Anti-factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome

Mihály Józsi[1], Stefanie Strobel[1], Hans-Martin Dahse[2], Wei-shih Liu[3], Peter F. Hoyer[3], Martin Oppermann[4], Christine Skerka[2], and Peter F. Zipfel[2,5]

From the [1] Junior Research Group Cellular Immunobiology and [2] Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute, Jena, Germany; [3] Department of Pediatric Nephrology, University Clinic Essen, University Duisburg-Essen, Germany; [4] Department of Cellular and Molecular Immunology, University of Göttingen, Göttingen, Germany; and [5] Friedrich Schiller University, Jena, Germany

Corresponding author:

Dr. Mihály Józsi

Junior Research Group Cellular Immunobiology, Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute, Beutenbergstr. 11a, D-07745 Jena, Germany;

Phone: +49 3641 656878; Fax: +49 3641 656879; e-mail: Mihaly.Jozsi@hki-jena.de.

Running title: Characterization of factor H autoantibody in HUS

Scientific heading: Hemostasis, Thrombosis, and Vascular Biology

Abstract

The atypical form of the kidney disease hemolytic uremic syndrome (aHUS) is associated with defective complement regulation. In addition to mutations in complement regulators, factor H (FH)-specific autoantibodies have been reported for aHUS patients. The aim of the present study was to understand the role of these autoantibodies in aHUS. First, the binding sites of FH-autoantibodies from five unrelated aHUS patients were mapped using recombinant FH fragments and competitor antibodies. For all five autoantibodies the binding site was localized to the FH C-terminus. In a functional assay, isolated patient IgG inhibited FH binding to C3b. In addition, autoantibody positive patient's plasma caused enhanced hemolysis of sheep erythrocytes, which was reversed by adding FH in excess. These results suggest that aHUS-associated FH-autoantibodies mimic the effect of C-terminal FH mutations, as they inhibit the regulatory function of FH at cell surfaces by blocking its C-terminal recognition region.

Introduction

Hemolytic uremic syndrome (HUS) is characterized by hemolytic anemia, thrombocytopenia and acute renal failure. HUS is most often caused by bacterial infection and is associated with diarrhea (D+ HUS), or arises rarely as atypical HUS (aHUS).[1] Mutations identified in complement genes in aHUS patients indicate that complement dysregulation is involved in disease development.[2] In addition, factor H (FH)-autoantibodies have been reported for three aHUS-patients.[3]

FH is a major regulator of the alternative complement pathway. FH is composed of 20 short consensus repeats (SCRs). The four N-terminal domains (SCR1-4) mediate cofactor and

decay accelerating activity, and the C-terminal domains (SCR19-20) form a recognition region, which contains binding sites for C3b, glycosaminoglycans and endothelial cells.[4-6] SCR19-20 represent a hot spot for aHUS-associated FH mutations,[2] several of which have been shown to impair FH binding to C3b and to cellular surfaces.[7-9] Thus suggesting that by affecting the C-terminal domains aHUS-associated mutations reduce FH regulatory activity on cells. Indeed, reduced FH binding leads to enhanced complement activation on host cells and results in cell lysis as shown by analysis of a mutant FH protein lacking most of SCR20,[10] by using blocking monoclonal antibodies (mAbs),[11] or recombinant SCR19-20 fragment.[12]

In contrast to the FH mutations, aHUS-associated FH-autoantibodies have not yet been analyzed in detail. In the present report we describe how five FH-autoantibodies affect FH function.

Materials and Methods

Identification and domain mapping of FH-autoantibodies

Patients' plasma was analyzed for FH-autoantibodies by ELISA.[3] For domain mapping, microtiter plates were coated with recombinant FH fragments at 5 µg/ml.[7,11] For inhibition experiments, immobilized FH was preincubated with FH-specific mAbs.[6,11]

C3b binding assay

IgG was isolated from plasma according to standard protocols. Microtiter plate wells were coated with 5 µg/ml FH (Merck Biosciences, Schwalbach, Germany). After blocking, the wells were preincubated with purified IgG, then C3b (2 µg/ml) was added and binding was measured using C3-specific antiserum (Merck Biosciences).

Hemolysis assay

Hemolysis assays were performed in 100 µl buffer (20 mM HEPES, 7 mM $MgCl_2$, 10 mM EGTA, 144 mM NaCl, 1% BSA, pH 7.4) containing 5×10^6 sheep erythrocytes (SRBC, BioTrend Chemikalien GmbH, Cologne, Germany) and 10-40% plasma. After incubation at 37°C for 30 min, optical density of the supernatants was measured at 414 nm.

