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Modeling How CD46 Deficiency Predisposes to Atypical Hemolytic Uremic Syndrome

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Abstract

Mutations in complement regulatory proteins predispose to the development of aHUS. Approximately 50% of patients bear a mutation in one of three complement control proteins, factor H, factor I, or membrane cofactor protein (MCP; CD46). Another membrane regulator that is closely related to MCP, decay accelerating factor (DAF; CD55) thus far has shown no association with aHUS and continues to be investigated. The goal of this study was to compare the regulatory profile of MCP and DAF and to assess how alterations in MCP predispose to complement dysregulation. We employed a model system of complement activation on Chinese hamster ovary (CHO) cell transfectants. The four regularly expressed isoforms of MCP and DAF inhibited C3b deposition by the alternative pathway. DAF, but not MCP, inhibited the classical pathway. Most patients with MCP-aHUS are heterozygous and express only 25–50 % of the wild-type protein. We, therefore, analyzed the effect of reduced levels of wild-type MCP and found that cells with lowered expression levels were less efficient in inhibiting alternative pathway activation. Further, a dysfunctional MCP mutant, expressed at normal levels and identified in five patients with aHUS (S206P), failed to protect against C3b amplification on CHO cells, even if expression levels were increased 10-fold. Our results add new information relative to the necessity for appropriate expression levels of MCP and further implicate the alternative pathway in disease processes such as aHUS.

Keywords

Hemolytic uremic syndrome; complement; CD46; MCP; CD55; DAF; alternative pathway of complement

1. Introduction

A failure to properly inhibit complement activation is increasingly recognized as a predisposing factor to human disease. An example is hemolytic uremic syndrome (HUS), a clinicopathologic entity characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure (Moake, 2002; Richards et al., 2002). It typically follows a diarrheal illness (primarily *E. coli* O157:H7 infection) but also occurs in nonenteropathic settings in association with pregnancy, malignancies, drugs, and transplantation. Such cases are called atypical HUS or

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aHUS and may occur as sporadic or familial forms (Taylor et al., 2004). Although most do not have an identifiable trigger, some follow infection (Constantinescu et al., 2004).

Deficiency of complement regulators is a major risk factor for development of aHUS (Dragon-Durey and Fremeaux-Bacchi, 2005; Esparza-Gordillo et al., 2005; Goodship et al., 2004; Noris and Remuzzi, 2005; Zipfel et al., 2006). More than 50% of patients with aHUS have mutations in one of three complement control proteins: factor H (fH), membrane cofactor protein (MCP; CD46) or factor I (fI) (Dragon-Durey and Fremeaux-Bacchi, 2005; Esparza-Gordillo et al., 2005; Goodship et al., 2004; Zipfel et al., 2006). Additionally, factor H auto-antibodies have been reported as a mechanism for the development of sporadic aHUS (Dragon-Durey et al., 2005). An interesting point is that approximately 90% of the MCP-aHUS patients are heterozygous, expressing 25–50% of the wild-type protein. Also, fH and MCP haplotypes, which predispose to this thrombomicroangiopathy, were identified in several large cohorts (Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2005). The genes for fH and MCP are located at 1q32 in the regulators of complement activation (RCA) genetic cluster (Rodriguez de Cordoba et al., 1985). Other proteins in this family are decay accelerating factor (DAF; CD55), complement receptors one (CR1; CD35) and two (CR2; CD21) and plasma protein C4b binding protein (C4BP). These proteins consist largely or entirely of repeating motifs called CCP modules that house the sites for C3b/C4b interactions. Except for CR2, these proteins share the functional task of regulating C3b and C4b in plasma or deposited on host cells. This is accomplished by two processes. One is known as *cofactor activity*, mediated by MCP, fH, CR1, and C4BP. This refers to the inactivation of C3b or C4b via limited proteolytic cleavage mediated by the serine protease factor I and one of the cofactor proteins previously noted. The other mechanism, *decay accelerating activity*, is a process whereby the catalytic serine protease domain is dissociated from the C3 and C5 convertases. Surprisingly, no mutations in DAF have been identified in aHUS thus far (Esparza-Gordillo et al., 2005 and David Kavanagh, personal communication).

