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Endothelial Microparticles and Systemic Complement Activation in Patients With Chronic Kidney Disease

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Background—Endothelial microparticles are associated with chronic kidney disease (CKD) and complement activation. We hypothesized that the complement pathway is activated in patients with CKD via endothelial microparticles and that complement activation correlates with endothelial dysfunction in CKD.

Methods and Results—We analyzed complement data of 30 healthy subjects, 30 patients with stage III/IV CKD, and 30 renal transplant recipients with stage III/IV CKD, evaluating the potential correlation of complement fragments with brachial artery flow–mediated dilation, Chronic Kidney Disease Epidemiology Collaboration glomerular filtration rate, and urinary albumin/creatinine ratio. Endothelial microparticles were characterized via proteomic analysis and compared between study groups. Complement fragment Ba was significantly increased in CKD and post–kidney transplant CKD. Plasma Ba levels correlated significantly with lower brachial artery flow–mediated dilation, lower Chronic Kidney Disease Epidemiology Collaboration glomerular filtration rate, and higher urinary albumin/creatinine ratio. Factor D levels were significantly higher in the plasma microparticles of patients with CKD versus healthy controls. Plasma microparticles isolated from patients with CKD and containing factor D activated the alternative pathway in vitro.

Conclusion—The alternative complement pathway is activated in CKD and correlates with endothelial dysfunction and markers of CKD. Future studies are needed to evaluate whether endothelial microparticles with increased factor D play a pathologic role in CKD-associated vascular disease.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT02230202. (J Am Heart Assoc. 2018;7:e007818. DOI: 10.1161/JAHA.117.007818.)

Key Words: chronic kidney disease • Microparticles complement activation

Chronic kidney disease (CKD) is a highly prevalent condition and an independent risk factor for cardiovascular disease (CVD). Approximately 50% of patients with CKD will die of cardiovascular complications. Indeed, patients with CKD are more likely to die of CVD than they are to reach end-stage renal disease. The increased risk of CVD in this patient population is only partially explained by traditional (Framingham) risk factors, and “nontraditional” factors likely influence the risk of CVD. Systemic inflammation has been proposed to contribute to the development of CVD in patients with CKD, as inflammatory markers, such as C-reactive protein and interleukin-6, are elevated in CKD. In...
Clinical Perspective

What Is New?

• In patients with chronic kidney disease, the alternative complement pathway is activated and correlates with endothelial dysfunction and with markers of kidney disease, and microparticles with increased factor D are more prevalent and may play an important role in the activation of the complement pathway.

What Are the Clinical Implications?

• Future studies are needed to evaluate complement fragments and microparticles as markers of cardiovascular and kidney disease and as potential therapeutic targets.

some observational studies, cytokines prospectively predicted the development of CVD.12

Greater understanding of the underlying molecular causes of inflammation in CKD may lead to biomarkers that more accurately predict the risk of CVD than those currently available and may reveal new therapeutic targets for preventing cardiovascular complications. Immunosuppressive drugs are commonly used in CKD to treat concomitant diseases, but little is known about the direct effects of these drugs on systemic inflammation. Several drugs that target specific immune targets have been tested in CKD, but these studies have generated conflicting results.13,14 Given the heterogeneity of CKD and the complexity of the immune system, the development of effective and safe therapies will require additional insights into pathogenesis and new mechanistic biomarkers of immune activation in this disease.

