RESEARCH ARTICLE | Mechanism and Treatment of Renal Fibrosis

Pericytes and immune cells contribute to complement activation in tubulointerstitial fibrosis

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Xavier S, Sahu RK, Landes SG, Yu J, Taylor RP, Ayyadevara S, Megyesi J, Stallcup WB, Duffield JS, Reis ES, Lambris JD, Portilla D. Pericytes and immune cells contribute to complement activation in tubulointerstitial fibrosis. Am J Physiol Renal Physiol 312: F516–F532, 2017. First published January 3, 2017; doi:10.1152/ajprenal.00604.2016.—We have examined the pathogenic role of increased complement expression and activation during kidney fibrosis. Here, we show that PDGFRα-positive pericytes isolated from mice subjected to obstructive or folic acid injury secrete C1q. This was associated with increased production of proinflammatory cytokines, extracellular matrix components, collagen, and increased Wnt3a-mediated activation of Wnt/β-catenin signaling, which are hallmarks of myofibroblast activation. Real-time PCR, immunoblots, immunohistochemistry, and flow cytometry analysis performed in whole kidney tissue confirmed increased expression of C1q, C1r, and C1s as well as complement activation, which is measured as increased synthesis of C3 fragments predominantly in the interstitial compartment. Flow studies localized increased C1q expression to PDGFRα-positive pericytes as well as to CD45-positive cells. Although deletion of C1qA did not prevent kidney fibrosis, global deletion of C3 reduced macrophage infiltration, reduced synthesis of C3 fragments, and reduced fibrosis. Clodronate mediated depletion of CD11bF4/80 high macrophages in UUO mice also reduced complement gene expression and reduced fibrosis. Our studies demonstrate local synthesis of complement by both PDGFRα-positive pericytes and CD45-positive cells in kidney fibrosis. Inhibition of complement activation represents a novel therapeutic target to ameliorate fibrosis and progression of chronic kidney disease.

Activated fibroblasts, termed myofibroblasts, are the cells most likely responsible for increased production and deposition of extracellular matrix, including collagens and matrix proteins (28). The source of interstitial myofibroblasts in kidney disease is an area of current controversy (13, 53). Recent studies have identified pericytes as a major source of precursors of scar-producing myofibroblasts during kidney fibrosis (12). When pericytes are activated, they migrate from the pericapillary area to the interstitial space and contribute to vascular instability, an important mechanism for the progression of kidney disease (27, 28, 47). Previous work by several investigators has identified numerous secreted factors that contribute to the persistence of fibrotic response, including transforming growth factor-β (TGFβ), VEGF, connective tissue growth factor, matrix metalloproteinases, WNT ligands, and platelet-derived growth factors (PDGFs) (12, 61, 63, 64). A recent study using Col1A2-Cre and tenascin Cre system to conditionally delete TGFβ type 2 receptor on collagen-producing fibroblasts and medullary interstitial cells in the model of unilateral ureteral obstruction (UUO) demonstrated reduced collagen production but no significant effect on kidney tissue fibrosis, suggesting that signaling pathways other than TGFβ could contribute to the pathogenesis of renal fibrosis (35).

Additional factors that could be involved in the pathogenesis of kidney fibrosis include the activation of the immune response. A component of the innate immune response that has been demonstrated to contribute to renal inflammation is the complement system, which can directly affect cell function and also influence the adaptive immune response (46, 51). The complement system has been characterized primarily as a host defense system that can be activated in the presence of injured cells, accumulating debris, or foreign materials (44). However, recent studies support the notion that an overly active complement system can turn this protective response to a destructive one that drives disease pathogenesis (14, 38, 45). Also, new studies show that complement is regulated not only in the intravascular space but also through local secretion of complement components by injured tissue and infiltrating cells as well as by intracellular complement turnover that contributes to the

THE PREVALENCE OF CHRONIC KIDNEY DISEASE (CKD) in the US is high and continues to rise in our aging population (6). Independent of the cause of CKD, tubulointerstitial fibrosis and glomerulosclerosis represent major pathways of progression of kidney disease; however, there are no approved drugs to treat kidney fibrosis and/or ameliorate progression of CKD (54).

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complement response in various pathological conditions (23, 66). Complement activation occurs through three distinct pathways: the classical pathway (CP) that involves antibody-mediated activation of the C1 complex comprising of C1q, C1r, and C1s, the lectin pathway (LP) triggered by carbohydrate signatures on cell surfaces, and the alternative pathway (AP), which involves direct activation of C3 through surface and properdin (FP) binding of C3b. Within the C1 complex, C1r and C1s are serine proteases responsible for initiating the classical complement pathway. Binding via C1q to immune complexed IgG or to certain pathogens induces a conformational change in C1q that activates the proteases in a stepwise fashion: C1r first autoactivates and then activates C1s. C1s subsequently cleaves substrates C4 and C4 bound C2 to form the C3 convertase (C4b2a), the downstream component of the reaction cascade. Seminal observations have provided a considerable understanding of the important role of AP in kidney disease (32, 36, 37, 55, 58). Upon activation of the complement system, C3 and C5 are among the proteins that are cleaved, thus generating several active downstream products, including anaphylatoxins C3a and C5a. In previous studies using animal models of ischemia reperfusion injury (IRI), complement activation was demonstrated primarily via activation of the alternate and the lectin pathway during acute kidney injury (AKI) (40, 56, 65). Additional studies have characterized the importance of tubulo-interstitial fibrosis in AKI by elucidating the functional role of complement inhibitor Crry expressed in the proximal tubule (57). In addition, recent studies suggest that interstitial fibrosis and macrophage infiltration are significantly reduced in C5-deficient (C5−/−) mice (2), and moreover, C3 deficiency limits renal fibrosis by suppressing EMT processes during degeneration of the nephrotubulus (67). Those above studies had demonstrated complement activation predominantly in renal tubular epithelial cells and proximal tubules (2, 67).

In the present study, we examined whether nonimmune interstitial cells such as PDGF receptor-β (PDGFRβ)-positive pericytes and immune cells isolated from UUO and folic acid-treated mice synthesize and secrete complement components when compared with sham pericytes. In addition, we used mice with deletion of specific complement genes C1qA or C3 to examine the role of complement activation in kidney fibrosis.

