Aliskiren inhibits renin-mediated complement activation

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Certain kidney diseases are associated with complement activation although a renal triggering factor has not been identified. Here we demonstrated that renin, a kidney-specific enzyme, cleaves C3 into C3b and C3a, in a manner identical to the C3 convertase. Cleavage was specifically blocked by the renin inhibitor aliskiren. Renin-mediated C3 cleavage and its inhibition by aliskiren also occurred in serum. Generation of C3 cleavage products was demonstrated by immunoblotting, detecting the cleavage product C3b, by N-terminal sequencing of the cleavage product, and by ELISA for C3a release. Functional assays showed mast cell chemotaxis towards the cleavage product C3a and release of factor Ba when the cleavage product C3b was combined with factor B and factor D. The renin-mediated C3 cleavage product bound to factor B. In the presence of aliskiren this did not occur, and less C3 deposited on renin-producing cells. The effect of aliskiren was studied in three patients with dense deposit disease and this demonstrated decreased systemic and renal complement activation (increased C3, decreased C3a and C5a, decreased renal C3 and C5b-9 deposition and/or decreased glomerular basement membrane thickness) over a follow-up period of four to seven years. Thus, renin can trigger complement activation, an effect inhibited by aliskiren. Since renin concentrations are higher in renal tissue than systemically, this may explain the renal propensity of complement-mediated disease in the presence of complement mutations or auto-antibodies.

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Renal diseases may be associated with activation of the alternative pathway of complement due to mutations in genes encoding complement regulators or proteins and circulating autoantibodies,[1,2] allowing uncontrolled complement activation and resulting in deposition of components of the alternative pathway and terminal–complement complex in the kidneys.[3,4] These conditions include atypical hemolytic uremic syndrome[1] and C3 glomerulopathy, the latter further subdivided into dense deposit disease (DDD) and C3 glomerulonephritis,[2] conditions that may be associated with circulating C3 nephritic factor (C3NeF), an autoantibody directed to the C3 convertase.[5,6] All these conditions ultimately lead to renal failure.

Complement-mediated disease has a propensity to affect the kidneys. In atypical hemolytic uremic syndrome disease activity subsides as renal failure progresses but recurs upon renal transplantation, indicating that viable renal tissue may contribute to disease activity. It is as yet unclear why the kidney is a target organ in these conditions. In this study we investigated whether renin, known to be secreted solely in the kidney,[7] activates the alternative pathway of complement.

Renin is an aspartate-protease released exclusively by cells of the renal juxtaglomerular apparatus.[8] Renin initiates the renin–angiotensin–aldosterone system (RAAS) by cleaving angiotensinogen into angiotensin I. To date, the only known substrate of renin is angiotensinogen.

Aliskiren is an orally active, nonpeptide renin inhibitor approved for treatment of hypertension. It has also been successfully used to reduce proteinuria in diabetic[9] and nondiabetic nephropathy[10] both in adults and children, as well as for the control of pediatric hypertension.[11] It may thus have a role in nephroprotection during chronic progressive nephropathies.

In this study we identified C3 as a novel substrate of renin and showed that renin-mediated C3 cleavage was identical to that induced by the C3 convertase *in vitro*. Renin-mediated C3 cleavage was specifically blocked by the renin inhibitor aliskiren. The effect of aliskiren on complement activation was assessed in 3 patients with DDD demonstrating evidence for decreased systemic and renal complement activation.

RESULTS

Renin cleaves C3 into C3b and C3a *in vitro*

C3 (100 μg/ml) combined with plasma renin (4.5 pg/ml, unless otherwise stated) resulted in C3 cleavage and the appearance of a band corresponding to C3b. The cleavage of

C3 by renin was confirmed using kidney renin and recombinant renin (Figure 1a). C3 cleavage by plasma renin commenced within 5 minutes and was complete between 2 and 5 hours (Figure 1b). C3 alone did not undergo spontaneous cleavage within 24 hours (Figure 1b, lane 11). C3 incubated with renin induced the release of C3a (Figure 1c and d). C3a levels induced by renin or C3 convertase-mediated C3 cleavage were comparable (Figure 1c), as were kinetics (Supplementary Table S1). The Km was 2.97 μM for plasma renin and 2.16 μM for C3 convertase (the previously published Km of the C3 convertase was 5.86 μM).[12]

N-terminal sequencing of the cleaved product confirmed that renin cleavage of C3 occurred at the same site as cleavage by C3 convertase,[13] because the N-terminal 9 amino acids of C3b α chain were identical (Figure 1e). The amino acid sequence at the cleavage site differed from the sequence within angiotensinogen.[14]

Experiments similar to those carried out with human proteins were carried out using murine renin and murine C3, but C3 cleavage could not be demonstrated (Supplementary Figure S1).

Inhibition of renin-mediated C3 cleavage by aliskiren, pepstatin, and zinc

C3 cleavage by plasma renin (0.45 pg/ml) was inhibited by the renin inhibitor aliskiren at concentrations of ≥0.01 M (Figure 1f). Pepstatin A, an inhibitor of aspartate proteases, also inhibited plasma renin (Figure 1f) and kidney renin–induced C3 cleavage at concentrations of ≥0.3 mM. Aliskiren and pepstatin alone had no effect on C3 (Figure 1f).

Cathepsin D is also inhibited by aliskiren[15] and pepstatin[16] but did not cleave C3 at a concentration of 10 μg/ml (data not shown). $ZnCl_2$, known to inhibit aspartate proteases,[17] inhibited plasma renin–induced C3 cleavage, but $MgCl_2$ and $NiCl_2$ did not (Supplementary Figure S2).

Aliskiren inhibits C3 cleavage by renin in the presence of serum

The cleavage reaction induced by plasma renin (2.7 pg/ml) was carried out in the presence of normal human sera ($n = 6$). A band corresponding to C3b was not demonstrated (Figure 2, lane 2). The lack of a band corresponding to C3b could be due to the presence of complement factor I (CFI) allowing C3b degradation. The incubation was therefore performed in CFI-depleted serum ($n = 3$). Under these conditions a band corresponding to C3b was visualized (Figure 2, lanes 4 and 7 in the presence of plasma renin and lane 8 in the presence of recombinant renin). The band corresponding to C3b was not visible when CFI was added to the CFI-depleted serum (Figure 2, lane 5). Aliskiren, at concentrations at or above 0.13 M, inhibited C3 cleavage induced by plasma renin visualized in CFI-depleted serum (Figure 2, lane 6).

Renin-mediated cleavage of a C3 peptide

Recombinant renin (0.27 μg/ml) was incubated with a fluorescence resonance energy transfer C3 peptide, consisting of 14 amino acids covering the cleavage site. Renin-mediated cleavage was compared with the C3 convertase at various time points, showing enhanced fluorescence over time (Figure 3).

Functional assays of the C3 cleavage product

C3a as a chemoattractant. C3a is a known mast cell chemoattractant.[18] The product of C3 incubation with plasma renin significantly increased mast cell migration, in comparison with C3 alone, and this effect was inhibited by pepstatin (Figure 4). The use of aliskiren was precluded owing to excessive dilution requirements.

Analysis of the interaction between C3 and renin by surface plasmon resonance. Experiments were designed to determine whether the cleavage products C3b and C3a were formed after C3 cleavage by plasma renin. Using an anti-C3b antibody that specifically recognizes the C3b neoepitope formed after C3 cleavage, binding was detected to C3 incubated with plasma renin but not to C3 alone (Figure 5a). Anti-C3a antibody bound to C3 but not to the C3–renin cleavage product (Figure 5b), indicating that C3a was released and washed away in the presence of renin.

