Regulation of complement activation on red blood cells

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Publication date
2019
Document Version
Other version
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Chapter 5

Blocking the binding domains 6-8 of FH inhibits its complement regulation function on human red blood cells

Manuscript in preparation

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ABSTRACT

Red blood cells (RBCs) are constantly exposed to complement and rely on several complement regulators to protect themselves from unwanted complement-mediated damage. While some of these regulators are expressed on the cell membrane, one of the most important complement regulators, complement factor H (FH), circulates in the bloodstream and binds to the RBCs upon complement activation. Currently, the binding of FH to the cell surface is mainly attributed to its C-terminal binding region, consisting of domains 19 and 20. Here, we describe 20 novel anti-FH monoclonal antibodies which we have characterized and used to study the binding of FH to human RBCs. By targeting specific domains with our antibodies, we show that blocking domain 20, but also blocking domain 6, independently, results in decreased FH function. This indicates that not only the binding region comprising domains 19-20 but also the simultaneous binding via domains 6-8 of FH is crucial for the protection of human RBCs against complement-mediated clearance. These results shed new light on the function of FH on human cell surfaces and the consequences of SNPs affecting either of its two binding regions in protecting RBCs from complement.

INTRODUCTION

The human complement system is a powerful protein cascade in blood plasma with a pivotal role within innate immunity, contributing to pathogen clearance by opsonisation with C3b, the induction of an inflammatory response and lysis through the formation of the membrane attack complex. The system partly relies on pattern recognition molecules such as C1q for targeted activation. However, the alternative pathway is driven by the spontaneous, a-specific activation of C3. This requires tight regulation on host surfaces to prevent unwanted complement activation and damage. While human RBCs express the membrane bound complement regulators complement receptor 1 (CR1), decay accelerating factor (DAF), and CD59. Also circulating complement regulators, such as factor H (FH), are pivotal in controlling the complement system[1-3]. This becomes apparent in paroxysmal nocturnal hemoglobinuria (PNH) in which somatic mutations in PIG-A result in aberrant glycosylphosphatidylinositol (GPI) anchor formation in a sub-population of the hematopoietic stem cell lineage[4,5]. As both DAF and CD59 are GPI-anchored complement regulators, PNH RBCs become vulnerable for complement-mediated clearance[6,7] and more dependent on FH for protection against complement[8].
FH consists of 20 domains, of which domains 1-4 contain the complement regulatory activity[9,10], and domains 6-8 and 19-20 are involved in the binding to surfaces[11-18]. FH binds to host surfaces via C3b and poly-anionic residues such as sialic acid and glycosaminoglycans[19]. The binding of FH to host cells is mainly attributed to its C-terminal binding region comprising domains 19 and 20, which are also a hotspot for disease-associated SNPs20. The importance of binding via domain 6-8 is unclear. The 6-8 region contains a binding site for poly-anionic residues and was shown to be involved in surface binding using deletion mutants of FH[11,21]. However, a recent study showed that recombinant fragments comprising 6-8 could not inhibit FH binding to cell surfaces, indicating that surface binding is mainly mediated via domains 19-20[19]. To circumvent possible differences in binding affinities of fragments to the cell surfaces compared to FH, we targeted FH directly with the use of monoclonal antibodies (mAbs) to further elucidate the function of FH on human RBCs.

MATERIAL AND METHODS

Immunization and antibody characterization

Mouse immunization and generation of mAbs was performed as described previously[22], using plasma purified human FH (CompTech, Tyler, TX, USA) as immunogen. Epitope mapping of the mAbs on FH was performed with the use of recombinant FH fragments[17,18]. Fluid phase co-factor activity[23] and binding of C3b[24]

were determined as described previously.

