Complement C5 Mediates Experimental Tubulointerstitial Fibrosis

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Renal fibrosis is the final common pathway of most progressive renal diseases. C5 was recently identified as a risk factor for liver fibrosis. This study investigated the role of C5 in the development of renal tubulointerstitial fibrosis by (1) induction of renal fibrosis in wild-type and C5−/− mice by unilateral ureteral ligation (UUO) and (2) investigation of the effects of a C5a receptor antagonist (C5aRA) in UUO. In C5−/− mice, when compared with wild-type controls, markers of renal fibrosis (Sirius Red, type I collagen, fibronectin, α-smooth muscle actin, vimentin, and infiltrating macrophages) were significantly reduced on day 5 of UUO. On day 10, fibronectin mRNA and protein expression were still reduced in the C5−/− mice. Cortical mRNA of all PDGF isoforms and of TGF-β1 (i.e., central mediators of renal disease) were significantly reduced in C5−/− mice when compared with controls. Renal tubular cell expression of the C5aR was sparse in normal cortex but markedly upregulated after UUO. Treatment of wild-type UUO mice with C5aRA also led to a significant reduction of cortical Sirius Red staining, fibronectin protein expression, and PDGF-B mRNA expression on day 5. Neither genetic C5 deficiency nor C5aRA treatment caused any histologic changes in the nonobstructed kidneys. In cultured murine cortical tubular cells, C5a stimulated production of TGF-β1, and this was inhibited by C5aRA. Using a combined genetic and pharmacologic approach, C5, in particular C5a, is identified as a novel profibrotic factor in renal disease and as a potential new therapeutic target.

in the kidney. We therefore chose a nonproteinuric model of primary tubulointerstitial damage, namely UUO, to investigate the effects of genetic C5 deficiency or pharmacologic inhibition of the C5aR on the development of tubulointerstitial damage and fibrosis.

Materials and Methods

C5aR Antagonist

The C5aR antagonist (C5aRA) JPE-1375 was provided by Jerini AG (Berlin, Germany) and has been characterized previously (17). In brief, JPE-1375 is a hexameric linear peptidomimetic molecule (molecular weight 955) that inhibits C5a binding to the human C5aR with an IC50 of 111 nM (in transfected HEK-293 cells). Functional inhibition of C5aR signaling was shown in vitro for the C5a-induced glucosaminidase release from human polymorphonuclear cells (IC50 41 nM) and for the inhibition of chemotaxis of murine macrophage-like J774A.1 cells (IC50 42 nM). JPE-1375 abolished the C5a-induced effect in an in vivo model of an immune complex–mediated peritonitis, the reverse passive Arthus reaction (17), and in experimental kidney transplantation (unpublished data, F. Gueler, 2006). Potent inhibition of human C5aR and 17-fold higher activity on murine C5aR (17) makes JPE-1375 particularly suitable for testing in murine models.

Cell Culture Experiments

Murine cortical tubular (MCT) cells were provided by F. Strutz (University of Göttingen, Göttingen, Germany). To address the expression of C5aR (CD88) in vitro, MCT cells were seeded on glass coverslides in six-well plates, grown to subconfluence, and subsequently serum-starved for 24 h. Then, the glass coverslides were removed, fixed in 100% acetone for 10 min, dried again, and then stained as described in Renal Morphology and Immunohistochemistry. For examination of the effect of C5a on TGF-β, production, MCT cells were seeded into 25-mm2 flasks, grown to subconfluence, and then serum-starved for 36 h. Cells were subsequently stimulated for 24 h with recombinant human C5a (rhC5a; 25 nM) or the combination of rhC5a (25 nM) and C5aRA (2.5 μM). Specific binding and activity of rhC5a on murine C5aR was demonstrated previously (unpublished data, H.H., 2004) (18,19). Thereafter, cell culture supernatants were collected for TGF-β measurements using a commercially available kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). All cell culture experiments were performed in triplicate.