Factor B (CFB) binding to cleaved C3 (incubated with plasma renin overnight to ensure total cleavage) was demonstrated by adding CFB to C3, C3b, and C3 incubated with renin (Figure 5c). The results show considerably more binding of CFB to C3b and C3 cleaved by renin than to uncleaved C3. Similar results were obtained when the C3 and C3b curves were aligned before injection of CFB (Supplementary Figure S3). In the presence of aliskiren CFB did not bind to C3 (Figure 5d). Factor D (CFD) cleaved CFB bound to the C3bB complex formed after adding CFB to the cleavage product of renin (Figure 5e).

Release of factor Ba. C3 or C3 cleaved by plasma renin overnight were incubated with CFB and CFD. The results indicate that C3b, generated by renin cleavage of C3, bound to CFB, and factor Ba was released in the presence of CFD. Levels corresponded to factor Ba released in the presence of the $C3(H_2O)$ convertase formed by binding of C3 to CFB (middle column in Figure 5f). Of note, C3 digested by renin (right column, Figure 5f) contains C3 totally cleaved to C3b but no C3.

Aliskiren decreases C3 deposition on renin-producing cells. Renin-producing Calu6 cells incubated with C3 for 4 hours exhibited C3 deposition, demonstrated in Figure 6a. Preincubation of the cells with aliskiren for 18 hours before addition of C3 reduced C3 deposition (Figure 6b), which was comparable to Calu6 cells, incubated with C3, in which renin was knocked out (Figure 6c).

C4 and C5 are not cleaved by renin

Incubation of human C4 with plasma or recombinant renin for 24 hours did not lead to cleavage (silver staining, data not shown). Incubation of human C5 with plasma renin for 2 and 24 hours did not lead to cleavage (see immunoblot, Supplementary Figure S4). Likewise, a band corresponding to C5a was not detected. C5a levels were also not increased

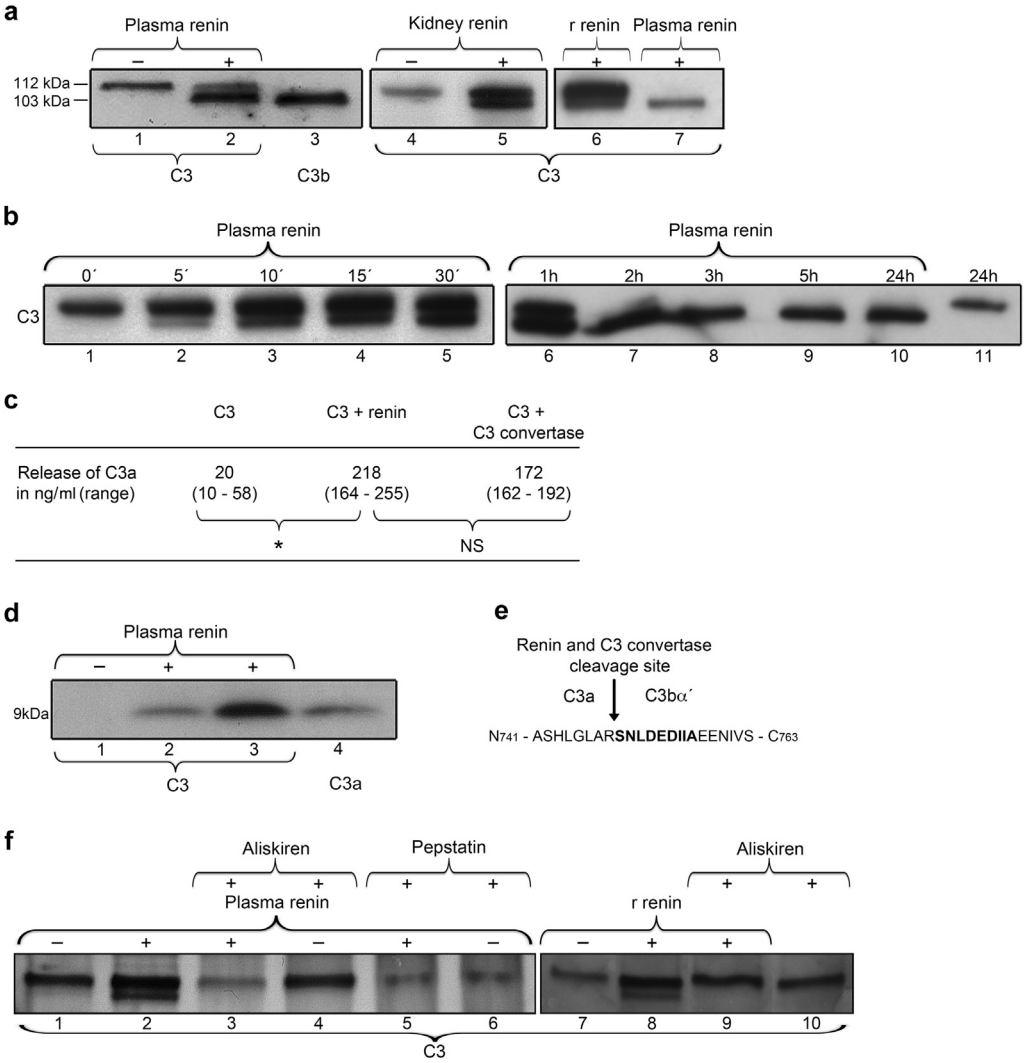


Figure 1 | C3 cleavage by renin and its inhibition. (**a**) C3 cleavage by renin. C3 (100 μg/ml, lane 1) incubated with plasma renin (4.5 pg/ml) for 1.5 hours and analyzed by immunoblotting showing cleavage to C3b (lane 2). Pure C3b, under the same conditions (100 μg/ml, lane 3). C3 (lane 4) incubated with kidney renin (5.7 ng/ml, lane 5) and recombinant renin (0.82 μg/ml, lane 6) overnight demonstrated the appearance of a C3b band. For comparison, C3 incubated overnight with plasma renin in lane 7. Results depict 1 representative experiment of a total of 3 experiments. (**b**) C3 cleavage by plasma renin over time depicting total cleavage within 2 hours. Reproducible results were obtained 4 times. Lanes 1–5 were run in the same gel and rearranged. (**c**) Release of C3a after C3 incubation with ($n = 3$) or without plasma renin ($n = 7$) for 60 minutes or with preformed C3 convertase ($n = 3$). (**d**) A band corresponding to C3a was demonstrated when C3 (500 μg/ml, lanes 1 and 2; 1000 μg/ml, lane 3) was incubated with plasma renin for 2 hours. Lane 4: C3a 5 μg/ml. (**e**) The C3 cleavage site generated by renin (4.5 pg/ml) was determined by N-terminal sequencing and confirmed the 9 N-terminal amino acids of C3bα chain (indicated in bold) identical to the sequence after cleavage by the C3 convertase. (**f**) Aliskiren (0.04 M) and pepstatin (0.3 mM) preincubated with plasma renin (0.45 pg/ml) for 3 hours inhibited C3 cleavage by renin during a 2-hour incubation demonstrated by silver staining (lanes 3 and 5, respectively). Aliskiren and pepstatin did not have an effect on C3 itself after 2 hours' incubation in the absence of renin (lanes 4 and 10 aliskiren, lane 6 pepstatin). Aliskiren (0.08 M) preincubated with recombinant renin (2 μg/ml) for 1 hour inhibited C3 cleavage by renin during a 2.5-hour incubation, demonstrated by immunoblotting (lane 9). Lanes were run on the same gel and rearranged. Full-length gels and immunoblots corresponding to panels (**a**), (**b**), and (**f**) are presented in Supplementary Figures S5 and S6. *$P < 0.05$. NS, not significant. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

(enzyme-linked immunosorbent assay [ELISA], data not shown).

