Original Paper

Membrane Attack Complex and Factor H in Humans with Acute Kidney Injury

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Key Words

AKI • Complement System • Membrane Attack Complex (MAC) • Factor H

Abstract

Background and Aims: Complement system seems to play an important role in the pathogenesis of Acute Kidney Injury (AKI). The aim of this study was to investigate the role of complement system in the pathogenesis of human AKI. For this purpose, we studied Membrane Attack Complex (MAC) and factor H in plasma and kidney tissue in AKI. **Methods:** Plasmatic concentrations of MAC and Factor H were studied in patients with hospital-acquired AKI and their respective controls. MAC and Factor H expression and localization within the kidney were studied by immunohistochemistry in kidney tissue samples from autopsies. Demographical, past medical, and laboratory data in patients on admission and 3 years after discharge were recorded. **Results:** Plasmatic MAC concentrations were significantly higher in AKI-patients (5848 ± 3604 vs 3703 ± 1483 mAU/mL, $p < 0.01$), mainly in the severe cases, as measured by the need of renal replacement therapy, non-recovery of renal function, RIFLE classification and CKD development. MAC deposition was observed in tubular epithelial cell basal membranes, showing a larger number of tubules with MAC deposition, larger perimeter of affected tubules and greater intensity of MAC immunostaining in AKI patients. Factor H concentrations were higher in AKI patients (0.86 ± 0.05 vs 0.60 ± 0.04 mg/mL, $p = 0.007$), showing a strong positive association with plasmatic MAC ($r = 0.7$, $p < 0.01$). Factor H immunostaining showed a tubular cytoplasmic pattern, with significant variations in the staining intensity, associated with the severity of histologic damage. **Conclusion:** Our data confirm that complement system is involved in human AKI, through the lytic action of MAC in tubular epithelial cells. These results suggest that complement system activation in AKI could be related with CKD development.

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Introduction

Acute kidney injury (AKI) is a common clinical syndrome caused by a wide range of kidney insults and it is associated with prolonged hospitalizations and increased risk of death in spite of optimum supportive care [1, 2]. There is a growing body of experimental evidence indicating that complement activation contributes to the pathogenesis of AKI [3].

The complement system is an important innate humoral defensive barrier comprised of more than 20 plasma proteins that may be activated in a cascade fashion by the classic, the alternative or the lectin pathways. It is a regulatory system composed of both plasma proteins and membrane bound proteins that acts to prevent the inappropriate activation of complement by autologous cells [4]. The main plasma regulatory protein of the alternative pathway is Factor H. Factor H mutations are associated with specific organs diseases, including kidney [5, 6] and eyes [7, 8], suggesting that these tissues are particularly liable to local Factor H dysregulation. Notably, the alternative pathway activation contributes to the disease even in patients without Factor H mutations; for example, injury after renal ischemia [9, 10], focal and segmental glomerulosclerosis [11], ANCA associated vasculitis [12], and haemolytic uremic syndrome [13]. Studies in mice [3] and rat [14] models demonstrate increased deposition of C3 along the tubular basement membrane after ischemia-reperfusion injury (I/R).

Human kidneys with acute tubular necrosis (ATN) also showed C3 deposits along the tubular basement membrane and patchy tubulointerstitial deposition [10]. Complement activation has been shown to be an important event in the development of ischemic AKI in mice through the generation of the anaphylatoxin C5a [15] and the membrane attack complex (C5b-C9 or MAC) [16]. MAC is a final multimeric product of complement activation that forms lytic pores in the outer membrane of target surfaces. The complex can lyse cells, and in sublytic quantities it causes cell activation. Studies using C6 deficient mice, which are not able to generate MAC, demonstrated that these mice are protected from renal I/R [17]. Given that complement activation is most prominent in the tubulointerstitium, the primary targets of the MAC are tubular epithelial cells. Exposure to sublytic doses of MAC activates renal parenchymal cells, which then release proinflammatory cytokines, reactive oxygen species vasoactive chemicals, and profibrotic factors [18, 19]. The aim of this study was to investigate the role of the complement system in the pathogenesis of human AKI. For this purpose, we studied MAC and factor H in plasmatic and renal tissue samples, and their relationship with the severity of AKI episodes.

Materials and Methods

Study Design

The following studies were performed to determine the role of MAC and Factor H in plasma and renal tissue in AKI patients: a) To ascertain the role of plasmatic MAC and Factor H an observational, prospective, single-centre cohort study was performed. b) To study the localization of MAC and Factor H in kidney tissue, immunohistochemistry study was conducted.