Results and Discussion

FH-autoantibodies of aHUS patients bind to the C-terminus of FH

Plasma samples from 60 patients with hemolytic uremic syndrome (51 with aHUS and 9 with D+ HUS) were screened for the presence of FH-autoantibodies. Five positive cases, children diagnosed with aHUS at 4-12 years of age, were identified using an ELISA assay. This corresponds to ~10% of the patients, which is similar to the ~6% reported recently.[3] FH specificity of the autoantibodies was verified by demonstrating dose-dependent binding to FH, reduced binding after IgG depletion, and by analyzing FH-specific IgG isotypes (Supplementary Figure 1).

In order to localize the binding domain of the FH-autoantibodies, their reactivity with recombinant FH fragments was measured. All five autoantibodies bound to FH fragments that include SCR20, i.e. SCR15-20 and SCR19-20 (Figure 1A). No specific binding of the autoantibodies to the N-terminal or middle region of FH was observed. A weak binding of plasma #503 to SCR8-11 was also detected, which was not further analyzed. To confirm the autoantibody binding site, a competition assay using domain mapped mAbs was performed.[6,11] In all five cases, binding of the autoantibody to FH was reduced by mAbs which bind to the FH C-terminus, namely mAbs C02 (binding in SCR19), C14 and C18 (both binding within SCR20). By contrast, mAbs that recognize the N-terminus (mAb N11) or the

middle region of FH (mAbs M12, M13, M15) did not affect autoantibody binding (Figure 1B). Moreover, binding was competed with FH SCR15-20 fragment (Supplementary Figure 2).

FH-autoantibody inhibits C-terminus mediated function of FH

Reduced C3b binding of FH and impaired C3b processing on the cell surface, which results in enhanced complement activation and cell damage, is caused by the mAbs C18 and C14,[11] which share binding epitopes with the autoantibodies of all five analyzed aHUS patients (Figure 1C). Therefore we measured whether FH-autoantibody has the same effect as mAbs C18 and C14.

To this end, the IgG fraction was isolated from plasma of three patients. Purified patient derived IgG showed the same reactivity with FH as the whole plasma (Supplementary Figure 3). C3b binding was strongly reduced when FH was preincubated with patient derived IgG, which contained FH-autoantibodies, whereas control IgG had no effect (Figure 2A). A previous report found no effect of the autoantibody on FH binding to C3b, which is likely due to the different experimental conditions.[3]

Sera derived from aHUS patients with heterozygous C-terminal FH mutations cause SRBC lysis.[13] When performing a similar assay, incubation with the autoantibody-positive plasma of patient #564 resulted in enhanced hemolysis of SRBC (Figure 2B). Furthermore, the hemolytic activity was reversed in a dose-dependent manner by addition of excess FH to the patient's plasma, and thus increasing the concentration of autoantibody-free FH (Figure 2C). This effect of the autoantibody is in line with the observation, that the C-terminally binding mAbs C18 and C14 cause enhanced SRBC lysis when added to normal human plasma (data not shown).

That the autoantibodies of all five patients do not bind to the N-terminus of FH, where the complement regulatory domains are localized, is in agreement with a previous report, and explains the normal FH activity in fluid phase in the presence of autoantibody (data not shown).[3]

The presence of C-terminally binding FH-autoantibodies, which influence FH activity, has relevance for diagnosis and treatment of aHUS patients. FH-autoantibody titer can be decreased by plasma exchanges,[3] which in the case of patient #564 were accompanied with improvement of clinical parameters (data not shown). However, for the autoantibody positive patients kidney transplant is likely at high risk, as the autoreactive antibodies will remain in plasma.

Domain mapping and functional analysis of additional aHUS-associated FH-autoantibodies are required to assess whether the mechanism suggested here applies in general. Other autoantibodies may have different binding site and affect e.g. FH stability or conformation. However, it is striking that all five FH-autoantibodies identified in the present study bind predominantly to the FH C-terminus. Thus, in aHUS FH dysfunction of autoimmune origin, i.e. caused by autoantibodies, seems analogous to the C-terminal mutations, and both scenarios result in an impaired recognition of host cells by FH during complement attack.

Acknowledgments

We thank Gerlinde Heckrodt, Ina Löschmann and Eva-Maria Neumann for technical assistance. Supported by grants from the Deutsche Forschungsgemeinschaft and the Kidneeds Foundation, Cedar Rapids, US. M.J. designed and performed research, interpreted data and wrote the paper; S.S. and H-M.D. performed research; P.F.H. and W-s.L. collected biological samples and developed clinical concept; M.O. contributed vital reagents; C.S. interpreted data; P.F.Z. designed research and wrote the paper. The authors declare no competing financial interests.