The effect of aliskiren on complement activation in DDD patients

Three pediatric patients with DDD (Table 1) were treated with the renin inhibitor aliskiren. Complement levels obtained before and during aliskiren treatment are summarized in Table 2 and Figure 7 and detailed in Supplementary Tables S2–5. Decreased systemic complement activation was demonstrated by significantly increased C3 levels in all 3 patients and decreased C3a and C5a levels in patients 1 and 3 (values were not available in patient 2 treated with repeated plasma exchanges before the onset of aliskiren treatment).

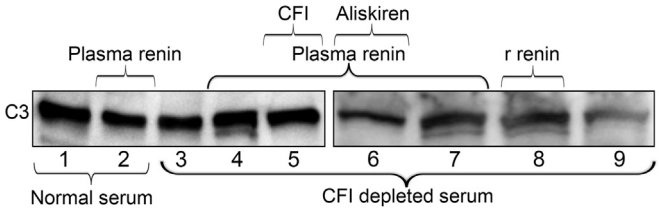


Figure 2 | C3 cleavage by renin and its inhibition by aliskiren in the presence of serum. Normal human serum or complement factor I (CFI)–depleted serum were incubated with plasma renin (2.7 pg/ml) or recombinant renin (1.2 μg/ml) for 24 hours and C3 cleavage analyzed by immunoblotting. C3 cleavage was not detected in normal serum with or without renin (lanes 1 and 2). C3 cleavage was not visible in CFI-depleted serum in the absence of renin (lanes 3 and 9). A C3b band was demonstrated in CFI-depleted serum incubated with plasma renin (lanes 4 and lane 7) or with recombinant renin (r renin, lane 8). The C3b band was not visualized when CFI (1 μg/ml) was added, suggesting that C3b was degraded (lane 5) to iC3b. The appearance of a degradation band corresponding to the alpha chain of iC3b is demonstrated in Supplementary Figure S7. Aliskiren (0.13 M) preincubated with plasma renin for 1 hour inhibited C3 cleavage by plasma renin in CFI-depleted serum (lane 6). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Decreased renal complement deposition was demonstrated in repeat kidney biopsies taken during aliskiren treatment. Decreased C3 deposition was demonstrated in all 3 patients (Figure 8a, biopsy from patient 2), and reduced C5b-9 deposition was demonstrated in patient 2 (Figure 8b and c). Furthermore, glomerular basement membrane (GBM) thickness was reduced in repeat biopsies in all 3 patients (Table 2; Supplementary Table S6; and Figure 8d biopsy from patient 1), suggesting a decrease in complement deposition.

Patient 1 showed a marked increase in serum C3 and a decrease in C3a and C5a, as well as a decrease in GBM thickness (Table 2; Supplementary Tables S2, S5, and S6; and Figures 7 and 8d), providing evidence of improvement while being treated with aliskiren. These findings were further supported by the decrease in C3 occurring during a temporary discontinuation of aliskiren (Supplementary Table S2). This patient has had stable renal function while receiving aliskiren treatment for 5 years (Tables 1 and 2), during which it was given as a monotherapy for 1 year (Supplementary Table S7). Treatment with i.v. Igs was initiated in 2015 to prevent recurrent infections. She developed drusen deposits in the retina in 2017.

Patient 2 was treated with plasma exchanges regularly starting in 2006 and at the time aliskiren was started in 2011. Between 2006 and 2010, each attempt to prolong intervals between plasma exchanges to more than 5 weeks resulted in decreased C3 levels and increased proteinuria, despite ongoing treatment with mycophenolate mofetil and prednisolone. After initiation of aliskiren, intervals between plasma exchanges were extended to 6–8 weeks without decreasing C3 levels (Supplementary Table S3) or increasing proteinuria. Plasma exchange was discontinued in 2012, 8 months after starting aliskiren. Ophthalmologic investigation demonstrated that this patient developed drusen deposits in the retina during this period. This patient underwent 2 biopsies before starting aliskiren treatment, with no change in glomerular complement deposits between the first and the second biopsy; a decrease was noted in the third biopsy while the patient was receiving aliskiren treatment and after discontinuation of plasma exchanges (Table 2; Supplementary Table S6; Figure 8a–c). The patient remains stable on aliskiren treatment (started in 2011).

Patient 3 was treated with eculizumab, mycophenolate mofetil, and prednisolone when aliskiren was begun (Table S7) and the second renal biopsy was performed. Treatment with eculizumab was terminated 3 months after initiation of aliskiren. The third renal biopsy was performed 22 months after eculizumab was discontinued. Both repeat renal biopsies showed, unexpectedly, that C5b-9 deposits were unchanged (eculizumab, directed to C5, would be expected to affect C5b-9), but C3 deposits decreased. GBM thickness decreased in the third biopsy (Table 2; Supplementary Table S6). C3 levels in sera increased (Figure 7), and C3a and C5a levels decreased (Supplementary

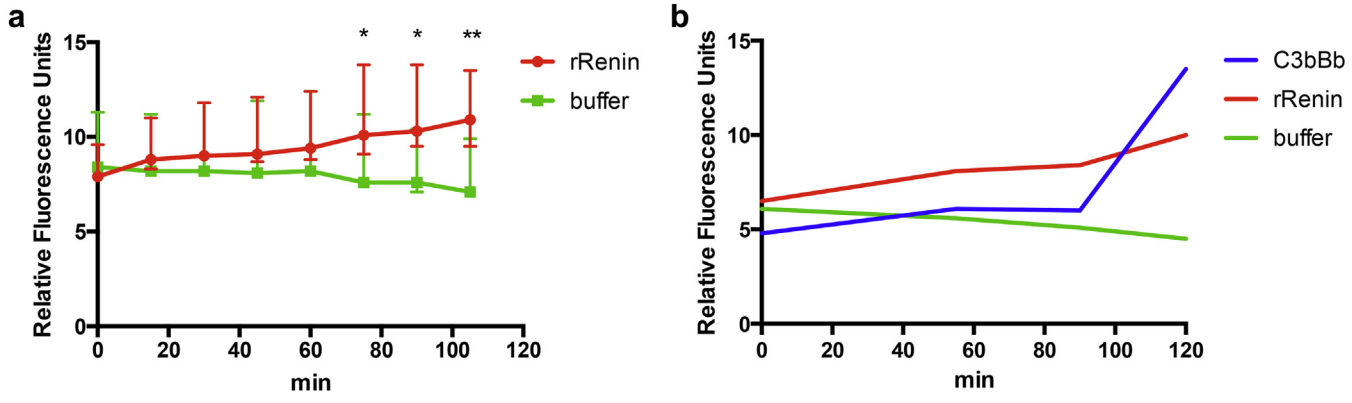


Figure 3 | C3 peptide–fluorescence resonance energy transfer (FRET) assay. The 14-amino-acid FRET peptide was incubated with recombinant renin (**a**, $n = 5$) or C3 convertase (**b**, $n = 1$) for up to 2 hours, showing increasing fluorescence release after cleavage. The buffer without enzymatic activity was incubated simultaneously and exhibited a decrease in fluorescence. *$P < 0.05$; **$P < 0.01$, assessed at each time point.