METHODS

Animals and reagents. C1qA−/− mice with targeted disruption of exon 1 of the C1qa gene in C57BL/6 background were obtained from Dr. Andrea Tenner’s laboratory (made by Dr. Marina Botto). C5−/− mice (strain B6.129S4-C3tm1Crr/J) of C57BL/6 genetic background and wild-type (WT) mice (C57BL/6) were obtained from The Jackson Laboratory (Bar Harbor, ME). Antibodies included monoclonal antibody to mouse C1q (Clone JL-1) from Hyclut Biotech (Plymouth Meeting, PA), rabbit polyclonal antibody to C1q from Abcam (Cambridge, MA), rat monoclonal antibody to mouse C3b/C3/C3e (clone 2/11, which was reported previously) (31), rabbit polyclonal α-SMA from Abcam (Cambridge, MA), mouse monoclonal PCNA from Biolegend (San Diego, CA), rabbit polyclonal F4/80 from Abcam, and mouse monoclonal GAPDH from Santa Cruz Biotechnology (Dallas, TX). For immunofluorescence staining, rabbit monoclonal antibody to C1q was conjugated with Alexa Fluor 488 using the antibody labeling kit (Molecular Probes; Life Technologies, Eugene, OR) according to the manufacturer’s instructions.

Renal fibrosis models. All experiments were performed in accordance with the National Institutes of Health’s (NIH) Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the University of Virginia Animal Care and Use Committee. For the UUO model, 8- to 10-wk-old male mice (C57BL/6 WT, C1qA−/−, or C3−/− mice) were used. The left kidney was exposed through a midline incision under sterile conditions, and the ureter was dissected and securely tied at two places with 6-0 silk sutures. Volume depletion was prevented by administration of 0.1 ml of saline into the peritoneal cavity. The midline incision was closed; the mice were returned to their cages and allowed free access to food and water. As control, sham surgery was performed the same way as UUO without tying the ureter. On days 3 and 10, mice were euthanized and the left kidneys from UUO and sham mice collected for protein, RNA isolation, and histological evaluation. For the folic acid model, 8- to 10-wk-old male mice (C57BL/6 WT, C1qA−/− or C3−/− mice) received intraperitoneal injection of folic acid (250 μg/g body weight). Mice were euthanized after 2 wk, and kidney tissue was harvested.

Isolation of pericytes/fibroblast from sham, UUO, and folic acid-treated mice. To isolate PDGFRβ-positive pericytes from sham and UUO kidneys, kidney tissue was minced and digested in Liberase/DNase mix for 30 min at 37°C with vigorous shaking (48). The mixture was filtered with a 40-μm nylon mesh, and single cells were spun down, resuspended in MACS buffer, and incubated with rabbit anti-mouse PDGFRβ antibody (39) for 15 min on ice, followed by incubation with goat anti-rabbit IgG Microbeads (Miltenyi Biotec, San Diego, CA). Cells were washed and applied to a MACS affinity column under a magnetic field. Magnetic bead column-purified cells were cultured in DMEM-F-12 with 10% FBS and insulin/transferrin/selenium on gelatin-coated dishes. Enrichment of CD45 cells from kidney cell suspension. Kidney cell suspension was prepared as described previously. The cells were suspended in PEB buffer (PBS + 2 mM EDTA + 0.5% bovine serum albumin) and incubated on ice for 30 min and blocked with Fc block. Then the cells were stained for CD45 and attached to magnetic beads as per the instructions of the manufacturer to positively select the CD45 cells using the Magisort mouse CD45.2 + ve selection kit (eBiosciences). The positive cells were separated using an AU-TOMACS system (Miltenyi Biotec). Positive cells were then washed briefly in phosphate buffer and lysed for RNA preparation, and real-time PCR analysis for various genes was done.

Protein analysis of UUO secretome. Proteins isolated from supernatants of sham and UUO mice were separated on two- or one-dimensional electrophoresis, and proteins excised from the gel were identified by mass spectrometry. Briefly, proteins isolated from the supernatant of sham or UUO mice (10 days) were suspended in Laemmli buffer containing 2% SDS (wt/vol) and 0.5% (vol/vol) β-mercaptoethanol and heated for 5 min at 95°C. Proteins were separated on SDS acrylamide gradient gels (4–12%) and then stained with SYPRO Ruby (ThermoFisher Scientific and Invitrogen) for visualizing total protein, and 1-mm slices were excised. For two-dimensional separation, protein was suspended in IEF loading buffer (8 M urea, 2% CHAPS, 40 mM DTT, and 0.2% Biolyte) and resolved by isoelectric focusing, pH 3–10, and then electrophoresed on 1% sodium dodecyl-sulfate (SDS) and 4–12% polyacrylamide gradient gels (Invitrogen) and visualized by staining with SYPRO Ruby (Invitrogen). Selective differential proteins were excised. Proteins were digested with trypsin, and peptides were analyzed by high-resolution LC-MS/MS with a Thermo Velos Orbitrap mass spectrometer coupled to a Waters nanoACQUITY LC system (4). Proteins were identified by Mascot (www.matrixscience.com) matching of peptide fragmentation patterns to a database of predicted patterns, as reported previously (4). For clustering, we used DAVID (version 6.7, https://david.abcc.ncifcrf.gov/) analysis to proteins that were either over- or underrepresented in UUO or control samples.
Luminex analysis. PDGFRβ-positive cells isolated from sham and UUO kidneys were cultured in six-well plates after isolation and grown to confluence in complete media. For measurements of cytokines produced, pericytes were grown for 24 h in serum-free media, and then supernatants were collected and concentrated using an AMICON 1K membrane (UFC800324) and centrifugation at 4,000 g for 40 min. The concentrated samples were used for analysis by Luminex Mag Pix; 25 μl was used for the luminex analysis in duplicate for each sample and the cytokine profile determined using the MAPKIT-31 (Millipex). The standards provided with the kit were used to calibrate the readings and for quality assurance.