Our goal is to characterize how decreased function of host regulatory proteins predisposes to complement dysregulation on cells. We compared the complement regulatory profile of MCP to that of DAF. MCP consists of a family of four regularly expressed isoforms that share an identical amino-terminal segment consisting of four CCP modules (Fig. 1A). DAF also contains four CCPs and, similar to MCP, is flanked by a domain for O-glycosylation (Fig. 1B), a region that is alternatively spliced in MCP. DAF is anchored via a phosphatidylinositol anchor. MCP is a transmembrane protein with a cytoplasmic tail of 16 or 23 amino acids, both of which possess signaling motifs (Liszewski et al., 2005).

We also evaluated an MCP mutant (S206P) that lies in a functionally important site in CCP4 and that has been linked to aHUS in two of the first families described with MCP mutations (Fig. 1C) (Richards et al., 2003).

To initially characterize MCP and DAF, we employed a model system in which human complement regulatory proteins are stably expressed in Chinese hamster ovary (CHO) cells (Barilla-LaBarca et al., 2002; Liszewski and Atkinson, 1996). These transfected cells are sensitized with antibody, exposed to complement and then assessed for their capacity to inhibit complement activation (Barilla-LaBarca et al., 2002; Liszewski and Atkinson, 1996).

In these studies, the four regularly expressed MCP isoforms and DAF inhibited C3b deposition by the alternative pathway while DAF, but not MCP, inhibited the classical pathway. Reduced expression of wild-type MCP, as seen in aHUS patients, decreased protection against C3b deposition by the alternative pathway. A dysfunctional, but normally expressed form of MCP (S206P) found in the two unrelated families with aHUS, was ~10% as effective as wild type against C3b amplification mediated by the alternative pathway or its feedback loop. Our studies

suggest that, during complement activation states, a delicate balance exists between MCP-dependent inactivation and alternative pathway driven amplification. These findings expand our knowledge of host complement regulation and further demonstrate how complement dysregulation could predispose to aHUS.

2. Materials and Methods

2.1 Cell lines and antibodies

CHO clones expressing MCP isoforms have been described (Liszewski and Atkinson, 1996) and are designated as: BC1, clone 23-9, or M100; BC2, clone M26; C1, clone 14-8; and C2, clone 15-5. A lower expressing MCP-BC1, clone MCP 3-10, was designated as M25. Mutant MCP (S206P) CHO clones were isolated by limiting dilution from a previously stably transfected cell line (Richards et al., 2003) and were designated as S206P-2, S206P-6, and S206P-7. We have previously cloned DAF cDNA (Medof et al., 1987). For expression studies, DAF cDNA was cloned into the EcoRI site of stable expression vector pH β Apr1.neo (Gunning et al., 1987) and transfected into CHO cells using Lipofectin (Life Technologies/Invitrogen, Carlsbad CA). Stable cells were selected using Geneticin (Life Technologies) at 0.5 mg/ml active concentration. Clones were isolated by limiting dilution and evaluated by flow cytometry using a mAb to DAF (BRIC 216, Serotec, Raleigh, NC). Flow cytometry for evaluation of MCP and mutant MCP (S206P) expression on CHO clones was performed with mAb TRA-2-10 that reacts with CCP1 (Liszewski et al., 2000).

2.2 Initiation and analysis of complement activation

Methods for initiation of the classical and alternative pathways have been described (Barilla-LaBarca et al., 2002; Liszewski and Atkinson, 1996). CHO cells bear minimal or no endogenous regulatory activity against human complement. Briefly, CHO cells were grown to 70–80% confluency and collected by trypsinization in 1% FCS-PBS. Collected cells were sensitized with anti-CHO antibodies (goat anti-CHO, Cygnus Technologies, Southport, NC or rabbit anti-hamster lymphocyte IgG, Sigma-Aldrich, St. Louis, MO) in PBS for 20 min at 4 °C. Cells were washed with gelatin veronal buffer (GVB⁺⁺, Sigma-Aldrich, St. Louis, MO) or GVB containing 10 mM EGTA and 7 mM magnesium chloride (MgEGTA-GVB)]. Next, C7-deficient (C7d) serum (gift of P. Densen, University of Iowa, Iowa City, Iowa) was diluted to 10% in the same washing buffer and 100 μ L was added to cell preparations. The samples were rotated at 37 °C for the time points indicated, washed twice in 1% FCS-PBS, and assessed by flow cytometry and Western blotting. For flow cytometry, murine mAbs to human complement component fragments, C3d, C4c, and C4d (Quidel, San Diego, CA) were added (5 μ g/ml) to the cell preparations. After 20 min incubation at 4 °C with appropriate FITC-conjugated secondary antibody, cells were washed and fixed in 0.5% paraformaldehyde and analyzed with a FACSCalibur system (BD Biosciences, Mountain View, CA).