Sheep RBC hemolytic assay

FH functionality was measured with a hemolytic assay as described previously[25]. Normal human serum (NHS, pool of 30 healthy donors, obtained with informed consent) was pre-incubated with the indicated mAbs and mixed in a 1-to-1 ratio with sheep RBCs to reach a final concentration of 10% (v/v) NHS with 1.05*108 cells/mL in Veronal buffer (VB) with 5 mM MgCl2 and 10 mM EGTA (ethyleneglycoltetraacetic acid), or VB with 10 mM EDTA (ethylenediaminetetraacetic acid) as blank, followed by incubation at 37°C for 75 minutes while shaking. Lysis was stopped by adding 100 µL ice-cold VB with 20 mM EDTA followed by centrifugation (2.5 minutes, 1800 rpm, 7°C). Hemolysis was measured as absorbance of the supernatants at 412 nm and expressed as percentage of the 100% lysis control (H2O with 0.6%, w/v, Saponin).

Table 1. Characteristics of the anti-FH mAbs used in this study

			residual activity of FH	
mAb	Isotype	epitope location	Fluid phase co-factor *	C3b binding †
αFH.02	IgG1	20 ‡	93 %	44 %
αFH.03	IgG1	unknown §	97 %	53 %
αFH.07	IgG1	18	87 %	55 %
αFH.09	IgG1	6	94 %	88 %
αFH.10	IgG1	20 ‡	99 %	72 %
αFH.11	IgG2a	1-4	15 %	38 %
αFH.15	IgG1	5	88 %	59 %
αFH.16	IgG1	16-17	100 %	59 %
αFH.19	IgG1	20 ‡	99 %	55 %

* Fluid phase co-factor activity was determined as described by Pechtl et al.[20] C3b alpha-chain degradation by FH and FI, visualized by SDS-PAGE and quantified using a ChemiDoc and Image Lab software, version 5.0 (Bio-Rad, Hercules, CA, USA), in the absence of mAb was set to 100%.

† Binding of C3b to FH was determined as described by Hebecker et al.21 C3b binding to coated FH in the absence of mAb was set to 100%.

‡ Anti-FH.02, anti-FH.10, and anti-FH.19 all bind to different epitopes on domain 20 as the mAbs do not cross-compete for binding of FH.

§ Anti-FH.03 did not bind to any FH fragment, while it did bind to plasma purified FH.

Flow cytometry analysis of C3 deposition on human RBCs

RBCs from three healthy donors were collected, with informed consent, by centrifugation of citrated blood (10 minutes, 2500 rpm) and washed 3 times with PBS. RBCs were re-suspended to a 50% cell suspension in saline-adenine-glucose-mannitol solution (SAGM, Fresenius Kabi, Zeist, the Netherlands), stored at 4°C and

washed with PBS before use. RBC suspensions of 0.16% were incubated in a final volume of 100 µL with 25% (v/v) NHS from one healthy donor, pre-incubated with the indicated mAbs, in VB supplemented with 0.05% (w/v) gelatine, 10 mM CaCl2 and 2 mM MgCl2, at 37°C for 1 hour followed by washing with PBS containing 0.5% (w/v) BSA (bovine serum albumin). C3 deposition was measured by flow cytometry using FITC-conjugated anti-C3.19 mAb (Sanquin)[26].

RESULTS AND DISCUSSION

We successfully generated and characterized 20 novel anti-FH mAbs which were sub-divided in 9 groups based on competition for FH binding. Of each group, one mAb was selected to map the binding site and study the effects on FH function as defined by C3b binding and co-factor activity (Table 1). Three non-competing mAbs were directed against domain 20, indicating non-overlapping epitopes. Two other mAbs were directed against an epitope in domains 16/17 and domain 18, while all other mAbs were directed against epitopes in domains 1-6, except for anti-FH.03, which did not bind to any FH fragment. The mAbs affected known FH functions, corresponding with the different roles known for the targeted domains[9,10,13-15], e.g. anti-FH.11 (domains 1-4) inhibited the fluid-phase co-factor activity and anti-FH.02 (domain 20) inhibited C3b binding.

