Hemolytic Uremic Syndrome: A Factor H Mutation (E1172Stop) Causes Defective Complement Control at the Surface of Endothelial Cells

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Defective complement regulation results in hemolytic uremic syndrome (HUS), a disease that is characterized by microangiopathy, thrombocytopenia, and acute renal failure and that causes endothelial cell damage. For characterization of how defective complement regulation relates to the pathophysiology, the role of the complement regulator factor H and also of a mutant factor H protein was studied on the surface of human umbilical vein endothelial cells. The mutant 145-kD factor H protein was purified to homogeneity, from plasma of a patient with HUS, who is heterozygous for a factor H gene mutation G3587T, which introduces a stop codon at position 1172. Functional analyses show that the lack of the most C-terminal domain short consensus repeats 20 severely affected recognition functions (i.e., binding to heparin, C3b, C3d, and the surface of endothelial cells). Wild-type factor H as well as the mutant protein formed dimers in solution as shown by cross-linking studies and mass spectroscopy. When assayed in fluid phase, the complement regulatory activity of the mutant protein was normal and comparable to wild-type factor H. However, on the surface of endothelial cells, the mutant factor H protein showed severely reduced regulatory activities and lacked protective functions. Similarly, with the use of sheep erythrocytes, the mutant protein lacked the protective activity and caused increased hemolysis when it was added to factor H–depleted plasma. This study shows how a mutation that affects the C-terminal region of the factor H protein leads to defective complement control on cell surfaces and damage to endothelial cells in patients with HUS. These effects explain how mutant factor H causes defective complement control and in HUS—particularly under condition of inflammation and complement activation—causes endothelial cell damage.

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gous; therefore, patients have one intact and one defective allele (for review, see reference [22]). The C-terminus of factor H represents a hotspot for mutations. A total of 70% of all mutations are located in domains 19 and 20 of the factor H gene and represent either premature stop codons or single amino acid substitutions. The C-terminus includes the recognition region and has binding sites for ligands such as heparin, C3b, and C3d and to the surface of endothelial cells.

Given the association of factor H gene mutations in HUS, it is of interest to elucidate how C-terminal recognition domain relates to the pathomechanism of HUS and endothelial cell damage. Here, we show for the first time, to our knowledge, that factor H mediates its protective, inhibitory activity at the surface of endothelial cells. In addition, we show that a mutant factor H protein, which has the C-terminal domain SCR20 deleted and was purified from serum of a patient with HUS, displays defective recognition and regulatory functions at the surface of endothelial cells and sheep erythrocytes but shows normal regulatory activity in the fluid phase (i.e., plasma). Therefore, modifications of the C-terminal recognition regions of factor H impair the regulatory functions on the surface of endothelial cells.

Materials and Methods

The Patient

The patient R043 is heterozygous for a mutation in the factor H gene nucleotide position G3587T within domain 20, introducing a stop codon at position 1172. The patient tested negative for C3 nephritic factor. All experiments were conducted with the patient’s informed consent. Hemolysis and renal function ameliorated after an intense course of plasma exchanges; however, in the subsequent 2 yr, the patient manifested three HUS recurrences. A renal biopsy that was performed in February 1997 showed irreversible changes of chronic nephropathy with severe thrombotic microangiopathy. Renal function rapidly worsened, and in 1998, the patient started hemodialysis. In 1999, he received a cadaveric kidney transplant, but the graft failed within 1 mo because of disease recurrence. The factor H concentration in the plasma of this patient was elevated (908 μg/ml; normal range 350 to 800 μg/ml), and C3 plasma levels were reduced (0.51 mg/ml; normal range 0.81 to 1.70 mg/ml).

Purification of Mutant Factor H

Approximately 10 ml of serum from patient R043 was treated with 13% polyethylene glycol (molecular weight 6000 Da) on ice. The pellet was dissolved in 1 ml of buffer A (10 mmol/L sodium phosphate and 100 mmol/L glycine [pH 7.3]) and applied to heparin affinity chromatography (HiTrap Heparin column; GE Health Care, Freiburg, Germany). After extensive washing with buffer A, proteins were eluted with a linear salt gradient (30 to 500 mmol/L NaCl). Factor H–containing fractions were combined, dialyzed with deionized water, and subjected to ion exchange chromatography (Resource Q column; GE Health Care) using buffer A. Again, bound proteins were eluted with a linear salt gradient (range 30 to 600 mmol/L NaCl), and factor H–containing fractions were combined and concentrated. The factor H concentration was determined by ELISA (23).