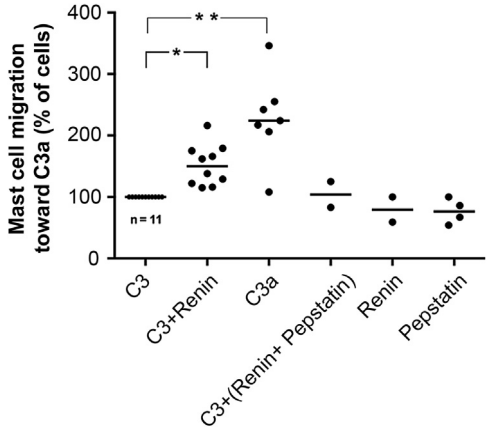


Figure 4 | Mast cell migration toward C3a. Mast cell migration was analyzed using C3 alone (defined as 100%), C3 incubated with plasma renin overnight, C3a alone (1 μg/ml, the positive control), C3 incubated with plasma renin that was preincubated with pepstatin (3 mM) for 2 hours, renin alone, and pepstatin alone. *$P < 0.05$; **$P < 0.01$. Multigroup comparison was significant, $P < 0.0001$.

Table S5). The patient remains stable on aliskiren treatment (4 years to date; Tables 1 and 2). Owing to the presence of persistent C3NeF and proteinuria, 2 doses of rituximab were given in late 2017.

All patients exhibited evidence of decreased complement activation (increased C3 levels, decreased systemic C3a and C5a, decreased renal C3 and C5b-9 deposition, and/or decreased GBM thickness) while on aliskiren treatment, and in addition, exhibited a stable course of renal disease (low-grade or stable proteinuria, normal or stable glomerular filtration rate; Table 1).

DISCUSSION

This study demonstrates that renin cleaves C3 into its physiological cleavage products C3a and C3b, thus triggering the alternative pathway. The enzymatic reaction is specific because cleavage was demonstrated using plasma and kidney renin, as well as recombinant renin, and activity was inhibited by the specific renin inhibitor aliskiren and by the aspartate protease inhibitor pepstatin. The cleavage product C3b is capable of binding CFB, thereby forming the C3 convertase, which could activate the amplification loop of the alternative pathway. This mechanism initiates complement activation, with specific tropism for the kidney, because renin concentrations are higher in the kidney than in the systemic circulation.[19] Furthermore, disproportionate renin release may occur during renal disease.[20] Renin is released by the juxtaglomerular apparatus and would therefore be present at the highest concentrations in structures relevant to complement-mediated renal disease (i.e., the afferent arteriole, glomerular capillaries, and mesangium). Under normal physiological conditions, with functional complement regulators, the effect of renin would be blocked by these inhibitors (schematically depicted in Figure 9), in parallel to the effect of the C3 convertase, which is inhibited by soluble and membrane-bound complement regulators.[21] During complement-mediated renal disease, such as DDD, complement activation triggered in the kidney would proceed in an uninhibited manner owing to mutations in complement components or circulating antibodies such as C3NeF, thus explaining why these diseases affect the kidney. Importantly, the effect of renin on C3 cleavage was completely inhibited by the renin inhibitor aliskiren. The contribution of renin-mediated complement activation was therefore investigated in 3 patients with DDD who were treated with aliskiren. Complement activation decreased significantly during treatment. We therefore propose that renin inhibition may reduce complement activation in complement-mediated renal disease.

In patients with DDD spontaneous remissions are uncommon, and approximately 50% develop end-stage renal failure within 10 years.[22] Thus multiple treatments have been attempted, and no single treatment has been effective in all patients.[23] Criteria for the success of treatment were defined as prevention of disease progression and a decrease in activation of the alternative complement pathway.[22] In the patients included in this study there was evidence of ongoing disease activity, as supported by the almost continuous presence of C3NeF, triggering complement activation, or low C3 levels, suggesting that the underlying disease process was still active. All patients exhibited decreased complement activation during aliskiren treatment, as demonstrated by significantly increased C3 levels and decreased C3 deposition in the kidneys. Patients also exhibited decreased circulating C3a and C5a, as well as decreased C5b-9 deposition and/or decreased GBM thickness in the kidneys. In addition, temporary discontinuation of aliskiren led to decreased C3 levels in patient 1, which improved after reinstatement of treatment (Supplementary Table S2). This patient was also treated with aliskiren as a monotherapy. The other 2 patients could discontinue multiple treatments, including plasma exchanges (patient 2) and eculizumab (patient 3; Supplementary Table S7) after initiation of aliskiren.

Of note, patients 2 and 3 are currently treated with mycophenolate mofetil, which they also received before aliskiren treatment, and thus the decrease in complement activation could not be attributed to this treatment. Mycophenolate mofetil is beneficial in patients with C3 glomerulonephritis,[24] a condition similar to DDD. Mycophenolate mofetil may affect C3 levels or complement deposits in the kidney by reducing C3NeF, but as shown in the supplementary tables, C3NeF activity was still present in all 3 patients. Thus, the reduction in C3 levels and complement deposits (C3 and C5b-9) in the kidneys should, most probably, be attributed solely to aliskiren.

Patients 1 and 2 developed drusen during the follow-up period. Drusen are extracellular deposits that accumulate beneath the retinal-pigmented epithelium and contain complement components.[25] The deposition of drusen at the same time that C3 and C5b-9 deposits in the GBM diminished could suggest that reduced complement activation induced by aliskiren is preferably localized to the kidney.[26] On the basis of the reduction of complement activation demonstrated in the