C3 fragment deposition was also characterized by Western blotting. After washing, the cells were lysed with nonionic detergent Nonidet P-40 and supernatants (1 x 10⁵ cell equivalents/lane) were applied to gels (Barilla-LaBarca et al., 2002). Following SDS-PAGE and transfer to a nitrocellulose membrane, a polyclonal goat anti-C3 Ab (CompTech, Tyler, TX) was added (1/7500). Detection, using Super Signal West Pico chemiluminescent substrate, was performed according to manufacturer's directions (Pierce, Rockford, IL).

2.3 Quantitation of MCP Expression

MCP expression was assessed by flow cytometry and ELISA (Barilla-LaBarca et al., 2002). Briefly, for flow cytometric analysis, cells were trypsinized, harvested and washed in PBS before transfer to microtiter plates (5 x 10⁵ cells/well) or lysis (1% NP-40/0.05% SDS/2 mM PMSF in PBS). Trypsinization did not alter expression levels. TRA-2-10, a murine mAb

(Andrews et al., 1985) that reacts with MCP as well as the MCP mutant S206P, was added (10 μ g/ml) followed by incubation with cells for 30 min at 4° C. Following centrifugation and washing, FITC goat anti-mouse IgG was added (Sigma-Aldrich, St. Louis, MO). After 30 min incubation at 4 °C, cells were resuspended in 1% FCS-PBS and analyzed by FACSCalibur. In the ELISA (Barilla-LaBarca et al., 2002), TRA-2-10 was employed as the capture antibody and polyclonal rabbit anti-MCP for detection. MCP clones employed express the following approximate copy number/cell: 1 x 10⁵, MCP isoforms BC1 (M100), BC2, C1, and C2; 2.5 x 10⁴, M25; 1 x 10⁵, S206P-2; 3 x 10⁵, S206P-6; and 1 x 10⁶, S206P-7.

3. Results

We have previously described a model system for characterizing regulation of complement activation on mammalian cells (Barilla-LaBarca et al., 2002; Liszewski and Atkinson, 1996). Stable clones derived from Chinese hamster ovary (CHO) cells expressing a complement regulator are sensitized with antibody and then challenged with human serum. In this cell-based system, higher levels of Ab sensitization primarily engage the classical pathway as similar levels of complement deposition occur in factor B depleted serum (Liszewski and Atkinson, 1996). The alternative pathway is assessed by incubating the Ab-sensitized cells in serum treated with MgEGTA or by lowering the sensitizing level of antibody. In the latter situation, the alternative pathway becomes a contributor to C3 fragment deposition via its feedback loop mechanism (Barilla-LaBarca et al., 2002; Liszewski and Atkinson, 1996). Flow cytometry and Western blotting are employed to monitor C3 fragment deposition. To begin, we isolated stable clones of MCP and DAF expressing ~ 100,000 copies per cell as determined by ELISA (not shown) and FACS (Fig. 2A) and also characterized by Western blotting (Fig. 2B).

3.1 DAF, but not MCP, inhibits C3b deposition by the classical pathway

None of the four regularly expressed MCP isoforms inhibited C3b deposition mediated by Ab activation of the classical pathway (Fig. 3A-E). This is consistent with previous data using the MCP-BC1 isoform (Liszewski and Atkinson, 1996) at this as well as lower levels of sensitization (Barilla-LaBarca et al., 2002; Devaux et al., 1999). In contrast, DAF inhibited C3 deposition by 76% $\pm$ 6 (mean $\pm$ SEM of three experiments) as compared to control. These data indicate that in this system DAF, but not MCP, regulates the classical pathway C3 convertase.