Microparticles (also called extracellular vesicles) are sub-micrometer-sized membrane vesicles (0.05–1 μm diameter) that are actively shed from cells in response to activation, injury, and apoptosis.15,16 A number of recent studies have shown that diseases of the vasculature and kidneys, including CKD, are associated with increased numbers of circulating endothelial microparticles.17–22 We have previously reported that the microparticles released from endothelial cells can, under some conditions, activate the complement system, and that generation of complement-activating microparticles is associated with vascular and renal injury in mice.23 The complement system is an important part of the body’s defense against pathogens, but complement activation also contributes to the pathogenesis of a broad range of diseases, including CVD, ischemia/reperfusion injury, atypical hemolytic uremic syndrome, and renal allograft injury.24–29 The production of complement-activating microparticles might trigger acute inflammatory diseases (such as atypical hemolytic uremic syndrome) in susceptible patients, and the continuous production of complement-activating microparticles might also be an important cause of chronic inflammation in CKD. As such, complement activation has been proposed to play a role in CKD.30 Consistent with this hypothesis, we previously observed elevated levels of complement activation fragments in a small number of patients with CKD.31

Based upon the observations discussed above, we hypothesized that microparticles generated in patients with CKD activate the complement system and are associated with vascular endothelial dysfunction. Endothelial dysfunction is common in CKD and has been shown to predict CVD prospectively in several groups, including healthy individuals,32,33 individuals with peripheral vascular disease,34 and CKD.35 To explore this hypothesis we recruited healthy control subjects and patients with stage III and IV CKD. We also recruited renal transplant recipients with renal dysfunction in order to examine the effects of immunosuppression on the complement system in the setting of CKD. We measured vascular endothelial function and the levels of complement activation fragments, and we examined the molecular composition of microparticles in plasma from these patients. The overall goal of these experiments was to identify novel biomarkers of vascular injury in patients with CKD and to explore potential novel mechanisms of vascular and renal injury in these patients.

Methods

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Patient Characteristics

This is a pilot study including 30 healthy subjects, 30 patients with stage III and IV CKD, and 30 renal transplant recipients with stage III and IV CKD. Healthy subjects were recruited by public advertisement. Patients with stage III and IV CKD and kidney transplant recipients receiving maintenance immunosuppression consisting of tacrolimus, mycophenolate mofetil, and corticosteroids were recruited from our CKD and transplant clinics, respectively, at the University of Colorado Hospital. Individuals with CKD and kidney transplant recipients were considered eligible for participation if they were at least 18 years of age, had stage III/IV CKD with a Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) estimated glomerular filtration equation (eGFR) of 20 to 59 mL/min per 1.73 m²,1 and were able to give informed consent. The only exclusion criteria for healthy subjects was pregnancy or breastfeeding. Exclusion criteria for the other groups included pregnancy or breastfeeding, uncontrolled hypertension, body mass index ≥40 kg/m², life expectancy <1 year, history of significant liver disease or significant congestive heart failure (ejection fraction <20%), hospitalizations within
the past 3 months, or active infection on antibiotic therapy. For individuals with CKD of their native kidneys, history of immunosuppressive therapy in the past year was an additional exclusion criteria. The study was approved by the Colorado Multiple Institutional Review Board. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation. All the study procedures were subsequently conducted during 1 visit at the Clinical and Translational Research Center at the University of Colorado Anschutz Medical Campus.

FMD Measurements
Brachial artery flow-mediated dilation (BA-FMD) was measured at the Clinical and Translational Research Center by a trained technician using high-resolution ultrasonography (GE Vivid 7 Dimension) as described originally by Celermajer et al.\(^3\) and subsequently by our group.\(^3\)\(^7\)\(^,\)\(^8\) ECG gated end-diastolic ultrasound images and Doppler flow of the artery were acquired during baseline and FMD conditions. Reactive hyperemia was produced by inflating a pediatric forearm cuff around the forearm to 250 mm Hg for 5 minutes followed by rapid deflation to measure FMD. A commercially available software package (Vascular Analysis Tools 5.8.1, Medical Imaging Applications) was used to concurrently acquire ECG gated brachial artery diameters. BA-FMD was determined and reported as the percent change from baseline. Doppler flow of the brachial artery was also measured, and peak shear rate was calculated as a potential covariate. The images were analyzed by an independent research assistant who was blinded to the study groups.

