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The molecular and structural bases for the association of complement C3 mutations with atypical hemolytic uremic syndrome

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ABSTRACT

Atypical hemolytic uremic syndrome (aHUS) associates with complement dysregulation caused by mutations and polymorphisms in complement activators and regulators. However, the reasons why some mutations in complement proteins predispose to aHUS are poorly understood. Here, we have investigated the functional consequences of three aHUS-associated mutations in C3, R592W, R161W and I1157T. First, we provide evidence that penetrance and disease severity for these mutations is modulated by inheritance of documented "risk" haplotypes as has been observed with mutations in other complement genes. Next, we show that all three mutations markedly reduce the efficiency of factor I-mediated C3b cleavage when catalyzed by membrane cofactor protein (MCP), but not when catalyzed by factor H. Biacore analysis showed that each mutant C3b bound sMCP (recombinant soluble MCP; CD46) at reduced affinity, providing a molecular basis for its reduced cofactor activity. Lastly, we show by electron microscopy structural analysis a displacement of the TED domain from the MG ring in C3b in two of the C3 mutants that explains these defects in regulation. As a whole our data suggest that aHUS-associated mutations in C3 selectively affect regulation of complement on surfaces and provide a structural framework to predict the functional consequences of the C3 genetic variants found in patients.

1. Introduction

Hemolytic uremic syndrome (HUS) is a severe renal disorder characterized by Coomb's negative microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Typical HUS usually follows a diarrheal episode associated with infection by Shiga toxin producing 0157:h7 *Escherichia coli*. Between 5 and 10% of HUS cases are not associated with infection; these have a poor prognosis with multiple recurrences and often progress to acute renal failure, with a 30% mortality rate, reduced significantly since the introduction of anti-complement therapeutic Eculizumab. The majority of cases of this atypical form of HUS (aHUS) are associated with mutations or polymorphisms in complement proteins. Renal injury in aHUS is caused by complement-mediated endothelial damage with formation of microthrombi that occlude kidney arterioles. Arteriole occlusion causes one of the hallmarks of HUS, the presence of schistocytes, fragments produced as erythrocytes break up when passing at high pressure through partially occluded vessels.

Complement is a major component of innate immunity with crucial roles in microbial killing, apoptotic cell clearance, immune complex handling and modulation of adaptive immune responses. Complement activation by three independent activation pathways, classical (CP), lectin (LP) and alternative (AP), results in formation of meta-stable protease complexes, C3-convertases

Abbreviations: AP, alternative pathway; aHUS, atypical hemolytic uremic syndrome; CP, classical pathway; CR1, complement receptor 1; DAF, decay accelerating factor; DDD, dense deposit disease; EM, electron microscopy; FB, factor B; FD, factor D; FH, factor H; FI, factor I; KD, equilibrium dissociation constant; LP, lectin pathway; MAC, membrane attack complex; MCP, membrane cofactor protein; mt, mutant; sMCP, soluble recombinant membrane cofactor protein (MCP, CD46); SCR, short consensus repeat; SPR, surface plasmon resonance; wt, wild-type.

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(AP, C3bBb; CP/LP, C4b2a), that cleave C3 to generate C3b. Convertase-generated C3b forms more AP C3-convertase, providing exponential amplification of initial activation. Binding of C3b to the C3-convertase generates a C5-cleaving enzyme (C5-convertase) that initiates formation of the lytic membrane attack complex (MAC). In health, activation of C3 is restricted and deposition of C3b and further activation of complement limited to the surface of pathogens by multiple regulatory proteins, including factor H (Sansbury et al., 2014), C4b-binding protein (C4BP), membrane cofactor protein (MCP), decay accelerating factor (DAF), complement receptor 1 (CR1) and CD59, that control complement activation and prevent consumption of components by inactivating C3b or C4b (factor I-cofactor activity), dissociating the C3/C5 convertases (decay accelerating activity), or inhibiting MAC formation (Walport, 2001a,b).

Mutations in genes encoding the regulatory proteins FH (*CFH*), MCP (*MCP*) and FI (*CFI*), and the complement activating components factor B (*CFB*) and C3, are associated with aHUS; importantly, aHUS-associated mutations in regulators are loss-of-function, while mutations in activators are gain-of-function (Richards et al., 2001, 2003; Pérez-Caballero et al., 2001; Goicoechea de Jorge et al., 2007; Caprioli et al., 2001, 2003; Esparza-Gordillo et al., 2005; Sartz et al., 2012; Roumenina et al., 2012; Noris et al., 2003; Sánchez-Corral et al., 2002; Warwicker et al., 1998; Frémeaux-Bacchi et al., 2004, 2008). We present here the functional and structural characterization of three C3 mutations found in the Spanish aHUS cohort. All three mainly impair regulation of the AP convertase on surfaces by MCP (CD46), an effect consistent with the pathogenic mechanisms that characterize aHUS, and we provide a structural basis for this impairment. Further, we show that concurrence of these C3 mutations with aHUS-conferring risk polymorphisms in the *CFH* and *MCP* genes modulates penetrance and clinical severity of disease in aHUS.

