Brief report

Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome


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Atypical hemolytic uremic syndrome (aHUS) is a disease of complement dysregulation. In approximately 50% of patients, mutations have been described in the genes encoding the complement regulators factor H, MCP, and factor I or the activator factor B. We report here mutations in the central component of the complement cascade, C3, in association with aHUS. We describe 9 novel C3 mutations in 14 aHUS patients with a persistently low serum C3 level. We have demonstrated that 5 of these mutations are gain-of-function and 2 are inactivating. This establishes C3 as a susceptibility factor for aHUS. (Blood. 2008;112: 4948-4952)

Introduction

Mutations in the genes encoding the complement regulators factor H, factor I, and membrane cofactor protein (MCP; CD46), as well as in the activating component factor B, have been detected in approximately 50% of patients with atypical hemolytic uremic syndrome (aHUS). A proportion of the remaining patients have persistently low serum levels of C3. In this study we have examined the hypothesis that mutations in the gene encoding C3 could be associated with aHUS in these patients.

C3 is the pivotal component of the complement system. Activation of the classical, lectin, and alternative pathways results in cleavage of C3 to generate C3b and the anaphylatoxin C3a. When C3b is produced, the thioester is cleaved, and then this highly reactive species may bind covalently to targets. Interaction of the zymogen factor B with C3b and subsequent cleavage of factor B by factor D results in formation of the alternative pathway C3 convertase C3bBb. This set of reactions represents an amplification loop.

A series of complement regulators including factor H and MCP prevent feedback via this loop by increasing the rate of dissociation of C3bBb and/or by serving as cofactors for the serine protease factor I to cleave C3b. Mutations in the gene encoding factor B were recently found to enhance formation of C3bBb or increase resistance to inactivation.

The importance of C3 as a susceptibility factor for human disease has been emphasized by recent studies documenting that a common nonsynonymous coding change in C3 (rs2230199, Arg80Gly, corresponding to C3S and C3F) is both a susceptibility factor for age-related macular degeneration and associated with long-term renal allograft survival.

Methods

Subjects

In 2 independent cohorts of aHUS patients (Paris, France and Newcastle upon Tyne, United Kingdom), 26 patients (17 Paris, 9 Newcastle) with a serum C3 level persistently below the lower end of the normal range of 680 to 1380 mg/L were identified. In these patients functionally significant mutations in CFH, MCP, CFI, and CFB had not previously been detected. Mutation screening of C3 was undertaken in these patients.

Approval for this study was obtained from the Departement de la Recherche Clinique et du Developement, DRRC Ile de France, France and the Northern and...
Yorkshire Multi-Center Research Ethics Committee, United Kingdom. Informed consent was obtained in accordance with the Declaration of Helsinki.

**Mutation screening**

The coding sequence of *C3* was amplified with flanking primers (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Direct sequencing was undertaken using a 96-capillary Sequencer 3700 (Applied Biosystems, Courtaboeuf, France) using the dye terminator method. For the genomic DNA sequence the first nucleotide A of the initiator ATG codon is denoted as nucleotide +1. The amino acid numbering does not include the 22 residues of the signal peptide.

**Functional studies**

*C3* cDNA (gift of David Isenman, University of Toronto, Toronto, ON) was sequenced and compared with the sequence published for the *C3* crystal structure. Two single basepair changes were found in the cDNA and altered (QuikChange Multi Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) to match the published sequence of the *C3* used in the structural analysis. Mutant clones were produced (QuikChange XL Site-Directed Mutagenesis Kit; Stratagene) and sequenced in both directions. The mutant and WT *C3* DNAs were transiently transfected into either 293T or COS-1 cells using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA). Two to 3 days after transfection, the supernatants were collected and concentrated. *C3* was quantitated by ELISA and examined by Western blotting (see Document S1). Conversion of *C3* to i*C3* was accomplished by storage at 4°C or repetitive freeze thawing and monitored by autolytic cleavage (see Document S1). *C3b* ligand-binding assays were undertaken using recombinant MCP (see Document S1 for protocol), factor H (Complement Technologies, Tyler, TX), soluble CR1 (gift of H. Marsh, Avant Immunotherapeutics, Needham, MA) and factor B (Complement Technologies). Cofactor assays were undertaken using wild-type and the mutant *C3* proteins, human factor I (Complement Technologies) and the above noted cofactor proteins.

See Document S1 for methodology relating to assays for cofactor protein binding to *C3* and cofactor activity.