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Figure legends

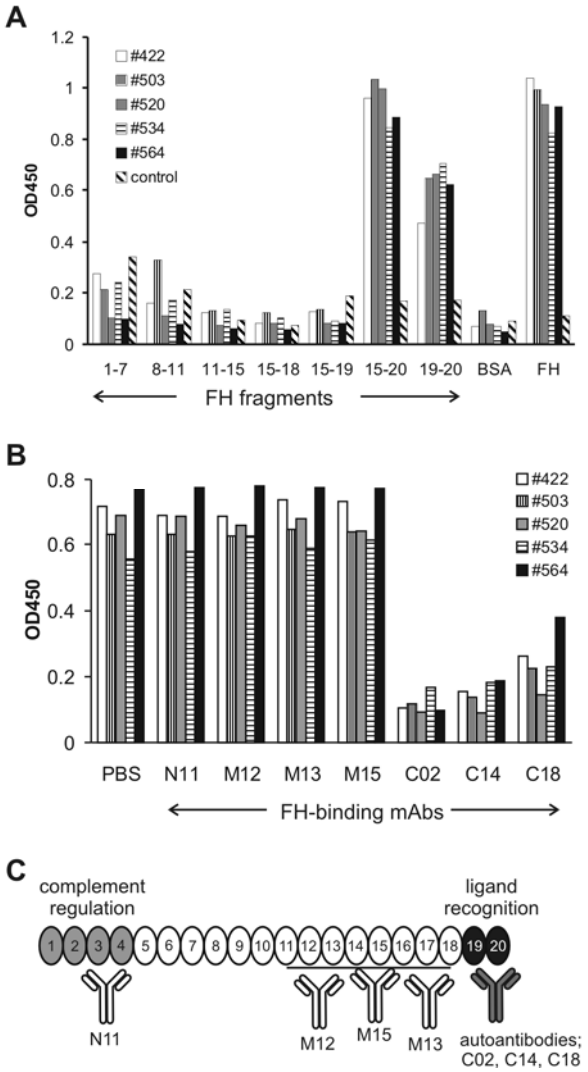
Figure 1. Autoantibodies bind to the C-terminus of FH.

(A) Reactivity of autoantibodies of five aHUS patients was tested with recombinant FH fragments, covering all domains of FH. BSA was used as negative control and FH as positive control. Normal human serum was included as additional control. Data are representative of three experiments with similar results. (B) Autoantibody binding to FH was blocked with mAbs (25 µg/ml) specific to the C-terminal domains SCR19-20 of FH, i.e. C02, C14 and C18, whereas mAbs N11, M12, M13 and M15, which bind in the N-terminal and middle regions of FH, did not inhibit autoantibody binding. Representative data of three experiments are shown. (C) Binding domains of the identified FH-autoantibodies and the mAbs used in this study. Important functional domains of FH are highlighted.

Figure 2. FH-autoantibody blocks C-terminal recognition function of FH.

(A) Patient derived IgG, added in the indicated concentrations, reduced binding of human C3b to immobilized FH in an ELISA assay, when compared with control IgG. Data represent mean ± SD from three experiments. Difference between samples was analyzed by Student's *t* test. *, $p<0.05$. (B) Plasma of patient #564 (filled circles) caused dose-dependent lysis of sheep erythrocytes, whereas normal human plasma (open circles) showed no effect. Hemoglobin release was measured as described in Methods. Mean ± SD of data from five measurements is shown. (C) Addition of excess FH rescued sheep erythrocytes from complement mediated lysis. Cells were incubated in 40% #564 plasma without FH added and in the presence of the indicated amounts of purified FH, and hemoglobin release was measured as above. Erythrocyte lysis in the absence of FH was set to 100%. Mean ± SD of data from four independent experiments is shown.