Experimental Model and Experimental Design

All animal experiments were approved by the local review boards. The animals were held in rooms with constant temperature and humidity, 12-h/12-h daylight cycle, with ad libitum access to drinking water and food. All animals were obtained from The Jackson Laboratory (Bar Harbor, ME).

In the first approach, 7-wk-old male C5 deficient (C5−/−) mice (strain B6.D2-Heβ/oSnJ) and age-matched wild-type controls (C57BL/10SnJ) underwent UUO. Renal tissue (for histologic examination and RNA isolation) from both the obstructed and contralateral kidney was obtained when the mice were killed, on day 5 (C5−/−, n = 7; wild-type, n = 8) and day 10 (C5−/−, n = 5; wild-type, n = 7) after UUO.

In the second experiment, 7-wk-old male C57BL/10SnJ mice underwent UUO with a subsequent intraperitoneal implantation of an AL-ZET micro-osmotic pump (model 1002; pump rate 0.25 μl/h; DURECT Corp., Cupertino, CA). The pumps were filled with either C5aRA (n = 8) or vehicle (0.9% NaCl in 15% vol/vol ethanol; n = 8). Daily C5aRA dosage was 0.63 ± 0.05 mg/kg body wt. A similar dosage was previously demonstrated to be effective in mice (17). Blood and kidney tissue were obtained as described on day 5 after disease induction.

Renal Morphology and Immunohistochemistry

For the evaluation of fibrosis, renal tissue was stained with Sirius Red in 4-μm sections of kidney biopsies, fixed in methyl Carnoy’s solution, and embedded in paraffin. For evaluation of Sirius Red and the various immunohistochemical stains listed in the next paragraph, computer-based morphometry (Soft Imaging System, Münster, Germany) was performed by an observer who was unaware of the origin of the slides. The percentage of positively stained area was calculated from 20 cortical fields (0.093 mm2 each) per section, representing nearly the whole cortex.

Indirect immunoperoxidase procedure was performed as described previously (20). The following primary antibodies were used: Horseradish peroxidase–coupled murine mAb (clone 1A4) to human α-smooth muscle actin (α-SMA; DAKO A/S, Glostrup, Denmark), rabbit polyclonal antibody to rat fibronectin (Chemicon, Temecula, CA), goat polyclonal antibody to human collagen type I (Southern Biotechnology Associates, Birmingham, AL), rat mAb to murine macrophage antigen F4/80 (MCA497, clone A3-1; Serotec Ltd., Oxford, UK), and chicken polyclonal antibody to mouse C5aR (BMA Biomedicals AG, Augst, Switzerland). All secondary antibodies used were biotinylated and affinity-purified (Vector, Burlingame, CA); Rabbit anti-goat IgG, goat anti-rabbit IgG, mouse-adsorbed rabbit anti-rat IgG, and donkey anti-chicken IgG. Negative controls were performed as described previously (21).

RNA Extraction and Analyses

Total RNA was extracted from renal cortex using the RNeasy Mini Kit (Qiagen, Hilden, Germany). From total RNA, cDNA was synthesized and real-time quantitative reverse transcriptase–PCR was performed as described previously (22). Sequences of primers and probes used are listed in Table 1. Messenger RNA for each sample was normalized to the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase. Results are given as relative mRNA expression normalized to the expression in contralateral healthy kidneys.

Statistical Analyses

All values are expressed as means ± SD. Groups were compared using unpaired t test or Mann-Whitney U test, where appropriate (both two-tailed). For in vitro experiments, ANOVA with least significant difference post hoc test was used. Statistical significance was defined as P < 0.05.