After characterizing each mAb, we investigated whether blocking the different domains affected the activity of FH on sheep RBCs[25]. As expected, blocking domain 20 with anti-FH.02, anti-FH.10 or anti-FH.19 resulted in complement-mediated lysis (Fig. 1A). However, blocking domain 6 (anti-FH.09) also resulted in a strong increase of complement-mediated lysis, suggesting that the first binding region (domains 6-8) is also required for complement regulation by FH. Remarkably, anti-FH.11, which blocks FH co-factor activity, did not result in a dose-dependent increase in hemolysis, which was explained by a complete consumption of native C3 in the fluid phase as measured by a C3b/c ELISA (data not shown).

Next, we determined whether the binding region in domains 6-8 is also crucial for FH function on human RBCs. As normal human RBCs express CR1, DAF and CD59 to prevent complement-mediated lysis, we measured C3 deposition as a read-out of disturbed FH function by FACS analysis. In line with the sheep RBCs, not only blocking domain 20 (with anti-FH.02, anti-FH.10 and anti-FH.19) but also blocking domain 6 (with anti-FH.09) resulted in increased C3 deposition in a dose-dependent manner (Fig. 1B-C), indicating that indeed both surface binding regions in domains 6-8 and 19-20 are truly involved in the complement regulating function of FH on human RBCs. As suggested by our findings, binding of FH via only one of these regions is not sufficient for optimal protection and FH needs both regions to

