Insights into hemolytic uremic syndrome: Segregation of three independent predisposition factors in a large, multiple affected pedigree

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Abstract

Mutations in the complement regulators factor H, membrane cofactor protein (MCP), and factor I are associated with atypical hemolytic uremic syndrome (aHUS, MIM 235400), suggesting that the disease develops as a consequence of the inefficient protection of the renal endothelium from damage by the complement system. Incomplete penetrance of the disease in individuals carrying these mutations is, however, relatively frequent. Here, we report the identification of a large, multiple affected aHUS pedigree in which there is independent segregation of three different aHUS risk factors: a MCP missense mutation (c.-598C > T; Pro165Ser) that decreases MCP expression on the cell surface, a dinucleotide insertion in the coding sequence of factor I (c.-1610insAT) that introduces a premature stop codon in the factor I protein, and the MCPggaac SNP haplotype block that was previously shown to decrease the transcription activity from the MCP promoter. Interestingly, individuals affected by aHUS in the pedigree are only those who have inherited the three aHUS risk factors. These data show an additive effect for mutations in MCP and factor I and provide definitive support to the conclusion that aHUS results from a defective protection of cellular surfaces from complement activation. Furthermore, they help to explain the incomplete penetrance of the disease, illustrating that concurrence of multiple hits in complement regulatory proteins may be necessary to significantly impair host tissue protection and to confer susceptibility to aHUS.

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1. Introduction

Hemolytic uremic syndrome (HUS) is clinically defined by thrombocytopenia, Coomb’s test negative microangiopathic haemolytic anaemia and acute renal failure. Most HUS cases occur associated to Escherichia coli infections leading to hemorrhagic diarrhea (Karmali, 2004). This typical form of HUS usually resolves satisfactorily and complete recovery of the renal function is achieved. However, 5–10% of HUS cases lack a particular relationship with infection and have a poorer prognosis (Moake, 2002). This idiopathic atypical form of HUS (aHUS) is frequently associated with immunosuppressive drugs, cancer therapies, oral contraceptives or develops during pregnancy or postpartum. The molecular mechanisms underlying atypical HUS are not completely understood. The complement system has been implicated in the pathophysiology of this syndrome for many years (Thompson and Winterborn, 1981), but only recently mutations in the genes for the complement proteins factor H (CFH) (Warwicker et al., 1998; Padrón et al., 2001; Caprioli et al., 2001; Richards et al., 2001; Sánchez-Corral et al., 2002; Manuelian et al., 2003, reviewed in Rodríguez de Córdoba et al., 2004), membrane cofactor protein (MCP) (Noris et al., 2003; Richards et al., 2003) and factor I (FI) (Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2005) have been shown to predispose to aHUS. Functional characterization of several of these mutations suggest that the disease likely develops as...
a consequence of a defective protection of cellular surfaces from complement activation due to an improper function of complement regulatory proteins (Sánchez-Corral et al., 2002; 2004; Manuelian et al., 2003; Richards et al., 2003).

Despite these advances in our understanding of the molecular basis of aHUS, incomplete penetrance of the disease in individuals carrying factor H, MCP or factor I mutations is relatively frequent, suggesting the existence of additional genetic factors contributing to aHUS. Recently, different groups have reported that relatively frequent CHF and MCP-SNPs are strongly associated with aHUS (Caprioli et al., 2003; Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2005). Among these SNPs, the MCP haplotype block MCPggaac is particularly interesting because it includes two SNPs, c.-547G/C and c.-261G/C, that influence transcription from the MCP promoter in transient transfection experiments (Esparza-Gordillo et al., 2005). Moreover, the observation that MCPggaac was especially frequent among patients who carry mutations in CFH, MCP or IF suggested that the concurrence of different mutations and polymorphisms in the complement regulatory genes increases predisposition to aHUS (Esparza-Gordillo et al., 2005). Here we provide further support to this conclusion identifying a pedigree in which the affected individuals carry three different genetic susceptibility factors in two different complement regulatory genes (IF and MCP).

2. Patients and methods

2.1. Family RCO

This study focuses on the characterization of aHUS genetic predisposition factors in a large, multiple affected Spanish pedigree, referred to as family RCO. This pedigree was initially selected because we found that two patients in the Spanish HUS registry, HUS 68 and HUS 84, were first cousins and the only affected members in their family. HUS 68 has been reported earlier (Esparza-Gordillo et al., 2005). All protocols included in these studies have been approved by national and/or local institutional review boards, and all subjects gave their informed consent.