We previously determined that > 95% of the C3b was deposited at 5 min if the classical pathway was activated by Ab on CHO cells (Barilla-LaBarca et al., 2002). Therefore, we assessed the kinetics of complement activation from 2 to 80 min on DAF-expressing CHO cells (Fig. 4). In this setting, the majority of C3b (~90%) was deposited within 2 min and was maximal by 5 min. As noted above, under these conditions DAF decreased overall C3b deposition (Fig. 3G). Consequently, DAF disassociated C3 convertases within this time frame and inhibited C3 deposition but did not influence the kinetics of activation.

3.2 Factor H mediates C3b cleavage following classical pathway activation

We next employed a Western blot to characterize C3b fragments generated by classical pathway activation on the control versus DAF or MCP expressing transfectants (Fig. 5). MCP and DAF cell lysates showed the expected cleavage fragments consistent with iC3b (i.e., loss of α' and generation of the $\alpha 1$ and $\alpha 2$ cleavage fragments). Approximately one-third of the $\alpha 1$ fragment is present at its expected mol wt, indicating a noncovalent interaction or binding to small molecules on the cell membrane. The high mol wt fragments represent $\alpha 1$ or α' fragments covalently bound to membrane proteins of 80 to 150 kDa. The similar signal strengths observed in parental CHO cells (CHO+) (lane 4) and in the four isoforms of MCP (lanes 6–9), as compared to the decreased signal of DAF (lane 5), correlate with the results obtained by FACS (Fig. 3). Additionally, the pattern of C3b cleavage is the same in CHO cells as well as in the

CHO cells expressing any of the four MCP isoforms. These findings confirm and extend our earlier observations that factor H, and not MCP, is primarily responsible for C3b cleavage on the cell surface in this experimental system (Barilla-LaBarca et al., 2002). Yet MCP can compensate. That is, in the same study, when the functional activity of factor H is blocked with a mAb, MCP begins to cleave the deposited C3b suggesting that the difference between the functional activity of factor H versus MCP on the cell surface is largely a matter of kinetics with factor H acting more rapidly. However, factor H does not block C3b deposition and primarily serves to cleave C3b to iC3b. With other cell types, such as in the case of injured renal endothelium, factor H may have other cell surface interactions that enhance regulatory activity. Additionally, although DAF diminishes overall deposition, the remaining C3b is also similarly cleaved by factor H and factor I to iC3b (lane 5 and the longer exposure of this same lane in 5b).

3.3 MCP and DAF inhibit C3b deposition mediated by the alternative pathway

Next, we assessed the role of MCP in the alternative pathway using serum treated with MgEGTA (which abrogates classical and lectin pathway activation). Ab sensitized control CHO cells not expressing MCP or DAF show a robust level of C3 deposition (Fig. 6A, curve on right side). However, in the presence of each of the MCP isoforms or DAF, C3b deposition was inhibited by > 97% (Fig. 6B-F). Thus, under these experimental conditions, all four MCP isoforms and DAF effectively and similarly inhibited alternative pathway mediated complement activation.

3.4 Reduced MCP expression decreases functionality

Most patients with aHUS express 25 to 50 % of the wild-type MCP levels (Caprioli et al., 2006; Fremeaux-Bacchi et al., 2006; Richards et al., In Press), so next we characterized how decreased expression of MCP might predispose to complement dysfunction. First, we isolated two clones with a four-fold difference in MCP expression levels (Fig. 7, M25 and M100). In the alternative pathway system, C3 deposition was inhibited by both the higher and lower expressing MCP clones, although the lower expressing clone was less efficient (Fig. 8, AP set: 95% versus 82% inhibition, respectively, for M100 and M25). Next, we reduced the level of sensitizing antibody such that activation of the classical pathway and of the feedback loop of the alternative pathway both contributed to C3 deposition (Fig. 8, AP + CP set). Under these conditions, C3 deposition was inhibited by both the high and low MCP expressing clones (57% and 34% inhibition for M100 and M25, respectively). We have previously shown that MCP minimally inhibits the classical pathway even at low concentrations of Ab (Barilla-LaBarca et al., 2002; Liszewski and Atkinson, 1996). Thus, these findings are consistent with previous studies demonstrating that MCP primarily inhibits the alternative pathway (Devaux et al., 1999; Kojima et al., 1993; Liszewski and Atkinson, 1996). Taken together, these studies indicate that either *de novo* activation or engagement of the feedback loop is regulated by MCP. Also, the lower expressing clone (M25) inhibited C3 deposition less efficiently than the higher expressing clone (M100) in both settings, demonstrating that the level of MCP expression impacts its complement regulatory activity.

