SPARC Accelerates Disease Progression in Experimental Crescentic Glomerulonephritis

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Podocytopenia characterizes many forms of glomerular disease, preceding the development of glomerulosclerosis. While detachment of viable podocytes from the underlying glomerular basement membrane is an important mechanism of podocyte loss, the underlying factors involved remain unclear. Secreted protein acidic and rich in cysteine (SPARC), a matricellular protein with counteradhesive properties, is normally expressed at low levels by the podocyte but is markedly increased following podocyte injury. Accordingly, we elucidate the role of SPARC in mediating experimental crescentic glomerulonephritis by inducing passive nephrotoxic nephritis in SPARC^{+/+} and SPARC^{-/-} mice. By days 4, 7, and 21 following disease induction, podocyte number is better preserved, glomerulosclerosis is ameliorated, and proteinuria is reduced in SPARC^{-/-} mice as compared with SPARC^{+/+} littermates. Moreover, the preserved podocyte number in SPARC^{-/-} mice correlates with reduced urinary levels of both nephrin and podocin. To establish a causal role for SPARC in mediating detachment, cultured SPARC^{+/+} and SPARC^{-/-} podocytes were subjected to mechanical strain as well as trypsin digestion, and detachment assays were performed. While podocytes lacking SPARC were more resistant to stretch-induced detachment, stable re-expression of SPARC restored detachment rates to levels comparable with SPARC^{+/+} podocytes. Taken together, this study proves that SPARC plays a causal role in mediating podocyte detachment and accelerating glomerulosclerosis in experimental crescentic glomerulonephritis. (*Am J Pathol* 2009, 174:1827–1836; DOI: 10.2353/ajpath.2009.080464)

Secreted protein acidic and rich in cysteine (SPARC), a highly conserved member of the family of matricellular proteins, has pleiotropic effects on modulating diverse

cellular responses. Physiological expression in adult vertebrates is largely limited to tissues undergoing remodeling, such as bone and healing wounds.¹ The role of SPARC in contributing to the organization of matrix in connective tissue is reinforced by the phenotype of the SPARC null mouse, which displays early cataractogenesis, disruption of collagen fibrillogenesis with impaired wound healing, and osteoporosis.² Disruption of focal adhesion assemblies promotes counteradhesive properties of SPARC, a characteristic exploited by many solid tumors to facilitate metastatic potential.³

Within the glomerulus, the role of SPARC has remained enigmatic. Abundantly expressed during development, glomerular expression of SPARC in the mature kidney is normally limited to the podocyte.⁴ Present in low levels under basal conditions, podocyte levels of SPARC are increased following both immune- and nonimmune-mediated glomerular disease.^{5,6} SPARC has been proposed as a “marker” of podocyte injury; however, its role in glomerular disease remains largely unknown. Recent studies in an experimental model of diabetic nephropathy demonstrate that increased SPARC is maladaptive and worsens renal disease.⁷ However, the effects of increased SPARC in an immune-mediated model of podocyte disease are yet to be elucidated. Accordingly, in this study we will establish that increased SPARC causes a progressive reduction in podocyte number, leading to the development of glomerulosclerosis in the passive nephrotoxic nephritis model.

Materials and Methods

Animals

SPARC wild-type (^{+/+}) and null (^{-/-}) mice maintained on a C57Bl/6 genetic background were obtained as a gift from Dr. E. Helene Sage (Benoroya Research Institute, Seattle WA). The generation and characterization of

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these mice has been previously reported.^{8–10} Mice were housed in a pathogen-free vivarium where they had unrestricted access to water and maintained on a diet of standard rodent chow. The treatment and use of mice in all studies was in accordance with protocols approved by the University of Washington Animal Care Committee.

Glomerulonephritis Model

Our group has previously reported a passive nephrotoxic nephritis model in mice, characterized by progressive glomerulosclerosis with crescent formation, in which podocyte injury is a prominent feature.¹¹ Accordingly, disease induction was achieved in 12-week-old male SPARC^{+/+} and SPARC^{-/-} mice by the intraperitoneal injection of sheep anti-rabbit glomerular antibody (12 mg/20 g of body weight) on 2 consecutive days, as previously reported.¹¹ SPARC^{+/+} and SPARC^{-/-} mice were sacrificed on days 4, 7, or 21 after the second injection of anti-glomerular antibody ($n = 6$ animals per group/time point). Urine was collected from individual normal animals (before disease induction) and just before sacrifice by placing animals in metabolic cages for 12 hour intervals. Urine protein excretion was measured by the sulfosalicylic acid method as previously reported by our group.¹² Renal tissue was embedded in optimal cutting temperature compound (Miles, Elkart, IN) and frozen at -70°C or fixed in either 10% neutral buffered formalin or methyl-Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid) for subsequent immunostaining studies.