2.2. Case reports

HUS 68 (Fig. 1, III-5) presented with acute renal failure, thrombocytopenia and Coombs’ test negative microangiopathic haemolytic anaemia at the age of 57. Plasma creatinine was 405 μmol/L. Renal biopsy was not performed due to the small size of her kidneys. Because of the rapid and progressive loss of renal function, haemodialysis was commenced and plasma exchanges was undertaken. The patient’s renal function was stabilized by this treatment and the dialysis could be stopped. Fifteen months later she presented again with microangiopathic anaemia and thrombocytopenia. Despite repeated plasma exchanges and fresh plasma infusions, she rapidly lost renal function and since 1994 she is being treated with haemodialysis. She is currently 68 years old.

HUS 84 (Fig. 1, III-8) presented with HUS at the age of 41 after an acute catarh episode. She was under periodic haemodialysis for 6 months and recovered a limited renal function that allowed her to abandon the haemodialysis program. However, progressive loss of renal function took her back to haemodialysis in 2004, 17 years later. HUS 84 is currently 59 years old.

2.3. The relatives of HUS 68 and HUS 84

Individuals II-5, III-1, III-2, III-3, III-4, III-6, III-7, III-9, III-10, III-11, IV-1, IV-2 and IV-3 (Fig. 1) are all alive and healthy with no history of renal disease. Current age for each of these individuals is depicted in Fig. 1. All other members of the pedigree RCO are deceased. Relatives I-1, I-2, II-1, II-2, II-3, II-4 and II-7 died at advanced age, most of them over 80 years old, with no record of renal disease. II-1 and II-8 died in their 50s from heart disease. II-6 is the only member of the RCO pedigree who died in her 30s and the only one who may have had an haemolytic anaemia related disorder. No samples were, however, available for analyses from this individual or from any of the deceased relatives of HUS 68 and HUS 84.

2.4. Complement analyses

C3, C4, factor H and factor I levels were measured in serum or plasma samples as previously described (Pérez-Caballero et al., 2001; Esparza-Gordillo et al., 2004; González-Rubio et al., 2001). The standard hemolytic assays CH50 and AP50, and a recently described hemolytic assay to test factor H function (Sánchez-Corral et al., 2004) were performed to assess complement function and regulation. Expression levels of the membrane regulators MCP (CD46) and decay accelerating factor (DAF or CD55) in peripheral blood lymphocytes (PBLs) were analyzed by flow cytometry in whole blood samples drawn in the presence of EDTA. Each of these analyses included at least three whole blood samples from control individuals drawn the same day in the same conditions. Cells were assessed using three-colour acquisition on a FACScanCalibur (Becton Dickinson, CA, USA). The monoclonal antibodies PE-anti-CD45, FITC-anti-CD46 and PerCP-anti-CD55, and the appropriate isotypic controls were purchased from BD Biosciences (Pharmingen, San Diego, CA, USA). PBLs were examined using side-scatter versus CD45 gating, and data analysed using CELLQUEST software (Becton Dickinson).

2.5. Genomic analyses of complement regulatory genes

Patients and their relatives were screened for mutations and polymorphisms in CFH, MCP and IF genes. DNA from these individuals was extracted from PBLs or from buccal mucosa cells collected with cheek brushes (MasterAmp buccal swab brush, Epicentre Technologies) using standard procedures. Each exon of the CFH, MCP and IF genes was amplified from genomic DNA of patients using specific primers derived from the 5’ and 3′ intronic sequences. The sequence of these primers and the PCR conditions used for the amplifications are described elsewhere (Pérez-Caballero et al., 2001; Richards et al., 2003; Fremeaux-Bacchi et al., 2004). Direct sequencing of PCR products was performed automatically in an ABI 3730
sequence using a dye terminator cycle sequencing kit (Applied Biosystems, NJ, USA). MCP c.-598C > T SNP (NCBI id rs7144) was used to identify the presence of the MCP-gaas haplotype. Genotyping of the MCP c.-2181T > C SNP was performed by allelic discrimination on 10 ng of genomic DNA using TaqMan probes (MCP assay id: 2784726; CFH assay id: 2530387; Applied Biosystems; Foster city, CA, USA) and real time PCR equipment (PE7700; Applied Biosystems; Foster city, CA, USA) following the manufacturer’s specifications.