Results

C5 Deficiency Reduces Interstitial Fibrosis and Macrophage Influx after UUO

On day 5 after UUO, wild-type mice were characterized by widespread renal tubulointerstitial damage and fibrosis, as evidenced by increased Sirius Red–positive area and increases in type I collagen, fibronectin, α-SMA, and macrophage staining (Figure 1). In comparison with wild-type mice, the C5−/− mice exhibited reduced renal cortical scarring as assessed by Sirius Red staining, collagen type I, and fibronectin immunostaining (−53, −52, and −42%, respectively; Figure 1). On day 10 after UUO, most of these differences persisted numerically but lost statistical significance as a result of high scatter, except for fibronectin expression, which remained significantly decreased (−43%) in the C5−/− mice (Figure 1). Consistent with the latter, renal cortical mRNA levels of fibronectin were significantly
Table 1. Sequences of primers and probes used for real-time reverse transcriptase–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Taqman Probe (5’ to 3’)</th>
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<tr>
<td>mCol IV</td>
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<tr>
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<tr>
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<td>TAGAGGCTGACTGGCGGATGAG</td>
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<tr>
<td>mPDGF-B</td>
<td>CCATCCGCTCTCCCTT</td>
<td>TGGTGTGCATCCAATTACC</td>
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<tr>
<td>mPDGF-C</td>
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<tr>
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<td>GCAGAGATTAATATCTAC</td>
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<tr>
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<td>SYBR Green Kit</td>
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*mGAPDH, mouse glyceraldehyde-3-phosphate dehydrogenase; mCol IV, mouse type IV collagen; mFibr, mouse fibronectin; mVim, mouse vimentin; mTGF-β1, mouse TGF-β1; mPDGF-A, -B, -C, and -D, mouse PDGF-A, -B, -C, and -D.

Figure 1. Representative images of obstructed kidneys at day 5 in wild-type (WT; A, C, and E) and C5 deficient (C5−/−; B, D, and F) mice: Staining for Sirius Red (A and B), fibronectin (C and D), and α-smooth muscle actin (α-SMA; E and F). Diagrams show data obtained by computer-based morphometric analysis on days 5 and 10 after the induction of unilateral ureteral obstruction (UUO) in obstructed and in healthy contralateral kidneys. Y axes give the relative area (%) of tissue that stained positively. Data are means ± SD. *P < 0.05 versus WT day 5; **P < 0.01 versus WT day 5; †P < 0.05 versus WT day 10. F4/80, staining for macrophages; contralat., contralateral healthy kidney. Magnification, ×100.
enchymal transition (EMT) was assessed by the tubulointerstitial protein level (Figure 1). After 10 d (Figure 2); however, no differences were found on C5 after 5 d. UUO reduced by approximately 50% of the type IV collagen mRNA was not affected by the lack of C5 in WT and C5−/− mice on days 5 and 10 after UUO. Y axes depict the mRNA expression of the appropriate gene relative to nonobstructed kidneys, which were pooled and arbitrarily set as 1. Data are means ± SD. *P < 0.05 versus WT day 5; **P < 0.01 versus WT day 5; #P < 0.05 versus WT day 10.

lower in C5−/− mice compared with wild-type mice, both on day 5 (−33%) and 10 (−76%) after UUO (Figure 2). Expression of the type IV collagen mRNA was not affected by the lack of C5 after 5 d of UUO but was reduced by approximately 50% after 10 d (Figure 2); however, no differences were found on protein level (Figure 1).

Activation of renal interstitial fibroblasts and epithelial-to-mesenchymal transition (EMT) was assessed by the tubulointerstitial de novo expression of α-SMA and vimentin. Compared with wild-type mice, C5−/− mice expressed significantly less renal cortical α-SMA on day 5, whereas significance of the difference was lost on day 10 (Figure 1). Relative cortical mRNA expression of vimentin was also reduced in C5−/− mice on both days 5 (−62%) and 10 (−55%); Figure 2). Lack of C5 after UUO did not affect the tubular expression of E-cadherin, another EMT marker (data not shown).

On day 5 of UUO, macrophage influx was reduced by 57% in C5−/− mice. In the later phase of the disease, no difference between the animals persisted (Figure 1).