Cell proliferation assay. PDGFRβ-positive cells from P1 passage were grown in 24 wells in complete medium for 7 days at a density of 15,000 cells/well. The cells were then cultured for 16 h in 3% FCS and DMEM-F-12. At the end of this period, complete medium was replaced. Cells grown for an additional 24 h. Staining for PCNA was carried out using an antibody against PCNA (1:200) and an AF488 conjugated secondary antibody in the wells. DAPI was used to locate and enumerate the nuclei. A Zeiss Axiovision microscopy and ×10 magnification were used for microscopic analysis of PCNA-positive cells. From each well at least five spots were recorded, and the percentage of PCNA-positive cells was calculated at each spot. The average value of the percentages for each sample was plotted.

Topflash reporter assays. Super 8xTopFlash and 8xFOPFlash were a gift from Randall Moon (Addgene plasmid nos. 12456 and 12457), and recombinant mouse Wnt-3a was from R & D Systems (Minneapolis, MN). Pericytes from sham and day 3 UUO kidneys were plated (75,000 cells/well) in a six-well gelatin-coated plate. After 24 h, cells were transfected with 2.5 μg of either TopFlash or FOPFlash reporter along with a renilla luciferase plasmid using lipofectamine 2000 (Invitrogen). The next day, cells were starved for 3 h before treatment with 10 ng/ml Wnt3a (R & D Systems) and lysed in passive lysis buffer 24 h later. Luciferase activity was measured using the dual luciferase assay system (Promega, Madison, WI) with the GloMax Multi Jr Detection System: Luminometer (Promega). Activity was expressed as relative light units.

Gene expression analysis by quantitative real-time PCR: Total RNA from pericytes and kidney tissue was isolated using a Spinsmart RNA Mini purification kit from Denville Scientific (Metuchen, NJ). Frozen kidney sections (6 μm) were paraffin embedded in paraffin were sectioned (5 μm). Sections were deparaffinized, dehydrated, and mounted with cytosol and viewed by light microscopy (Carl Zeiss Axioskop). Photographs were taken with an SPOT RT camera (software version 3.3; Diagnostic Instruments, Sterling Heights, MI). The extent of picrosirius red-positive area was quantitated using the grid method (266 squares) applied to images in Photoshop (Adobe Systems, San Jose, CA) that were obtained at either ×200 or ×400 magnification. Fibrosis was expressed as a percentage of the total area. A total of four to five images were evaluated per kidney, and the mean values were calculated. For immunohistochemistry, antigen retrieval was performed using the antigen unmasking solution from Vector Laboratories (Burlingame, CA). Sections were blocked with

**Protein analysis and Western blotting.** Pericytes from sham UUO and folic acid-treated kidneys were grown to confluence in six-well dishes and serum starved for 24 h. Cells were lysed in RIPA lysis buffer with protease inhibitor cocktail (Roche, Mannheim, Germany). Culture supernatants were concentrated using Amicon Ultra (EMD Millipore, Billerica, MA). Kidney tissue lysates were prepared by homogenizing in ice-cold RIPA buffer for 30 s using an ultrasonic processor (Cole-Parmer). Lysates were clarified by centrifugation, and protein concentration was determined by the BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). The membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and then incubated with primary antibodies rabbit polyclonal anti-C1q (1:1,000), mouse monoclonal anti-C1q (1:50), rabbit polyclonal α-smooth muscle actin (1:1,000), rat monoclonal anti-C3b/IC3b/C3c (1:1,000), and mouse GAPDH (1:200) overnight at 4°C and then with goat anti-rabbit IRDye 800CW-, goat anti-mouse IRDye 680LT- labeled secondary antibody (1:15,000) (LI-COR Biosciences) in Odyssey blocking buffer with 0.1% Tween for 1 h at room temperature. Membranes were imaged, and densitometric analysis of bands was performed using the LI-COR Odyssey Infrared Imaging System with Image Studio Lite 4.0 analytical software.

**Native gel electrophoresis for C3 fragments.** Native gels of 8%, cast in Tris-HCl (pH 8.0), were run with Tris-glycine buffer to analyze kidney lysates; 120 μg of the protein quantified by the BCA method was run in each lane. Samples were run under nonreducing conditions, blotted, and probed using the procedure or the method of Mastellos et al. (31). Densitometry analysis of the signals was done, and the average values for sham or UUO were plotted.

**Immunofluorescence microscopy.** Kidneys were fixed in 2% PLP (1% paraformaldehyde, 1.4% α-d-methylene blue, and 0.2% sodium periodate in 0.1 M phosphate buffer, pH 7.4) overnight at 4°C, incubated in 30% sucrose for 24 h at 4°C, and embedded in OCT compound (Fischer Scientific, Houston, TX). Frozen kidney sections (6 μm) were permeabilized with 0.3% Triton X-100 and blocked with 15% horse serum and 15% chicken serum for 1 h at room temperature, followed by blocking with anti-mouse CD16/32 (clone 2.4G2; StemCell Technologies, Vancouver, BC, Canada) with 0.02% sodium azide overnight at room temperature. Sections were labeled with FITC-labeled anti-C1q, mouse monoclonal anti-smactin smooth muscle-Cy3 (Sigma-Aldrich, St. Louis, MO), and PE-conjugated anti-F4/80 (Clone BM8; Biologend, San Diego, CA) all at 1:25 PM at room temperature for 2 h. Sections were mounted with ProLong Gold Antifade reagent with DAPI (Molecular Probes, Life Technologies, Eugene, OR). Images were acquired using the Carl Zeiss Axiovert 200 microscope system with ApoTome imaging and Axiosview software (Carl Zeiss Microscopy, Thornwood, NY).

