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A novel mutation in the complement regulator clusterin in recurrent hemolytic uremic syndrome☆

Anne-lie Ståhl[a], AnnCharlotte Kristoffersson[a], Anders I. Olin[b], Martin L. Olsson[c], Anne-Marie Roodhooft[d], Willem Proesmans[e], Diana Karpman[a,*]

[a] Department of Pediatrics, Clinical Sciences Lund, Lund University, Lund, Sweden
[b] Division of Infection Medicine, Clinical Sciences Lund, Lund University, Lund, Sweden
[c] Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University, Lund, Sweden
[d] Department of Paediatrics, St. Jozef Hospital, Malle, Belgium
[e] Renal Unit, Department of Paediatrics, University Hospital, Leuven, Belgium

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ABSTRACT

A novel heterozygous mutation in the clusterin gene, nucleotide position A1298C (glutamine > proline Q433P), was detected in exon 7 of a child with recurrent hemolytic uremic syndrome (HUS). The same mutation was found in the child's two siblings and mother but not in 120 controls. In addition, a previously described heterozygous mutation was detected in the gene encoding membrane cofactor protein (MCP) causing a 6 base-pair deletion 811-816delGACAGT in exon 6. It was found in the patient, both siblings and the father. One sibling had recovered from post-streptoccocal glomerulonephritis. Clusterin levels in the patient, siblings and parents were normal as was the migration pattern in a gel. Patient serum induced C3 and C9 deposition on normal washed platelets, and platelet activation, as detected by flow cytometry. The same phenomenon was found in serum taken from the siblings and the mother but not in the sample from the father and controls. Addition of clusterin to patient serum did not inhibit complement activation on platelets. The Q433P mutant, in isolated form, was further studied by binding to the components of the terminal complement complex. The mutant did not bind to C5b-7 that was immobilized onto a BIAcore chip, whereas wild-type clusterin did, indicating that the mutation could lead to defective inhibition of formation of the membrane attack complex under these conditions. Hemolysis of rabbit erythrocytes was inhibited by wild-type clusterin but not by the mutant. Mutated clusterin could thus not prevent assembly of the membrane attack complex on platelets and erythrocytes.

1. Introduction

Mutations in complement regulators factor H (Warwicker et al., 1998; Ying et al., 1999), factor I (Kavanagh et al., 2005; Nilsson et al., 2007) and membrane cofactor protein (MCP/CD46) (Richards et al., 2003) have been associated with recurrent atypical hemolytic uremic syndrome (aHUS). In addition, mutations in C3 have been associated with HUS (Fremeaux-Bacchi et al., 2008) as well as gain-of-function mutations in factor B (Goicoechea de Jorge et al., 2007). Mutations in factor H are usually localized at the C terminal in aHUS (Caprioli et al., 2001). These mutations have been shown to affect host recognition and allow uninhibited complement activation via the alternative pathway on endothelial cells (Manuelian et al., 2003) and platelets (Ståhl et al., 2008), which could explain the endothelial injury and platelet consumption seen during this condition. This phenomenon has been characterized in mice with a homozygous deletion of the C terminal of factor H causing a condition resembling human HUS (Pickering et al., 2007).

The defective activity of mutated MCP (Richards et al., 2003), factor I (Kavanagh et al., 2005; Nilsson et al., 2007), C3 (Fremeaux-Bacchi et al., 2008) and factor B (Goicoechea de Jorge et al., 2007) leading to alternative pathway activation have also been documented. Most mutations are heterozygous and certain patients may have more than one mutation and/or risk-associated polymorphisms in these factors (Caprioli et al., 2003). A pattern of incomplete penetrance has been demonstrated in families (Esparza-Gordillo et al., 2006) suggesting that other factors, which may be environmental, infectious and/or genetic, may trigger disease induction. The known mutations account for disease in

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* Corresponding author. Tel.: +46 46 2220747; fax: +46 46 2220748.
E-mail address: diana.karpman@med.lu.se (D. Karpman).

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approximately 50% of aHUS patients (Loirat et al., 2008). All mutations found to date affect the initial steps of the alternative pathway. So far, no mutations in regulators of membrane attack complex (MAC) assembly have been reported.

Formation of the MAC can result from activation of the classical, lectin or the alternative pathways of the complement system. MAC assembly is initiated by the generation of C5b, followed by sequential addition of one C6, C7, C8 and numerous C9 molecules. Host cells are protected from cell lysis by regulatory proteins that inhibit the formation or integration of the MAC into the cell membrane. S protein (vitronectin) (Podack and Muller-Eberhard, 1979) and clusterin (Murphy et al., 1989) are the two major inhibitors in the fluid phase while CD59 regulates MAC formation on cell membranes (Meri et al., 1990).

Clusterin is transcribed from a single copy gene of nine exons located on chromosome 8 (8p21) (Fink et al., 1993). Clusterin is a widely expressed and multifunctional protein (Jenne and Tschopp, 1992). There are two isoforms, nuclear and secreted. The nuclear form is pro-apoptotic, while the secretory form is considered pro-survival with properties that can regulate DNA repair (Shannan et al., 2006). Clusterin is mainly produced by the liver (Högåsen et al., 1996) but also by megakaryocytes and thus released from platelets upon activation (Tschopp et al., 1993b). Clusterin circulates in association with lipoproteins (de Silva et al., 1990) at a concentration of approximately 250–420 μg/mL (Högåsen et al., 1993). Mutations in clusterin have, as yet, not been associated with human disease although disease-associated polymorphisms have been described (Miwa et al., 2005; Tycko et al., 1996).