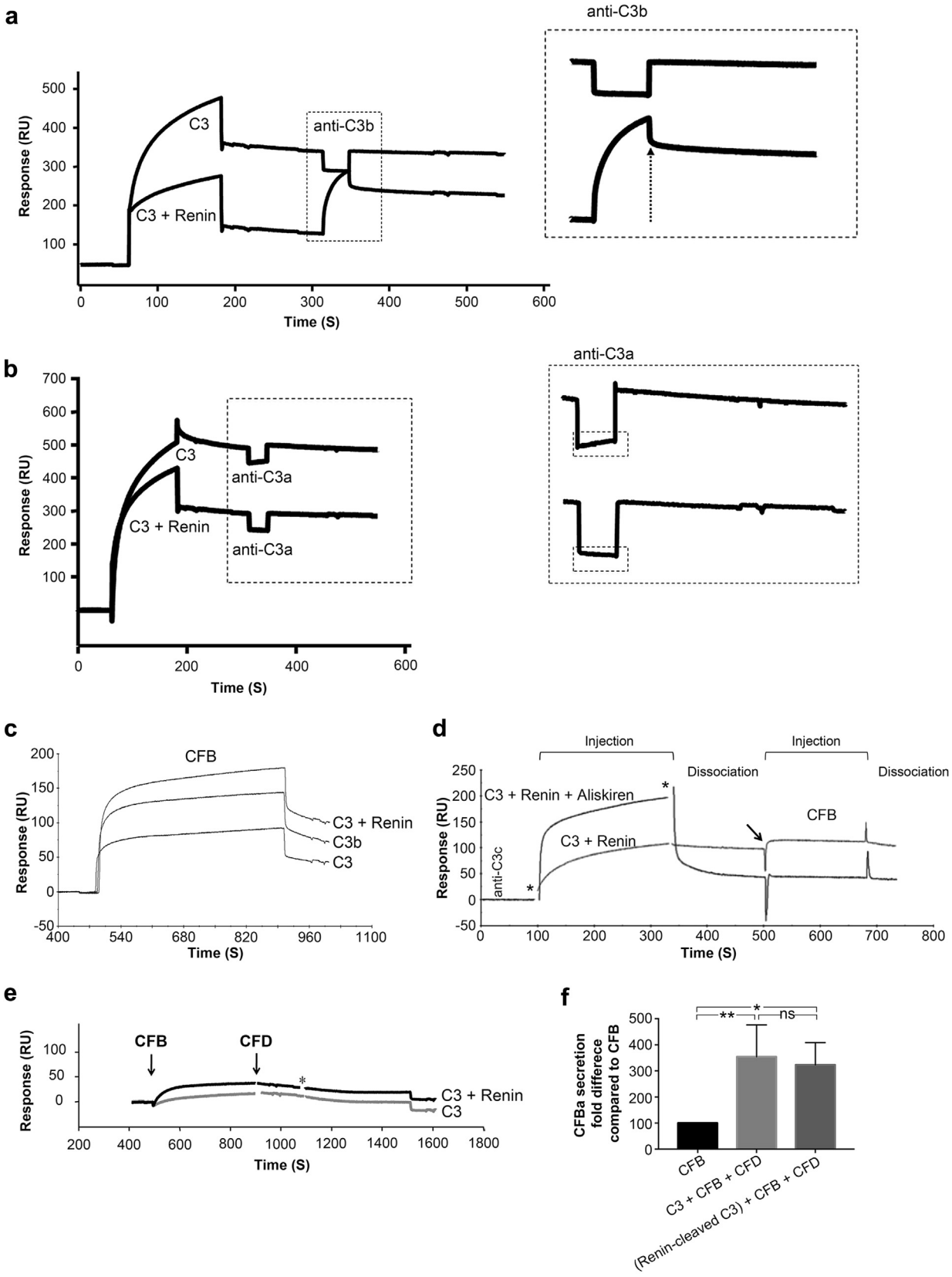


Figure 5 | C3 cleavage product detected by surface plasmon resonance and factor Ba release. (**a–d**) Surface plasmon resonance analysis of the interaction of C3 with renin (representative results from 2 experiments). In all experiments C3 (100 μg/ml) and plasma renin (4.5 pg/ml) were used. (**a**,**b**) C3 or C3 incubated with renin for 5 hours were injected over the immobilized anti-C3c antibody surface. (**a**) The anti-C3b antibody (detecting the neoepitope formed after C3 cleavage) bound to the renin cleavage product (arrow) but not to uncleaved C3. (Continued)

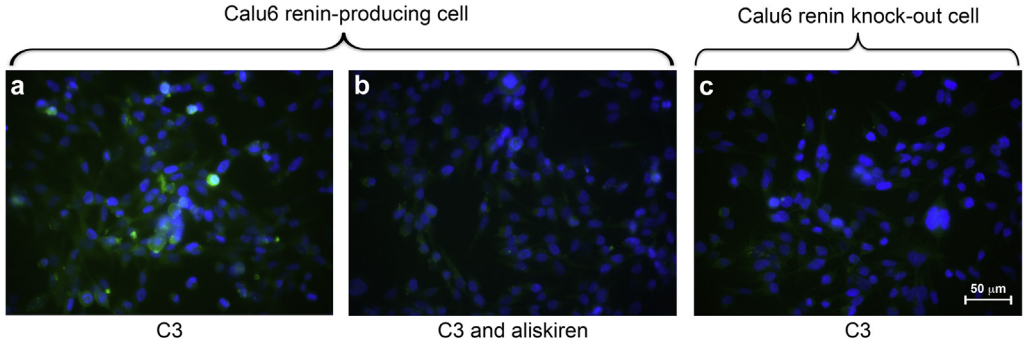


Figure 6 | Aliskiren prevents C3 deposition on Calu6 renin-producing cells. (**a**) C3 deposition was observed on Calu-6 renin-producing cells. Average fluorescent intensity of 2 representative fields was 5643 pixels. Cells were counterstained with 4′,6-diamidino-2-phenylindole (blue). (**b**) Cells preincubated with aliskiren for 18 hours before addition of C3 exhibited decreased C3 deposition. Average fluorescent intensity of 2 representative fields was 4244 pixels. (**c**) Calu6 cells in which the renin gene was knocked out exhibited an average fluorescent intensity of 2 representative fields of 4018 pixels. Bar = 50 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

patients, we suggest that aliskiren can induce partial complement inhibition, in addition to its previously demonstrated effect on proteinuria,[10] and thus be beneficial for complement-mediated renal disease with or without other anti-inflammatory therapies. We cannot rule out that the beneficial effect observed in our patients was also partly related to the known nephroprotective antifibrotic and anti-proteinuric effects of aliskiren,[27] but these would not explain the reduced systemic and local complement activation demonstrated here. Aliskiren treatment should, however, be reserved for patients without ongoing deterioration of renal function.

The terminal complement inhibitor eculizumab has been used in certain patients with C3 glomerulopathy,[28,29] although it has not been effective in all cases. It has therefore been suggested that complement blockade at the level of C3 could be preferential.[23] Patient 3 described herein was initially treated with eculizumab with a beneficial effect. After a period of combined therapy affecting both C3 (aliskiren) and C5 (eculizumab), the latter treatment was discontinued (Supplementary Table S7), and the patient remains stable. Use of a renin inhibitor may thus be considered, to inhibit complement activation at the C3 level.

Aliskiren has been shown to reduce renal complement deposition in a previous study using double transgenic rats carrying the human renin and angiotensinogen genes.[30] Although that study addressed other mechanisms of renal disease, the finding that aliskiren reduced C5b-9 expression strengthens our *in vitro* and *in vivo* findings in DDD patients. Unfortunately, murine renin did not cleave murine C3, precluding the testing of aliskiren in a murine DDD model. This may be due to significant differences between murine and human renin. Murine renin does not cleave human angiotensinogen,[31] and mice may possess either 1 (*Ren-1C*) or 2 (*Ren-1D* and *Ren-2*) renin genes, whereas humans possess only 1 with an additional exon (*Ren-1*).[32]

A reactive rise in circulating renin occurs during treatment with the renin inhibitor aliskiren owing to removal of angiotensin II–mediated negative feedback on renin release. Nevertheless enzymatic activity of renin is blocked.[33] A similar increase of renin levels is well established during treatment with RAAS-blockade, in which the enzymatic activity is not inhibited. The findings described here could indicate that RAAS blockade without renin inhibition could have an adverse effect in complement-mediated kidney disease. RAAS blockade is used in chronic renal disease because