**Results and discussion**

In 11 patients, we identified a heterozygous *C3* mutation. Three additional family members were also affected by HUS, 2 from a
pedigree (Figure S1) in which an unaffected individual also carried the same change, and another from an affected sibling pair. Therefore, 14 affected persons were found to harbor a mutation. There were a total of 9 distinct mutations identified in the initial 11 patients, the same mutation was carried by 2 unrelated persons. There were 8 heterozygous missense mutations (R570W, R570Q, R713W, A1072V, D1093N, C1136W, Q1139K, and H1142D) and one heterozygous nonsense mutation (Y832X). The positions of these are shown in Figure 1. None of these variants was present in 200 chromosomes from healthy persons.

Clinical and laboratory data are presented in Table S2. Median age at presentation was 6.5 years (range, 8 months to 40 years). Seven of the 14 recovered renal function after their initial presentation, and 4 of these had recurrences. Among the 14 patients there have been 12 renal transplantations (9 cadaveric and 3 live-related), 5 of which have been affected by recurrent disease.

Next, 8 of 9 C3 mutant constructs (except for the missense mutation) were prepared and transfected into mammalian cells. The C1163W mutant was either minimally or not detectably expressed. Of the remaining 7, each was expressed in approximately comparable amounts, and protein bands in gels migrated identically to wild-type C3 (Figure S2).

Assays were performed to assess the interaction of the 7 secreted C3 mutants with MCP. Binding of R570Q, R570W, A1072V, D1093N, and Q1139K was 22%, 30%, 18%, 17%, and 69% of wild-type, respectively (Figure 2A). Binding of R713W and H1442D was not statistically different from wild-type. The results of the cofactor assays paralleled the ligand binding data (Figure 2B). The 2 mutants with normal MCP binding also had normal cofactor activity, whereas the 5 mutants with decreased MCP binding had decreased cofactor activity. Specifically, there were no detectable cleavage fragments in 4 and modest cleavage by mutant R570Q. Based on the loss of the α-chain and appearance of major α1 and α41 kDa cleavage fragments, there

Figure 2. Ligand binding and cofactor activity of the C3 proteins. Proteins were transiently expressed in 293T cells, concentrated and quantified before analysis (see Document S1). (A) Binding to MCP, factor H, factor B, and soluble CR1 (sCR1) in ELISA. For MCP and factor H, C3 protein was incubated at 15 ng/mL and for factor B, 200 ng/mL. Detection was made with chicken anti-human C3 and HRP-linked donkey anti-chicken IgY. * indicates a significant reduction in binding (P < .05). (B) Kinetic analysis of cofactor activity. C3 preparations were incubated for 0 to 30 minutes at 37°C with factor I and a cofactor protein (MCP, factor H, or sCR1). The zero control is before the addition of factor I. The last lane is an iC3b control. Samples were reduced and analyzed by Western blotting using either chicken anti-human C3 or goat anti–human C3 (see Document S1). Cofactor activity is assessed by the loss of the α chain and appearance of the α1 and α41 kDa major cleavage fragments. The minor α43 kDa cleavage fragment is more variable but no pattern was observed that was specific for a mutant. Data are representative of 5 similar experiments.
was more than 90% reduction in cofactor activity for these 5 mutants. Thus, the reduced interaction of 5 of the 7 secreted C3 mutant proteins with MCP (the major membrane cofactor regulator of C3b) is likely to lead to a gain of function relative to complement activation.

We next evaluated the interaction of the 7 secreted mutants with complement receptor 1 (soluble CR1, sCR1) and the plasma regulatory protein factor H. Both of these inhibitors also bind C3b and are cofactors for its cleavage by factor I. There were no statistically significant differences between binding of sCR1 to wild-type C3 versus that of the 7 mutants. For factor H, however, the binding profile generally resembled that of MCP except that the magnitude of the decrease in binding was less. However, there was a statistically significant decrease in IC3 binding of A1072V, D1093N, and Q1139K, while R570Q and R570W were modestly (10%-20%) but not statistically significantly decreased. Cofactor activity for Q1139K was also decreased compared with native C3 (Figure S3).

We also assessed binding to factor B. Factor B interacts with C3b or iC3 to begin the formation of the alternative pathway C3 convertase. There were no statistically significant alterations in binding of these mutants to factor B. Taken together, these results further suggest that it is a modification in interactions with regulators that leads to a secondary gain of function in these C3 mutants.

Homozygous C3 deficiency in association with recurrent bacterial infections has been recognized for many years. The functional significance of R713W and H1442D mutations in C3 and aHUS is likely to lead to a gain of function relative to complement activation for a given degree of cellular injury as the fundamental predisposing event for aHUS.

In summary, this study establishes a new association between heterozygous mutations in C3 and aHUS.

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Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

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