**Immunohistochemistry and picrosirius red staining.** Kidneys embedded in paraffin were sectioned (5 μm). Sections were deparaffinized, fixed in 4% paraformaldehyde, rinsed in PBS, and stained with picrosirius red solution (Polysciences, Warrington, PA) for 1 h at room temperature. Slides were washed in acid water, dehydrated, and mounted with picrosirius red-positive area was quantitated using the grid method (266 squares) applied to images in Photoshop (Adobe Systems, San Jose, CA) that were obtained at either ×200 or ×400 magnification. Fibrosis was expressed as a percentage of the total area. A total of four to five images were evaluated per kidney, and the mean values were calculated. For immunohistochemistry, antigen retrieval was performed using the antigen unmasking solution from Vector Laboratories (Burlingame, CA). Sections were blocked with...
5% goat serum containing 10% Triton X-100 for 1 h at room temperature and incubated with rat monoclonal anti-C3b/C3i/C3c or rabbit polyclonal F4/80 antibodies both at 1:100 at 4°C overnight, followed by incubation with biotinylated secondary antibodies (1:250; Vector Laboratories) for 1 h at room temperature. Color was developed using diaminobenzidine (Vector Laboratories), and sections were counterstained with hematoxylin, rinsed, air-dried, and mounted using cytoseal. Quantification of F4/80 positive areas in four to five images per kidney obtained at ×400 magnification was done using the grid method and expressed as percentage of F4/80-positive areas.

Flow cytometry. Phosphate-buffered saline-perfused kidneys were obtained from sham and 10-day UUO mice and single-cell suspensions prepared by digesting the tissue with Liberase (Roche Diagnostics) and DNase for 30 min at 37°C, with constant shaking and vortexing every 5 min for 30 s. The suspension was filtered and centrifuged, and RBC lysis was done on the pellet, followed by neutralization and filtration through a 40-μm filter. The filtrate was centrifuged and the cell pellet washed in PBS and resuspended in PBS for live dead staining using the Live Dead Kit (Life Technologies). The cell suspension was then blocked with Fc Block (BD PharMingen). The cells were stained for surface markers, followed by fixation and permeabilization. The permeabilized cell suspension was stained with AF488-conjugated anti-mouse C1q or an isotype control. Data acquisition was done using a Cyteck upgraded FACS Calibur, and data were analyzed using the Flowjo software. Single color controls were prepared from spleen cell suspension for the various fluorochromes used. The following antibodies were used: CD 45.2 (clone 104) from eBiosciences; Ly6c (clone AL-21) and Fc block CD16/32, both from BD Biosciences; F4/80 (clone BMS), CD11c (clone418), CD8α (clone 53-6.7), CD4 (clone GK1.5), MRC1 (clone C068C2), PDGFRβ (clone APB5), Ly6G (clone IA8), CD11b (clone M/170), and CD31 (clone 390), all from Biologend; and Collectrin rabbit polyclonal antibody from Dr. Thu Le, Department of Medicine, University of Virginia.

Macrophage depletion. Clophosome-neutral liposomal clodronate and control liposome (7 mg/ml) obtained from FormuMAX Scientific (Palo Alto, CA) were injected intraperitonially in 8- to 10-wk-old male mice 2 days before UUO surgery. Following surgery, mice were injected with 75 μl of liposomes on days 1, 3, and 5. On day 7, left kidneys from UUO and sham mice were collected for flow cytometry, RNA isolation, and histological evaluation.

Statistics. All experiments were performed at least three times. All values are expressed as means ± SD. Statistical analysis was performed using an unpaired Student’s t-test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Increased secretion of extracellular matrix components and complement in secretome of UUO pericytes. Previous studies have shown that platelet-derived growth factor receptor-β (PDGFRβ) is expressed on all renal myofibroblasts (18). To investigate the cellular pathways involved in UUO-mediated pericyte to myofibroblast transition, we isolated PDGFRβ-positive cells from sham and UUO mice and established primary cell cultures as described (48). The scheme of isolation of these cells is shown in Fig. 1A. These cell cultures have been well characterized in earlier studies by Dr. Jeremy Duffield, who has shown that these PDGFRβ-positive pericytes lack epithelial, endothelial, leukocyte, and podocyte markers (48). We collected supernatants of these cells grown in serum-free media, concentrated them, and performed a proteomic analysis on selected protein spots that were different when comparing supernatants from UUO and control samples (Fig. 1, B and C). Proteins identified by mass spectrometry from differential spots from 2D gels were serotransferrin, fibronectin, thrombospondin, vinculin, and myosin 1, 6, 7, and 8 isoforms. The entire secretome identified a total of 750 proteins, and from these a total of 214 proteins showed differences between levels of expression when comparing cells isolated from sham and UUO secretome. Supplemental Table S1 (all Supplemental Material for this article is available online at the AJP-Renal Physiology website) shows that PDGFRβ-positive pericytes isolated from UUO mice secrete increased amounts of collagens, including α1, α2, (I and XII), fibrillin-1, proteoglycans like fibronectin, cathepsins B and D, fibulin-2, osteopontin, thrombospondin B, and several complement components. Among the proteins that had increased phosphorylation in UUO secretome compared with sham are osteopontin, vimentin, latent TGF-β, insulin-like growth factor-binding protein 3 and 5, vinculin, and several others, as shown in Supplemental Table S2. The most significantly enriched categories from DAVID gene ontology (GO) (https://david.ncifcrf.gov/) terms or pathways are

![Fig. 1. Analysis of the secretome of platelet-derived growth factor receptor-β (PDGFRβ)-positive pericytes isolated from sham and unilateral ureteral obstruction (UUO) mice. A: schematic representation of the procedure used for isolation of cells. B and C: SDS-PAGE separation of proteins isolated from pericyte supernatants from sham and day 3 UUO. Regions sliced for protein mass spectrometry analysis are marked in rectangular boxes. M, molecular weight marker.](http://ajprenal.physiology.org/)

Kidney tissue Homogenates

Passed through MACS columns after adding PDGFRβ ab and anti-rabbit IgG microbeads

Single cell suspension of PDGFRβ positive cells

Primary cell culture of pericyte (sham mice) and activated myofibroblasts (UUO mice)

Gel electrophoresis and LC/MS Identification of proteins from cell culture supernatants.
Complement C1q was one of the enriched GO term glycoproteins, with a 6.6-fold increase in the UUO secretome.