Immunostaining

To demonstrate that disease induction was similar between the SPARC^{+/+} and SPARC^{-/-} groups, we performed immunofluorescence staining for sheep IgG (heterologous phase of disease) and mouse IgG (autologous phase of disease) as previously reported.¹³ Briefly, frozen sections were rehydrated in PBS and following fixation in -20°C methanol, incubated with streptavidin AlexaFluor 594-conjugated antibody to sheep IgG (Molecular Probes Inc, Eugene, OR), to determine glomerular antibody deposition in the two groups. The autologous phase of the disease was similarly assessed by immunostaining with a fluorescein isothiocyanate-conjugated antibody to mouse IgG (Cappel, Durham, NC).

Immunohistochemistry for SPARC was performed as we have previously reported.⁵ Briefly, methyl-Carnoy's fixed tissue sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA), rehydrated with ethanol, and treated with hydrogen peroxide to neutralize endogenous peroxidase. Tissue sections were incubated overnight at 4°C with primary rabbit polyclonal anti-mouse SPARC antibody from antiserum 5944 (gift from Dr. E. Helene Sage) This antibody was affinity-purified as described previously.¹⁴ Tissue sections were next incubated with a biotinylated goat anti-rabbit secondary antibody (1:100 dilution, Promega, Madison, WI) for 60 minutes at room temperature, followed by ABC reagent

(Vector Laboratories Inc.) for 20 minutes at room temperature. Color development was achieved by incubating in diaminobenzidine solution at 37°C for 6 minutes and counterstaining in methyl green for 2 minutes. Substitution of the primary antibody with an irrelevant rabbit IgG served as a negative control. Immunohistochemistry for WT-1 was performed using a primary polyclonal rabbit anti-WT-1 antibody (Santa Cruz, Santa Cruz, CA) as outlined above; however, diaminobenzidine was supplemented with nickel to optimize nuclear staining.

Assessment of Glomerulosclerosis

Glomerulosclerosis determined on periodic acid Schiff-stained sections was graded quantitatively based on the percentage of glomerular tuft area involvement as follows: grade 0 = all loops patent with 0% tuft involvement; grade 1 = $<25\%$; grade 2 = 25 to 50%; grade 3 = 50 to 75%; grade 4 = 75 to 100% or evidence of crescent formation. Slides were viewed using a Leica confocal microscope (Leica, Deerfield, IL). A minimum of 20 glomeruli were examined per tissue section and a total of 6 animals were studied in each group at each time point.

Transmission Electron Microscopy

Randomly selected cases of each experimental group at Day 14 following disease induction were used for electron microscopy and processed as described previously in detail.¹⁵ Grids were scanned using a Philips 410 microscope (Philips, Eindhoven, The Netherlands).

Immunoblotting

Urine was collected via bladder puncture on day 14 following induction of passive nephrotoxic nephritis in SPARC^{+/+} and SPARC^{-/-} mice and each sample centrifuged at $14,000 \times g$ for 7 minutes at 4°C using 10,000-Da centrifugal filters (Millipore, Bedford, MA). Reducing buffer was added to 40 μl aliquots of concentrated urine and boiled for 5 minutes. The samples were then resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred onto polyvinylidene difluoride membrane (GE Health care, Piscataway, NJ). Blots were stained with primary guinea pig polyclonal anti-nephrin antibody (Fitzgerald Industries, Concord, MA) and rabbit polyclonal anti-podocin antibody (Abcam, Cambridge, MA) used at dilutions of 1:500 and 1:1000 respectively. HRP-conjugated secondary antibodies (Abcam) were used at dilutions of 1:2000 and 1:4000 respectively. Detection was performed using enhanced chemiluminescence detection system (GE Health care).

For cell culture studies, protein was harvested from growth-restricted SPARC^{+/+}, SPARC^{-/-}, SPARC^{-/-} transfected with SPARC cDNA (SP^{-/-} cDNA), and SPARC^{-/-} transfected with the empty plasmid vector (SP^{-/-} empty) cultured podocytes. Cells were scraped on ice using lysis buffer [1% Triton X-100, 50 mmol/L Tris-HCL, 5 mmol/L EDTA, 150 mmol/L NaCl, 2.5 mmol/L Na_4PO_7 (pH 7.4)] that contained 100 mmol/L NaF, 2

mmol/L Na_3VO_4 , and protease inhibitors (Roche). Following an overnight freeze-thaw cycle, lysates were cleared by centrifugation at $17,000 \times g$ for 10 minutes at 4°C and protein concentration determined by BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's directions. Reducing buffer was added to the protein extract and boiled for 5 minutes. Ten-microgram samples were then resolved by SDS-polyacrylamide gel electrophoresis. Electro-transfer and antibody staining were performed as described above using goat polyclonal anti-SPARC antibody (R&D industries, Milford, IA) and mouse monoclonal anti- β -actin antibody (Abcam) as a loading control.