3.5 HUS MCP mutants protect poorly against C3b amplification

Lastly, we characterized how the expression of a mutant form of MCP may predispose to aHUS. We previously demonstrated that this mutant was expressed at normal levels in five aHUS patients as well as in transfected cell lines; however, despite having similar C4b regulatory activity compared to wild-type MCP, transfectant lysates possessed ~15% of the C3b binding capability and no C3b cofactor activity (Richards et al., 2003). For the current study, we examined cell surface regulatory activity. We isolated a clone with an expression equivalent to wild-type MCP clone M100 (i.e., HUS MCP clone 206P-2, see Fig. 7) and found that the

HUS mutant protected minimally against C3b amplification on the cell surface (Fig. 9). That is, wild-type MCP (+ MCP) inhibited C3b deposition by 95% while that for 206P-2 mutant was nearly superimposable with the profile of control (-MCP or non-MCP expressing) CHO cells. We next isolated two additional clones expressing higher quantities of the S206P mutant (206P-6 and 206P-7, see Fig. 7). Even at a 10-fold higher expression level (206P-7 as compared to wild-type M100), the HUS MCP mutant was not as efficient as native MCP (Table 1).

4. Discussion

Atypical hemolytic uremic syndrome is recognized as a disease of complement dysregulation in which complement amplification outstrips its regulation. The lack of appropriate complement control in aHUS leads to a procoagulant state with microthrombi formation in the renal vasculature (Atkinson et al., 2005; Dragon-Durey and Fremeaux-Bacchi, 2005; Goodship et al., 2004; Noris and Remuzzi, 2005).

Mutations in complement regulatory proteins account for ~ 50% of aHUS cases (Dragon-Durey and Fremeaux-Bacchi, 2005; Esparza-Gordillo et al., 2005; Pirson et al., 1987; Richards et al., 2002; Zipfel et al., 2006). Factor H was the initial complement regulatory protein in which mutations were linked to the development of aHUS (Warwicker et al., 1998). Subsequently, mutations in MCP (Caprioli et al., 2006; Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2006; Kavanagh et al., 2005; Noris et al., 2003; Richards et al., 2003) and factor I (Caprioli et al., 2006; Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2005) have been linked to aHUS. Several studies have also screened patients for DAF deficiency, but no association has been identified yet (Esparza-Gordillo et al., 2005) (David Kavanagh, personal communication).

Heterozygous individuals of all three regulators are affected, suggesting that half-normal levels of these proteins are insufficient to protect kidney vessels. In the case of MCP, more than 75% of the mutations cause a reduction in expression (Caprioli et al., 2006; Dragon-Durey and Fremeaux-Bacchi, 2005; Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2006; Noris et al., 2003; Richards et al., 2003; Richards et al., In Press). Kidney transplantation is a treatment strategy primarily effective for patients with MCP mutations since the transplanted kidney should express normal levels of MCP. Currently, 90% of renal transplant patients with MCP-aHUS have not had a recurrence of disease (Richards et al., In Press). This is not the case for a deficiency in plasma factors H or I in which the transplanted kidney cannot compensate for deficiency of a protein produced primarily in the liver.

Our aim was to provide a better understanding of how MCP expression levels impact complement regulation. The approach taken was to characterize how decreased quantity of a wild-type regulatory protein or a mutant form of MCP (S206P, found in two independent aHUS families, one heterozygous and one homozygous) predisposes to complement dysregulation (Richards et al., 2003).