Clinical Variables
Race/ethnicity were evaluated by questionnaire. Diabetes mellitus status was defined as history of diabetes mellitus according to the medical record, current treatment with oral hypoglycemic agents or with insulin, or fasting glucose ≥126 mg/dL. Weight and height were measured, and body mass index was calculated and expressed as kilograms per square meter. Blood pressure was measured via automated cuff after 10 minutes of rest at the beginning of each visit. We measured clinical labs including serum creatinine and albumin-to-creatinine ratio (ACR) at the University of Colorado Hospital clinical lab. eGFR was calculated based on the CKD-EPI formula.\(^1\) Urinary ACR was reported as milligrams per gram.

Materials
Complement ELISAs were purchased from Quidel (San Diego, CA). For flow cytometry, sizing beads (Life Technologies, Carlsbad, CA), counting beads (Life Technologies) and compensation beads (Invitrogen) were used for gating and counting. Antibodies to identify endothelial microparticles included anti-CD41a (eBiosciences, Thermo Fisher, Waltham, MA), anti-CD105 (Novus Bio, Littleton, CO), anti-immunoglobulin G (Abcam, Cambridge, UK), and anti-C3b/iC3b (mAb 3E7, generated as previously described).\(^3\) Factor D–depleted serum and purified factor D were purchased from Complex Technologies (Tyler, TX).

Complement Fragment Measurements
Plasma was collected into EDTA-containing tubes, which were inverted several times and placed immediately on ice. Within 10 minutes the samples were centrifuged at 1000g at 4°C, and the layer of plasma was removed with a Pasteur pipette. For urine samples, 9 mL of freshly voided urine was immediately mixed with 1 mL of 10 mmol/L Tris buffer, pH 8.6, with 0.05% Tween 20, 0.01% NaN₃, and protease inhibitors (10 mmol/L benzamidine, 10 mmol/L e-aminocaproic acid, 20 mmol/L EDTA and 100 kallikrein inhibitor units of aprotinin) to prevent protein degradation after collection.\(^4\)\(^0\)\(^,\)\(^4\)\(^1\) The sample was centrifuged at 1000g for 10 minutes at 4°C, and the supernatant was removed. Plasma and urine samples were stored at −80°C until use. Complement activation fragments (Ba, C4a, C3a, C5a, sC5b-9) in plasma and urine were measured using commercial ELISAs according to the manufacturer’s instructions (Quidel). The plasma and urine samples were diluted 1:10 and 1:15, respectively.

Microparticle Isolation
Plasma microparticles were isolated as previously published.\(^3\)\(^\text{I}\) The samples were thawed in a 37°C water bath and then centrifuged at 400g for 15 minutes at 4°C. The supernatants were collected and the volume recorded. Samples were then centrifuged at 20 000g for 2.5 hours. The pellets were resuspended in MP buffer (Hank’s buffered saline solution containing 20 mmol/L HEPES and 5 mmol/L glucose) at a volume of 40% of the initial plasma volume.

Flow Cytometry
Similar to work previously published by our group,\(^3\)\(^\text{I}\) after microparticles were isolated and resuspended, they were stained using the antibodies to CD105, CD41a, immunoglobulin G, and C3b/iC3b. Bound antibodies were detected at the University of Colorado Flow Cytometry Shared Resource using a MoFlo Astrios EQ flow cytometer (Beckman Coulter, Brea, CO). Sizing beads were used to identify particles in the 0.2 to 1 μm range. Equivalent numbers (0.6 × 10⁵) of counting beads were added to the microparticles before antibody staining to
Human serum, and C3 deposition on the particles was measured as previously described. Briefly, 10 μL of serum was incubated with 10^9 zymosan particles at 37°C for 30 minutes in a master mix containing 5 mmol/L MgCl₂ and 10 mmol/L EGTA. In some experiments, factor D–depleted serum was used. Purified factor D, microparticles from patient samples, and inhibitory antibody to factor D were added to some samples as described in the text.