Cohort Study

Patients with AKI diagnosis in the Hospital del Mar, Barcelona, between 2010 and 2012 were enrolled in the study. Inclusion criteria were age >18 years-old, and meet the criteria for AKI according the consensus of the Acute Disease Quality Initiative [1]. Patients with prior known chronic kidney disease (CKD), or unknown baseline renal function, were excluded from the study.

Different etiologies of AKI were included in the study, among them sepsis, ischemia-reperfusion, nephrotoxicity, and multifactorial AKI subgroups. Within the septic group patients admitted in the Intensive Care Unit (ICU) with sepsis or septic shock diagnoses were recruited. In the ischemia-reperfusion group, renal allograft recipients with delayed graft function (DGF) from the Transplant Unit were recruited. Finally,

in the nephrotoxicity group domiciliary hospitalization patients under colistin treatment, a well-known nephrotoxic drug, were included [20, 21].

The control cohort group was performed including patients at risk for AKI: patients admitted in hospital with sepsis or septic shock at diagnosis, patients under colistin treatment with normal renal function, and patients with different risk factors for AKI, such as contrast administration, and renal allograft recipients with immediate graft function. AKI was defined as an increase in serum creatinine (sCr) by ≥ 0.3 mg/dl within 48 hours or an increase in sCr by ≥ 1 , 5 times baseline [1]. Patients were included in the study 48 hours after AKI diagnosis following the ADQI recommendations. With this approach we ensure that they achieved a correct hemodynamic status, and under-resuscitated patients were avoided. For analysis purpose, AKI-patients were staged according to the maximum increase in serum creatinine using the RIFLE classification. Baseline renal function was defined as the lowest sCr value during the 3 months before admission either in our center or in the relevant basic health area.

Obstructive AKI was excluded by radiological examination. Complete recovery of renal function was defined as renal function normalization before hospital discharge when sCr reaches baseline; and partial recovery of renal function was defined as sCr decrease $>50\%$ before hospital discharge. Urine output criteria were not used for diagnosis or staging because these data were not available in all studied patients.

In the ischemia-reperfusion group, plasma samples were collected 1 week after kidney transplant, delayed graft function (DGF) was defined as hemodialysis requirement during the first week after renal transplantation.

Patients were followed for 3 years after hospital discharge. Demographical, past medical, and laboratory data on hospital admission and 3 years after discharge were recorded.

Plasma samples, MAC and Factor H measurements

Blood samples were collected at the moment of AKI diagnosis; in patients who needed renal replacement therapy during hospitalization, samples were collected before starting dialysis treatment. In a small group of patients, plasma samples were taken the day of renal recovery. Blood samples were collected in EDTA tubes to prevent complement activation. Plasma samples were stored in aliquots at -80°C until its use. Sandwich ELISAs were used to measure MAC (Hycult Biotech) and Factor H (MyBiosource) according to the manufacturer's protocol. Samples and standards were incubated in microtiter wells coated with antibodies recognizing human MAC. Streptavidin- peroxidase conjugate was used to bind the biotinylated tracer antibody, and the enzyme reaction was stopped by the addition of oxalic acid. The absorbance was measured with a spectrophotometer at a wavelength of 450 nm (Tecan Infinite 200, TECAN Instruments). A standard curve by plotting the absorbance versus the corresponding concentrations of the respective biomarker was obtained. Plasmatic MAC concentrations (pMAC) were determined from the standard curve. Plasma samples run out with different previous experiments (Data not shown in this work). For this reason, Factor H was only measured in a small subgroup of AKI patients.

Immunohistochemistry in kidney tissue samples

Kidney tissue samples were obtained from clinical autopsies performed between 2011 and 2013 in our center. Samples with a pathologic diagnosis of ATN corresponding to patients with AKI diagnosis ≥ 3 days before death were selected. Kidney tissue samples from patients who died with normal kidney function were used as controls.

Kidney samples were fixed and embedded in paraffin, 2-4 μm sample sections were cut, deparaffined in xylene, and rehydrated through a graded ethanol series. Antigen retrieval method was performed using Proteinase-K. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide and non-specific binding was blocked using bovine serum albumin fraction (BSA). Tissue sections were then stained with primary antibodies against MAC (anti- human terminal complex complement; Hycult Biotech, the Netherlands) and Factor H (gift of Dr. S.de Córdoba, CSIC).