Initially, we characterized the functional profile of the four MCP isoforms and contrasted them to DAF. Previous studies have examined primarily only one of the four regularly expressed isoforms of MCP (Barilla-LaBarca et al., 2002; Richards et al., 2003). While the MCP isoforms and DAF equivalently and efficiently inhibited the alternative pathway, only DAF inhibited the classical pathway in this experimental system. That MCP preferentially inactivates the alternative pathway agrees with previous findings (Barilla-LaBarca et al., 2002; Devaux et al., 1999; Kojima et al., 1993; Seya and Atkinson, 1989). Interestingly, DAF was also effective in inhibiting the alternative pathway and yet no mutations have thus far linked DAF to aHUS (Esparza-Gordillo et al., 2005) and personal communication, D. Kavanagh).

We found that reduced MCP expression led to decreased ability to inhibit alternative pathway or its feedback loop. Further, a mutant of MCP (S206P) failed to adequately protect against the alternative pathway, even at 10-fold higher levels than wild-type MCP. The S206P mutation lies within a hypervariable loop in CCP4 that is important for C3b regulation (see Fig. 1C) (Liszewski et al., 2000; Richards et al., 2003). Currently, ~20 different MCP mutations have been identified and partially characterized in aHUS (Caprioli et al., 2006; Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2006; Noris et al., 2003; Richards et al., 2003). Many result in a premature stop codon or produce a mutant protein that is retained in the endoplasmic reticulum and degraded intracellularly [reviewed (Richards et al., In Press)].

That heterozygotes develop aHUS suggests a critical level of expression is required for adequate complement regulation. Some patients with the MCP deficiency show reduced expression levels on peripheral blood mononuclear cells (PBMC) (Esparza-Gordillo et al., 2005; Noris et al., 2003; Richards et al., 2003). Our data demonstrate that reduced MCP expression correlates with increased complement dysregulation.

An intriguing and relevant question is why other control proteins such as factor H and DAF are not adequate to inhibit C3b deposition in the setting of lowered expression of MCP such as occurs in MCP-aHUS. Along this line, it is also surprising that DAF deficiency has so far not been associated with aHUS (Esparza-Gordillo et al., 2005 and David Kavanagh, personal communication). One possibility is that the convertase dissociating capacity of DAF is not a permanent inactivation process. Instead, alternative pathway C3 convertases may reform. In vivo, cofactor activity may be necessary to prevent the build up of C3b and alternative pathway C3 convertases. Since a deficiency of either factor H or MCP predisposes to aHUS, both appear to be required to protect host tissue and do not have overlapping functions *in vivo* in this pathologic situation. For example, a likely scenario is that MCP patrols cell surfaces to prevent the feedback amplification loop, whereas factor H protects similarly in plasma and on acellular tissue matrix. In a setting of either factor H or MCP deficiency, excessive complement activation occurs on endothelial cells or exposed basement membranes to initiate a procoagulant state that leads to renal damage.

However, the general consensus is that mutations of complement regulators more predispose rather than cause the thrombotic microangiopathy (Esparza-Gordillo et al., 2006; Goodship et al., 2004). In this setting, endothelial activation that occurs secondary to injury is undesirably enhanced by excessive complement activation. A characteristic feature of MCP and factor H associated HUS is a variable penetrance and inheritance. In one panel of families, disease penetrance was ~50% (Richards et al., 2001; Richards et al., 2003), and these results are consistent with several other series (Fremaux-Bacchi et al., 2006; Noris et al., 2003). Further, these patients are healthy prior to their first episode of aHUS and many recover only to have another attack years later.

Our studies demonstrate that MCP primarily controls the alternative pathway. In the process of classical or lectin pathway activation on cell surfaces, however, the feedback loop of the alternative pathway is often engaged. In these settings, our results indicate that MCP should also function as an effective inhibitor.

It is becoming increasingly clear that the alternative pathway plays a role in many diseases (Thurman and Holers, 2006). Relative to kidney disease, factor B deficient mice (*fB* $-/-$ MRL/*lpr*) as well as factor D deficient mice (*fD* $-/-$) are both protected from renal disease in a systemic lupus erythematosus model (Elliott et al., 2004; Watanabe et al., 2000). Forms of membranoproliferative glomerulonephritis (MPGN) are associated with C3 deposition in the glomerulus. More than 80% of patients with type II MPGN have circulating autoantibodies, called nephritic factors, that bind and stabilize the alternative pathway convertase (Thurman

and Holers, 2006). Additionally, age-related macular degeneration (AMD) is the most common cause of visual loss in the elderly (Hageman et al., 2005). Four separate groups recently described a polymorphism of factor H associated with the development of AMD (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). The retinal lesions are structurally similar to those seen in type II MPGN (Mullins et al., 2001). Thus, enhanced alternative pathway activity or a reduction in complement regulatory activity is implicated in several forms of human kidney disease.