**Statistical Analysis**

Descriptive statistics are reported according to study group as n (%) for categorical variables and mean (standard deviation) or median (interquartile range) for the continuous variables. The potential correlation between complement activation fragments and kidney function (including CKD-EPI eGFR and urinary ACR) and BA-FMD was evaluated by Spearman correlation coefficients. To account for the difference in age between the healthy controls and the subjects in both CKD stage III/IV and posttransplant recipients we applied linear regression models to the relation between complement activation fragments and markers of kidney function and BA-FMD. Comparisons between multiple groups were

**Table 1. Clinical Characteristics According to Study Group**

<table>
<thead>
<tr>
<th></th>
<th>Healthy Control (n=30)</th>
<th>CKD Stage III &amp; IV (n=30)</th>
<th>Posttransplant CKD Stage III &amp; IV (n=29)</th>
<th>P Value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>38±13</td>
<td>59±15</td>
<td>52±14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (Male)</td>
<td>6 (20%)</td>
<td>18 (60%)</td>
<td>17 (57%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Race (White)</td>
<td>27 (90%)</td>
<td>25 (76%)</td>
<td>30 (100%)</td>
<td>0.01</td>
</tr>
<tr>
<td>History of DM</td>
<td>0 (0%)</td>
<td>12 (36%)</td>
<td>6 (20%)</td>
<td>~0.0001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.3±5.5</td>
<td>29.2±5.2</td>
<td>25.9±5.0</td>
<td>0.035</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>116±10</td>
<td>130±12</td>
<td>136±14</td>
<td>~0.001</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>72±8</td>
<td>77±10</td>
<td>82±11</td>
<td>0.0004</td>
</tr>
<tr>
<td>CKD-EPI eGFR, mL/min per 1.73 m²</td>
<td>82±17</td>
<td>37±8</td>
<td>44±10</td>
<td>~0.0001</td>
</tr>
<tr>
<td>ACR, mg/g</td>
<td>0.08±0.09</td>
<td>5±14</td>
<td>3±6</td>
<td>0.11</td>
</tr>
<tr>
<td>FMD % Δ</td>
<td>9.9±7.1</td>
<td>6.4±5.5</td>
<td>6.95±3.8</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Healthy controls were younger, mostly women, had lower BMI and blood pressure and higher BA-FMD compared with healthy. Values are expressed as means±standard deviation or %–percent of patients. ACR indicates urinary albumin/creatinine ratio; BMI, body mass index; CKD, chronic kidney disease; CKD-EPI eGFR, Chronic Kidney Disease Epidemiology Collaboration estimated glomerular filtration rate; DBP, diastolic blood pressure; DM, diabetes mellitus; FMD % Δ, percent change in brachial artery flow mediated dilation; SBP, systolic blood pressure.
performed using 1-way ANOVA with a Tukey’s multiple comparison test or Wilcoxon test with Critchlow-Fligner (DSCF) multiple comparison analysis as appropriate. A $P$ value of $<0.05$ was considered statistically significant. Statistical analysis was carried out using SAS version 9.4 (SAS Institute, Cary, NC).

Figure 1. Levels of complement activation fragments are higher in patients with stage III/IV CKD compared with healthy controls. A, Plasma Ba and C5b-9 levels were significantly higher in stage III/IV CKD and transplant patients compared with healthy controls. There was also a trend toward higher levels of the other complement fragments in CKD patients, but these levels were not significantly higher than in healthy controls. B, Urine Ba levels were higher in patients with stage III/IV CKD compared with healthy controls. ***$P$<0.001, *$P$<0.05.
Results
Clinical Characteristics

Compared with CKD stage III/IV patients and posttransplant recipients, healthy controls were younger, mostly women, and had lower body mass index and blood pressure and higher BA-FMD (Table 1). Patients with CKD had significantly lower BA-FMD (6.4±5.5%) compared with healthy controls (9.9±7.1%; P=0.05) but not compared with posttransplant subjects (6.95±3.8%). When evaluating the whole group of participants, BA-FMD correlated with higher eGFR and lower urinary ACR, although this did not achieve statistical significance (data not shown).