Renal Cortical Expression of all PDGF Isoforms and TGF-β1 Are Reduced in C5−/− Mice after UUO

PDGF is an important mediator of renal fibrosis (22–26). In comparison with nonobstructed kidneys, the expression of PDGF-A, -B, and -D mRNA increased 4.5-, 8.3-, and 3.2-fold, respectively, in obstructed kidneys of wild-type mice on day 5 and 6.3-, 11.4-, and 5.5-fold, respectively, on day 10 (Figure 3). In contrast, PDGF-C mRNA exhibited very little change (Figure 3). C5 deficiency in the obstructed kidney led to reduced cortical mRNA expression of both PDGF receptor β agonists (PDGF-B and -D [−61 and −29%, respectively]) as compared with wild-type mice. On day 10, the relative expression of all PDGF isoform mRNA was reduced in C5−/− mice by −20% (PDGF-C) to −50% (PDGF-D; Figure 3). The expression of mRNA for TGF-β1, another key mediator of fibrosis, was also reduced in C5−/− mice when compared with wild-type mice on day 5 by 27% and on day 10 by 30% (Figure 3).

Renal Expression of C5αR Is Upregulated after UUO

The expression of the C5αR in healthy mouse kidneys was sparse and localized to the apical side of tubular epithelial cells. Five days after UUO, a >16- and 22-fold increase in C5αR expression was detected in vehicle- and C5αRA-treated mice, respectively (Figure 4). Upregulated C5αR expression was mainly localized to the luminal side of tubular cells, but some tubular cells showed also lateral or diffuse positivity. Upregulated expression was particularly prominent in dilated tubules but occasionally also occurred in tubule segments that appeared normal by light microscopy (Figure 4, H and I). Treatment with C5αRA did not significantly affect the expression of the receptor in healthy or obstructed kidneys (Figure 4, G and I).

Treatment with C5αRA Ameliorates Renal Scarring and PDGF-B Expression after UUO

Treatment with C5αRA after UUO reduced the Sirius Red-positive area and fibronectin expression by 41 and 21%, respectively (Figure 4, A through F). C5αRA-treated mice, when compared with vehicle-treated mice, also exhibited numerically lower renal cortical accumulation of type I collagen (6.9 ± 2.2 versus 9.1 ± 2.3% positively stained area, respectively; P = 0.06) and lower tubulointerstitial expression of α-SMA (5.1 ± 3.1 versus 8.8 ± 6.0% positively stained area, respectively; P = 0.14). C5αRA treatment did not significantly influence renal cortical macrophage influx (2.4 ± 0.5 versus 3.0 ± 1.4% positively stained area in C5αRA- and vehicle-treated mice, respectively) or collagen type IV expression (3.5 ± 0.7 versus 4.1 ± 0.7% positively stained area in C5αRA- and vehicle-treated mice, respectively).

At the mRNA level, renal cortical expression of type IV collagen and fibronectin was not significantly influenced by C5αRA treatment (type IV collagen 6.7 ± 3.2 versus 8.1 ± 4.8 relative mRNA expression in C5αRA- and vehicle-treated mice, respectively; fibronectin 7.6 ± 3.1 versus 11.1 ± 4.6, respectively). PDGF-B mRNA was decreased in mice that were treated with C5αRA when compared with vehicle-treated mice (5.7 ± 2.4 versus 12.3 ± 7.0 relative mRNA expression; P < 0.05), whereas the other PDGF isoforms were not affected by the treatment. The expression of TGF-β1 mRNA decreased mildly in mice that received C5αRA (3.7 ± 2.5 versus 4.5 ± 3.3 relative mRNA expression in C5αRA-treated versus vehicle-treated mice, respectively; NS).
Effect of C5 Deficiency and C5aRA Treatment on Nonobstructed Kidneys and on Body Weight