Samples were evaluated by two independent observers (ER and JG) in a blinded manner with a semiquantitative method, based on the percentage of stained tubules and the percentage of stained perimeter of the average stained tubule. The intensity of staining was graded as 0 (no staining), +1 (stain visible at 40X magnification), +2 (at 20X), +3 (at X10), and +4 (at 2-4X).

Statistical Analyses

Data are presented as mean $\pm$ standard deviation. Student t-test or paired t-test was used. Multiple-group comparisons were performed using ANOVA test. Linear correlation was studied using the Pearson coefficient. Statistics were performed using SPSS v21 statistics software.

Ethics approval

This study is adhered to the Principles of Helsinki Declaration and was approved by the hospital's Ethics Committee (CEIC-IMAS) with the number 2010/3777/1.

All participants gave written informed consent. This study adhered to the Principles of Helsinki Declaration, and the Ethics Committee of the Hospital del Mar approved the protocol.

Results

Study population

156 patients were included in the study, 82 (52.6%) with an AKI diagnosis. Of the overall sample, 102 (65.4%) patients were men, and mean age was 63 ± 15.4 years. Patients' clinical characteristics are described in Table 1, sCr at admission was 4.1 ± 2.7 mg/dl, average length of AKI episode was 18.2 days, and 27 patients (32.9%) needed renal replacement therapy.

At hospital discharge, 61 patients (74.3%) showed partial or complete recovery of renal function while 20 patients (24.4%) did not recover; 6 of the patients needed dialysis therapy. All of them recovered renal function in less than 4 weeks after hospital discharge.

Overall mortality was 18% (n=28), 17 patients belonged to the AKI group and 11 to the non-AKI group.

Plasmatic MAC Concentration

Twenty patients did not have enough sample to measure pMAC. Thus, pMAC was finally measured in 136 patients, seventy-one (52%) were AKI patients. pMAC was significantly higher in AKI-patients than in those with normal renal function (5848 ± 3604 vs 3703 ± 1483 mAU/mL, $p < 0.01$) (Fig. 1), regardless the AKI etiology (Fig. 2). pMAC showed a significant decrease when AKI was recovered as compared to the initial levels (n=22; 4555 ± 525 vs 3423 ± 325 mAU/mL, $p < 0.05$) (Fig. 3); and no changes in pMAC in controls were observed when the acute episode was resolved (sepsis, colistin administration ...) (n=20; 3601 ± 1287 vs 2789 ± 1187 mAU/mL, $p = 0.7$).

Direct correlation between sCr and pMAC ($r = 0.4$, $p < 0.01$) and indirect correlation between glomerular filtration rate and pMAC ($r = -0.7$, $p < 0.01$) were observed.

pMAC was associated with AKI severity, pMAC concentration was higher in AKI-patients who needed dialysis as compared with AKI-patients that did not need dialysis (6159 ± 528 [n=24] vs 5818 ± 590 [n=47] mAU/mL, $p < 0.001$). pMAC was significantly lower in AKI-patients with early renal recovery (during hospitalization) as compared with late

Table 1. Demographic and clinical characteristics in patients with and without AKI. SD, standard deviation

Parameter	AKI (n=82)	Non-AKI (n=74)	p
Age, years (mean $\pm$ SD)	64 ± 14.3	61.5 ± 16.5	0.16
Gender (male/female), n	51/31	51/23	0.25
Arterial hypertension, n (%)	55 (68.8%)	44 (58.7%)	0.54
Type 2 Diabetes mellitus, n (%)	21 (26.3%)	14 (18.7%)	0.32
Ischemic Heart disease, n (%)	6 (7.5%)	16 (21.3%)	0.24
Mortality, n (%)	17 (21%)	11 (14.7%)	0.32
Serum creatinine (mg/dl)	4.1 ± 2.6	0.8 ± 1.2	< 0.01
RIFLE classification, n (%)		-	
Risk	15 (18%)		
Injury	13 (16%)		
Failure	33 (40%)		
Loss	21 (25.6%)		
Etiology of acute kidney injury (n)			
Ischemic	29	22	
Septic	14	13	
Nephrotoxicity	20	29	
Multifactorial	19	11	

renal recovery patients (4197 ± 762 [n=51] vs 6123 ± 516 [n=51] mAU/mL, $p < 0.05$) (Fig. 4). Interestingly, pMAC increased as the severity of AKI worsened: 4965 ± 847 mAU/mL in “risk” patients, 5246 ± 952 in “injury” ones, 5729 ± 461 in “failure” ones, and 6970 ± 888 in “loss” ones, but these differences did not reach statistical significance ($p = 0.07$).