UUO pericytes are activated myofibroblasts. Previous studies have established that myofibroblasts are inflammatory cells that can generate cytokines and chemokines and promote injury (12, 43). Pericyte activation, detachment, and transition to myofibroblasts are driven primarily by Wnt/β-catenin signaling in vivo in response to kidney injury (29). Whereas transient Wnt activation in epithelial cells is associated with repair mechanisms, Wnt activation in pericytes/myofibroblasts leads to fibrotic changes (30). To establish that the cultures we obtained from UUO kidneys are indeed activated myofibroblasts, we characterized our cell populations for the positivity of all of the hallmarks of myofibroblasts. Pericytes isolated from UUO kidneys showed elevated mRNA expression of α-smooth muscle actin (α-SMA), collagen 1A1, and TGF-β (Fig. 2A) and increased α-SMA protein (Fig. 2B). Luminex analysis of cell culture supernatants from UUO pericytes shows increased secretion of proinflammatory chemokines IL-6, KC, MCP-1, and MIP-1α when compared with pericytes isolated from sham mice (Fig. 2C). The Wnt/β catenin signaling pathway activity measured using a TOPflash reporter assay in the presence of Wnt3a showed increased Wnt signaling in UUO pericytes (Fig. 2D). UUO pericytes had enhanced cell proliferation when compared with sham (Fig. 2E). Altogether, these results suggest that PDGFRβ-positive pericytes isolated from UUO mice have several features of activated myofibroblasts.

C1q expression is increased in whole kidney tissue and PDGFRβ-positive pericytes isolated from UUO and folic acid-treated kidneys. We observed by mass spectrometry analysis of the secretome that UUO pericytes have increased C1q production. To confirm these findings, we analyzed cell lysates of PDGFRβ-positive pericytes isolated from sham and UUO mice. The mRNA levels of C1qa, -B, and -C subunits were elevated in UUO pericytes (Fig. 3A) as well as in whole kidney tissue lysates obtained from mice subjected to UUO for 3 and 10 days (Fig. 3B). Immunostaining localized C1q expression to the interstitium, with no significant staining in the tubular

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### Table 1. GO analysis: enrichment of functional annotation terms for proteins differentially represented in UUO compared with control secretome

<table>
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<th>Count</th>
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GO, gene ontology; UUO, unilateral ureteral obstruction; EGF, epidermal growth factor; GlcNAc, N-acetyl-D-glucosamine; FDR, false discovery rate. GO terms are listed along with Bonferroni-corrected FDR and corresponding fold changes in differential proteins in UUO. *These proteins are in both the “up” and “down” lists; i.e., separate subsets of proteins in these GO categories increased and decreased with UUO.

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**Fig. 2.** Characterization of PDGFRβ-positive pericytes isolated from sham and UUO mice. A: real-time PCR analysis of fibrotic gene expression in sham and day 10 UUO pericytes (n = 3). B: Western blot for α-smooth muscle actin (α-SMA) in cell lysates from sham and day 10 UUO pericytes (n = 4). C: Luminex analysis for chemokines and cytokines in culture supernatants from sham and day 10 UUO pericytes (n = 5). D: TOPflash reporter assay for Wnt signaling in sham and day 10 UUO pericytes (n = 3). E: PCNA proliferation assay showing %proliferating cells in sham and day 10 UUO pericytes (n = 3). *P < 0.05. TGFβ1, transforming growth factor-β1; NS, not significant.
epithelium of UUO mice, whereas sham kidneys showed little or no staining (Fig. 3C). Kidney tissue sections were also stained with anti-α-SMA antibodies, and the majority of the staining was detected in the interstitium, with significant costaining with C1q shown as double-positive cells (Fig. 3C).

We confirmed increased C1q protein expression and secretion in UUO pericytes by Western blotting of cell lysates and culture supernatants (Fig. 3D). In whole kidney lysates, C1q protein was elevated in UUO mice on day 3 and day 10 (Fig. 3E). To determine whether increased synthesis of C1q was also present in other models of kidney fibrosis, we performed similar measurements of C1q in whole kidney tissue as well as in PDGFRβ-positive pericytes isolated from folic acid-treated mice. After 2 wk of folic acid given intraperitoneally, there is significant proteinuria (26), tubulointerstitial fibrosis with reduced fatty acid oxidation (19), and reduced mitochondrial biogenesis (52), which are metabolic signatures of progressive kidney disease. The mRNA levels of C1qA, -B, and -C were also elevated in kidney tissue of folic acid-treated mice and in PDGFRβ-positive pericytes (Fig. 4A). Western blotting showed an increased amount of C1q protein in culture supernatants from pericytes isolated from folic acid-injected mice and in whole kidney tissues (Fig. 4B). Similar to the UUO model, in the folic acid mice, staining for C1q was localized to the interstitium with significant colocalization with α-SMA staining (Fig. 4C). These observations confirm that the increased complement C1q expression occurs predominantly in the interstitial space in two different models of kidney fibrosis.