In contralateral, nonobstructed kidneys, C5 deficiency did not affect most histologic parameters in comparison with non-manipulated wild-type mice (data not shown); however, on day 5, decreased macrophage counts and fibronectin immunoreactivity were noted in the nonobstructed kidneys of C5\(^{-/-}\) mice (0.12 \(\pm 0.11\) versus 0.33 \(\pm 0.18\)% positively stained area for F4/80 in C5\(^{-/-}\) and wild-type mice, respectively; 0.89 \(\pm 0.35\) versus 1.75 \(\pm 0.63\)% positively stained area for fibronectin in C5\(^{-/-}\) and wild-type mice, respectively; both \(P < 0.05\)). These results probably represent a statistical chance observation.

Treatment with C5aRA did not induce any detectable changes in nonobstructed kidneys (data not shown).

C5\(^{-/-}\) mice exhibited between 13 and 19% lower body weight when compared with wild-type mice before UUO and on days 5 and 10 after UUO (data not shown; \(P < 0.01\) for all groups). The body weight of wild-type mice that were treated with vehicle or C5aRA was similar before disease induction (25 \(\pm 2\) versus 25 \(\pm 2\) g, respectively) but significantly higher in the vehicle group after 5 d of UUO (24 \(\pm 1\) versus 22 \(\pm 1\) g, in vehicle- and C5aRA-treated mice, respectively; \(P < 0.01\)).

Effects of C5a on Murine Cortical Tubular Cells In Vitro

We first showed by immunocytochemistry that cultured MCT cells expressed C5aR (Figure 5, A and B). Addition of C5a to the cell culture medium of serum-starved MCT cells stimulated their production of TGF-\(\beta_1\) to a level comparable to cells that were grown in full medium, and this effect was inhibited by addition of C5aRA (Figure 5C). In contrast to TGF-\(\beta_1\) production, mRNA levels of PDGF-B and -D in MCT cells were not affected by C5a (data not shown).
Discussion

The major and novel finding of this study was that in the early phase of nonproteinuric renal damage, C5 deficiency led to a significant amelioration of major components of renal fibrosis: Fibroblast activation, extracellular matrix protein deposition, morphage influx, and some markers of EMT. Our findings in mice that had UUO and were treated with a C5aRA additionally suggest that the anaphylatoxin C5a is the central mediator of this C5 effect. Therefore, the interaction of C5a with the C5aR (CD88) seems to be a key mediator of fibrosis in nonproteinuric conditions.

Our data are consistent with earlier findings showing that C6 deficiency does not affect the course of UUO (15) in contrast to proteinuric renal diseases, where C6 and thus C5b-9 contributed to tubulointerstitial damage (8–10). These latter findings, however, do not exclude an additional role of C5a in mediating renal interstitial fibrosis.

On day 10 after UUO, many of the C5-mediated effects lost statistical significance. This may relate to the progressive course of renal damage in the UUO model and to the redundancy of the various biologic systems involved in renal fibrosis (reviewed in Klahr and Morissey [27]). Indeed, many previous intervention studies in the UUO model have shown as well that the effect of the therapeutic intervention was ultimately lost as postrenal obstruction led to progressive destruction of the renal architecture (28–30).

Until now, there were no published data on the role of C5a in renal fibrosis. However, the first data emerged recently in liver and lung fibrosis models. We previously described that specific C5AR inhibition in a murine model of liver fibrosis significantly ameliorated the progress of fibrosis and reduced hepatic collagen content and expression as well as mRNA levels of tissue inhibitor of matrix metalloproteinases type 1 (16). Similar to our results, fibrosis was markedly attenuated in C5-/- mice with bleomycin-induced pulmonary fibrosis (31). In this study, pulmonary accumulation of collagen types I and III was significantly decreased in C5-/- mice, and the expression of TGF-β1 and matrix metalloproteinase-3 were downregulated as well (31). These findings confirm and extend our hypothesis that C5a has a central role in the process of fibrogenesis in general (i.e., independent of the tissue or organ) (16).

