Soluble CR1 Therapy Improves Complement Regulation in C3 Glomerulopathy


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ABSTRACT
Dense deposit disease (DDD) and C3 glomerulonephritis (C3GN) are widely recognized subtypes of C3 glomerulopathy. These ultra-rare renal diseases are characterized by fluid-phase dysregulation of the alternative complement pathway that leads to deposition of complement proteins in the renal glomerulus. Disease triggers are unknown and because targeted treatments are lacking, progress to end stage renal failure is a common final outcome. We studied soluble CR1, a potent regulator of complement activity, to test whether it restores complement regulation in C3 glomerulopathy. In vitro studies using sera from patients with DDD showed that soluble CR1 prevents dysregulation of the alternative pathway C3 convertase, even in the presence of C3 nephritic factors. In mice deficient in complement factor H and transgenic for human CR1, soluble CR1 therapy stopped alternative pathway activation, resulting in normalization of serum C3 levels and clearance of iC3b from glomerular basement membranes. Short-term use of soluble CR1 in a pediatric patient with end stage renal failure demonstrated its safety and ability to normalize activity of the terminal complement pathway. Overall, these data indicate that soluble CR1 re-establishes regulation of the alternative complement pathway and provide support for a limited trial to evaluate soluble CR1 as a treatment for DDD and C3GN.


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Dense deposit disease (DDD) and C3 glomerulonephritis (C3GN) are two widely recognized subtypes of C3 glomerulopathy.1,2 These ultra-rare renal diseases are caused by fluid-phase dysregulation of the alternative complement pathway (AP) of complement, with variable concomitant dysregulation of the C5 convertase. Consistent with complement-mediated disease acting through the AP, C3G is strongly positive for C3 and notably negative for IgGs by immunofluorescence microscopy.2 Electron microscopy distinguishes DDD from C3GN, with the former characterized by pathognomonic electron-dense transformation of the lamina densa of the glomerular basement membrane (GBM).3 In C3GN, the electron microscopy deposits are lighter in color, and are more often mesangial and/or subendothelial, intramembranous, and subepithelial in location.4 In both diseases, mass spectroscopy of laser dissected glomeruli is highly enriched for proteins of the AP and terminal complement cascade.4,5
Although long-term outcome data are not available for C3GN, nearly half of all DDD patients progress to end-stage renal failure (ESRF) within 10 years of diagnosis. In virtually all cases of DDD, transplantation is associated with histologic recurrence, explaining the 5-year graft failure rate of 50%. There are no target-specific treatments for C3G; however, its pathophysiology suggests that therapeutic approaches to restore C3 convertase control, impair C3 convertase activity, or remove C3 breakdown products from the circulation warrant consideration.

Similar to human DDD, the complement factor H (CfH)–deficient mouse, reclassified recently as a murine model of C3GN, accumulates C3 fragments within glomeruli as the first pathologic abnormality to develop. Systemic administration of C3GN, accumulates C3 fragments within glomeruli as the first pathologic abnormality to develop. Systemic administration of C3GN, accumulates C3 fragments within glomeruli as the first pathologic abnormality to develop. Systemic administration of C3GN, accumulates C3 fragments within glomeruli as the first pathologic abnormality to develop.

We assessed the efficacy of sCR1 as a complement regulator in vitro in normal and DDD sera. In normal pooled human sera, it prevented classic pathway (CP) and AP complement activation (IC₅₀ values of 2.55±0.55 nM and 0.71±0.08 nM, respectively; Figure 1A). Confirmatory hemolytic assays were performed with rabbit and sheep erythrocytes. Rabbit erythrocytes are a complement-activating surface in human sera; however, lysis could be prevented by the addition of sCR1 (IC₅₀=29.46±4.64 nM). In comparison, fH did not prevent hemolysis even at high concentrations (Figure 1B). Sheep erythrocytes do not activate complement in normal sera; however, hemolysis occurred when tested against DDD sera. The addition of sCR1 restored AP control in a dose-dependent manner (Figure 1C) and prevented hemolysis even when DDD sera contained C3 convertase-stabilizing autoantibodies called C3Nefs (Figure 1D).

