeled proteins in the conditioned medium were resolved by SDS-PAGE on an 8% gel under reducing conditions and were visualized by autoradiography. Prior to separation, aliquots of the supernatants containing equivalent amounts of labeled protein were digested with bacterial collagenase. Lanes 1 and 3, undigested proteins; lanes 2 and 4, digested proteins.

**Fig. 3. Reduced expression of collagen type I mRNA and protein by SPARC-null mesangial cells.** A, 1 μg of total RNA extracted from wild-type and SPARC-null cells was reverse-transcribed and amplified in the presence of specific primers for the α1(I) collagen chain, for SPARC, and for β-tubulin as a control for gel loading. The products were subjected to agarose gel electrophoresis. Lane 1, wild-type cells; lane 2, SPARC-null cells. B, Western blot analysis with an anti-collagen type I antibody of 40 μg of total cellular protein (resolved by SDS-PAGE on a 7% gel under reducing conditions) derived from wild-type (lane 1) and SPARC-null cells (lane 2). α-Enolase was used as an internal control.

mRNA relative to that of wild-type cells, with a mean value of 44 ± 3%, a decrease of 2.2-fold (Table I). Similar decreases in α2(I) mRNA were seen in SPARC-null cells by RT-PCR. Relative to wild-type cells, levels of α2(I) mRNA in SPARC-null cells were diminished by 53 ± 4% (1.9-fold). Since the changes in expression of α2(I) were quantitatively similar to those of the α1(I) chain, only the latter has been shown.

---

**TABLE I**

Expression of collagen type I mRNA in wild-type and SPARC-null mesangial cells

| Addition | Changes in α1(I) mRNA expression |
|----------|----------------------------------|
| 1. Untreated control | 100% |
| 2. rhSPARC (0.9 μg/ml) | 1.3-Fold* increase |
| 3. rhTGF-β1 (5 ng/ml) | 1.4-Fold increase |
| 4. Anti-TGF-β1 antibodies (30 μg/ml) | 1.3-Fold decrease |
| 5. rhSPARC + anti-TGF-β1 antibodies | 1.0-Fold increase |
| 6. rhTGF-β1 + anti-TGF-β1 antibodies | 1.1-Fold increase |

* Level of α1(I) mRNA in treated cells relative to that of untreated cells (untreated wild-type cells are set at 100%, in bold). In comparison to wild-type cells, SPARC-null cells expressed 44% collagen type I mRNA (2.2-fold decrease, Row 1). Treatment with rhSPARC or rhTGF-β1 increased the expression of collagen type I mRNA by 1.3- and 1.4-fold, respectively (Rows 2 and 3) in wild-type cells, whereas in SPARC-null cells the expression increased 1.6- and 1.9-fold, respectively (Rows 2 and 3). Treatment with anti-TGF-β1 antibodies alone decreased the endogenous production of collagen type I mRNA 1.3-fold in the wild-type cells and 1.6-fold in the SPARC-null cells (Row 4). The stimulatory effect of rhSPARC or rhTGF-β1 on the expression of collagen type I mRNA was reduced to nearly control levels by anti-TGF-β1 antibodies in wild-type cells (Rows 5 and 6). In SPARC-null cells, however, the stimulatory effect of rhSPARC or rhTGF-β1 was not blocked completely by anti-TGF-β1 antibodies (i.e. it was decreased from 1.6- to 1.2-fold, or 67% for rhSPARC, and from 1.9- to 1.3-fold, or 67% for rhTGF-β1) (Rows 5 and 6). The numbers represent means ± S.D. For all values, the standard deviation did not exceed 11%. Results are averaged from five independent mesangial cell preparations, and experiments were repeated four times on each preparation (n = 20).

The diminished expression of collagen type I mRNA shown in Fig. 3a was consistent with the amounts of protein synthesized by the mesangial cell strains. The SPARC-null cells expressed significantly less collagen type I relative to wild-type cells (Fig. 3B). By scanning densitometry with α-enolase as an internal control, we found the level of expression of collagen type I protein in SPARC-null cells to be diminished 1.4-fold (70 ± 5%), relative to wild-type cells. By RT-PCR, levels of other collagen types that are known to be expressed in the kidney, e.g. types III, IV, and VIII, were not altered significantly in SPARC-null cells, in comparison to wild-type cells (data not shown).