Cell Culture Studies

Conditionally Immortalized Mouse Podocyte Line

Female SPARC^{-/-} mice were crossed with a male H-2K^b-tsA58 transgenic mouse (ImmortoMouse; Jackson Laboratory, Bar Harbor, ME) and the F1 generation intercrossed. Conditionally immortalized mouse podocytes were derived from SPARC^{+/+} and SPARC^{-/-} littermates as described previously.^{16,17} Experiments were performed using early passage⁸⁻¹⁶ growth-restricted, podocytes as we have previously reported.^{18,19} When grown under permissive conditions (in the presence of interferon- γ at 33°C), cells proliferate and display characteristics of undifferentiated podocytes. This is required to propagate the cells in culture. However, under growth-restricted conditions (absence of interferon- γ at 37°C), proliferation is markedly reduced and cells undergo cytoskeletal rearrangement with the formation of arborizing cellular processes and express podocyte-specific proteins, resembling the morphological appearance of mature differentiated podocytes *in vivo*.^{20,21} Cells were grown on collagen type 1-coated plates in RPMI 1640 media containing 10% fetal bovine serum (Summit Biotechnology, Ft Collins, CO), penicillin (100U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), glutamine (2mmol/L) sodium pyruvate (1 mmol/L, Irvine Scientific, Santa Ana, CA), HEPES buffer (10 mmol/L, Sigma Chemical Co., St Louis, MO), and sodium bicarbonate (0.075%, Sigma). Cells were growth-restricted for greater than 10 days at 37°C in 95% air/5% CO_2 .

Stable Transfection of SPARC Expression Plasmid

SPARC^{-/-} podocytes were stably transfected with full-length murine SPARC expression plasmid (gift from Dr. E. Helene Sage), or the empty plasmid inserted into the Nhe1/BamH1 sites of pcDNA3.1/Zeo (+) vector (Invitrogen, Carlsbad, CA) using N-Fect reagent (Neuromics, Edina, MN) according to manufacturer's instructions. Transfected cells were selected in RPMI 1640 media supplemented with Zeocin, 250 $\mu\text{g}/\text{ml}$ (Invitrogen), and then the cells were maintained in media with Zeocin, 125 $\mu\text{g}/\text{ml}$. Medium was changed every three to four days. Podocytes were characterized by staining and localization of the podocyte proteins WT-1 (Santa, Cruz), nephrin

(Fitzgerald Ind.), podocin (Abcam), CD2AP (Santa Cruz), podocalyxin (Abcam), and ezrin (Millipore).

Detachment Assay

Mechanical strain: Growth-permissive, conditionally immortalized podocytes were seeded on flexible six-well plates coated with bovine collagen type-1 (Flexcell International Corporation, Hillsborough, NC) at a density of approximately 75,000 cells per well. Cells were switched to growth restrictive conditions for 12 days, yielding a final confluency of 25% to 40%. Mechanical strain was induced using a computer-assisted stretch apparatus (FlexerCell Strain Unit 3000T) as we previously reported.²² Intermittent negative pressure was applied to the biomembrane by a vacuum, resulting in cyclic stretch and relaxation of the adherent cell layer. Based on prior studies by our group, a regimen of 60 cycles of stretch and relaxation per minute with an amplitude of 10% bi-axial surface elongation was uniformly applied across the membrane. Cells grown under identical conditions (within the same incubator as the strain unit), but not exposed to stretch, served as controls. To measure cell detachment, supernatant was pooled from three wells from each experimental condition, and pelleted by centrifugation (1200 rpm for 5 minutes at 4°C). Cells were then resuspended in 0.45% Trypan Blue and quantitated by hemacytometer. To determine the percentage of detached cells, total viable cell number was determined by performing MTT assay (Promega) as we have previously reported.²³

Trypsin digest: Growth-restricted, conditionally immortalized podocytes at 90% confluence were exposed to a solution of $1 \times$ trypsin (Irvine Scientific, Santa Ana, CA) and 0.1% collagenase (Sigma-Aldrich) for 2, 5, 8, 10, and 12 minutes' duration at 37°C . The supernatant was collected at each time point and detached cells were counted via Trypan Blue dye exclusion as described above. The remaining adherent cells were quantified following prolonged exposure to trypsin collagenase solution and vigorously aspirated (to ensure all cells were removed from the tissue culture plate).

Statistical Analysis

All results are expressed as mean $\pm$ SD. Statistical analysis was performed using paired *t*-test or analysis of variance with a Bonferroni-Dunn correction (Statview 5.0, Abacus Concepts, Berkeley, CA). A *P* value <0.05 was considered statistically significant.