Figure 5 | (Continued) The arrow indicates an increase of 117 resonance units (RU) binding of the anti-C3b antibody after subtraction of the control. The down-slope indicates slow dissociation from the surface. (**b**) The C3a antibody bound to C3 (an increase of 10 RU, upper boxed area in the inset) but did not bind to the renin cleavage product (lower boxed area in the inset), confirming that C3a was released and washed away. Controls were subtracted in the viewed graphs. (**c**) C3 was incubated with renin overnight to ensure total C3 cleavage. C3 incubated with renin, pure C3b, or C3 alone were injected over the immobilized anti-C3c surface, followed by injection of factor B (CFB). Increased CFB binding was demonstrated to the renin cleavage product (180 RU) and to C3b (145 RU) compared with uncleaved C3 (93 RU). (**d**) In these experiments renin was preincubated with aliskiren 0.08 M for 2 hours. CFB bound to C3 incubated with renin overnight (15 RU, see arrow), but no binding was demonstrated in the presence of aliskiren. Asterisk depicts deleted spikes occurring at the start or end of injection. (**e**) C3 incubated with renin overnight or C3 were injected over the immobilized antibody surface (not shown), followed by injection of CFB that bound to the renin cleavage product with higher affinity (46 RU) compared with uncleaved C3 (12 RU). After discontinuation of CFB flow, factor D (CFD) was injected. CFD cleaved CFB bound to the C3bB complex, demonstrated by a declining curve. Asterisk depicts discontinuation of CFD flow. (**f**) Release of factor Ba assayed by enzyme-linked immunosorbent assay. C3 (middle column) or C3 totally cleaved to C3b by overnight incubation with renin (right column) were incubated with CFB (0.5 μM) and CFD (0.17 μM). The middle column shows release of factor Ba in the presence of the C3(H2O) convertase. The results depict CFBa release compared with CFB alone, defined as 100% (mean value and SD from 6 separate experiments). $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 1 | Clinical and laboratory findings in the patients included in this study

			Clinical and laboratory findings						
Patient	Age at presentation (yr/sex)	Disease duration (yr)	Proteinuria[a] presentation/latest value	Hematuria[b]	Ophthalmological findings (drusen)	Creatinine/GFR	C3[b]	Complement mutations	C3 nephritic factor[c]
1	7/F	5	456/170	+	+[d]	41[e] N/90-103	↓	None[f]	+
2	10/F	12	526/35	+[g]	+[h]	57[i] N/89-137	↓	None[j]	+
3	12/F	5	3067/163	+	−	247[k] ↑/40–38	↓	None[f]	+

F, female; GFR, glomerular filtration rate (ml/min per 1.73m^2), at presentation and the latest value; N, normal; ↓, decreased; ↑, elevated.
Creatinine levels presented were taken at debut.
[a]Urine albumin/creatinine ratio at presentation and the latest value. Normal reference value: <3.8 g/mol.
[b]At presentation.
[c]Analyzed by crossed immunoelectrophoresis, hemolytic assay, and/or ELISA[39] (for details regarding C3 levels and C3 nephritic factor see Supplementary Tables S2–4).
[d]Drusen developed 4 years after presentation.
[e]Normal creatinine value for age: 28–57 μmol/l.
[f]Gene sequencing performed for complement factor H, complement factor H-related protein 5, and C3.
[g]Macroscopic hematuria.
[h]Drusen developed 6 years after presentation.
[i]Normal creatinine value for age: 50-90 μmol/l.
[j]As in (f) plus complement factor I.
[k]Normal creatinine value for age: 37-72 μmol/l.

of its antiproteinuric and other favorable effects on the renal and systemic vasculature. The cumulative effect of RAAS blockade may be beneficial even if complement activation is sustained.

During complement-mediated diseases, regulators of complement may be dysfunctional owing to mutations and/or the presence of autoantibodies.[1,2] All patients described here had C3NeF, an antibody shown to stabilize the C3 convertase, thus promoting activation of the alternative pathway. Complement is activated by triggers, such as infections. We envisage that renin continuously cleaves C3 and that further complement activation will be blocked by complement regulators. When complement regulation is dysfunctional, such as during DDD, complement activation via the alternative pathway will proceed in an uninhibited manner, thus allowing excessive complement activation, as depicted in Figure 9. Aliskiren treatment would primarily have a local effect, because it accumulates in the kidney,[26] thus reducing complement activation in the kidney while systemic C3 levels could still be reduced by the continuous presence of C3NeF or mutations. Systemic inhibition of C3 activation might cause severe side effects. Thus, C3 inhibition at the kidney level may have considerable advantages.

In summary, this study demonstrates a novel kidney-specific mechanism of complement activation by renin cleavage of C3 and its inhibition by the renin inhibitor aliskiren and shows, in 3 patients with DDD, a beneficial effect of aliskiren on complement activation. The findings could provide the basis for a clinical trial including more patients.

Table 2 | Complement profiles in patients during aliskiren treatment

		Complement activation					
		Systemic			Local, repeat kidney biopsy		
Patient	Duration of aliskiren treatment (yr)	C3	C3a	C5a	C3	C5b-9	GBM thickness
1	5	↑[a]	↓[a]	↓[a]	↓	UC	↓[b]
2	7	↑[c]	NA[d]	NA[d]	↓[e]	↓[f]	↓[b]
3	4	↑[g]	↓[g]	↓[g]	↓[h]	UC[h]	↓[b]

NA, not available; UC, unchanged.
[a]Values are given in Supplementary Tables S2 and S5 and in Figure 7.
[b]Values are given in Supplementary Table S6. Fewer ribbon-like deposits in the glomerular basement membrane (GBM) were found in the repeat biopsy shown in Figure 8d.
[c]Values are given in Supplementary Table S3 and in Figure 7.
[d]Not investigated owing to plasma exchange administered before the start of aliskiren treatment.
[e]This patient underwent 2 biopsies before the start of aliskiren treatment and 1 biopsy 15 months after the start of treatment. Comparison was carried out between the second and third biopsies. Decreased C3 demonstrated in Figure 8a.
[f]Demonstrated in Figure 8b, c.
[g]Values are given in Supplementary Tables S4 and S5 and in Figure 7.
[h]This patient underwent 2 repeat biopsies, the first during eculizumab treatment shortly after starting aliskiren treatment, the second 22 months after discontinuation of eculizumab. Comparison was carried out between the pre-aliskiren biopsy and the 2 biopsies during aliskiren treatment.

METHODS

C3 cleavage by renin and the C3 convertase

Human C3 was obtained from 3 separate sources, and human renin was acquired from 3 separate sources (plasma renin, kidney renin, and recombinant renin). C3 was incubated with human renin at 37°C for 5 minutes to 24 hours. Cleavage of the C3α chain (112 kD) was detected by the appearance of a band corresponding to the C3bα chain (103 kD) or to C3a (9 kD) detected by sodium dodecylsulfate–polyacrylamide gel electrophoresis with silver or Coomassie staining or immunoblotting, under reducing conditions, or by an increase in C3a detected by ELISA as described in the Supplementary Methods. C3 was also cleaved by the C3 convertase after incubation for 5–60 minutes, as described in the Supplementary Methods. The enzymatic Km, based on the release of C3a, was calculated using the Michaelis-Menten enzyme kinetics test.

Renin-induced cleavage of C3 was also tested in the presence of serum and CFI-depleted serum with or without added CFI (1 μg/ml); see Supplementary Methods.