Alternative pathway activation has also been implicated in aHUS. Decreased levels of C3 and factor B as well as increased levels of C3 activation fragments have been described, whereas C4 levels are unaffected (Noris et al., 1999; Stuhlinger et al., 1974; Zipfel et al., 2006). C3, but not C4, is deposited in the glomerulus and arterioles of aHUS patients (Hammar et al., 1978). For the factor H mutations, there is evidence for a reduced ability to bind to polyanion-rich surfaces (such as the glomerular basement membrane) (Manuelian et al., 2003; Perez-Caballero et al., 2001). Such binding may be required at sites of injury to prevent alternative pathway activation. That MCP mutations have been linked to development of aHUS and given its ability to preferentially inactivate the alternative pathway further emphasizes the role of this pathway in the disease process.

Our results add new information about the necessity for appropriate expression levels of complement regulator MCP and further implicate the alternative pathway in disease processes. Thus, therapies that reduce alternative pathway-mediated complement activation may be important strategies for ameliorating many types of human disease.

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Abbreviations

MCP	membrane cofactor protein or CD46
DAF	decay accelerating factor or CD55
HUS	hemolytic uremic syndrome
aHUS	atypical hemolytic uremic syndrome
CHO	Chinese hamster ovary cells

Figure 1.

(A) Schematic diagram of membrane cofactor protein (MCP; CD46). MCP is composed of four repeating units called complement control protein repeats (CCPs) that house the sites for complement regulation. The CCP region is followed by an O-glycosylation domain that is alternatively spliced. All MCP isoforms possess the C module, while the B module which carries approximately 3-fold more O-linked sugars than the C module is alternatively spliced. MCP expresses a cytoplasmic tail of 16 or 23 amino acids. The four common MCP isoforms are called BC1, BC2, C1 and C2 and are co-expressed on most cell types. (B) DAF also consists of four CCPs followed by a segment that is O-glycosylated. DAF is tethered by a glycosylphosphatidyl inositol (GPI) anchor. (C) An MCP mutation in CCP4 that is associated with aHUS (S206P) lies within the hypervariable loop as modeled on CCP1 of MCP.

(A) MCP and DAF Clones: FACS **(B) Western Blot** **Figure 2.**

Flow cytometry (A) and Western blot (B) of CHO cloned cell lines. These clones express ~ 100,000 copies of MCP or DAF per cell as determined by ELISA. Mean fluorescence intensity (in arbitrary units) of all clones is within ~25 units of each other (BC1, 152; BC2, 146; C1, 171; C2, 155; DAF, 168). For flow cytometry, a primary MCP mAb (TRA-2-10) or DAF mAb (BRIC 216) was utilized followed by the same secondary FITC-conjugated goat anti-mouse IgG. In the Western blot analysis, a rabbit polyclonal antiserum to MCP was used and for DAF mAb BRIC 216 was used. The light bands at ~ 40,000 in the MCP lanes represent the precursor form. Representative experiment of three is shown.

Figure 3.

DAF, but not MCP isoforms, inhibits C3 deposition by the classical pathway. FACS analysis of C3 deposition on sensitized cells (8.5 mg rabbit IgG/ml) exposed to 10% C7d human serum for 45 min. **A.** CHO control. The thin line shows cells treated with an isotype control while the thick line detects binding by a mAb to C3d (MFI 1390). **B-F** histograms of MCP isoforms. MFI for C3d: 1270, 1294, 1310, 1206, and 405, respectively. **G.** Overlay. Heat-inactivated serum showed no C3 deposition (not shown). A representative experiment of three is shown.

Figure 4.