Figure 1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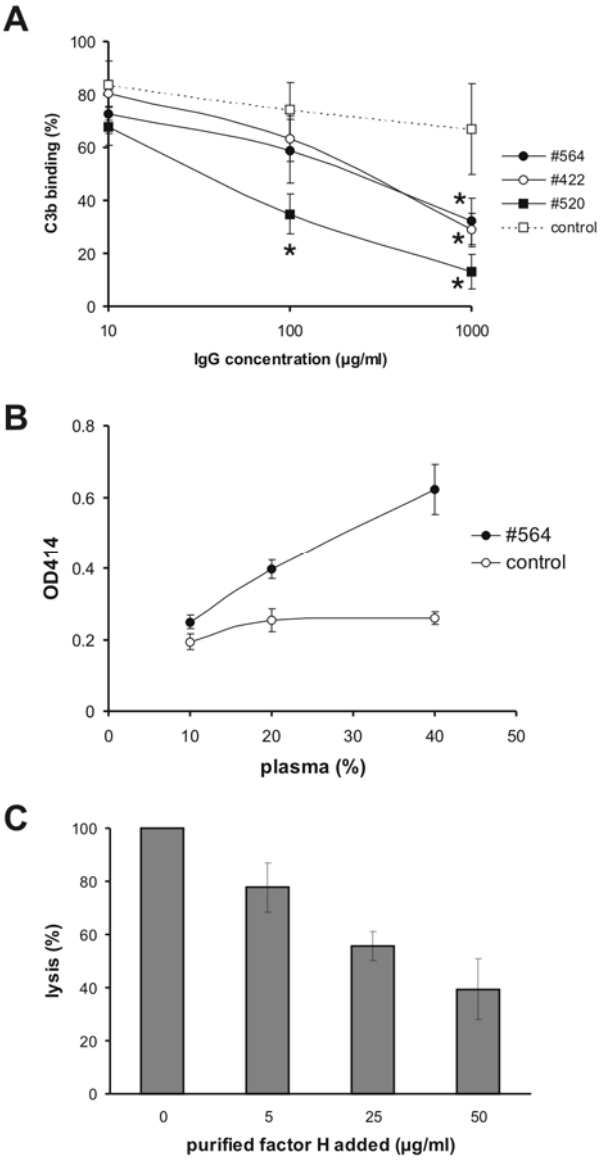
autoantibodies; C02, C14, C18

Figure 2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B
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Legends to Supplementary Figures

Supplementary Figure 1. Identification of autoantibody positive patients.

(A) Plasma samples of aHUS (n = 51) and D+ HUS (n = 9) patients as well as healthy donors (n = 30) were analyzed for binding on immobilized purified FH in an ELISA assay. The samples having an OD value above the mean + 2 SD of those in the control group were considered positive. (B) Dose-dependent binding of the autoantibody positive plasma samples of five aHUS patients to immobilized FH, indicating different autoantibody titers. (C) Adsorption of IgG from the patients' plasma by incubation with Protein G beads removed FH autoreactivity. (D) Isotypes of FH-specific IgG in the plasma samples were determined using mouse antibodies specific to human IgG1, IgG2, IgG3 and IgG4.

Supplementary Figure 2. Competition assays show C-terminal specificity for autoantibody binding.

C-terminally binding mAbs, i.e. C02, C14 and C18 (dotted lines), but not mAbs binding outside SCR19-20 (continuous lines), exhibited dose-dependent inhibitory activity on autoantibody binding to FH as shown for plasma of patients #564 (A) and #520 (B). (C) The FH SCR15-20 fragment (dotted lines), but not the SCR15-18 fragment (continuous lines), inhibited autoantibody binding to FH in a dose-dependent manner as shown for three patients.

Supplementary Figure 3. Characterization of the IgG fraction purified from patient's plasma

(A) Purified IgG of patient #564 bound specifically to the FH fragments SCR15-20 and SCR19-20, i.e. those containing the most C-terminal domains (filled bars). Control IgG purified from a healthy donor showed no specific binding (open bars). (B) Dose-dependent binding of purified IgG from patients #422, #520 and #564 to immobilized FH.