We next measured the systemic effect of sCR1 in vivo in both CfH−/− and CfH−/−/huCR1-Tg mice (the latter express CR1 on erythrocytes only) by introducing sCR1 by the tail vein or intraperitoneally, respectively, after first verifying that it prevented hemolysis of rabbit erythrocytes in mouse sera (IC₅₀=6.15±0.18 nM; Figure 2 and Supplemental Figure 3). After a single tail vein injection of sCR1 in CfH−/− mice (50 mg/kg, n=5; Figure 2A, blue), serum C3 levels rose dramatically within 24 hours from 12.03±12.29 mg/L to 219.77±42.32 mg/L but fell to near preinjection levels by 48 hours (41.84±24.29 mg/L; Figure 2B, blue). Single-dose intraperitoneal injections in CfH−/−/huCR1-Tg mice at concentrations of 5 mg/kg, 25 mg/kg, or 50 mg/kg showed that changes in serum C3 levels were sCR1 dose dependent (Figure 2, A and B, red). In all sCR1-treated animals, glomerular C3 deposition was markedly reduced (Figure 2C, top panel) with only trace glomerular staining for sCR1 (Figure 2C, bottom panel). In PBS-treated and unmanipulated animals, glomerular C3 deposition was clearly evident (Figure 2C, top panel) and sCR1 was undetectable (Figure 2C, bottom panel).

We calculated the serum t₁/₂ of sCR1 in mice to be approximately 18 hours. On the basis of the dose-response curves to single intraperitoneal injections, we treated four CfH−/−/huCR1-Tg mice with three intraperitoneal injections (25 mg/kg, n=2 mice; 50 mg/kg, n=2 mice) at 24-hour intervals (Figure 3A). In all sCR1-treated animals, serum C3 levels rose to near normal by 12 hours and remained high throughout the experiment (Figure 3B). Glomerular immunofluorescence studies showed that there was a significant decrease in new C3 deposition, with clearance of old C3 demonstrated by the marked reduction in C3c staining. C3d staining also decreased and sCR1 staining was absent (Figure 3D). To verify that control of the C3 convertase had been restored and that it was an increase in serum C3 and not its degradation products that we were measuring, we performed Western blotting under reducing conditions with a C3α-chain–specific antibody to document the appearance of the 106-kD C3α chain. In PBS-treated and untreated animals, serum C3 levels remained nearly undetectable and the 106-kDa C3α chain was not seen (Figure 3C). Thus, sCR1 treatment restored fluid-phase AP regulation in these murine models of C3G.
On the basis of these data, we sought permission from the US Food and Drug Administration (FDA) for a limited multidose safety trial with sCR1 when presented with a C3G patient in ESRF. The patient was an 8-year-old girl with biopsy-proven DDD (Figure 4A) who had presented 3 months earlier with lower extremity swelling, nephritic urine sediment, nephrotic-range proteinuria, positive C3Nefs, and a low serum C3 level (21.6 mg/dl; normal range, 90–180 mg/dl). She transitioned to rapidly progressive GN (eGFR <10 ml/min per 1.73 m²) necessitating dialysis. Approval for a seven-dose trial of sCR1 was obtained from the FDA and from the University of Iowa Institutional Review Board.

The patient received a 10 mg/kg loading dose of sCR1 followed by six maintenance doses at 5 mg/kg every 48 hours. During treatment, serum C3 rose to a peak of 48 mg/dl but dropped to pretreatment levels by the end of the trial. Soluble C5b-9 levels were elevated before treatment, normalized after the sixth dose of sCR1, and became abnormal after terminating therapy (Figure 4D). Biopsies taken before and after treatment were similar (Figure 4, B and C, and Table 1). The patient continues on dialysis at this time.