Results

Induction of Passive Nephrotoxic Nephritis Is Equivalent in SPARC^{+/+} and SPARC^{-/-} Mice

To determine the biological role of SPARC in the progression of immune-mediated experimental glomerulonephritis, the passive nephrotoxic nephritis model was used as

Figure 1. Induction of passive nephrotoxic nephritis is equivalent in $SPARC^{+/+}$ and $SPARC^{-/-}$ mice. Immunofluorescence staining was performed to confirm that disease induction was similar in $SPARC^{+/+}$ and $SPARC^{-/-}$ mice. Deposition of sheep IgG at day 7 was used to assess the heterologous phase of disease induction and revealed similar intensity and pattern of staining in $SPARC^{+/+}$ (A) and $SPARC^{-/-}$ (B) mice. Staining for mouse IgG as an indicator of the autologous phase of disease likewise revealed comparable staining in the $SPARC^{+/+}$ (D) and $SPARC^{-/-}$ (E) mice. The absence of sheep and mouse IgG deposition (C and F respectively) before disease induction confirms specificity of staining.

we have previously reported.¹¹ Injection of sheep anti-rabbit glomerular antibody results in severe glomerular injury, notable for crescent formation and proteinuria by day 7. Podocyte damage is an early prominent feature of this model, and a progressive reduction in podocyte number is implicated in the subsequent development of glomerulosclerosis and tubulointerstitial fibrosis. To ensure induction of disease was comparable between the $SPARC^{+/+}$ and $SPARC^{-/-}$ groups, immunofluorescence staining was performed to assess glomerular antibody deposition. As shown in Figure 1, linear deposition of sheep IgG and mouse IgG was noted, consistent with the heterologous (Figure 1, A and B) and autologous (Figure 1, D and E) phases of disease induction respectively. Furthermore, the intensity of glomerular staining was similar in both $SPARC^{+/+}$ and $SPARC^{-/-}$ mice, suggesting no differences with respect to disease induction between the two groups. The absence of IgG deposition in normal mice confirmed specificity of staining (Figure 1, C and F).

Podocyte Levels of SPARC Are Markedly Increased in the Passive Nephrotoxic Nephritis Model

While injury and reduced podocyte number are prominent features of the passive nephrotoxic nephritis model, the underlying mechanisms are not known. To test the hypothesis that increased SPARC was in part causal, we performed immunohistochemistry in nephritic $SPARC^{+/+}$ and $SPARC^{-/-}$ mice, and the results are shown in Figure 2. While faint immunostaining for SPARC was detected in the glomeruli of $SPARC^{+/+}$ mice before disease induction (Figure 2A, arrowheads), there was a marked increase in intensity of staining for SPARC at day 4 (data not shown) and day 7 (Figure 2C, arrows). Whereas occasional staining of the parietal epithelial cell layer was noted, increased SPARC staining was predominantly localized to the periphery of the glomerular tuft, consistent with a podocyte distribution (Figure 2C, arrows). The absence of glomerular staining in $SPARC^{-/-}$ at baseline and day 7 following disease induction (Figure 2, B and D respectively) confirms specificity of SPARC staining.

7 following disease induction (Figure 2, B and D respectively) confirms specificity of SPARC staining.

Increased SPARC Leads to Accelerated Podocyte Loss in Passive Nephrotoxic Nephritis

To test the hypothesis that increased SPARC reduced podocyte number, podocytes were identified in glomerular sections based on positive staining for the transcription factor WT-1, as previously reported by our group and others.^{24,25} Notably, as shown in Figure 3, A and B, the

Figure 2. Podocyte levels of SPARC are increased in the passive nephrotoxic nephritis model. Immunohistochemistry for SPARC was performed in mice following induction of passive nephrotoxic nephritis. While low levels of SPARC immunostaining were detected in the glomerulus of $SPARC^{+/+}$ mice before disease induction (A, arrowheads), a marked increase in intensity of staining for SPARC is evident at day 7 in primarily a podocyte distribution (C, arrows). The absence of glomerular staining in $SPARC^{-/-}$ at baseline and day 7 following disease induction (B and D, respectively) confirms specificity of SPARC staining.

Figure 3. Podocyte number is better preserved in SPARC^{-/-} mice following induction of passive nephrotoxic nephritis. **A:** Representative sections of WT-1 immunostaining are shown at days 0 and 21 following disease induction in SPARC^{+/+} and SPARC^{-/-} mice. **B:** The average number of podocytes per glomerulus was determined by counting WT-1 staining in 20 consecutive glomeruli in SPARC^{+/+} and SPARC^{-/-} mice. The number of WT-1 positive cells was similar between the two groups at baseline (day 0). While a progressive reduction in WT-1 positive cells was noted in the SPARC^{+/+} group at days 4, 7, and 21 following disease induction (* $P < 0.05$, ** $P < 0.001$ vs. baseline), podocyte number was over-all better preserved in the ^{-/-} group. Furthermore, the number of WT-1 positive cells in SPARC^{+/+} mice was significantly reduced at days 7 and 21 (* $P < 0.05$, ** $P < 0.003$) when compared with their SPARC^{-/-} counterparts.