In this study a novel mutation in clusterin was demonstrated in one family in which one child suffered from recurrent aHUS and another had recovered from one episode of post-streptococcal glomerulonephritis. The study investigated the function of this mutated protein with regard to regulation of MAC assembly leading to complement deposition and activation of platelets.

2. Methods

2.1. Subjects

The proband is a currently 11-year-old boy admitted at 4 years of age to the University Hospital of Leuven due to fever, edema, pallor, petechiae, reduced consciousness and macroscopic hematuria. He was normotensive and laboratory assays revealed hemoglobin 7.7 g/dL, platelet count 40,000/μL, haptoglobin 0.2 g/L (reference value 0.3–2.0), negative direct antiglobulin (Coombs') test, urea 257 mg/dL, creatinine 4.95 mg/dL, C3 0.57 g/L (0.79–1.52) and normal C4. Urinalysis showed hematuria and proteinuria. He was diagnosed with aHUS, treated with fresh-frozen-plasma for five days and hemodialysis, and made a full recovery but was readmitted one year later with a very similar picture except that C3 was normal. He was treated with fresh-frozen-plasma again for five days and again made a full recovery with a creatinine clearance of 116 mL/min/1.73 m^2. He remains healthy. ADAMTS13 activity assayed as described (Gerritsen et al., 1999) was normal. The currently 13-year-old brother was admitted at 8 years of age for post-streptococcal glomerulonephritis. Laboratory values showed hemoglobin 12.7 g/dL, platelets 253,000/μL, anti-streptolysin 657 IU/mL (26–150), creatinine 0.8 mg/dL, C3 0.57 g/L. Urinalysis revealed hematuria and proteinuria. He made a full recovery. A younger sister and the parents are healthy. Blood and serum samples were obtained from the child (three times during remission), his family members and healthy adult controls ($n = 22$). DNA analysis was performed on samples from 120 apparently healthy blood-donor controls. Samples from patients, parents and controls were obtained with the informed consent of the parents and the controls and the study was approved by the Ethics Committees of Lund and Leuven Universities.

2.2. Blood samples and serum

Whole blood was drawn by venipuncture into ethylenediamine tetraacetic acid tripotassium salt (EDTA K3, Becton Dickinson, Franklin Lakes, NJ) or sodium citrate vacutainer tubes (0.109 M, Becton Dickinson). Platelet rich plasma, washed platelets and serum were obtained as previously described (Ståhl et al., 2008).

2.3. Mutation analysis

DNA was extracted from whole blood as previously described (Miller et al., 1988; Vaziri-Sani et al., 2006). All exons of complement regulators factor H (Richards et al., 2001), factor I (Fremeaux-Bacchi et al., 2004), membrane cofactor protein (Fremeaux-Bacchi et al., 2006), factor B (Goicoechea de Jorge et al., 2007), C3 (primers available upon request) and clusterin (primers as in (Tycko et al., 1996) except exon 7 forward 5′-TCTCACTTGCGTTTCTTCCA-3′, reverse 5′-TCTGCCGTGTGATAAATGCT-3′) were sequenced.

2.4. Clusterin, wild-type and Q433P constructs

Recombinant clusterin was obtained from ProSpec, Rehovot, Israel. Full-length clusterin cDNA was subcloned into the pRC-CMV vector (a gift from Professor Martin Gleave, The Prostate Centre at VHG, Vancouver, BC). Mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratgene, La Jolla, CA). Primers used were forward 5′-CCT CAC GCA AGG CGA AGA CCC GTA CTA TCT GCG GGT CAC CAC-3′ and reverse 5′-GTG GTG ACC CGC AGA TAG TAC GGG TCT TCG CCT TGC GTG AGG-3′. The Q433P mutant was constructed into the pRC-CMV vector and the mutation verified by digestion with *Afe*I (New England BioLabs, Ipswich, MA) and by sequencing.

2.5. Transient transfection

COS-7 cells were seeded onto 6-well culture plates and grown in DMEM (Invitrogen GmbH, Karlsruhe, Germany), supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (all from Invitrogen GmbH) to approximately 95% confluence prior to transfection. A total amount of 4 μg plasmid DNA was added to each well (wild-type, Q433P mutant or wild-type co-transfected with the Q433P mutant) and transfection performed with Lipofectamin (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the media was changed to serum-free and the cells were cultured for an additional 48 h. The media were collected and supplemented with protease inhibitors complete Mini EDTA (Roche Diagnostic GmbH, Mannheim, Germany) and centrifuged to remove cell debris.

2.6. ELISA for determination of clusterin concentrations in cell media and serum samples

Pooled samples (cell media) were concentrated 6-fold using Centricon concentrators (Millipore Corp., Bedford, MA). Cell media or sera (from the patient, siblings, the parents or normal controls, diluted 1:4000 or 1:8000 in PBS-Tween) were coated onto microtiter plates (NUNC, Roskilde, Denmark) and incubated over-night at 4 °C (Högåsen et al., 1993). After washing in Phosphate-buffered saline (PBS)-Tween (Medicago, Uppsala, Sweden), clusterin was detected by mouse anti-human clusterin (1:2000, Quidel, San Diego, CA) followed by goat anti-mouse HRP