Follow-up data from 60 AKI patients were recorded three years after the AKI episode, excluding renal allograft recipients. Twenty-nine (48.3%) of AKI patients suffered a new AKI episode and 23 patients (38.3%) developed CKD during the follow-up period. pMAC at first AKI episode was higher in patients with a new AKI episode as compared with patients without new AKI episode (6614 ± 940 vs 5791 ± 527 mAU/mL; $p = 0.064$). pMAC concentration was significantly higher in AKI episode in patients who developed CKD as compared with non-CKD patients at follow-up (7786 ± 959 vs 4839 ± 434 mAU/mL; $p < 0.05$) (Fig. 4).

MAC immunohistochemistry in kidney tissue

Thirty-three kidney tissue samples obtained from clinical autopsies were included in the study: 21 (63.6%) from AKI patients and 12 (36.4%) from patients with normal kidney function. MAC immunohistochemistry showed linear staining

Fig. 1. Plasmatic MAC concentration. * $p < 0.05$. AKI: Acute Kidney Injury.

Fig. 2. Plasmatic MAC concentrations according AKI etiology.

Fig. 3. Plasmatic MAC concentrations pre-post renal recovery. * $p < 0.01$.

Fig. 4. Plasmatic MAC concentration and AKI severity measured as A) renal function recovery or not, and B) renal replacement therapy needed or not, C) recurrent acute kidney injury episode, D) Chronic kidney disease development at follow-up. * $p < 0.05$. RRT: renal replacement therapy.

in tubular basal membrane. There was no staining in cytoplasmic, apical, and/or lateral membranous in tubular cells (Fig. 5).

Tubular basal membrane MAC staining was significantly increased in AKI samples as compared to non-AKI samples: 13 (63%) AKI tissue samples showed MAC staining in >50% of the tubules, whereas in non-AKI samples, MAC staining was only observed in 34% of them ($p < 0.05$). In addition, MAC staining affected a larger tubular perimeter in AKI samples as compared to non-AKI patients: AKI-tissue samples showed MAC staining >50% of the tubular perimeter, whereas non-AKI tissue samples ($n = 12$) showed MAC staining in <25% ($p = 0.02$) (Fig. 5). MAC staining intensity was strong (+3, +4) in the majority ($n = 14$, 66.7%) of AKI-tissue samples and weak (+1, +2) in the majority ($n = 12$, 63.2%) of non-AKI samples ($p < 0.005$) (Fig. 6). Plasmatic Factor H concentrations. Plasmatic Factor H concentration was measured in a subgroup of 28 patients, 18 (64.2%) of AKI patients and 10 (35.8%) of controls. Factor H was significantly higher in AKI patients as compared to controls (0.86 ± 0.05 vs 0.60 ± 0.04 mg/mL, $p = 0.007$). In addition, plasmatic Factor H was directly correlated with pMAC in AKI patients ($r = 0.709$, $p < 0.01$).

Factor H immunohistochemistry in kidney tissue

Twenty-eight renal cortical tissue samples coming from autopsies were included in the study, 21 from AKI patients and 12 from patients with normal kidney function. Factor H immunohistochemistry staining showed a tubular cytoplasmic pattern (Fig. 7), with significant variations in the staining intensity. 77.8% of AKI tissue samples showed strong staining (+3, +4), whereas 70% of control tissue samples showed weak (+1, +2) or no staining ($p=0.02$). In AKI patients, staining intensity was associated with the severity of histologic damage. All AKI samples showed classical tubular vacuolar hydropic degeneration; a less severe form of ATN, showed staining +3, whereas 62.5% of the samples with coagulative necrosis, the most severe ATN, showed staining +4 ($p<0.05$).

Discussion

Our results demonstrated that plasmatic MAC concentrations are significantly higher in AKI patients regardless of AKI etiology. These results are in agreement with previous studies focused on the activation of C3 in different murine and cellular models of AKI [10, 22]. To our knowledge, this is the first report that describes, in humans, the association between increased plasmatic MAC concentrations in AKI and its severity in humans.