number of WT-1 positive cells was identical in SPARC^{+/+} and SPARC^{-/-} mice at baseline (day 0). However, following disease induction, there was a significant progressive reduction in the number of WT-1 positive cells at day 4 $\times 14\%$ ($P < 0.05$), day 7 $\times 24\%$ ($P < 0.001$), and day 21 $\times 33\%$ ($P < 0.001$), as compared with ^{+/+} animals without disease. In contrast, the number of cells staining positive for WT-1 did not decrease significantly in nephritic SPARC^{-/-} mice at similar time points following disease induction. Furthermore, podocyte number was significantly reduced in SPARC^{+/+} mice at days 7 ($P < 0.05$) and 21 ($P < 0.003$), as compared with their SPARC^{-/-} counterparts. Taken together, these results demonstrate that an increase in SPARC levels correlated with reduced podocyte number, whereas absence of SPARC in diseased animals preserved podocyte number.

Proteinuria Is Absent in Diseased SPARC^{-/-} Mice

Given the critical role of podocytes in maintaining integrity of the permselective glomerular filtration barrier, we

Figure 4. Proteinuria is less severe in SPARC^{-/-} mice following induction of passive nephrotoxic nephritis. Total urinary protein excretion in SPARC^{+/+} and SPARC^{-/-} was measured in a 12-hour urine collection. Proteinuria at baseline was similar between the two groups. While proteinuria was significantly increased in SPARC^{+/+} mice at day 7 and day 21 following disease induction (* $P < 0.01$ vs. baseline), no significant increase was noted in SPARC^{-/-} mice. Furthermore, proteinuria in the SPARC^{+/+} group was significantly increased at day 7 versus the SPARC^{-/-} group (* $P < 0.05$), and this trend persisted at day 21 ($P = 0.06$).

queried whether the accelerated loss of podocytes in SPARC^{+/+} mice was associated with more severe proteinuria compared with their SPARC^{-/-} counterparts. Baseline proteinuria, albeit it very low, was similar between normal SPARC^{+/+} and SPARC^{-/-} mice (Figure 4). However, following disease induction, a sixfold increase in urinary protein excretion was noted in the SPARC^{+/+} mice at day 7 vs. baseline ($P < 0.01$), and the increase remained persistently elevated at day 21 ($P = 0.06$). In contrast, there was no significant elevation in proteinuria at either time point in diseased SPARC^{-/-} mice.

Glomerulosclerosis Is Less Severe in SPARC^{-/-} Mice

Compelling experimental evidence supports the notion that a progressive reduction in podocyte number contributes to the future development of glomerulosclerosis.^{26–30} Because podocyte number was still preserved following experimental podocyte injury in the absence of SPARC, we next determined whether glomerular injury was milder in diseased SPARC^{-/-} mice (Figure 5A). As shown in Figure 5B, glomerulosclerosis was absent SPARC^{+/+} and SPARC^{-/-} mice without injury. In diseased SPARC^{+/+} mice, there was a progressive increase in glomerulosclerosis by 4.4-fold at day 7 and 6.6-fold at day 21 ($P < 0.0001$ vs. normal). Though glomerulosclerosis was also increased in diseased SPARC^{-/-} mice at each time point, the degree of injury was milder when compared with diseased SPARC^{+/+} mice (day 7: 1.01 ± 0.27 vs. 1.65 ± 0.18 ; day 21: 1.43 ± 0.38 vs. 2.49 ± 0.29 , $P < 0.005$). Electron microscopy revealed increased matrix accumulation with obliteration of capillary loops in glomeruli from SPARC^{+/+} mice compared with glomeruli from SPARC^{-/-} mice where capillary loops were patent. At higher power, diffuse foot process effacement was

Figure 5. Glomerular injury is less severe in SPARC^{-/-} mice following induction of passive nephrotoxic nephritis. **A:** Periodic acid Schiff staining from representative sections in SPARC^{+/+} and SPARC^{-/-} mice at days 0, 7, and 21 following disease induction is shown. **B:** Glomerular injury was scored in SPARC^{+/+} and SPARC^{-/-} mice in 20 consecutive glomeruli per animal. The degree of glomerulosclerosis at baseline in unmanipulated animals was equivalent in both groups. While a progressive increase in the glomerulosclerosis score was noted at day 7 and day 21 following disease induction in both groups, the degree of glomerular injury was attenuated in the SPARC^{-/-} mice when compared with their SPARC^{+/+} counterparts (**P* < 0.005). **C:** Transmission electron microscopy of glomeruli from SPARC^{+/+} and SPARC^{-/-} mice is shown. Capillary loops are obliterated by matrix accumulation in SPARC^{+/+} mice (**C, a, arrowhead**; magnification $\times 1600$) whereas capillary loops are patent in glomeruli from SPARC^{-/-} mice (**c, arrowhead**). At higher power (magnification $\times 7100$), foot process effacement is demonstrated in glomeruli from SPARC^{+/+} mice (**b, long arrow**) compared with SPARC^{-/-} mice where foot process architecture is better preserved (**d, short arrow**). P = podocyte cell body.