Flow cytometry study shows increased synthesis of C1q by PDGFRβ-positive pericytes and immune cells during UUO. To define the cell types that synthesize C1q in kidney tissue during fibrosis, we performed flow cytometry experiments only in UUO mice. Since the majority of C1q protein synthesized in UUO pericytes was secreted into the supernatant, and no surface staining could be obtained, suspended single-kidney cells were permeabilized to detect C1q by flow cytometry. The mRNA levels of C1qA, -B, and -C were also elevated in kidney tissue of folic acid-treated mice and in PDGFRβ-positive pericytes (Fig. 4A). Western blotting showed an increased amount of C1q protein in culture supernatants from pericytes isolated from folic acid-injected mice and in whole kidney tissues (Fig. 4B). Similar to the UUO model, in the folic acid mice, staining for C1q was localized to the interstitium with significant colocalization with α-SMA staining (Fig. 4C). These observations confirm that the increased complement C1q expression occurs predominantly in the interstitial space in two different models of kidney fibrosis.
both C1q and F4/80 in kidney sections of UUO mice, which was predominant in the interstitial compartment. C1qA deficiency does not prevent UUO-mediated kidney fibrosis. C1qA mRNA levels after UUO were increased only in wild-type but not in C1qA−/−/− mice; however, C1qB, C1qC, C1r, C1s, and C3 levels were persistently elevated in kidney tissue of both wild-type and C1qA-null mice after UUO (Fig. 6A). Previous studies characterizing complement activation in kidney tissue have used complement C3 antibodies that do not recognize complement C3 fragments generated upon complement activation. To address the issue of whether complement activation occurs in kidney tissue after obstructive injury in wild-type and C1q-null mice, we used in our studies rat monoclonal antibodies (clone 2/11) that preferentially react with the cleaved C3 fragments (31). These antibodies have been well characterized in previous studies (31). Western blot analysis using this antibody showed that protein levels for C3 fragments were increased to the same extent after UUO in both wild-type and C1qA−/−/− mice (Fig. 6B). C1qA−/−/− mice also showed similar levels of fibrosis as WT mice when assessed by picrosirius red staining (Fig. 6C). Fibrogenic gene expression, including α-SMA, fibronectin, and collagen 1A1, was also significantly increased after UUO in both wild-type and C1qA−/−/− mice, as shown in Fig. 6D. A recent study demonstrated that targeted deletion of C1q in mice alters pulmonary vascular homeostasis by augmenting the expression of endothelial adhesion markers associated with enhanced susceptibility of the lung endothelium to injury (49). To examine the potential mechanism by which fibrosis was still observed in C1qA−/−/− mice, we measured mRNA levels of two adhesion molecules involved in endothelial cell injury, ICAM-1 and VCAM-1. As shown in Fig. 6E, we found that transcript levels for ICAM-1 and VCAM-1 were significantly enhanced after UUO, suggesting increased endothelial damage in kidney tissue from C1qA−/−/− mice. Our results support the presence of complement C3 activation in kidney tissue during obstructive injury leading to kidney fibrosis, an effect that cannot be prevented solely by the use of C1q-deficient mice. Similar to the model of UUO injury, we also found lack of protection against kidney fibrosis in C1q-null mice subjected to folic acid injury (results not shown). Our results showing that increased expression of endothelial cell adhesion markers occurs in C1q-null mice during obstructive injury points toward a potential protective role of C1q on vascular endothelium. This role of C1q has been suggested to be independent from its role in classical complement activation (24). C3 deficiency reduces infiltration of inflammatory cells and fibrosis in obstructive and folic acid injury. Compared with wild-type mice, C3−/− mice had reduced expression of C1qA, C1r, and C1s as well as C3a and C5a receptors (Fig. 7A).
Immunohistochemistry analysis of kidney sections from wild-type and C3−/− UUO mice indicated that complement activation measured by increased C3 fragments after UUO in wild-type mice was localized predominantly in the interstitium, with no significant staining in the tubular epithelium (Fig. 7B). No staining for C3 fragments was seen in C3−/− mice subjected to UUO. Staining for α-SMA was also reduced in C3−/− UUO kidneys. Of importance is the observation that C3−/− mice had marked reduction in macrophage infiltration when compared with wild-type mice after UUO (Fig. 7B, bottom). Western blotting for C3 fragments showed increased C3 protein in WT UUO kidneys, which was absent in C3−/− mice (Fig. 7C). Fibrogenic gene expression after UUO in C3−/− mice was reduced compared with wild-type mice (Fig. 7D). We also examined the effect of C3 deficiency on kidney fibrosis in the folic acid injury model, and again we observed significant protection against fibrosis by C3−/− mice. There was reduced synthesis of the complement genes C1q, C1r, and C1s in C3-deficient mice (Fig. 8A) and reduced expression of fibrogenic genes, including α-SMA, fibronectin, and collagen1A1 (Fig. 8B), as well as collagen staining by picrosirius red (Fig. 8C). Our study is the first one to demonstrate increased complement activation in...
kidney tissue after obstructive injury using antibodies that recognize complement C3 fragments generated upon complement activation and significant protection from fibrosis in two different animal models of fibrosis when using C3-deficient mice.

Flow cytometry shows C3 complement activation in inflammatory cells during UUO. Our immunohistochemistry studies showed increased staining for C3 fragments in the interstitium (white arrows) on day 7 UUO by immunohistochemistry; increased staining for α-SMA on day 7 UUO on frozen sections and increased infiltration of F4/80 cells in WT day 7 UUO mice (white arrows) when compared with WT sham and C3−/− UUO mice (histogram shows quantitation of F4/80-positive areas).

Bottom: picrosirius red staining for collagen deposition in WT day 7 UUO mice (histogram shows quantitation of fibrotic areas; original magnification ×400).

C, native gel electrophoresis for C3b expression in kidney lysates from sham and day 7 UUO mice (n = 3). D: real-time PCR analysis for expression of fibrotic genes in WT (gray bars) and C3−/− (black bars) mice (n = 4). *P < 0.05; **P < 0.001.

Fig. 7. C3 deficiency reduces complement expression, macrophage infiltration, and fibrosis during UUO. A: real-time PCR analysis for expression of complement genes in WT (gray bars) and C3−/− (black bars; sham and day 7 UUO) mice (n = 3). B: images showing increased staining for C3 fragments in the interstitium (white arrows) on day 7 UUO by immunohistochemistry; increased staining for α-SMA on day 7 UUO on frozen sections and increased infiltration of F4/80 cells in WT day 7 UUO mice (white arrows) when compared with WT sham and C3−/− UUO mice (histogram shows quantitation of F4/80-positive areas).

Bottom: picrosirius red staining for collagen deposition in WT day 7 UUO mice (histogram shows quantitation of fibrotic areas; original magnification ×400).

C, native gel electrophoresis for C3b expression in kidney lysates from sham and day 7 UUO mice (n = 3). D: real-time PCR analysis for expression of fibrotic genes in WT (gray bars) and C3−/− (black bars) mice (n = 4). *P < 0.05; **P < 0.001.
positive cells isolated from UUO kidneys show increased expression of C1r, C1s, C3, and C5 as well as increased expression of the C3a and C5a receptors (Fig. 9C). Interestingly we did not find increased expression of α-SMA in these UUO-derived CD45-positive cells (Fig. 8C). These observations suggest that immune cells, in addition to pericytes, are a major source for complement synthesis in kidney tissue, but unlike PDGFRβ-positive pericytes, CD45-positive cells do not express significant amounts of α-SMA after UUO.