3. Results

Blood samples obtained from patients HUS 68 and HUS 84 showed normal complement activity through the classical (CH50) and the alternative (AP50) pathways. Similarly, plasma levels of C3, C4 and factor H were within the normal reference range of variation (Table 1). However, both patients presented half-normal plasma levels of factor I, and also showed a 50% reduction in the MCP expression levels in PBLs (Table 1). These observations prompted us to search for mutations in the MCP and IF genes in both patients.

MCP sequencing revealed a novel heterozygous mutation (c.-598C > T; Pro165Ser) in exon 5, encoding short consensus repeat 3 (SCR3), in both aHUS patients (Fig. 2a). This missense mutation shows a perfect segregation with the 50% reduction in the MCP expression levels in PBLs that present different members of the RCO pedigree (Table 1 and Fig. 1). Pro165Ser results in a partial MCP deficiency because it affects a fully conserved proline residue that is characteristic of the SCR consensus sequence (Law and Reid, 1995) and likely interferes with the proper folding and transport of MCP to the cell membrane (Fig. 2b and c). The 50% reduction in MCP levels place the carriers of the Pro165Ser MCP mutation outside the normal range of MCP levels (Table 1). The pathogenic consequences of a 50% reduced MCP expression in the context of aHUS have been demonstrated in earlier work (Naris et al., 2003; Richards et al., 2003).

Sequencing of the IF gene led to the identification of a heterozygous c.-1610insAT mutation in both patients and some of their relatives (Figs. 1 and 3 b). This dinucleotide insertion, located in IF exon 13, causes a frameshift that generates a truncated factor I protein at position 538 that lacks 45 amino acids (19% of the functional serine protease region). In addition, as illustrated in Fig. 3b, this truncated form of factor I (expected MW = 83,000 Da) is likely not secreted to the plasma, as it cannot be detected in Western blots of whole plasma from the patients using anti factor I polyclonal antibodies. A total of five IF mutations leading to factor I deficiency have been reported thus far in aHUS patients (Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2005). The factor I mutation described here is a novel mutation located in the C-terminal region of the protein, eight residues upstream of another HUS-associated mutation (c.-1637G > A; Trp546Stop) that also leads to partial factor I deficiency (Fremeaux-Bacchi et al., 2004).

Since HUS 68 and HUS 84 are first cousins, the presence of identical MCP (1q32) and IF (4q25) mutations in both patients clearly indicate a germline transmission from a common ancestor. Analysis of all available relatives in family RCO showed segregation of the MCP and IF mutations, supporting this conclusion (Fig. 1). Furthermore, as expected, we observed that members of the RCO pedigree carrying the Pro165Ser MCP mutation have reduced MCP levels on their lymphocytes and...
Table 1

<table>
<thead>
<tr>
<th>MCP</th>
<th>Pro165Ser (c.-598C &gt; T)</th>
<th>IF</th>
<th>c.-1610insAT</th>
<th>C3 (77–210 mg/dl)</th>
<th>C4 (14–47 mg/dl)</th>
<th>Factor H (12–56 mg/dl)</th>
<th>Factor I (75–115 %)</th>
<th>MCP (91–109%)</th>
<th>Factor H hemolytic assay (2–25% of total lysis)</th>
</tr>
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<tbody>
<tr>
<td>III-5</td>
<td>III-5/III-6/III-9</td>
<td>−</td>
<td>−</td>
<td>106</td>
<td>59</td>
<td>99</td>
<td>85</td>
<td>100</td>
<td>2–25%</td>
</tr>
<tr>
<td>III-9</td>
<td>III-9/III-10/IV-1</td>
<td>−</td>
<td>−</td>
<td>102</td>
<td>24</td>
<td>30</td>
<td>50</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>IV-1</td>
<td>IV-1/IV-2/IV-3</td>
<td>−</td>
<td>−</td>
<td>128</td>
<td>16</td>
<td>31</td>
<td>95</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>IV-2</td>
<td>IV-2/IV-3/IV-4</td>
<td>−</td>
<td>−</td>
<td>112</td>
<td>25</td>
<td>24</td>
<td>101</td>
<td>61</td>
<td>4</td>
</tr>
<tr>
<td>IV-3</td>
<td>IV-3/IV-4/IV-5</td>
<td>−</td>
<td>−</td>
<td>124</td>
<td>26</td>
<td>32</td>
<td>100</td>
<td>54</td>
<td>3</td>
</tr>
</tbody>
</table>