SPARC Induces Collagen Type I in Mesangial Cells—Since the data above were derived from SPARC-null cells, we asked whether exogenous SPARC would rescue the expression of collagen type I to levels typical of wild-type cells. Consequently, we added rhSPARC to a final concentration of 30 μg/ml (0.9 μM) to wild-type and SPARC-null cells grown to 80% confluence. The levels of α1(I) mRNA were increased 1.3-fold (25%) in wild-type cells and 1.6-fold (60%) in SPARC-null cells, relative to those of unstimulated cells, after exposure to rhSPARC for 6 h (Fig. 4A). We were not able to restore completely the expression of collagen type I in SPARC-null cells to levels typical of wild-type cells. It is therefore possible that SPARC might not regulate the expression of collagen type I directly but might exert its effect via an additional factor. The differences in mRNA expression were calculated as described above and are shown in Table I. These results were confirmed at the protein level by immunoblotting for collagen type I (Fig. 4B); the addition of rhSPARC increased collagen type I by 40 ± 4% (1.4-fold) in wild-type cells and by 34 ± 2% (1.3-fold) in SPARC-null cells. Interestingly, the addition of rhSPARC was also associated
showed a 55% SPARC-null and wild-type cells. By RT-PCR, SPARC-null cells

Effect of Exogenous SPARC on the Expression of Collagen Type I and TGF-$\beta_1$—To determine whether the effects of exogenous SPARC on collagen type I expression were exerted through a TGF-$\beta_1$-dependent pathway, we treated cells for 0–6 h with or without anti-TGF-$\beta_1$-blocking antibodies (30 $\mu$g/ml), rhSPARC (0.9 $\mu$g), or rhTGF-$\beta_1$ (1, 5, and 10 ng/ml). Fig. 7A shows the expression of collagen type I and TGF-$\beta_1$ after stimulation of the cells with rhSPARC at different time points. The expression of TGF-$\beta_1$ mRNA was induced significantly after 1 h (lanes 2 and 7) and preceded the induction of collagen type I mRNA expression, which was first apparent at 4 h (lanes 4 and 9). Therefore, there was delayed induction of collagen type I after treatment with rhSPARC. In comparison, unstimulated cells did not show any significant changes in the steady-state levels of collagen type I or TGF-$\beta_1$ mRNA at these time points. Fig. 7B shows the levels of $\alpha(1)$ mRNA after treatment of cells with different concentrations of rhTGF-$\beta_1$. As expected (21), TGF-$\beta_1$ induced the expression of $\alpha(1)$ mRNA in a concentration-dependent manner. Fig. 7C shows the levels of $\alpha(1)$ mRNA after treatment of cells with the respective reagents and TGF-$\beta_1$-blocking antibodies. The effects of rhSPARC and rhTGF-$\beta_1$ on the expression of $\alpha(1)$ mRNA were diminished significantly in the presence of TGF-$\beta_1$-blocking IgG (lanes 5 and 6; wild-type; lanes 11 and 12, SPARC-null). TGF-$\beta_1$-blocking antibodies alone also diminished the expression of $\alpha(1)$ mRNA (Fig. 7C, lanes 4 and 10). The effects of rhSPARC, rhTGF-$\beta_1$, and anti-TGF-$\beta_1$ IgG on the expression of $\alpha(1)$ mRNA are more
with the remodeling process that occurs after kidney injury (14). Characteristics of this proliferative response are the elevated production of matricellular proteins (e.g. SPARC), matrix proteins (collagen type I), and cytokines (TGF-β1). The secretion of these factors and the accumulation of extracellular matrix proteins lead to an expansion of the glomerular basement membrane (39), an infiltration of immunocompetent cells (32), and in some cases, to a reversal of the pathological condition. Therefore, maintenance of glomerular integrity appears to be important because mesangial dysfunction often leads to excessive proliferation of the mesangial cells, overproduction of cytokines and growth factors, and an accumulation of extracellular matrix. These factors contribute collectively to renal fibrosis, glomerulosclerosis, glomerulonephritis, and the eventual loss of kidney function.

In this study we have identified two regulated targets of SPARC in mesangial cells, collagen type I and TGF-β1. The use of cells derived from normal mice and from mice with a disrupted SPARC gene indicated that the observed changes in collagen type I and TGF-β1 were attributable to the absence of endogenous SPARC. Confirmation of these results was provided by rescue experiments with rhSPARC. Furthermore, our data indicate that SPARC stimulates collagen type I production via a TGF-β1-dependent pathway. At this point, the data do not allow us to conclude whether the effect of SPARC depletion on collagen type I and TGF-β1 is a primary or derivative event.