2. Patients, materials and methods

2.1. Patients

Our series of aHUS patients comprises 237 unrelated individuals, including 214 Spaniards, seven from other European countries, six from the USA, six from South America and four from Tunisia. aHUS was diagnosed by the presence of one or more episodes of microangiopathic hemolytic anemia and thrombocytopenia defined on the basis of hematocrit (Ht) < 30%, hemoglobin <10 mg/dl, serum lactate dehydrogenase (LDH) > 460 U/L, undetectable haptoglobin, fragmented erythrocytes in the peripheral blood smear, and platelet count <150,000/μl, associated with acute renal failure. Patients with Stx-HUS, defined as the presence of Shiga toxin in the stools (by the Vero cell assay) and/or of serum antibodies against Shiga toxin (by ELISA) and/or LPS (O157, O26, O103, O111 and O145, by ELISA) were excluded. ADAMTS13 functional levels were used to exclude thrombotic thrombocytopenic purpura.

2.2. Clinical data from the families and the sporadic patient carrying C3 mutations

2.2.1. Family HUS19

There are three affected individuals (HUS19, II-1, HUS19M, I-1 and HUS19T, I-2) in this Spanish pedigree, all of them alive. Patient HUS19 is a 12y-old female who first presented at the age of 14 months after a respiratory infection. She has had 8 recurrences thus far. In all these occasions she was treated with peritoneal dialysis, plasmapheresis and plasma infusion and recovered renal function. The family history revealed that the mother (HUS19M) and a maternal aunt (HUS19T) also suffered an episode of aHUS of unknown origin without recurrence. The mother presented with aHUS when she was 6 years old and recovered completely her renal function. The aunt had developed aHUS of unknown origin when she was 14 months and also recovered her renal function. However, some neurological deficits remained (epilepsy secondary to hypertensive crisis) in this patient.

2.2.2. Family HUS107

Patient HUS107 is a female who belongs to another Spanish pedigree. Her mother was also affected. Patient HUS107 has a healthy daughter and two unaffected brothers; one of them also has two healthy daughters. HUS107 presented with aHUS at the age of 35 years associated with the intake of oral contraceptives. This episode was complicated by neurological disorders and was treated with antibiotics, immunosuppressive drugs (vincristine), plasmapheresis and plasma infusions. Currently, she has chronic renal insufficiency and is supported on peritoneal dialysis. Her mother presented at the age of 23 and also suffered aHUS associated with neurological complications. She received a cadaveric kidney graft at the age of 28 and died at the age of 31 with no evidence of recurrence in the grafted kidney.

2.2.3. Patient HUS193

This patient presented with aHUS without a clear triggering factor. The biopsy showed thrombotic microangiopathy and he recovered completely after 8 months with haemodialysis. However, 16 years later he developed terminal renal disease. He is currently on haemodialysis waiting for a transplant. This patient shows permanent hypocomplementemia with low levels of C3 and CH50 but normal levels of C4.

2.3. Mutation screening/genotyping

The patients were screened for mutations and polymorphisms in *CFH*, *MCP*, *CFI*, *THBD*, *CFB* and *C3* genes by automatic DNA sequencing of PCR amplified fragments. Genomic DNA was prepared from peripheral blood cells according to standard procedures (Miller et al., 1988). Each exon of those genes was amplified from genomic DNA by using specific primers derived from the 5′ and 3′ intronic sequences as described (Richards et al., 2003; Pérez-Caballero et al., 2001; Fremeaux-Bacchi et al., 2004; Miller et al., 1988; Delvaeye et al., 2009). Automatic sequencing was performed in an ABI 3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Copy number variations in the *CFHR1-R3* genes were analyzed by MLPA as described elsewhere (Abarrategui-Garrido et al., 2009).

2.4. Purification of complement components and activation fragments

FH and factor B (FB) were purified from plasma of healthy donors by a two-step method as described before (Tortajada et al., 2009). Briefly, filtered plasma was applied to a 5 ml affinity column to which 10 mg of mouse anti-human FHmAb (35H9) or anti-human FBmAb (D2) was coupled. Bound protein was eluted and polished by gel filtration on a SuperoseTM 6 10/300 column (GE Healthcare, Chalfont St. Giles, UK). sMCP (standing for soluble MCP) is a recombinant variant of MCP (also known as CD46) containing the first four SRCs (SCR1-4), lacking the membrane GPI anchor. sMCP was a generous gift from our collaborator Prof. Susan Lea, Oxford University. sMCP has been previously used to determine binding affinity with immobilized C3b (Heurich et al., 2011). All mutant and native C3 were purified from C3102R homozygotes as described before (Martínez-Barricarte et al., 2010). Briefly, C3 was