Supplementary Figure 1
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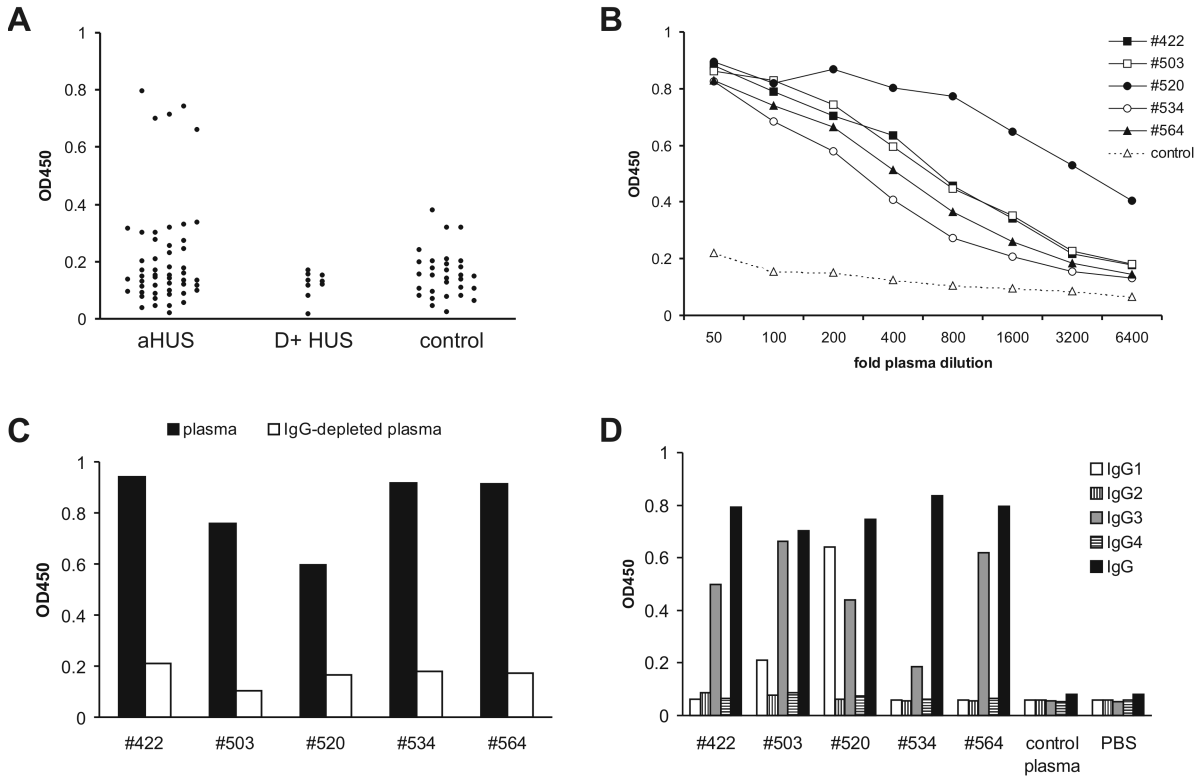
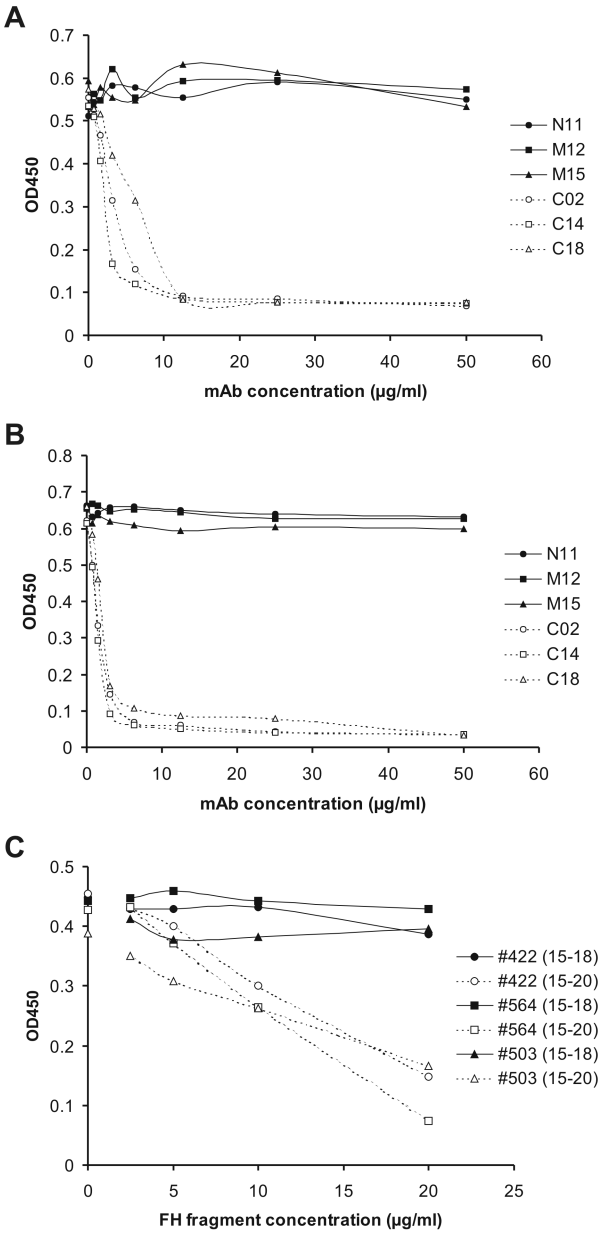


Supplementary Figure 2
Józsi et al., 2007



Supplementary Figure 3
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