SPARC modulates interactions among cells, extracellular matrix proteins, and growth factors and both colocalizes with and binds to collagen type I (1, 5). However, no induction of collagen type I by SPARC has been described. We now show that the levels of collagen type I mRNA and protein (both secreted and cellular forms) are significantly diminished in mesangial cells from mice with a disrupted SPARC gene. Furthermore, we demonstrate that exogenous rhSPARC was able to reverse the diminished production of collagen type I mRNA and protein. Since collagen types III, IV, and VIII appeared unchanged in SPARC-null cells compared with their wild-type counterparts, the regulatory effect of SPARC seems specific for collagen type I.

How might SPARC exert its effects on the expression of collagen type I protein? One possibility is the direct binding of SPARC to collagen (5). SPARC might be needed for alignment of the α chains into the triple helix. If SPARC is missing, proper processing and/or posttranslational modification might occur more slowly, and the single chains would be a target for degradation. Therefore, the overall amount of intact collagen type I might be diminished in the cells that are not expressing SPARC. This hypothetical function of SPARC is comparable to that of HSP47, a heat-shock protein that acts as a collagen chaperone (40).

Another explanation for the diminished collagen expression in SPARC-null cells could be related to their altered cell morphology (35). Since SPARC is not available to bind to collagen type I, the cells might modulate their expression of other extracellular matrix proteins (and thereby the composition of the extracellular matrix) to compensate for the lack of a collagen type I-SPARC complex. This response would be compromised by the addition of exogenous SPARC; therefore, the expression of collagen type I would be augmented as we have described here. Interestingly, it has been observed that cells lacking collagen type I do not deposit SPARC in the extracellular matrix of certain connective tissues, whereas wild-type cells show colocalization of SPARC and collagen type I (24). If cell shape is important in the regulation of protein expression (29), and SPARC alters cell morphology, the SPARC-null cells might
SPARC Regulates Collagen I and TGF-β1 Expression

3251

A second question is how SPARC might regulate the transcription of collagen type I. A possible mechanism involves the interactions of SPARC with growth factors. Previous reports have shown that SPARC exerts some its effects through direct binding to PDGF (4) and to vascular endothelial growth factor (VEGF) (41) and thereby abrogates their interaction with cognate receptors on cells. Moreover, in the case of VEGF, SPARC inhibits the phosphorylation of VEGF-receptor 1 and the mitogen-activated protein kinases extracellular signal-regulated kinase-1 and -2 (41). Thus, we would predict that signaling pathways governing the transcription of certain genes might be affected by SPARC. Since the proliferation of SPARC-null cells is enhanced significantly (5–10-fold) (35), we also investigated the expression of growth factors themselves that are known to be inducers of proliferation and gene transcription in mesangial cells (FGF-1, FGF-2, and PDGF). We were unable to show any differences in mRNA levels for these mitogens between wild-type and SPARC-null cells.

Interesting results were obtained, however, with an inhibitor of mesangial cell proliferation, TGF-β1. There was a significant decrease in the expression of TGF-β1 mRNA and protein in SPARC-null cells. Since it is known that minimal differences in the concentration of TGF-β1 can cause diverse biological responses in various cell types (17) and in mesangial cells (19), we suggest that the decreased collagen type I expression that was observed might be due to the diminished levels of TGF-β1. Addition of rhSPARC restored the levels of TGF-β1 in SPARC-null cells to those of wild-type cells. Additionally, it induced the accumulation of TGF-β1 mRNA to levels similar to those of wild-type cells that were exposed to rhSPARC. In our study, the level of collagen type I by rhSPARC was incomplete after 6 h. This result might be due to the indirect induction of collagen type I transcription by rhSPARC, similar to the reported induction of metalloproteinases by SPARC (7). We propose that TGF-β1 acts as a mediator. Therefore, SPARC would first activate the TGF-β1 system, and second, TGF-β1 would induce the transcription of collagen type I as reported (20). Thus, we observed an early induction of TGF-β1 expression and a delayed induction of collagen type I expression after treatment with rhSPARC, an observation consistent with the incomplete rescue of collagen type I transcription after 6 h. It has also been reported that TGF-β1 regulates the expression of SPARC (25, 42), but ours is the first report that SPARC induces the expression of TGF-β1. Furthermore, it has been demonstrated that TGF-β1 is regulated in mesangial cells in an autocrine manner (43). These findings collectively indicate a positive autocrine feedback loop between SPARC and TGF-β1.