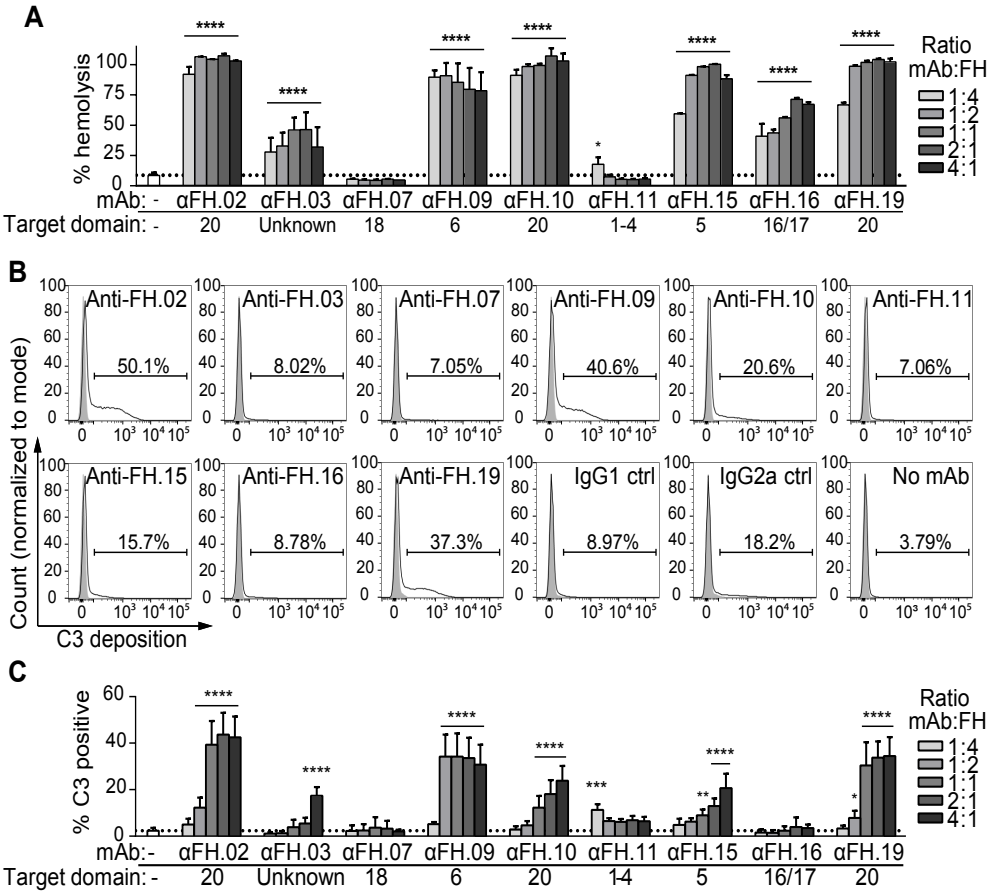

Figure 1. Anti-FH mAbs inhibit FH function on RBCs. (A) Sheep RBCs were incubated with 10% (v/v) NHS with a titration of the indicated anti-FH mAbs. Hemolysis was determined by measuring the supernatants at 412nm and expressed as percentage of the 100% lysis control. Bars represent the mean with error bars, indicating the SD of 2 independent experiments. Dashed line indicates hemolysis after incubation with NHS without addition of a mAb, also indicated by the white bar. **(B) C**3 deposition on human RBCs was determined by flow cytometry after incubation with 25% (v/v) NHS, containing ~0.5 μM FH, with the addition of a titration of the indicated mAb. Representative results o**f** RBCs from one experiment with the addition of a 2-times molar excess of the indicated mAbs are shown. Grey: RBCs without serum, black lines: RBCs incubated with serum. **(C)** Results from the flow cytometry analysis of C3 deposition on human RBCs as performed in B. Bars represent the mean percentage of C3 positive human RBCs from 3 individual healthy donors with error bars indicating the SD. Dashed line indicates the level of C3 deposition after incubation with NHS without addition of a mAb, also indicated by the white bar. Two-way ANOVA with Dunnett's multiple comparison post-test were performed, comparing to NHS with no mAb. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

effectively interact with and fully protect the RBC cell surface. Remarkably, only a sub-population of human RBCs seemed to be sensitive for C3 deposition when FH was blocked. This was evident in multiple experiments using different donors and was not correlating with the expression of CR1, DAF or CD59 (data not shown).
In conclusion, our results with epitope-mapped mAbs against human FH demonstrate a crucial role for the binding region in domains 6-8 of FH, next to the well-established role for domains 19-20. Simultaneous binding via both binding regions is required for FH to protect human RBCs against complement activation.

Acknowledgements
R.B.P. and T.W.K. receive funding from the European Union's seventh Framework program under EC-GA no. 279185 (EUCLIDS; www.euclidsproject.eu). The rest of this work was supported by an internal grant of Sanquin (PPOC-12-038-PRG).

Conflict-of-interest disclosure
R.B.P., M.C.B., T.W.K., and D.W. are inventors of a patent application (PCT/NL2015/050584) describing the therapeutic use of potentiating anti-FH antibodies. C.Q.S is an inventor of a patent application that describes the use of mini-FH for therapeutic applications. A.J.F.T declares no competing financial interests.