Macrophage depletion reduces complement expression and fibrosis. Our above results suggest that during UUO, in addition to pericytes, infiltrating immune cells and more specifically macrophages contribute significantly to complement activation. To assess the functional role of infiltrating macrophages in kidney fibrosis, we depleted F4/80hi macrophages using clodronate. Using the strategy depicted in Fig. 10A, we show that the levels of F4/80hi population were reduced in UUO mice receiving clodronate to levels comparable with sham (Fig. 10B). Macrophage depletion by clodronate was accompanied by significant reduction in C1r and C1s expression as well as C3, C4, and C5 (Fig. 10C). The reduction in complement gene expression was associated with reduced fibrotic gene expression (Fig. 10D) and reduction in tubulointerstitial fibrosis measured by picrosirius red staining (Fig. 10E). Our results further support the pathogenic role of macrophage infiltration, with increased complement activation leading to kidney fibrosis.

DISCUSSION

Increased synthesis of complement C1 components by kidney interstitial cells. In the current study, we examine the pathogenic role of increased complement expression and activation in kidney interstitial cells during kidney fibrosis. Our studies are the first to report local synthesis and secretion of C1q by mouse kidney PDGFRβ-positive pericytes isolated from two different animal models of fibrosis. Complement component C1q (460 kDa) is the first subcomponent of the classical pathway with three polypeptide chains (A, B, and C). We find that all three C1q subunits are synthesized during kidney
fibrosis by whole kidney tissue and by individual interstitial cells, including PDGFRβ-positive pericytes and CD45-positive inflammatory cells. C1q is involved in innate immunity and several modulatory processes, including pathogen clearance, apoptosis, angiogenesis, chemotaxis, autoimmunity, isotype switching, and tolerance (20, 21, 24). Based on the observation that C1q expression was increased both in kidney tissue as well as in interstitial cells during fibrosis, we were expecting that by reducing the levels of C1q alone using the C1q-null mice we would have protection from fibrosis. This was not the case, suggesting that the activation of C1 complex during fibrosis was more complex. Recent studies suggest the existence of noncomplement functions of C1q (22). For example, elevated levels of serum C1q have been observed during aging, leading to impaired regeneration of skeletal muscle and activation of the Wnt signaling pathway (33). C1q has been shown to promote permeability, proliferation, and migration of endothelial cells. C1q-deficient mice show impaired angiogenesis in a wound-healing model (3). In Alzheimer’s disease, C1q activation by brain microglia triggers amyloid plaque deposition (34). Our data showing C1q synthesis by PDGFRβ-positive pericytes and simultaneous increased Wnt signaling could represent a potential signal to trigger angiogenic responses in vivo in damaged endothelial cells in the interstitium. In future studies, the development of cell type-specific conditional deletions of C1q should help us understand better the contributions of increased C1q expression in kidney interstitial cells isolated from fibrotic kidneys, several important conclusions can be drawn from our studies:

1) increased C1q contributes to complement activation since we also found expression of the proteases C1r and C1s as well as increased expression of C3aR and C5aR (Fig. 9C); 2) increased C1q in PDGFRβ-positive pericytes contributes to increased inflammation via increased cytokine production, and we found that PDGFRβ-positive pericytes isolated from UUO mice secrete increased amounts of IL-6, MCP-1, and MIP1-α (a previous study in endothelial cells in culture showed that exogenous
administration of recombinant C1q led to increased cytokine production, including IL-6 and MCP-1 (59); this C1q-dependent cytokine production was abolished when endothelial cells were incubated with C1q in the presence of an inhibitory antibody to C1q receptor, and recent studies have begun to unravel the potential physiological role of the C1q receptor in cancer, but its role in kidney injury is currently unknown) (Fig. 2C) (9); and 3) C1q-mediated increased synthesis of cytokines by PDGFRβ-positive pericytes could further contribute to fibrosis by amplifying the interstitial inflammatory environment via recruitment of macrophages from increased MCP-1 expression.

In addition to C1q, our studies demonstrate that in wild-type mice there was increased expression of C1r and C1s in whole kidney tissue as well as in inflammatory CD45-positive cells isolated from diseased kidneys (Fig. 9C), demonstrating an important contribution to complement activation by immune cells during fibrosis. Within the C1 complex, C1r and C1s are serine proteases responsible for initiating the classical complement pathway. Flow studies of kidney cells confirmed that C1q, C1r, and C1s expression was increased in inflammatory cells isolated from UUO mice compared with sham mice (Figs. 5C and 9C). Interestingly, Ghebrehiwet et al. (8) and Hosszu et al (16) have shown that monocytes/macrophages express C1q, C1r, and C1s components on their surface and are able to initiate the classical complement cascade and regulate dendritic cell maturation. Because of the lack of reliable antibodies to localize the source of increased C1r and C1s protein expression during fibrosis in whole kidney tissue, we performed in situ hybridization studies (data not shown) and found increased expression of fibrotic gene expression in vehicle (gray bars) and clodronate-treated (black bars) mice (n = 4). D: quantification of fibrotic gene expression in vehicle (gray bars) and clodronate-treated (black bars) mice (n = 4). E: images showing picrosirius red staining for collagen deposition in vehicle and clodronate-treated mice (original magnification, ×200). Histogram shows quantitation of fibrotic areas. *P < 0.05; NS, not significant.