There are several possible mechanisms for how blockade of the C5a-C5aR interaction might ameliorate renal tubulointerstitial fibrosis. First, in mouse kidneys, tubular cells seem to be the only renal cell type that expresses C5aR, as shown in Figure 4 and previously described in BALB/c mice (32). This is similar to the C5aR expression pattern described in human kidney tissues (33,34). Our observation of a pronounced upregulation of tubular cell C5aR expression after UUO (Figure 4) provides a mechanistic basis in situ for exaggerated C5a signaling. Our data extend previous observation in an in vitro model of liver fibrogenesis using hepatic stellate cells, the principal cells of hepatic fibrosis, where C5aR was also greatly upregulated (approximately nine-fold) on day 5 of secondary culture of these cells, and the high C5aR levels were maintained thereafter (16).

A second profibrotic mechanism downstream of the C5aR may involve the production of mediators, such as TGF-β and PDGF-B and -D, all of which have been shown to be crucially involved in renal scarring (22–26,35). In vivo, at least at the mRNA level, the considerable overexpression of both PDGF-B and -D and TGF-β1 in obstructed kidneys was potently downregulated in C5-/- mice and in C5aRA-treated mice. Similar observations were made in vitro, where murine tubular cells that were stimulated with C5a produced significant amounts of TGF-β1 protein. The amounts produced were in the same range as after stimulation with angiotensin II (36). We recently described that in mesangiproliferative glomerulonephritis, PDGF-B acts downstream of TGF-β (37). Given the lack of a C5a effect on tubular cell PDGF-B and -D mRNA expression, our data suggest that C5a induces TGF-β1 on injured tubular cells, which in turn mediates profibrotic effects directly or via the upregulation of PDGF-B and -D chains in neighboring cells such as fibroblasts. The expression of PDGF chains and receptors by interstitial fibroblasts in obstructed murine kidneys has been documented previously (38). The potent effects of C5a on renal fibroblast overproduction and accumulation after UUO (Figures 1 and 4) is in line with downstream effects of PDGF. In cultured hepatic stellate cells, activation of the C5aR induced fibronectin production, which was not mediated through TGF-β1 (39).

A third mechanism that contributes to C5a-mediated tubulointerstitial renal fibrosis may be the inhibitory effect of C5a on the polarization of T-helper cells to TH1 cells (40,41). The following shift to TH2 cells, with their cytokine response pattern (e.g., IL-13 and TGF-β release), acts in a profibrotic manner (42).

The chemotactic factors, such as monocyte chemoattractant protein-1 and osteopontin, are overexpressed as well (27), and their action could have substituted for that of C5a. Another explanation that might contribute to a differential effect of C5 deficiency versus C5aRA is the...
chemotactic activity of C5b-9, as described in lungs (49) as well as in kidney (9,10). Although the numbers of infiltrating macrophages did not differ, their activation state might have done so. C5a was shown to induce TNF-α production in inflammation-primed macrophages (50). In turn, macrophages that produce TNF-α, as well as TGF-β and PDGF-B, promote fibrotic remodeling in UUO (35,51).

In addition to decreasing markers of renal fibrosis and affecting macrophage influx, the decreased cortical vimentin mRNA levels in obstructed kidneys of C5−/− mice supports the notion of decreased EMT. In contrast to vimentin, we observed no difference in E-cadherin expression between the groups. This is similar to our recent data with specific PDGF-D inhibition in established tubulointerstitial fibrosis (23) and consistent with other studies showing that development of EMT is not proceeding uniformly (52).

**Conclusion**

This is the first study to identify C5, in particular the anaphylatoxin C5a, as a novel profibrotic factor in renal disease. Our study points to a new therapeutic target in the treatment of tubulointerstitial fibrosis, a common end-condition characteristic for all progressive renal diseases.

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**Disclosures**

None.

**References**