Figure 6. Nephrin and podocin as markers of urinary podocyte loss are attenuated in SPARC^{-/-} mice following induction of passive nephrotoxic nephritis. Representative Western blots for nephrin and podocin of urine collected from three sets of diseased SPARC^{+/+} and SPARC^{-/-} mice. Samples were collected via bladder puncture at day 14 following induction of passive nephrotoxic nephritis and 40 μ l of concentrated urine per each animal was resolved by SDS-polyacrylamide gel electrophoresis. The intensity of the nephrin and podocin bands is weaker in SPARC^{-/-} relative to SPARC^{+/+} mice in all 3 groups.

demonstrated in affected glomeruli from SPARC^{+/+} mice whereas podocyte foot processes were well preserved in glomeruli from SPARC^{-/-} mice (Figure 5C). These findings demonstrate that glomerulosclerosis was more severe in disease associated with increased SPARC levels in podocytes.

Nephrin and Podocin as Markers of Urinary Podocyte Loss Are Attenuated in Diseased SPARC^{-/-} Mice

Podocyuria has been reported in several forms of experimental and clinical glomerular disease.^{25,31–35} We queried whether a potential mechanism underlying the reduced podocyte number in SPARC^{+/+} mice as compared with SPARC^{-/-} was detachment from the underlying glomerular basement membrane. Accordingly, urinary Western blot analysis was performed for specific podocyte markers as has been recently reported by others.^{24,36} Urine was collected from animals 14 days following induction of passive nephrotoxic nephritis and immunoblotting for nephrin was performed. As shown in Figure 6, a distinct single band at ~ 185 kDa consistent with nephrin was seen in urine of diseased SPARC^{+/+} mice, but not in diseased SPARC^{-/-} littermates. Probing for podocin as a second marker of podocyte loss in the urine yielded similar results. These findings suggest that podocyte detachment occurring in a SPARC-dependent fashion may be the underlying mechanism responsible for the progressive reduction in podocyte number seen in passive nephrotoxic nephritis.

SPARC Has a Causal Role in Promoting Primary Podocyte Detachment

To establish a causal role for increased SPARC in mediating podocyte detachment, an *in vitro*-based model system was used. We have previously shown that mechanical strain of podocytes, characteristic of

Figure 7. SPARC^{-/-} podocytes detach less as compared with SPARC^{+/+} podocytes. **A:** Mechanical strain-induced detachment is attenuated in SPARC^{-/-} podocytes. SPARC^{+/+} and SPARC^{-/-} growth-restricted podocytes were cultured on a flexible collagen-coated biomembrane and subjected to cyclical stretch of 10% amplitude. Supernatant was collected at 24 hours and detachment assay for viable cells performed based on Trypan blue dye exclusion. When compared with SPARC^{+/+} cells, SPARC^{-/-} podocytes were 26% more resistant to stretch-induced detachment (**P* < 0.05). When SPARC was stably re-expressed in SPARC^{-/-} cells (SPARC^{-/-} cDNA) detachment rate was comparable with SPARC^{+/+} cells, whereas transfection of SPARC^{-/-} cells with the empty plasmid vector only had no effect (SPARC^{-/-} empty). **B:** SPARC^{-/-} podocytes resist trypsin digest. SPARC^{+/+} and SPARC^{-/-} growth-restricted podocytes were exposed to a solution of 1× trypsin and 0.1% collagenase at various time points. Detachment of viable cells was quantified via Trypan blue dye exclusion. Trypsin-induced detachment was significantly less in SPARC^{-/-} podocytes compared with SPARC^{+/+} podocytes at 2 to 8 minutes. **P* < 0.05, ***P* < 0.01. Repeating detachment assays in SPARC^{-/-} cDNA podocytes restored detachment rates to that of SPARC^{+/+} podocytes, whereas detachment rates in SPARC^{-/-} empty did not differ from SPARC^{-/-} podocytes. **P* < 0.05, ***P* < 0.01 SPARC^{-/-} cDNA vs. SPARC^{-/-} empty. Experiments were performed in triplicate and on at least three separate occasions. **C:** A representative Western blot for SPARC confirmed the presence of SPARC at 43 kDa in SPARC^{+/+} podocytes (top panel). β-actin (42 kDa) was used for protein loading control (bottom panel).