Although the trial was very short, there were no adverse effects associated with the administration of sCR1 and immunogenicity was not detected. Assays for anti-sCR1 antibodies on plasma obtained before dosing and at days 2, 4, 6, 8, 10, 12, and 14 and 1 month after dosing remained consistently negative (A450 values ≤0.076 at dilutions of 1:50 at all time points; positive control, A450 1.10, 0.68, and 0.40 at 1:100,
Figure 2. Human sCR1 restores serum C3 levels and reduces C3 glomerular staining in Cfh<sup>−/−</sup> and Cfh<sup>−/−</sup>/huCR1-Tg mice. (A) Plasma levels of sCR1 dropped rapidly following a single tail-vein injection in 5 Cfh<sup>−/−</sup> mice (blue lines; t1/2 approximately 18 hours). After a single IP injection of sCR1 (5 mg/kg, 25 mg/kg or 50 mg/kg) in Cfh<sup>−/−</sup>/huCR1-Tg mice, plasma levels rose and then fell slowly (red lines). Each line represents a single mouse. (B) Serum C3 levels in Cfh<sup>−/−</sup> mice (blue lines) increased 24 hours after a single tail-vein injection of sCR1 (50 mg/kg) but returned to baseline 48 hours later. Serum C3 levels in Cfh<sup>−/−</sup>/huCR1-Tg mice (red lines) also increased 12 hours after a single IP injection (5 mg/kg, 25 mg/kg, or 50 mg/kg) and closely followed the sCR1 concentration curves in A. All data (A and B) represent the mean of triplicate assays. (C) C3 glomerular deposition decreased after 48 hours in sCR1-injected Cfh<sup>−/−</sup> mice (50 mg/kg by tail-vein injection) but not in PBS-injected or non-injected controls (top panel). Trace glomerular staining for human CR1 (CD35) was seen in sCR1-injected mice 48 hours after treatment (bottom).
Figure 3. Multi-dose sCR1 treatment clears iC3b glomerular deposition in mouse. (A) Daily dosing (arrows) maintained sCR1 serum levels (blue, 25mg/kg; red, 50mg/kg, black, PBS). (B) Serum C3 levels in Cfh<sup>−/−</sup>/huCR1-Tg mice increased and remained high with daily IP dosing (0, 24, and 48 hours) of 25 (blue) or 50 (red) mg/kg sCR1 (black, PBS; normal range: 300–1500 mg/L). Data represent the mean of triplicate assays. (C) Intact C3α (992 amino acids) was detected by Western blotting in reducing conditions using a murine C3α-specific antibody. The 106-kDa band (equivalent to the C3α chain in wild-type mice) appeared 12 hours after sCR1 treatment but was absent before treatment and in untreated controls. (D) IF of glomeruli harvested 60 hours after injection with relative fluorescence intensity (%).
DISCUSSION

The in vitro activity of sCR1 in complement inhibition is dependent on the specific conditions of the assay and varies with the concentration and hemolytic potential of the serum complement source and the efficiency of the complement-activating mechanisms. In the assays we used, sCR1 prevented C3 convertase activity in normal (rabbit erythrocyte hemolytic assay) and pathologic conditions (sheep erythrocyte hemolytic assay with DDD sera). In vivo experiments in two mouse models of C3G confirmed the in vitro data—sCR1 stopped AP dysregulation and restored plasma C3 levels to normal. These changes were accompanied by reduced deposition of new iC3b and clearance of old iC3b in the GBM.

Our results are consistent with the known role that CR1 plays as a central complement regulator of both the C3 and C5 convertases. In addition to regulating these convertases, CR1 is the only cofactor of fH that can cleave iC3b into smaller fragments (C3c and the thioester-containing fragment C3dg), thus explaining the rapid clearance of iC3b from the GBM. The slower clearance of C3d is consistent with its surface-binding properties (Figure 3D). Thus, by bringing complement dysregulation under control, the deposition of new iC3b is arrested (Figure 3).

These observations provide strong evidence that iC3b is deposited in the GBM and is an important component of the glomerular dense deposits, a conclusion consistent with work done by Pickering and colleagues demonstrating that the presence of fH is an absolute requirement in Cfh−/− mice for the development of a C3GN renal phenotype. In mice deficient in both fH and fI (Cfh−/−.Cfi−/− mice), although excess C3b is present in the circulation, no iC3b forms and GBM dense deposits are not seen.27 The Cfh−/−/huCR1-Tg mouse also provides an excellent animal model in which to study long-term gene therapy to assess the effect of constitutive hepatic expression of sCR1 on the renal phenotype.

The patient with DDD who we treated received only seven doses of sCR1; however, we demonstrated that multidose treatment was safe and nonimmunogenic in this limited study. We did not expect to, nor did we, see a histologic improvement with a transient improvement in serum C3 and soluble C5b-9 quantitation (averaged control value set to 100% with the exception of CD35 where averaged C3 control was set to 100%; scale bar: average of 10 glomeruli ± SD). From top to bottom: C3 was dramatically decreased; CD35 (CR1) was absent; C3d decreased significantly with the higher dose of sCR1; C3c was greatly reduced. *P<0.05, **P<0.001; controls versus experiments.