Complement Activation Fragments Are Elevated in Patients With CKD

Complement activation fragments were elevated in patients with stage III/IV CKD, but this achieved statistical significance only for fragment Ba and C5b-9 (Figure 1). Ba, a marker of activation of the alternative pathway of complement, was also increased in posttransplant recipients compared with healthy controls but not with individuals with stage III/IV CKD. Complement fragments C4a and C5a were mildly higher in patients with stage III/IV CKD compared with healthy controls and posttransplant subjects, but this was not statistically significant. Importantly, as shown in Table 2, there was significant correlation between the plasma levels of the different measured complement activation fragments, suggesting that elevation of these fragments was attributable to increased complement activation and not simply decreased renal clearance of any one fragment. This is additionally supported by the finding of higher Ba levels in the urine of those with stage III/IV CKD versus healthy controls.

Table 2. Plasma Levels of Complement Activation Fragments Correlate With Each Other

<table>
<thead>
<tr>
<th></th>
<th>LN_C3a*</th>
<th>C4a</th>
<th>C5a</th>
<th>sC5b_9</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN_Ba*</td>
<td>0.20</td>
<td>0.22</td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.036</td>
<td>0.17</td>
<td>0.006</td>
</tr>
<tr>
<td>LN_C3a*</td>
<td>0.27</td>
<td>0.02</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.9</td>
<td>0.06</td>
<td>0.6</td>
</tr>
<tr>
<td>C4a</td>
<td></td>
<td>0.29</td>
<td>0.007</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.001</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>C5a</td>
<td></td>
<td></td>
<td>0.24</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>
Complement activation fragments shown: Ba, C3a, C4a, C5a, and sC5b_9. Data are shown as Spearman correlation coefficient followed by the P value. *Variable not normally distributed and presented as natural log (LN).

Table 3. Plasma Levels of Complement Activation Fragment Ba Correlate With Biomarkers of CKD

<table>
<thead>
<tr>
<th></th>
<th>LN_Ba*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKD-EPI eGFR, mL/min per 1.73 m²</td>
<td>−0.82</td>
</tr>
<tr>
<td>Urinary ACR, mg/g</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Data are shown as Spearman correlation coefficient followed by the P value. ACR indicates urinary albumin/creatinine ratio; CKD-EPI eGFR, Chronic Kidney Disease Epidemiology Collaboration estimated glomerular filtration rate. *Variable not normally distributed and presented as natural log (LN).
measures >1100 proteins and can measure the abundance of most proteins in the fmol/L to pmol/L range).

We next examined those proteins whose abundance was different among the different patient groups. One of the strongest differences between the microparticle proteome of healthy controls and patients with CKD was the level of factor D, the rate-limiting protease of the alternative pathway. Factor D was significantly more abundant in the microparticles of CKD and transplant patients compared with healthy controls (Figure 3A), whereas the level of factor B was lower in the microparticles of patients with CKD versus healthy controls. Factor B is cleaved during alternative pathway activation, and lower levels of the protein may reflect its consumption during this process.

The SOMAscan can distinguish C3, iC3b, and C3d and measure their concentrations. The levels of all 3 forms of C3 were similar in microparticle samples from patients with CKD and transplant patients compared with healthy controls (Figure 3B). Levels of DAF and CD59 were both higher in microparticles from patients with CKD and patients with CKD with transplants compared with healthy controls. DAF and CD59 are complement regulatory proteins, and higher levels of these proteins may explain why elevated factor D levels in the microparticles of patients with CKD is not associated with greater C3 deposition on the surface of the microparticles.