A normal range of variation in controls is shown between brackets for each variable.

b C3 and C4 were determined by nephelometry.
c Factor H and factor I plasma levels were determined by ELISA. Factor I levels are referred to a reference serum; each value corresponds to the mean of three independent determinations.
d Levels of MCP in PBLs (MFI, mean fluorescence intensity) were determined by flow cytometry and referred to a series of control samples drawn and analysed the same day as indicated in Section 2. The average MFI value for the control samples was set to 100%. Range of variation for all control samples (n = 17) was within 10% of the average value (91–109%).
e Lysis observed in samples from control individuals varies from 2 to 25% of total lysis (Sánchez-Corral et al., 2004).

The MCP mutation here is exceptional because mutations in genes encoding complement regulatory proteins.

in incomplete penetrance of the disease in carriers of mutations in surfaces from complement activation and help to understand the concept that aHUS results from a defective protection of cellular members of the pedigree for the presence of CFH and MCP aHUS-associated SNPs (Caprioli et al., 2003; Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2005). DAF levels and factor H activity were normal and all individuals in the pedigree carry the CFH-257T risk allele (not shown). Therefore, differences among carriers of the MCP and IF mutations cannot be attributed to these parameters. Interestingly, the MCPggaac risk allele was present in family RCO and segregated independently of the Pro165Ser MCP mutation, demonstrating that these two genetic traits are carried by different MCP alleles. Segregation analyses in family RCO also revealed that the patients HUS 68 and HUS 84, but not III-11, IV-1 and IV-2 carried the MCPggaac risk allele, strongly suggesting that concurrence of the three aHUS susceptibility factors greatly influences the disease manifestation in this family.

4. Discussion

We report here the identification of three independent aHUS risk factors in a large Spanish pedigree with two members affected. These factors are: a MCP missense mutation (c.-598C > T; Pro165Ser) that decreases MCP expression on the cell surface, a dinucleotide insertion in the coding sequence of factor I (c.-1610insAT) that introduces a premature stop codon in the factor I protein, and the MCPggaac SNP haplotype block that was previously shown to influence the transcription activity from the MCP promoter (Esparza-Gordillo et al., 2005). Segregation analysis demonstrated that the three aHUS risk factors segregate independently and, most important, that individuals, thus far affected from aHUS in this pedigree are only those who inherited the three risk factors (Fig. 1). The HUS pedigree reported here is exceptional because mutations in MCP and IF are very rare. Together, our findings provide further support for the concept that aHUS results from a defective protection of cellular surfaces from complement activation and help to understand the incomplete penetrance of the disease in carriers of mutations in genes encoding complement regulatory proteins.
Fig. 2. Mutation in membrane cofactor protein: (a) identification of MCP mutations in patients HUS 68 and HUS 84. The chromatogram corresponding to the DNA sequence surrounding the mutated nucleotide in MCP exon 5 is shown for the HUS68/HUS84 patients and for a control sample. The corresponding amino acid numbering is referred to the translation start site (Met +1) and the nucleotide nomenclature is referred to the transcription start site described by Cui et al. (1993); (b) diagram of the MCP molecule with four extracellular SCR domains, a transmembrane region and an intracytoplasmic tail. The location of the Pro165Ser mutation in the consensus sequence of a prototypic SCR domain (circled amino acids) (Law and Reid, 1995) is indicated by an arrow; (c) flow cytometry analysis of peripheral blood lymphocytes from the HUS 68/HUS 84 patients (red) and from normal control samples (blue). Isotypic control is shown in black.