CONCISE METHODS

Patient

An 8-year-old patient with rapidly progressive DDD was enrolled under an FDA-approved compassionate-use investigational new drug study designed to administer seven doses of sCR1 under IRB-approved guidelines. DDD was diagnosed by renal biopsy.

Patient and Normal Sera

Ten patients with biopsy-proven DDD consented to provide sera under IRB-approved guidelines. All patients were positive for C3Nefs except for patients DDD-07 and DDD-08. Patient DDD-07 carries a genetic rearrangement between CFH and CFHB, and patient DDD-08 carries a genetic variant in CFHR5 (c.1541 T>G; p.Met514-Arg). No other genetic mutations were identified in CFH, CFH, CFB, MCP, or C3. Pooled normal human sera were obtained from Innovative Research (Novo, MI); pooled murine sera were collected from five 2-month-old C57BL/6 animals by cardiac puncture.

sCR1

sCR1 (also called TP10 or CDX-1135) was produced by recombinant DNA technology using Chinese hamster ovary cells and purified using standard filtration and chromatography methods as a single-chain polypeptide of 1931 amino acids with a protein molecular mass of 212 kD (Celldex Therapeutics, Needham, MA). Due to N-linked glycosylation, the final total molecular mass was 247 kD.28

Complement Activity Assays

AP and CP activity were evaluated using the appropriate Wieslab complement assay (Wieslab AB, Lund, Sweden) following the manufacturer’s protocols (serum dilution 1:34 for AP and 1:100 for CP). To measure complement inhibitory effects, increasing amounts of sCR1 were added to diluted sera before conducting these assays.

Hemolytic Assays

AP hemolytic activity was measured using rabbit or sheep erythrocytes. Rabbit erythrocytes activate AP-mediated lysis in human and mouse sera; sheep erythrocytes do not. For the rabbit erythrocyte hemolytic assay, increasing concentrations of sCR1 (0.2 nM to 200 nM) or fH (0.2 nM to 2000 M) were mixed with normal human or mouse sera (20% v/v) and incubated for 30 minutes at 37°C with 1×10⁶ rabbit erythrocytes in the presence of 10 mM EGTA/0.15 mM MgCl²/gelation veronal buffer (GVB) or EDTA-GVB as a
Figure 4. sCR1 restores soluble MAC levels in a DDD patient. (A) Biopsy at presentation showed a proliferative-appearing glomerulus with cellular crescents, endocapillary and mesangial hypercellularity and capillary loop “double contours” (light microscopy [LM] ×400). No global glomerulosclerosis was noted, and interstitial fibrosis and tubular atrophy were minimal. Glomerular “ribbon-like” electron-dense deposits were present on electron microscopy (EM, ×8200) with discrete, coarsely granular C3 immunofluorescence of the mesangium and
Table 1. Patient biopsies at presentation and before and after treatment with sCR1

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<th>Histology</th>
<th>Presentation</th>
<th>Before Treatment</th>
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<td>Crescents (n)</td>
<td>21 of 25</td>
<td>6 of 18</td>
<td>3 of 13</td>
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<tr>
<td>Glomerular sclerosis (n)</td>
<td>0 of 25</td>
<td>9 of 18</td>
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<td>Interstitial fibrosis</td>
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<td>C3 immunofluorescence</td>
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Nonhemolytic control. The reaction was stopped by adding 150 µl of 20 mM EDTA-GVB. After centrifugation, the supernatant was transferred to a clean 96-well plate and absorbance was recorded at 415 nm. Percent lysis was calculated as a fraction of the maximum A415 absorbance in the absence of sCR1 [(A415sCR1-EGTA−A415EDTA)/(A415EGTA) × 100]. To test whether sCR1 prevents C3Nef stabilization of C3 convertase, 10 µl of patient serum was added to 10 µl of sheep erythrocytes (1 × 10^8/ml) coated with preformed C3 convertase in a total 50 µl (EGTA-Mg-GVB) reaction as described. The mixture was allowed to decay at 30°C (water bath) for 20 minutes. Hemolysis was assayed by adding 50 µl of rat serum (1:9 diluted in GVB-EDTA buffer) as a source of C5-9. Sheep red blood cells were lysed in the presence of nondecayed C3 convertase stabilized by C3Nefs. The reaction was stopped and the percent of hemolysis was measured as described above. In parallel tests, varying concentrations of sCR1 (0 nM, 40 nM, 120 nM, 160 nM) were added to patient serum before mixing with sheep erythrocytes and incubating on ice for 15 minutes. Repetition of this experiment was done with sera from 10 patients with DDD.