TGF-β1 is a potent inducer of collagen type I gene expression (25). We were able to verify these findings in our system, and we observed a significant, concentration-dependent induction of collagen type I expression in both wild-type and, to a greater extent, in SPARC-null cells. Since the levels of collagen type III, IV, or VIII appeared to be unchanged in the SPARC-null cells in comparison to the wild-type cells, our findings furthermore imply that the presence of SPARC is essential for a preferential expression of collagen type I in mouse mesangial cells. In addition, there are numerous reports indicating that the expression of various types of collagen, especially types III and IV in cultured cells, are not solely dependent on TGF-β1 but on other factors such as cell density (44) and serum factors (45).

The expression patterns of collagen type I and TGF-β1 in mesangial cells derived from SPARC-null animals indicate that SPARC mediates its effects on the production of collagen type I, in part, through TGF-β1. In support of this hypothesis, there was a decrease in steady-state levels of α1(I) mRNA induced by SPARC in the presence of anti-TGF-β1 antibodies. These results provide strong evidence that SPARC regulates the production of collagen type I via a TGF-β1-dependent pathway in mouse mesangial cells.

Several alternatives exist (some of which are not mutually exclusive) for the mechanism(s) by which SPARC affects the production of collagen type I and TGF-β1: (i) SPARC could exert its effects through its own (yet unidentified) receptor(s). (ii) Similar to thrombospondin-1, another matricellular protein (46), SPARC, could be involved in the TGF-β1 activation cascade, in which latent TGF-β1 is processed to its active form. (iii) SPARC could modulate the activity or the conformation of the TGF-β1 receptor complex by its direct binding to the complex or to an extracellular matrix component (47). (iv) SPARC could change the configuration of the extracellular matrix and/or the shape of the cell and could thus affect binding between a ligand and its cell-surface receptor. (v) Since SPARC has been shown to be associated with the nuclear matrix (48), it could be involved in the activation or suppression of gene expression and/or in the modulation of nuclear shape. These hypotheses are currently under investigation in our laboratory.

Acknowledgments—We thank Drs. Kourois Motamed, Christine Kuporion, David Graves, and Robert Vernon for many helpful discussions throughout this study. We gratefully acknowledge the technical assistance of Juliet Carbon and Timothy Dale McClure.

REFERENCES

1. Lane, T. F., and Sage, E. H. (1994) FASEB J. 8, 163–173
2. Bornstein, P. (1995) J. Cell Biol. 130, 503–506
3. Motamed, K., and Sage, E. H. (1998) J. Cell Biochem. 70, 543–552
4. Raines, R. E., Lane, T. F., Iruela-Arispe, M. L., Ross, R., and Sage, E. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1281–1285
5. Sasaki, T., Hohenester, E., Göhring, W., and Timpl, R. (1998) EMBO J. 17, 1625–1634
6. Lane, T. F., Iruela-Arispe, M. L., and Sage, E. H. (1992) J. Biol. Chem. 267, 16746–16754
7. Trelle, P. M., Lane, T. F., Sage, E. H., and Werb, Z. (1992) J. Cell Biol. 121, 1433–1444
8. Engelmann, G. L. (1993) Cardiovasc. Res. 27, 1598–1605
9. Reed, M. J., Puolakainen, P., Lane, T. F., Dickerson, D., Bornstein, P., and Sage, E. H. (1993) J. Histochem. Cytochem. 41, 1467–1477
10. Shankavaram, U. T., DeWitt, D. L., Funk, S. E., Sage, E. H., and Wahl, L. M. (1997) J. Cell Physiol. 172, 237–247
11. Porte, H., Triboulet, J. P., Katelevets, L., Carrat, F., Prévot, S., Nordlinger, B., Di Gioia, Y., Wurtz, A., Comoglio, P., Guespin, C., and Chastre, E. (1998) Clin. Cancer Res. 4, 1375–1383
12. Umemori, E. N., and Amento, E. P. (1991) Curr. Opin. Rheumatol. 3, 953–959
13. Flocq, J., Johnson, R. J., Alpers, C. E., Fatemi-Nainie, S., Richardson, C. A., Gordon, K., and Coussen, G. W. (1993) Am. J. Pathol. 142, 657–650
14. Pickel, R. H., Bassuk, J. A., Hage, C., Reed, M. J., Eng, E., Gordon, K. L., Pippin, J., Alpers, C. E., Coussen, G. W., Sage, E. H., and Johnson, R. J. (1998) Am. J. Pathol. 148, 1153–1167
15. Ivanov, M., Akut, Y., Fujii, Y., Doi, Y., Matsumura, N., and Doi, K. (1994) Clin. Exp. Immunol. 97, 309–314
16. Pepper, M. S. (1997) Cytokine Growth Factor Rev. 8, 21–43
17. Massagué, J. (1990) Annu. Rev. Physiol. 52, 351–377
18. Frank, R., Aedelmann-Grill, B. C., Herrmann, K., Haustein, U. F., Petri, J. B., and Heckmann, M. (1996) J. Invest. Dermatol. 106, 36–41
19. Flocq, J., Topley, N., and Resch, K. (1991) Am. J. Kidney Dis. 18, 673–676
20. Fontelet, A. C., and Schnaper, H. W. (1988) Am. J. Physiol. 255, F458–F466
21. Gilbert, R. E., Wilkinson-Berka, J. L., Johnson, D. W., Cox, A., Soulis, T., Wu, L.-L., Kelly, D. J., Jerums, G., Pollock, C. A., and Cooper, M. E. (1998) Kidney Int. 54, 1052–1060
22. Yamamoto, T., Nohle, N. A., Cohen, A. H., Nast, C. C., Hishida, A., Gold, L. I., and Border, W. A. (1996) Kidney Int. 49, 461–469
23. Kasugami, S., Kuhara, T., Yamasaki, K., Okada, K., Laster, K., Reutter, W., and Kuroda, Y. (1996) Exp. Cell Res. 229, 1–6
24. Iruela-Arispe, M. L., Vernon, R. B., Wu, H., Jaenisch, R., and Sage, E. H. (1996) Dev. Dyn. 207, 171–183
25. Reed, M. J., Vernon, R. B., Abrams, I. B., and Sage, E. H. (1994) J. Cell Biol. 125, 158, 169–179
26. Lloyd, C. M., Minto, A. W., Dorf, M. E., Proudfoot, A., Wells, T. N., Salant, D. J., and Gutierrez-Ramos, J. C. (1997) J. Exp. Med. 185, 1317–1326