N-terminal sequencing of the cleaved C3 product

N-terminal amino acid sequencing of the renin-induced C3 cleavage product was performed by Edman degradation[34] and compared with

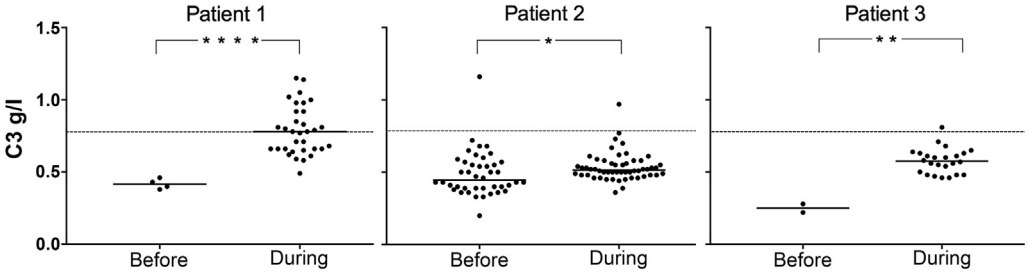


Figure 7 | C3 levels in patients with dense deposit disease (DDD) before and during aliskiren treatment. C3 levels in patients 1–3 before and during aliskiren treatment. The dotted line depicts the lower normal level of C3 (0.77 g/l). In patients 2 and 3 samples taken during intensified plasma exchange treatments (every other week or more frequently, as per Supplementary Tables S3 and S4) were not included. *$P <$ 0.05; **$P < 0.01$; ****$P < 0.0001$.

the known cleavage product of the C3 convertase[35]; see Supplementary Methods.

Interaction between murine renin and murine C3

The interaction between mouse renin and murine C3 (a kind gift from S. Rodriguez de Cordoba, Centro de Investigaciones Biologicas, Madrid, Spain) was investigated by silver staining, as described in the Supplementary Methods.

Inhibition of C3 cleavage induced by renin

Renin inhibition was investigated using the specific renin inhibitor aliskiren hemifumarate (Selleck, Houston, TX) 1 nM to 0.08 M, pepstatin A, an inhibitor of aspartate proteases (0.3–0.625 mM), $ZnCl_2$, $MgCl_2$, (both at 10 mM), or $NiCl_2$ (1 mM).

Aliskiren inhibition of renin-mediated cleavage was also performed in CFI-depleted serum at 37 °C for 24 hours. The inhibitory effect was assayed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblotting; see Supplementary Methods.

Cathepsin D interaction with C3

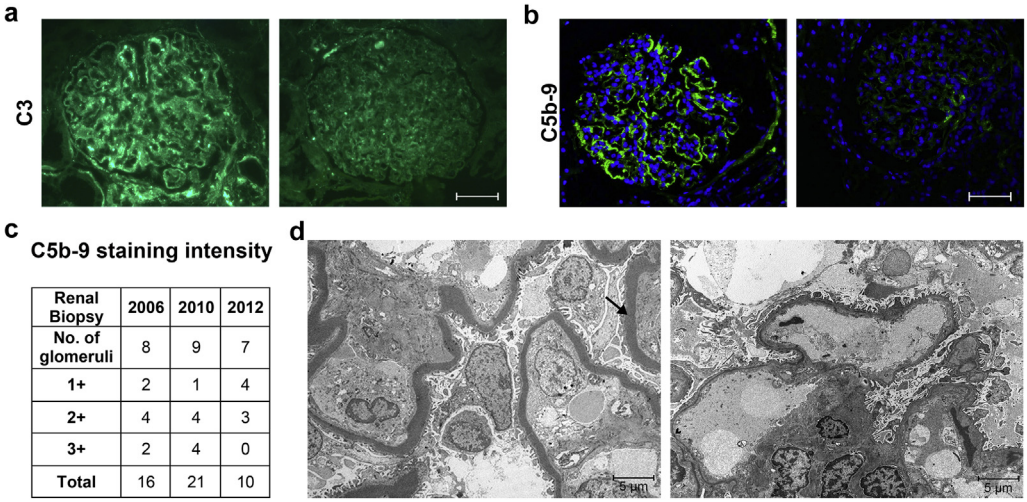
Human recombinant cathepsin D was incubated with C3 for 2 hours at 37 °C, and proteins were detected by silver staining; see Supplementary Methods.

Fluorescence resonance energy transfer C3 peptide

Recombinant renin or C3 convertase-mediated cleavage of a 14-amino-acid peptide, with terminal fluorophores, containing the R-S cleavage site of C3[36]; see Supplementary Methods.

Mast cell chemotaxis assay

A chemotaxis assay was performed to show migration of human mast cells toward C3a induced by renin-mediated C3 cleavage. In



Renal Biopsy	2006	2010	2012
No. of glomeruli	8	9	7
1+	2	1	4
2+	4	4	3
3+	2	4	0
Total	16	21	10

Figure 8 | Complement deposition and glomerular basement membrane thickness in the kidneys of patients with dense deposit disease before and during aliskiren treatment. (**a**,**b**) Immunofluorescence of renal biopsies from patient 2 showing (**a**) C3 labeling in a glomerulus found in the patient's second biopsy (left) before beginning aliskiren treatment and 14 months after the start of treatment (right). (**b**) C5b-9 staining in a glomerulus in the second biopsy (left, fluorescent intensity +++) and 14 months after start of treatment (right), showing a lower grade (1+) of fluorescent intensity (original magnification ×200). Bars = 50 µm. (**c**) Comparison of C5b-9 staining intensity in 3 biopsies taken from patient 2 (2006, 2010, both before start of aliskiren treatment, and 14 months after start). A total score was given to each biopsy by multiplying the number of glomeruli by the specific score of fluorescence intensity (1–3, low to high) and adding all scores. (**d**) Electron microscopy image from the renal biopsy from patient 1 performed before the start of aliskiren treatment, showing intramembranous ribbon-like electron-dense deposits (left, arrow), which decreased in the biopsy performed 14 months after the start of aliskiren (right). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

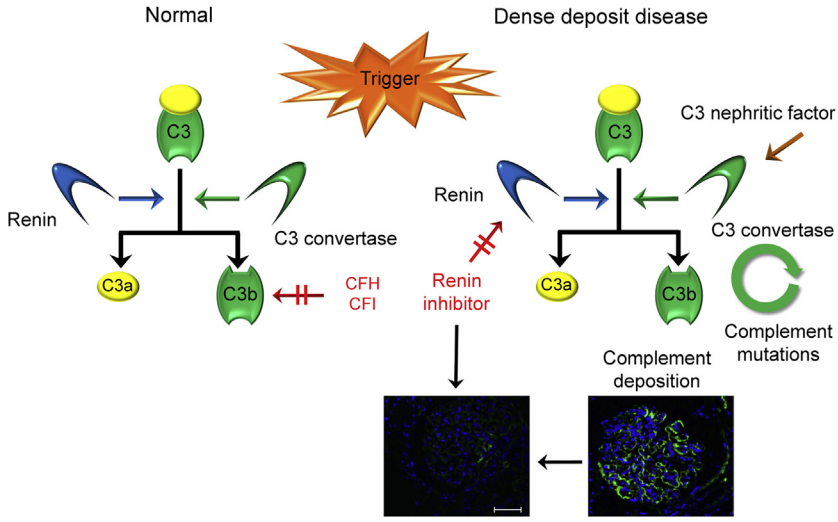


Figure 9 | Schematic depiction of the plausible role of renin in complement activation and inhibition by a renin inhibitor. A trigger, such as infection, induces complement activation. In the physiological setting (left), renin, secreted in the kidney, will cleave C3, but the amplification of the cleavage process via the alternative pathway will be inhibited by functional complement regulators. In dense deposit disease (right), renin will also cleave C3, and further complement activation will be amplified by the C3 convertase. This interaction will be perpetuated in the presence of the C3 nephritic factor, an autoantibody stabilizing the C3 convertase, or by complement loss-of-function mutations in regulators. Complement activation will lead to glomerular complement deposition. Renin-mediated complement activation can be blocked by a renin inhibitor and thereby decrease complement activation in the kidney, shown to be beneficial in 3 patients with dense deposit disease. CFH, complement factor H; CFI, complement factor I. Bar = 50 μm.

certain experiments pepstatin was preincubated with renin. Details in the Supplementary Methods.