**Mice**

The Cfh−/− mutant mouse, obtained from Dr. Matthew Pickering, was generated as described. On this background, human CR1 was introduced to create the Cfh−/−/huCR1-Tg mutant. The latter mouse line expresses CR1 only on mouse erythrocytes. The line was used to avoid any murine immunoresponse. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

**sCR1 and C3 Concentrations**

sCR1 concentrations were measured by sandwich ELISA in a microtiter plate format using two mouse mAbs specific for CR1. Microtiter plates were coated with mAb 6B1.H12; the detection antibody was horseradish peroxidase–conjugated mAb 4D6.1. Murine total C3 serum levels were measured using a two-site ELISA (Kamiya Biomedical, Seattle, WA); human total C3 was measured by radial immunodiffusion (The Binding Site, San Diego, CA). Data were collected in three independent assays and expressed as the mean for each group.

**Immunohistochemical Analyses**

Kidneys were harvested at the time of euthanasia and imbedded in Tissue-Tek OCT medium for routine processing (Sakura Finetek, Torrance, CA). For glomerular C3 deposition, sections were stained with FITC-conjugated mouse C3 antibody (MP Biomedicals, Solon, OH) at a dilution of 1:800 for 1 hour. For glomerular sCR1, C3c, and C3d staining, sections were treated with goat anti-human CR1 antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human C3c (1:8000; Abcam, Cambridge, MA), or goat antimouse C3d (1:400; R&D Systems, Minneapolis, MN) for 1 hour, respectively, followed by staining with the corresponding Alexa Fluor 488 (1:500; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Fluorescence images were acquired on a Leica model TCS SP5 laser system (Leica Microsystems, Buffalo Grove, IL). Relative fluorescence intensities (average of 16 glomeruli; ± SD) were quantified by ImageJ software (http://rsbweb.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

**Immunoblot Analyses**

Mouse sera were diluted 1:40 in Laemmlli buffer, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose membranes (Whatman Schleicher and Schuell Inc., Dassel, Germany). Membranes were blocked with 5% skim milk in PBS at room temperature for 30 minutes and incubated with anti-mouse C3 antibody (1:500; in-house antibody targeting the fl cleavage site on C3). Each membrane was washed three times with 0.05% Tris-buffered saline Tween-20 followed by incubation with horseradish peroxidase–conjugated anti-mouse secondary antibody (Jackson ImmunoResearch, Inc., West Grove, PA). Specific protein bands were visualized with an Enhanced Chemiluminescent System (GE Healthcare, Piscataway, NJ).

**sCR1 Dose Simulations**

Pharmacokinetic data from adult cardiac surgery patients treated with sCR1 were used to estimate sCR1 serum concentrations over time using empirical calculations that assumed concentrations from multiple doses to be additive because sCR1 pharmacokinetics has
been shown to be dose proportional (dose versus area under the curve are linear). Averaged data from 26 cardiac surgery patients receiving a single 10 mg/kg bolus of sCR1 were used to simulate the dosing regimen used in the single DDD patient. The dose regimen was designed to keep trough levels of sCR1 above 20 μg/ml throughout the dosing period.

Immunogenicity Assays
Patient plasma samples were screened for anti-sCR1 antibodies using a microtiter plate ELISA format. Microtiter plates were coated with sCR1 and blocked with BSA in PBS. Patient samples were diluted 1:50 in PBS 10 mM EDTA 0.5% Tween and incubated in coated plates. Antibodies were detected using horseradish peroxidase-conjugated F(ab)₂ goat anti-human IgG antibody (KPL Inc., Gaithersburg, MD). Normal human serum was used as a negative control. Knops-McCoy blood group antiserum, which is specific for CR1, served as a positive control. Positive patient samples were confirmed specific for sCR1 by preincubating with excess sCR1 and rerunning the assay.

Statistical Analyses
IC₅₀ values were calculated by GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance was calculated with the t test.

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