Fig. 10. Macrophage depletion protects against fibrosis. A: scheme of clodronate-mediated macrophage depletion. B: kidney cell suspension of vehicle and clodronate-treated mice gated by CD11b. CD11b-positive cells were then gated for F480 and CD11c. Histogram shows F480hi population in kidneys as % live singlet cells (n = 4). C: real-time PCR analysis for expression of complement genes in vehicle (gray bars) and clodronate-treated (black bars) mice (n = 3). D: quantification of fibrotic gene expression in vehicle (gray bars) and clodronate-treated (black bars) mice (n = 4). E: images showing picrosirius red staining for collagen deposition in vehicle and clodronate-treated mice (original magnification, ×200). Histogram shows quantitation of fibrotic areas. *P < 0.05; NS, not significant.
kidney tissue as markers of complement activation in kidney tissue during fibrosis has not been documented previously. In the present studies, we used a rat monoclonal antibody, clone 2/11, which specifically recognizes cleaved C3 fragments as a result of complement activation and breakdown of complement C3. These antibodies have been well characterized (31) and were used for our flow cytometry analysis, immunohistochemical studies, and Western blot analysis using native gels. We demonstrate clearly the accumulation of C3 fragments in whole kidney tissue homogenates in UUO model of fibrosis as well as accumulation of C3 fragments in CD45-positive inflammatory cells derived from UUO mice. Furthermore, our results using C3-deficient mice support a previous report of reduced fibrosis in these mice (67) but also extend this observation by showing that the lack of C3 expression in kidney tissue was accompanied by reduced deposition of active C3 fragments in the interstitial space as well as reduced infiltration of inflammatory cells, including macrophages. Reduction in macrophage and neutrophilic leukocyte infiltration in C3-deficient mice has been observed previously during development of arthritis in mice (15). Moreover, skin infiltration by eosinophils was also shown to be impaired in C3−/− mice in a model of skin inflammation (62). These observations support our findings that reduced macrophage infiltration plays an important role in the pathogenesis of kidney fibrosis and highlight a potential role of kidney tissue C3 in recruiting inflammatory cells at the site of injury. Although our studies did not examine the potential role of complement regulatory proteins in fibrosis, recent studies also suggest that lack of expression of either factor H or Crry can lead to widespread complement C3 activation in kidney tissue (25). Our studies showing that depletion of macrophages by clodronate reduces not only complement expression and inflammation but also fibrosis in wild-type mice further support the results of the experiments done using C3−/− mice. Bao et al. (1) used kidneys from Crry−/−/C3−/− mice and transplanted them into hosts lacking the C3aR and/or C5aR. Whereas unrestricted complement activation in the tubules was not affected by receptor status in the transplant recipient, C3aR deficiency in the recipients led to significantly reduced renal leukocyte infiltration as manifested in reduced tubulointerstitial inflammation and fibrosis. These authors concluded that manipulating C3a receptor signaling is a possible treatment to reduce inflammation and to preserve renal function.

What activates complement in kidney interstitial cells? Our studies do not allow us to draw any definite conclusions regarding the mechanisms that led to increased expression of C1q, C1r, and C1s resulting in complement activation in whole kidney tissue; however, our results demonstrate that this increased expression of C1 components on both pericytes and immune cells is intimately linked to C3 complement activation, which is defined by increased accumulation of C3 fragments in kidney tissue during fibrosis. IgM or IgG immune complexes are the best physiological C1 activators identified to date, especially in the presence of the C1 inhibitor. Although it has been known for a long time that C1q binds to the IgG Fc domain and that activation requires multivalent binding, recent studies in this area have begun to describe the details of how the C1 complex is assembled when it is activated (7). To address the issue of immunoglobulins being the potential mechanism for increased complement activation during fibrosis, we performed experiments using the UUO model in Rag1−/− mice that are deficient in immunoglobulins. We did not observe any differences in complement expression or activation during fibrosis, suggesting that the level of immunoglobulins does not affect complement activation during fibrosis. In addition, we performed experiments in isolated PDGFRβ-positive pericytes isolated from sham mice, and we did not see any effect on complement expression levels when these cells were stimulated with TGFβ, indicating that cellular mechanisms other than TGFβ could account for increased complement expression in UUO mice. We postulate that the increased generation of pathogen-associated or danger-associated molecular patters in the interstitium, as well as an increased inflammatory environment, is likely to contribute to complement activation during kidney fibrosis.

Many of the biological functions of anaphylatoxins C3a and C5a generated during kidney fibrosis are mediated through activation of their receptors C3aR and C5aR. Our studies also demonstrate increased expression of anaphylatoxin receptors
C3αR and C5αR in whole kidney tissue as well as in CD45-positive cells (Figs. 7A and 9C). In contrast to our findings, a previous study found C5αR expression to be upregulated in tubular cells during UUO injury and when blocked with an antagonist had minimal effects on reducing renal macrophage influx (2) while affording significant protection against fibrosis. Most recently, in a hypertensive model, expression of C5αR1 on infiltrating and resident renal immune cells was demonstrated, and the loss of C5αR1 did not change the numbers of infiltrating immune cells in the course of hypertension while exhibiting less renal injury (60). Another study by Peng et al. (41) also shows that deficiency of either or both of the receptors protects mice from IRI injury accompanied with the reduced infiltration of immune cells and suggests that C3αR and C5αR on both renal and circulating leucocytes contributes to the pathogenesis of renal ischemia-reperfusion injury. At the moment, we do not know the cellular mechanism(s) that leads to increased expression of C3αR or C5αR expression in kidney interstitial cells but likely depends on the inflammatory environment present during fibrosis. Previous studies also suggest the presence of epigenetic mechanisms at the level of C3 promoter that could contribute to increased transcriptional response of C3 (17, 42). We propose a model by which complement activation could contribute to interstitial fibrosis (Fig. 11). C1q produced by myofibroblasts and immune cells and C1r and C1s produced by immune and epithelial cells allow the formation of the C1 complex in the interstitium. This in turn contributes to complement activation with the breakdown of C3 by a convertase and increased generation of C3 fragments in interstitial cells, which further exacerbates the process of extracellular matrix accumulation and fibrosis. Tubular epithelium could potentially contribute with increased expression of C3α and C5α and activation of C3αR and C5αR.

In summary, complement activation during fibrosis offers ample opportunity for targeted therapeutic modulation. Although only two complement-targeted drugs have been used in clinical studies, the therapeutic anti-C5 antibody eculizumab and various preparations of the C1 esterase inhibitor C1-INH, several new candidate drugs targeting various components of the cascade are currently being developed. Among them is a peptide inhibitor, Compsatin, that selectively binds to native C3 and its bioactive fragments C3b and iC3b/C3c, thus blocking C3 activation by all convertases. Future studies aimed at examining the function of complement receptors in kidney interstitial cells as well as the potential mechanisms by which tubular epithelium contributes to inflammation and fibrosis may allow for the identification of well-defined targets to ameliorate progressive kidney disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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