Kinetic analysis of DAF inhibition of C3 deposited by the classical pathway. Methods as per Fig. 3. The control shown on the left in the histogram was not incubated with the anti-C3d monoclonal antibody. For DAF-expressing cells, the shaded curve is a 2 min serum treatment. The curves for 5, 15, and 80 min are coincident. The non-DAF expressing CHO cell is indicated on the far right. C3 deposition was maximal in < 5 min. A representative experiment of three is shown. In the three experiments, mean $\pm$ SEM for C3 deposition at 5 min was (left to right) 3 ± 1 (neg ctl), 159 ± 5 (DAF- expressing CHO cells), and 995 ± 22 (non-expressing CHO cells).

Figure 5.

Factor H degrades C3b to iC3b following classical pathway mediated deposition of C3b. CHO cells were challenged with Ab and 10% C7d serum as described in Fig. 3. The solubilized cell preparations were electrophoresed on an 8% PAG under reducing conditions. Following transfer, the blot was developed with a goat polyclonal anti-C3 Ab. Markers (kDa) are shown on the left. Lanes 1 and 2 are purified proteins. Lanes 3 and 4 represent untreated (CHO-) and Ab/serum treated (CHO+) cell lysates. The fragments with an M_r greater than that of the α' -chain (lanes 4–9) represent α' or α_1 covalently bound to membrane constituents (labeled as high molecular weight, HMW). Lane 5b is a longer exposure of lane 5. Representative Western blot of three.

Figure 6.

MCP and DAF inhibit C3 deposition by the alternative pathway. FACS analysis of C3 deposition on sensitized cells (8.5 mg rabbit IgG/ml) exposed to C7d serum (10% diluted in MgEGTA) for 45 min. **A.** CHO cell control. The thin line on the left shows the isotype control while the thick line demonstrates binding by a mAb to C3d following complement challenge. **B-F** histograms indicate that MCP isoforms and DAF each decrease deposition by > 97%. A heat-inactivated serum control showed no C3 deposition (not shown). A representative experiment of three is shown. MFI $\pm$ SEM for the three experiments (each condition in duplicate) was: CHO 627 ± 28 ; BC1, 20 ± 4 ; BC2, 21 ± 3 ; C1, 24 ± 3 ; C2, 22 ± 2 ; DAF, 23 ± 3 .

Figure 7.

Expression levels of wild-type MCP and S206P mutant CHO clones. FACS was performed using a mAb to MCP (TRA-2-10) that recognizes CCP1. The expression levels were determined by ELISA. Representative experiment of three is shown.

Figure 8.

Effect of reduced expression of MCP on C3 deposition. Two clones of MCP, M25 and M100, bearing 25,000 and 100,000 copies, respectively, were employed. In these experiments, the sensitizing antibody was goat anti-CHO at 0.25 mg IgG/ml. Following sensitization, the cells were incubated in 10% C7d serum at 37 °C for 45 min. At this level of sensitization both the classical and the alternative pathways are activated. In the presence of serum treated with MgEGTA, only the alternative pathway is activated. This data demonstrates that MCP effectively regulates deposition mediated by the alternative pathway and can regulate the feedback loop even when it is triggered by the classical pathway. Data represent the mean $\pm$ SEM from three experiments with each condition performed in duplicate.

Figure 9.

A dysfunctional form of MCP (aHUS mutant S206P) fails to protect against amplification by the alternative pathway. Wild-type and mutant MCP CHO clones (MCP M100 and mutant 206P-2, respectively, with equivalent expression levels $\sim 100,000$ molecules/cell) were sensitized with Ab, challenged with complement and analyzed as per Fig. 8. In three experiments, the mean $\pm$ SEM for inhibition of C3 deposition was $95\% \pm 4$ for the MCP-expressing clone and $2\% \pm 2$ for the S206P mutant.

Table 1

Comparison of inhibition of C3 deposition by wild-type MCP versus mutant S206P clones

Cell Line	Expression Level	Inhibition AP (%)	Inhibition CP + AP (%)
M100	1×10^5	95 ± 4^a	57 ± 3
206P-2	1×10^5	2 ± 2	4 ± 2
206P-6	3×10^5	50 ± 2	34 ± 2
206P-7	1×10^6	62 ± 3	43 ± 3

^aData represent the mean $\pm$ SEM from three experiments with each condition performed in duplicate. Methods per Fig. 8.