Surface plasmon resonance analysis of the interaction between C3 and renin

Two experimental settings were performed using surface plasmon resonance. The first experimental setting was designed to detect C3b and cleaving off of C3a after incubation of C3 with plasma renin. The second experimental setting was used to investigate CFB binding to the C3–renin cleavage product, in the presence or absence of aliskiren, and its interaction with CFD. Details in Supplementary Methods.

Factor Ba detection by ELISA

Factor Ba was detected by ELISA; see Supplementary Methods.

Calu6 renin-producing cells

Calu6 cells (ATCC-HTB-56) were previously shown to synthesize renin.[37] Renin production was knocked out using a renin CRISPR/Cas9 KO plasmid and renin homology-directed repair plasmid. Cells were incubated with or without C3 and aliskiren 10 μM at 37°C. Detection of C3 deposition was carried out as previously described[38]; see Supplementary Methods.

Renin incubation with C4 and C5

Human C4 and human C5 were incubated with plasma renin or recombinant renin at 37°C; see Supplementary Methods.

Patients and controls

Three patients with DDD were included. All patients had low C3 levels, and all were positive for C3NeF. The diagnosis was based on renal biopsies, and all patients underwent repeat biopsies after initiation of treatment with the renin inhibitor aliskiren (Rasilez; Novartis, Basel, Switzerland). For details see Table 1 and the Supplementary Methods. Serum samples from healthy adult controls ($n = 14$) were used. The study was performed with the approval of the Ethics Review Board at Lund University and informed written consent of the patients' parents, patient 2, and healthy controls.

Complement analysis in patient samples

C3, C3a, C5, and C5a levels were assayed; see Supplementary Methods.

Immunofluorescence for detection of C3 and C5b-9 in kidney biopsies

C3 and C5b-9 labeling by immunofluorescence in paraffin-embedded sections was performed in a blinded fashion. For antibodies and conditions, see Supplementary Methods.

Measurement of GBM thickness

GBM thickness in coded biopsies was investigated in a blinded fashion by electron microscopy; see Supplementary Methods.

Statistical analysis

Differences between groups were assessed by the 2-tailed Mann-Whitney U test or by the Kruskal-Wallis multiple-comparison test, followed by the Dunn procedure. P values ≤ 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 7.0.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods.

Table S1. Kinetics of C3a release by renin or C3 convertase.

Table S2. Complement profiles in patient 1.

Table S3. Complement profiles in patient 2.

Table S4. Complement profiles in patient 3.

Table S5. C3a and C5a levels before and during aliskiren treatment.

Table S6. Glomerular basement membrane (GBM) thickness in relation to aliskiren treatment.

Table S7. Medications given at the time of kidney biopsy.

Figure S1. Interaction between murine renin and murine C3. Purified murine C3 (66 μg/ml, lane 1) was incubated overnight with recombinant murine renin (12.5 μg/ml, lane 2) or 25 μg/ml (lane 3) and analyzed by silver staining. No cleavage of C3 was demonstrated.

Figure S2. Inhibition of renin-induced C3 cleavage by zinc. C3 alone (100 μg/ml, lanes 1 and 5) or incubated with plasma renin (4.5 pg/ml, lanes 2 and 6) for 2 hours; bands were detected by silver staining showing cleavage to C3b in the presence of renin. This reaction was inhibited by ZnCl2 (10 mM, lane 3) but not by NiCl2 (1 mM, lane 7) or MgCl2 (10 mM, lane 9). ZnCl2 (lane 4), NiCl2 (lane 8), or MgCl2 (lane 10) alone did not exhibit an effect on C3.

Figure S3. Factor B (CFB) binding to C3 or C3b analyzed by surface plasmon resonance. C3 and C3b (both at 100 μg/ml) were injected over the immobilized anti-C3c antibody in titrated amounts to an equal response, followed by injection of CFB. The captured C3b demonstrated an increased CFB binding (resonance units [RU] = 21) compared with C3 (RU = 4).

Figure S4. Renin incubation with C5. Incubation of human C5 (100 μg/ml) with plasma renin (4.5 pg/ml) for 2 hours did not lead to cleavage because a band corresponding to C5b could not be visualized by immunoblotting.

Figure S5. C3 cleavage by renin over time. C3 (100 μg/ml) incubated with plasma renin (4.5 pg/ml) and analyzed by silver staining (left: C3 incubation with renin for 5–30 minutes, representative of results from 3 experiments) or Coomassie staining (right: C3 incubation with renin for 1–5 hours, representative of results from 2 experiments). C3 cleavage to C3b is visible in the dotted boxes). C3 without renin is shown in lane 6 (30 minutes incubation) and in lane 7 (1.5 hours incubation).

Figure S6. Full-length gels and immunoblots corresponding to Figure 1. (**A**) C3 cleavage by renin. C3 (100 μg/ml, lane 1) incubated with plasma renin (4.5 pg/ml) for 1.5 hours and analyzed by immunoblotting showing cleavage to C3b (lane 2). Pure C3b, under the same conditions (100 μg/ml, lane 3). C3 (lane 4) incubated with kidney renin (5.7 ng/ml, lane 5) and recombinant renin (0.82 μg/ml, lane 6) overnight demonstrated the appearance of a C3b band. For comparison, C3 incubated overnight with plasma renin in lane 7. Dotted boxes correspond to the same lanes in Figure 1a. (**B**) C3 cleavage by plasma renin over time depicting total cleavage within 2 hours. Dotted boxes correspond to lanes with the same numbers in Figure 1b. (**C**) Aliskiren (0.04 M) and pepstatin (0.3 mM) preincubated with plasma renin (0.45 pg/ml) for 3 hours inhibited C3 cleavage by renin during a 2-hour incubation demonstrated by silver staining (lanes 3 and 5, respectively). Aliskiren and pepstatin did not have an effect on C3 itself after 2 hours' incubation in the absence of renin (lanes 4 and 10 aliskiren, lane 6 pepstatin). Aliskiren (0.08 M) preincubated with recombinant renin (2 μg/ml) for 1 hour inhibited C3 cleavage by renin during a 2.5-hour incubation, demonstrated by immunoblotting (lane 9). Dotted boxes correspond to lanes with the same numbers in Figure 1f.

Figure S7. Renin induced C3b degradation into iC3b by complement factor I (CFI). CFI-depleted serum was incubated with plasma renin (2.7 pg/ml) for 24 hours and C3 cleavage analyzed by immunoblotting. C3 cleavage was not visible in CFI-depleted serum in the absence of renin (lane 1). A C3b band was demonstrated in CFI-depleted serum incubated with plasma renin (lane 2). When CFI (1 μg/ml) was added the C3b band disappeared, but a band corresponding to the alpha chain of iC3b (39 kDa) was demonstrated, suggesting that C3b was degraded into iC3b (lane 3). Lanes were run on the same gel and rearranged.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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