3 A. Francki, unpublished observations.
27. Sato, N., Beitz, J. G., Kato, J., Yamamoto, M., Clark, J. W., Calabresi, P., Raymond, A., and Frackelton, A. R. (1993) Am. J. Pathol. 142, 1119–1130
28. Grande, J. P., Melder, D. C., and Zinsmeister, A. R. (1997) J. Lab. Clin. Med. 130, 476–486
29. Vareli, M., Ghahary, A., Scott, P. G., and Tredget, E. E. (1997) J. Cell. Physiol. 172, 192–199
30. Lenz, O., Striker, L. J., Jacot, T. A., Elliot, S. J., Killen, P. D., and Striker, G. E. (1998) J. Am. Soc. Nephrol. 9, 2040–2047
31. Norose, K., Clark, J. I., Syed, N. A., Basu, A., Heber-Katz, E., Sage, E. H., and Howe, C. C. (1998) Invest. Ophthalmol. & Visual Sci. 39, 2674–2680
32. Radeke, H. H., and Resch, K. (1992) Clin. Invest. 70, 825–842
33. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
34. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
35. Bassuk, J. A., Baneyx, F., Vernon, R. B., Funk, S. E., and Sage, E. H. (1996) Biochem. Biophys. Res. Commun. 325, 8–19
36. Funk, S. E., and Sage, E. H. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2648–2652
37. Goswami, P. C., Albee, L. D., Spitz, D. R., and Ridnour, L. A. (1997) Cell Proliferation 30, 271–282
38. Johnson, R. J., Raines, E. W., Floege, J., Yoshimura, A., Pritzl, P., Alpers, C., and Ross, R. (1992) J. Exp. Med. 175, 1413–1416
39. Satoh, M., Hiyoshi, K., Yakota, S., Hosokawa, N., and Nagata, K. (1996) J. Cell Biol. 133, 469–483
40. Kupprion, C., Motamed, K., and Sage, E. H. (1998) J. Biol. Chem. 273, 29635–29640
41. Wrama, J. L., Overall, C. M., and Sodek, J. (1991) Eur. J. Biochem. 197, 519–528
42. Ziyadeh, F. N., Sharma, K., Ericksen, M., and Wolf, G. (1994) J. Clin. Invest. 93, 536–542
43. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
44. Abrass, C. K., Spicer, D., and Raugi, G. J. (1995) Kidney Int. 47, 25–37
45. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouch, N. (1998) Cell 93, 1159–1170
46. Rosenblatt, S., Bassuk, J. A., Alpers, C. E., Sage, E. H., Timpl, R., and Preisssner, K. T. (1997) Biochem. J. 324, 311–319
47. Gooden, M. D., Vernon, R. B., Bassuk, J. A., and Sage, E. H. (1999) J. Cell. Biochem. 74, 152–167