Our results in kidney tissue are in concordance with the circulating MAC in human AKI. We described immunohistochemistry MAC deposition in basal membrane of tubular epithelial cells, showing larger number of tubules with MAC deposition, larger perimeter of affected tubules and greater intensity of MAC immunostaining in AKI samples. These data are in agreement with previous studies performed in experimental animal models. Zhou et al. described the lytic action of MAC in tubular cells from a murine model deficient of C3 and C6 [17]. These studies using C6 deficient mice, unable to generate MAC, demonstrated that these mice are protected from renal I/R [17]. Subsequently, other groups showed that tubular injury depends on the number of MAC multimers placed in tubular basal membrane, in such way that “sublytic MAC concentrations” induced apoptosis and cell activation, while

Fig. 5. MAC immunohistochemistry in kidney tissue. A) Anti-MAC (40X). Detail of some cortical tubules showing intense (+4) and global basement membrane staining. B) Anti-MAC (20X). Renal cortex showing variable peritubular staining. Some tubules show intense (+4) focal and global basement membrane staining. C) Anti-MAC (20X). Renal cortex without peritubular staining for MAC. Unspecific staining is seen in arteriolar endothelium.

Fig. 6. MAC Immunochemistry in kidney samples from patients with acute kidney injury (AKI) vs. non-AKI patients. a) Percentage of MAC stained-tubules in each sample. b) Percentage of MAC stained perimeter of each cortical tubules. c) Intensity of MAC staining intensity (in percentage).

Fig. 7. Anti-Factor H immunohistochemistry staining. A) Anti-Factor H (40X). Detail of cortical tubules showing granular diffuse cytoplasmatic Factor H staining and signs of acute tubular injury with microvacuolation. B) Anti-Factor H (40X). Detail of cortical tubules without cytoplasmatic Factor H staining from patient without AKI.

“lytic MAC concentrations” drilled cellular membranes producing cell lysis or necrosis [23, 24].

We have found that plasmatic Factor H concentrations are higher in AKI patients, showing a strong positive association with plasmatic MAC concentrations. Factor H is the principal alternative pathway regulator and its synthesis is mainly produced in the liver [25, 26]. Extrahepatic synthesis has been described in a variety of cells including tubular epithelial cells [27, 28] like a local defense mechanism against MAC lytic function [29]. Factor H immunohistochemistry showed tubular cytoplasmatic pattern, with significant variations in the staining intensity, associated with the

severity of histologic damage. Taken together, plasmatic and immunochemistry Factor H staining, our data regarding the Factor H increase could be interpreted as a response in front of the increase in circulating MAC and deposition of MAC in the kidney tissue.

During follow-up, 38% of AKI patients developed CKD, and pMAC was significantly higher in this subgroup as compared with those who maintained normal kidney function. An association between AKI and development of CKD has been previously documented in several studies [30, 31]. This deleterious evolution could be ascribed to a subclinical damage after different insults, previous studies have also demonstrated that even after fully recovered AKI episodes, some patients develop CKD [32, 33]. Some authors described that the intratubular assembly of MAC is one of the principal mediators of chronic tubulointerstitial damage in proteinuric renal disease [34, 35].

Our data suggest that further investigation of the association between complement system and CKD could be of great interest to understand its possible role in the pathophysiology of CKD.

One of the main limitations of our study is the use of kidney tissue samples from autopsies for the immunohistochemistry study. However, ATN is an exclusion diagnosis; thus, routine kidney biopsy is not indicated. For this reason, the use of kidney tissue from autopsies is thoroughly accepted [36].

Conclusion

Our study demonstrates that circulating and renal MAC and Factor H are increased in AKI patients. In addition, circulating and renal MAC increased as the severity of AKI disease worsens. Further investigations focused on pMAC, Factor H, and other complement compounds will help us to dissect the complement alterations in AKI, and find out the disrupted step in the complement cascade that leads to MAC activation in these patients.

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The authors contributed in the following forms:

ER: conception, design, analysis of data, carry out plasmatic and kidney tissue tests and drafting manuscript; JG: providing intellectual content mainly in pathologist area; CA: providing intellectual content mainly in complement system; CB: interpretation of data, revising manuscript; AS: interpretation of data, revising manuscript; DR: interpretation of data, revising manuscript; MS: interpretation of data, revising manuscript; MC: interpretation of data, revising manuscript; MR: design of the study, carry out plasmatic and kidney tissue tests; JP: design, interpretation of data, drafting and revising the manuscript and final approval of the version to be published.

Disclosure Statement

The authors listed certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in the manuscript. Neither the manuscript nor any significant part of it, have been published previously in whole or part except in abstract form.

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