**Endothelial Microparticle Numbers Are Not Significantly Increased in CKD**

Microparticle numbers and surface C3 fragment deposition were also analyzed by flow cytometry. Microparticles in the 0.2 to 1 μm size range were identified using sizing beads, and microparticles derived from endothelial cells were identified by detection of surface CD105 (Figure 4A). Using forward scatter to compare microparticles before and after purification, the average size was slightly greater after purification in both healthy control samples and CKD samples (Figure 4B and Figure S1). The size of the purified microparticles was the same in all 3 groups of patients, however (Figure 4C). There was a trend toward higher numbers of endothelial microparticles in the plasma of CKD and transplant patients compared with healthy controls, but the difference was not statistically significant (Figure 4D). Similarly, deposited C3 fragments on the microparticle surface did not differ significantly among the 3 groups (Figure 4E).

**Factor D in Microparticles Activates the Alternative Pathway in Serum**

Because we identified greater levels of factor D in the microparticles of patients with CKD and elevated alternative
pathway activation fragments in the plasma of these patients, we next sought to determine whether factor D in plasma microparticles is functionally active. To do this we tested the ability of purified microparticles to restore alternative pathway activity to factor D depleted serum. Zymosan particles activate the alternative pathway when incubated with serum. As expected, no alternative pathway activity was seen when factor D depleted serum was used in this assay (Figure 5A). Alternative pathway activity was restored, however, when purified factor D protein was added to the reaction. We also generated a novel inhibitory monoclonal antibody to factor D (Figure S2). Addition of the anti–factor D antibody reduced alternative pathway activity in factor D depleted serum that was reconstituted with purified factor D. We next isolated microparticles from 250 μL of plasma from a patient with CKD. When the microparticle pellet was added to factor D deficient serum, alternative pathway activity increased (Figure 5B). We did not see a similar increase in alternative pathway activity when microparticles from a healthy control subject were added to the reaction. The addition of the anti–factor D antibody to the reaction blocked the increase in alternative pathway activity when the microparticles were added, confirming that complement activation was caused by factor D in the microparticle pellet.

Figure 3. Complement proteins in microparticles from patients with CKD are altered compared with microparticles in healthy controls. We used an aptamer-based assay (SOMAscan) to measure microparticle proteins with high sensitivity. A, Factor D levels were significantly higher in the microparticles of patients with CKD and patients with transplants with CKD compared with healthy controls. Factor B levels were lower in these groups compared with healthy controls. B, Levels of C3, iC3b, and C3d were not significantly different among the patient groups. C, Levels of the complement inhibitory protein CD59 was higher in patients with CKD than in healthy controls. Levels of factor H and decay accelerating factor were not statistically different among the 3 groups. ***P<0.001, *P<0.05.
Staining of the microparticles with the anti–factor D antibody confirmed that factor D protein is detectable on the microparticle surface (Figure 5C). Proteins can adhere to the surface of microparticles in a calcium-dependent fashion or by binding of the proteins to phospholipids on the microparticle.43 We tested whether factor D levels on the microparticles are reduced by the addition of 20 mmol/L EDTA to chelate calcium or 50 U/mL heparin to compete with phospholipids on the microparticle surface. We did not see a reduction of factor D levels in either of these conditions (data not shown).

**Discussion**

In this study, we show evidence of systemic complement activation in patients with stage III/IV CKD. Notably, we have
found that complement activation fragment $\text{Ba}$ and $C5b-9$ are significantly increased in the plasma of patients with stage III/IV CKD and that plasma levels of $\text{Ba}$ correlated inversely with CKD-EPI GFR. Urinary levels of $\text{Ba}$ were mildly increased, suggesting that the higher levels of plasma $\text{Ba}$ are related to increased alternative pathway activation and are not simply due to reduced renal clearance in the setting of reduced GFR. The conclusion that elevated levels of $\text{Ba}$ are caused by complement activation is further supported by the correlation with levels of multiple complement activation fragments. In addition, plasma levels of $\text{Ba}$ were correlated with urinary ACR, an important marker of kidney damage and endothelial dysfunction. Importantly, the significant correlation between plasma levels of $\text{Ba}$ and BA-FMD is consistent with a functional role of the alternative complement pathway in vascular and kidney disease in this patient population.