REFERENCES

1. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11(9):785-797.
2. Baines AC, Brodsky RA. Complementopathies. Blood Rev. 2017;31(4):213-223.
3. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nat Rev Immunol. 2009;9(10):729-740.
4. Mastellos DC, Ricklin D, Yancopoulou D, Risitano A, Lambris JD. Complement in paroxysmal nocturnal hemoglobinuria: exploiting our current knowledge to improve the treatment landscape. Expert Rev Hematol. 2014;7(5):583-598.
5. Devalet B, Mullier F, Chatelain B, Dogne JM, Chatelain C. Pathophysiology, diagnosis, and treatment of paroxysmal nocturnal hemoglobinuria: a review. Eur J Haematol. 2015;95(3):190-198.
6. Wilcox LA, Ezzell JL, Bernshaw NJ, Parker CJ. Molecular basis of the enhanced susceptibility of the erythrocytes of paroxysmal nocturnal hemoglobinuria to hemolysis in acidified serum. Blood. 1991;78(3):820-829.
7. Lin Z, Schmidt CQ, Koutsogiannaki S, et al. Complement C3dg-mediated erythrophagocytosis: implications for paroxysmal nocturnal hemoglobinuria. Blood. 2015;126(7):891-894.
8. Ferreira VP, Pangburn MK. Factor H mediated cell surface protection from complement is critical for the survival of PNH erythrocytes. Blood. 2007;110(6):2190-2192.
9. Gordon DL, Kaufman RM, Blackmore TK, Kwong J, Lublin DM. Identification of complement regulatory domains in human factor H. J Immunol. 1995;155(1):348-356.
10. Kuhn S, Skerka C, Zipfel PF. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H1. J Immunol. 1995;155(12):5663-5670.
11. Blackmore TK, Sadlon TA, Ward HM, Lublin DM, Gordon DL. Identification of a heparin binding domain in the seventh short consensus repeat of complement factor H. J Immunol. 1996;157(12):5422-5427.
12. Blackmore TK, Hellwage J, Sadlon TA, et al. Identification of the second heparin-binding domain in human complement factor H. J Immunol. 1998;160(7):3342-3348.
13. Jokiranta TS, Hellwage J, Koistinen V, Zipfel PF, Meri S. Each of the three binding sites on complement factor H interacts with a distinct site on C3b. J Biol Chem. 2000;275(36):27657-27662.
14. Jokiranta TS, Cheng ZZ, Seeberger H, et al. Binding of complement factor H to endothelial cells is mediated by the carboxy-terminal glycosaminoglycan binding site. Am J Pathol. 2005;167(4):1173-1181.
15. Jozsi M, Oppermann M, Lambris JD, Zipfel PF. The C-terminus of complement factor H is essential for host cell protection. Mol Immunol. 2007;44(10):2697-2706.
16. Prosser BE, Johnson S, Roversi P, et al. Structural basis for complement factor

H linked age-related macular degeneration. J Exp Med. 2007;204(10):2277-2283.
17. Schmidt CQ, Herbert AP, Kavanagh D, et al. A new map of glycosaminoglycan and C3b binding sites on factor H. J Immunol. 2008;181(4):2610-2619.
18. Morgan HP, Schmidt CQ, Guariento M, et al. Structural basis for engagement by complement factor H of C3b on a self surface. Nat Struct Mol Biol. 2011;18(4):463-470.
19. Hyvarinen S, Meri S, Jokiranta TS. Disturbed sialic acid recognition on endothelial cells and platelets in complement attack causes atypical hemolytic uremic syndrome. Blood. 2016;127(22):2701-2710.
20. de Cordoba SR, Tortajada A, Harris CL, Morgan BP. Complement dysregulation and disease: from genes and proteins to diagnostics and drugs. Immunobiology. 2012;217(11):1034-1046.
21. Sharma AK, Pangburn MK. Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. Proc Natl Acad Sci U S A. 1996;93(20):10996-11001.
22. Pouw RB, Brouwer MC, Geissler J, et al. Complement Factor H-Related Protein 3 Serum Levels Are Low Compared to Factor H and Mainly Determined by Gene Copy Number Variation in CFHR3. PLoS One. 2016;11(3):e0152164.
23. Pechtl IC, Kavanagh D, McIntosh N, Harris CL, Barlow PN. Disease-associated N-terminal complement factor H mutations perturb cofactor and decay-accelerating activities. J Biol Chem. 2011;286(13):11082-11090.
24. Hebecker M, Jozsi M. Factor H-related protein 4 activates complement by serving as a platform for the assembly of alternative pathway C3 convertase via its interaction with C3b protein. J Biol Chem. 2012;287(23):19528-19536.
25. Sanchez-Corral P, Gonzalez-Rubio C, Rodriguez de Cordoba S, Lopez-Trascasa M. Functional analysis in serum from atypical Hemolytic Uremic Syndrome patients reveals impaired protection of host cells associated with mutations in factor H. Mol Immunol. 2004;41(1):81-84.
26. Meulenbroek EM, de Haas M, Brouwer C, Folman C, Zeerleder SS, Wouters D. Complement deposition in autoimmune hemolytic anemia is a footprint for difficult-to-detect IgM autoantibodies. Haematologica. 2015;100(11):1407-1414.