states of glomerular capillary hypertension, increases SPARC expression *in vivo* and *in vitro*.⁵ Accordingly, growth-restricted conditionally immortalized SPARC^{+/+} and SPARC^{-/-} podocytes were subjected to cyclical stretch and detachment assay for viable cells performed at 24 hours. As shown in Figure 7, A and C, when compared with their SPARC^{+/+} counterparts, podocytes lacking SPARC were significantly more resistant to the effects

of mechanical strain as a 26% reduction in stretch-induced detachment was noted (*P* < 0.05). To demonstrate that the difference between SPARC^{-/-} and SPARC^{+/+} cells was not due to issues of cell clone or passage number, SPARC was reintroduced in the SPARC^{-/-} cells and studies repeated. Stable transfection of SPARC^{-/-} cells with SPARC-expressing vector (SPARC^{-/-} cDNA) restored detachment rates to comparable levels of SPARC^{+/+} cells, whereas transfection with empty vector alone (SPARC^{-/-} empty) had no effect.

As a second method to evaluate podocyte detachment *in vitro*, trypsin digests were performed. Growth-restricted SPARC^{+/+} and SPARC^{-/-} podocytes were exposed to a solution of trypsin and collagenase and detachment assays for viable cells were performed serially. A 44% reduction in the rate of detachment of SPARC^{-/-} podocytes was observed at 2 minutes compared with SPARC^{+/+} counterparts. Furthermore, the increased resistance of SPARC^{-/-} podocytes to trypsin-induced detachment was sustained as shown in Figure 7B. When SPARC was stably re-expressed in SPARC^{-/-} podocytes, detachment rates following trypsin exposure were restored to that of SPARC^{+/+} podocytes, while transfection of the empty vector had no effect. To account for possible artifacts related to clonal variations, all detachment studies were validated in a second SPARC^{+/+} and SPARC^{-/-} cell line. Taken together, the results clearly demonstrate a causal role for SPARC in mediating podocyte detachment following injury *in vitro*.

Discussion

The podocyte is a known target of the immune system in many forms of chronic progressive glomerular disease in man. Increased levels of SPARC following immune-mediated injury of the podocyte are well documented experimentally and clinically.^{5,6} The role of SPARC in regulating glomerular damage is likely dependent on the site and type of injury. In diseases of the mesangium, which typically involve a proliferative response, SPARC, through its anti-mitogenic properties, has a beneficial effect and contributes to disease resolution.^{4,37,38} However, in diseases of the podocyte, increased levels of SPARC are likely maladaptive and worsen renal disease. Studies in an experimental model of diabetic nephropathy revealed that in the absence of SPARC, glomerular injury was attenuated with less severe glomerulosclerosis and matrix accumulation, associated with reduced albuminuria.⁷ In a clinical study of type II diabetes, increased levels of SPARC correlated with the severity of albuminuria.⁶ Furthermore, in a murine model of lupus nephritis, SPARC was significantly increased early in the disease course specifically in podocytes, and preceded the onset of proteinuria.³⁹ However, the mechanisms by which SPARC worsens diseases of the podocyte are yet to be elucidated.

In the current study, we have established a causal role for SPARC in the progression of glomerular disease following immune-mediated injury of the podocyte. We have specifically chosen the passive nephrotoxic nephritis

model, an established murine model of chronic glomerular disease associated with significant podocyte injury and loss. Following injection of heterologous anti-glomerular antibody, a marked increase of glomerular SPARC levels was noted predominantly in podocytes, suggesting that the podocyte indeed is a primary target in this disease model. By day 4, a reduction in podocyte number was seen, associated with development of proteinuria and glomerulosclerosis by day 7. In contrast, when passive nephrotoxic nephritis model was recapitulated in SPARC^{-/-} mice, though disease induction was similar to the SPARC^{+/+} counterparts, podocyte number was better preserved and associated with a notable absence of proteinuria and less severe glomerular scarring. Taken together, our findings support a causal role for induction of the matricellular protein SPARC in mediating podocyte loss, thereby accelerating glomerular injury in the passive nephrotoxic nephritis model.

SPARC binds to several collagens (including types I, III, IV, and VIII), and mediates extracellular matrix deposition through regulation of several secreted proteins and matrix metalloproteinases.⁴ Increased SPARC levels in fibroblasts have been implicated in the pathogenesis of fibrosis in several rodent models of chronic tubulointerstitial disease.^{40,41} Could glomerulosclerosis in our model of passive nephrotoxic nephritis result from direct action of SPARC on matrix components rather than an accelerated reduction in podocyte number? Wharram et al convincingly demonstrated the causal role of podocyte depletion in the subsequent development of glomerulosclerosis. In this model, diphtheria toxin was used to selectively knock out podocytes in transgenic rats. A reduction in podocyte number of 20% or greater was associated with synechia formation and focal segmental glomerulosclerosis.³⁰ In our model, we found a 24% reduction in podocyte number in SPARC^{+/+} mice at day 7 with associated glomerulosclerosis and proteinuria. These results indicate that podocyte loss in response to increased SPARC may be an important cause of glomerulosclerosis in passive nephrotoxic nephritis. Future studies with conditional SPARC knockout mice (podocyte specific deletion of SPARC) will be helpful in further establishing the role of SPARC in progression of immune-mediated podocyte disease.