Using an unbiased proteomics approach, we found that levels of factor D in plasma microparticles of patients with CKD were significantly higher than levels in microparticles from control patients. Factor D is a serine protease synthesized in the adipose tissue in humans, and it is the rate-limiting catalytic enzyme of alternative pathway activation. It was previously shown that factor D accumulates in CKD, but our data indicate that factor D is specifically increased in microparticles of patients with stage III/IV CKD. Our in vitro data show, for the first time, that plasma microparticles containing factor D cause alternative pathway activation. An inhibitory monoclonal antibody to factor D that we developed blocked alternative pathway activation by the microparticles in vitro, confirming that factor D in the microparticles is functional and that it can be pharmacologically blocked.

Collectively, these data indicate that microparticle-associated factor D increases in CKD and is associated with systemic activation of the alternative complement pathway. Alternative pathway activation, in turn, correlates with vascular endothelial dysfunction. Another study in patients who did not have CKD also recently linked factor D levels with CVD, further supporting a functional link between alternative pathways and vascular disease.

**Figure 5.** Microparticle-associated factor D activates plasma complement proteins. To test whether FD in plasma microparticles is catalytically active, we examined whether it could restore complement activity to FDS. A, Using an alternative pathway assay, we confirmed that purified FD restored activity to FDS. We also confirmed that an anti-FD reduced complement activity in the FDS reconstituted with FD. B, Similarly, CKD MPs restored activity to the FDS. The addition of an inhibitory antibody to factor D prevented this response, however, confirming that the effect was due to factor D protein contained within the isolated microparticles. C, Staining of the microparticles for factor D confirmed that it was present on the surface. Anti-FD indicates anti-factor D antibody; CKD MPs, microparticles purified from the plasma of a patient with CKD; FD, factor D; and FDS, factor D–depleted serum. ***$P<0.001$, **$P<0.01$, *$P<0.05$. DOI: 10.1161/JAHA.117.007818
microparticles, and future studies should focus on evaluating the methodology and whether in fact microparticles may be a valid marker of CVD.

In conclusion, we have found that the alternative pathway of complement is activated in patients with CKD. Ba levels in plasma correlate with vascular dysfunction, suggesting that plasma Ba is a biomarker of CVD in these patients. Microparticles in the plasma of patients with CKD also have increased levels of factor D, the activating enzyme of the alternative pathway. The micro-particle associated protein is functional and activates the alternative pathway in the plasma, demonstrating that altered factor D homeostasis may be an important mechanism of alternative pathway activation and systemic inflammation. These complement perturbations were similar in patients with kidney transplants with CKD, indicating that the immunosuppressive drugs routinely used in patients with transplants do not attenuate complement activation. Future longitudinal studies can confirm an association of alternative pathway activation with clinical outcomes and test whether use of complement inhibitory drugs provides an effective means of preventing CVD in this patient population.

Acknowledgments

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Disclosures

Thurman and Holers receive royalties from Alexion Pharmaceuticals, Inc, and are also consultants for AdMiRx, Inc, a company that develops complement inhibitors. They also hold stock and will receive royalty income from AdMiRx. The remaining authors have no disclosures to report.

References


Figure S1. The size of microparticles was analyzed in patient plasma before and after purification.

Although the percentage of microparticles that fell within the microparticle gate used for further analysis (MPs) decreased, the average size of the microparticles within the gate increased after ultracentrifugation.
Figure S2. Generation of an inhibitory antibody to factor D.

We immunized a factor D deficient mouse with purified human factor D. We generated hybridomas from the mouse and screened them for those that bound factor D by ELISA. A) One of the reactive antibodies was purified by affinity chromatography with protein G examined by Coomassie staining. B) The antibody also bound to factor D by Western blot analysis. C) Alternative pathway activity in serum was measured by a zymosan activation assay. The addition of increasing concentrations of the antibody to normal human serum led to nearly complete inhibition of the alternative pathway.