The efficiency of the complement system as an innate defense mechanism against microbial infections depends on a fine control that avoids the wasteful consumption of its components and restricts its activation to the surface of microorganisms, thus preventing non-specific damage to host tissues. Control of the complement system is performed by a set of plasma and membrane-associated regulatory molecules acting as a protein network (Law and Reid, 1995). Genetic and functional analyses have shown that this critical control of complement activation may be impaired in aHUS patients. Accordingly, it is generally accepted that the mutations in CFH, MCP or IF found in many patients predispose to aHUS because they generate a situation unable to provide efficient protection to the host cellular surfaces in the case of complement activation (Warwicker et al., 1998; Pérez-Caballeri et al., 2001; Caprioli et al., 2001; Richards et al., 2003; Sánchez-Corral et al., 2002, 2004; Manuelian et al., 2003; Noris et al., 2004; Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2005). IV-2 (Fig. 1) could be an example of this situation. Despite carrying the c.-1610insAT IF mutation, IV-2 shows levels of factor I in plasma that are comparable to those found in the reference control sample (Table 1). Like many other complement components, factor I shows a relatively large (two-fold) normal range of variation (González-Rubio et al., 2001; Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2005). IV-2 could have inherited a paternal IF allele with high expression and, thus, compensate the effect of the c.-1610insAT IF mutation. Similar quantitative variations have been observed in carriers of CFH mutations in other aHUS pedigrees with incomplete penetrance of the disease (Sánchez-Corral et al., 2002).

The case of HUS68 and HUS84 in family RCO and previous findings describing concurrence of different predisposition factors in HUS patients provides an explanation for the incomplete penetrance of the disease in carriers of CFH, MCP and IF mutations. They suggest that multiple hits, probably involving plasma and membrane-associated complement regulatory proteins, are required to impair protection to host tissues significantly. Factor I and MCP are proteins acting in the same complement regulatory pathway (Law and Reid, 1995). Therefore, it is likely that
genetic variants decreasing the levels of MCP and factor I have an additive effect in the context of complement regulation and aHUS susceptibility. Similarly, concurrence of the Pro165Ser mutation with MCPggaac may have an additive effect reducing further the expression of MCP in carriers of the MCP mutation. Although we have not been able to document a further decrease of MCP levels in PBLs in members of the RCO pedigree carrying both the Pro165Ser mutation and the risk allele MCPggaac (Table 1), our earlier observation that two SNPs, c.-547G/C and c.-261G/C, included in the MCPggaac haplotype block decreases transcription from the MCP promoter in transient transfection experiments (Esparza-Gordillo et al., 2005) supports this conclusion. MCPggaac is an important HUS risk factor that has been found strongly associated with the disease in three different HUS cohorts (Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2005). Future experiments should address whether carriers of the MCPggaac allele show reduced expression of MCP locally in the kidneys or in conditions of infection or inflammation.

We found remarkable that in family RCO the individuals affected from aHUS are only those who inherited the three risk factors segregating in this pedigree (Fig. 1). We are aware that HUS68 and HUS84 presented with the disease at the ages of 57 and 41, relatively late for aHUS, and that there is always the possibility that those in the kindred with one or two risk alleles may get disease in the future. However, considering that many of the individuals carrying one or two risk alleles are at present over the age of onset of HUS 68 and HUS84, we suggest that the concurrence of different genetic risk factors influences the onset of the disease and the penetrance of aHUS in carriers of MCP and IF mutations (Fig. 1).

We believe that these findings provide clues to understand the incomplete penetrance of the disease in other aHUS pedigrees and, therefore, we suggest that all genes currently known to be aHUS risk factors should be analyzed routinely in aHUS patients. Furthermore, analysis of additional proteins involved in complement activation, such as C3, factor B or properdin, may unravel novel aHUS risk factors and provide further insights into genetic factors predisposing to aHUS in the still high percentage of aHUS patients in whom mutations in CFH, MCP or IF have been excluded.

In conclusion, our data indicate that concurrence of different susceptibility alleles affecting complement regulator expression greatly influences predisposition to aHUS and provides an explanation for the incomplete penetrance of aHUS in carriers of mutations in the complement regulatory genes. They also
consolidate the hypothesis that an inefficient protection of the cellular surfaces from complement activation is a general feature of patients with aHUS. As a whole our current knowledge of the molecular mechanisms underlying aHUS reinforce the concept that complement inhibition therapies to prevent or reduce tissue damage by complement activation is indicated for the treatment of aHUS patients.

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