To elucidate the potential mechanism by which SPARC causes podocyte loss *in vivo*, we focused our attention on primary detachment. Tethered to the glomerular basement membrane via a network of integrins along the basolateral aspect of foot processes, podocytes are suspended in the urinary space. When damaged, podocytes predictably detach from the underlying glomerular basement membrane to be ultimately washed away in the urine. Indeed, "podocyuria" has been reported in various proteinuric states and proposed as a marker of disease severity in glomerular diseases clinically.^{25,32,35,42} Reports by our group and others have confirmed that podocytes isolated from the urine may be cultured *ex vivo* and retain expression of differentiation markers.^{33,35} These observations underscore a very important concept. Detachment may not merely represent a terminal event resulting from irreversible damage, but may occur early in

the response to injury, when the cell is still viable. Data from patients with glomerular disease are consistent with this hypothesis.^{31,35,42-44} Thus, these observations argue that primary cellular detachment is an under-appreciated, yet critical mechanism of reduced podocyte number in chronic glomerular disease.

In the current study, we demonstrated that while nephrin and podocin were detectable in the urine of SPARC^{+/-} mice following induction of passive nephrotoxic nephritis, levels were significantly attenuated in diseased animals lacking SPARC. These findings suggest that SPARC-dependent detachment of podocytes plays a causal role in podocytopenia in states of immune-mediated podocyte injury. A limitation of urinary westerns for podocyte biomarkers is the inability to distinguish podocyte fragments retaining podocyte specific proteins from intact viable podocytes in the urine. To definitively establish a causal role for SPARC in mediating primary detachment of podocytes, an *in vitro* based system was used. Our group has previously shown that in states of glomerular capillary hypertension, the resultant mechanical strain leads to a maladaptive podocyte response associated with increased SPARC expression both *in vitro* and *in vivo*.⁵ Accordingly, when conditionally immortalized mouse podocytes derived from SPARC^{+/-} and SPARC^{-/-} mice were subjected to cyclical stretch, fewer cells detached in podocytes lacking SPARC compared with their wild-type counterparts. Stable transfection of SPARC^{-/-} podocytes to re-express SPARC restored the wild-type phenotype with comparable rates of detachment, confirming a causal role for SPARC in mediating detachment in response to mechanical strain. Furthermore, exposure of podocytes to trypsin as a second method of inducing detachment yielded similar results. Taken together, these findings implicate a central role for the matricellular protein SPARC in mediating a progressive reduction in podocyte number in states of podocyte disease through the mechanism of primary detachment.

Why should SPARC be increased in various forms of podocyte disease? In addition to its counteradhesive properties, SPARC has also been identified to promote cell survival through ILK activation in glioma cells and cultured lens epithelial cells.^{45,46} We speculate that SPARC may have pro-survival effects in podocytes as well and have observed that rates of apoptosis in cultured podocytes are increased in the absence of SPARC (unpublished data). Therefore, increased SPARC following podocyte injury may be pro-survival; however, if SPARC levels are increased beyond a certain threshold, the counteradhesive effects may prevail leading to podocyte detachment and a reduction in podocyte number.

How does SPARC cause detachment in podocyte injury? Cell attachment to underlying matrix is a dynamic and fluid process whereby the cell may transition between an adhesive state and de-adhesive state. During the intermediate state of adhesion, restructuring of focal adhesions and stress fibers occurs while still maintaining cell shape.⁴⁷ This intermediate state of adhesion is thought to favor cell migration, an important role for SPARC in tumor metastasis.³ De-adhesion further involves transition from an intermediate state of adherence

to a state of weak adherence marked by a change in cell shape from a spread to rounded morphology. SPARC is known to mediate this activity, having been shown to induce cell rounding in a variety of cell types.⁴⁸ With restructuring of focal adhesions and disruption of extracellular matrix-integrin interaction, a rounded cell can no longer bind to its substrate favoring detachment. Weaver et al have shown that SPARC inhibits adherence of lens epithelial cells by several potentially related mechanisms including down-regulation of focal adhesions and $\alpha 6$ -integrin heterodimer formation as well as reduced phosphorylation of paxillin, a key focal adhesion protein.⁴⁹ The biochemical signaling that mediates this process, however, has yet to be elucidated.

In summary, using a combined *in vivo-in vitro* approach, we demonstrated that SPARC plays a causal role in mediating detachment of podocytes thereby accelerating glomerulosclerosis in an experimental model of crescentic glomerulonephritis. Further studies will be required to elucidate the precise biochemical mechanism by which SPARC mediates detachment, and will serve to broaden our understanding of the podocyte-glomerular basement membrane interaction in states of glomerular health and disease.

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