Surface Plasmon Resonance Binding

Protein–protein interactions were analyzed by surface plasmon resonance using a BIACore 3000 system (BIACore AB, Uppsala, Sweden) as described previously (24).

Cultivation of Endothelial Cells and Binding Experiments

Human umbilical vein endothelial cells (HUVEC; American Type Culture Collection, Rockville, MD) express the membrane-bound regulators MCP (CD46), decay-accelerating factor (DAF) (CD55), and CD99 but not complement receptor 1 (CR1) (CD35) and were cultured as described previously (24). Binding experiments with flow cytometry and confocal microscopy were performed as described previously (25).

Co-Factor Activity

Co-factor assays for fluid phase activity were performed as described previously (26). For determination of co-factor activity on the surface, HUVEC were cultivated in serum-free medium, washed thoroughly, and detached from the surface. Cells were incubated with purified truncated mutant factor H (factor H20Δ) or wild-type factor H (factor Hwt;2 2 μg) for 1 h at 37°C under gentle agitation. Then cells were washed three times with cold Dulbecco’s phosphate-buffered saline and incubated at 37°C in the presence of 120 ng of C3b (Merck Biosciences, Schwalbach, Germany) and 240 ng of factor I (Merck Biosciences). At each time point, 2 × 10⁶ cells were sedimented, the supernatant was transferred to sample buffer and separated by SDS-PAGE, and C3b degradation fragments were detected by Western blotting.

Depletion of Factor H of Plasma

Factor H–depleted human plasma was prepared using affinity column as described previously (27). Successful depletion was determined by Western blotting, and complement activity was confirmed by assay of lysis of sheep erythrocytes.

Hemolysis Assay

Hemolytic experiments were conducted in the presence of veronal buffered saline (144 mmol/L NaCl, 7 mmol/L MgCl₂, and 10 mmol/L EGTA [pH 7.4]). Increasing amounts of purified factor Hwt or factor H20Δ were added to the depleted plasma together with 2 × 10⁷ sheep erythrocytes. The mixture was treated for 30 min at 37°C. After centrifugation, hemolysis was determined by measurement of the absorbance at 414 nm.

Cross-Linking

The homobifunctional cross-linker dimethyladipimidate-dihydrochloride (DMA) was used (Pierce Perbio Science, Bonn, Germany). Purified protein (1 μg) or human plasma (1 μl) was treated in cross-linking buffer (200 mmol/L triethanolamine [pH 9.0]) in the presence of 1 mmol/L DMA. The mixture was incubated at 25°C for 60 min in the dark. Reactions were stopped by addition of 10 μl of 1 mol/L Tris buffer (pH 9.0).

SDS-PAGE and Western Blotting

Human serum, individual chromatography fractions, cell supernatants, and cell lysates were separated by 10% SDS-PAGE under nonreducing conditions (28).

Mass Spectroscopy

The PepClean C18 Spin columns kit (Pierce Perbio Science) was used to remove the contaminants from the PBS buffer in which the purified
proteins were stored. The samples were mixed 1:1 with saturated sinapinic acid (sinapinic acid in 0.1% [vol/vol], trifluoroacetic acid, and H2O [2:1]). Part of the sample (1 to 3 μl, depending on the protein concentration) was transferred onto the target (MTP384 ground steel; Bruker Daltonics, Bremen, Germany). Samples were let dry at room temperature (dried droplet procedure). The measurements were conducted with an Ultraflex TOF/TOF (Bruker Daltonics).

Results

Identification of a Mutated Factor H Protein in the Plasma of Patient R043

Patient R043 is heterozygous for a nonsense factor H gene mutation at nucleotide position G3587T. This mutation introduces in SCR20 at position 1172, a premature stop codon. Consequently, the mutant factor H20 protein has most of SCR20 deleted. Plasma of this patient was separated by SDS-PAGE and subjected to Western blot analysis. Two bands of 150 and 145 kD were identified, representing factor H and factor H20 (Figure 1A, top, lane 2). Both the 150- and 145-kD bands have similar intensities, indicating that the wild-type and mutant proteins are expressed in equal levels. Absence of the most C-terminal SCR domain in factor H20 was confirmed with an epitope-mapped mAb that binds within SCR20. This mAb D24 detected the 150-kD factor Hwt but not the mutant factor H20 protein (Figure 1A, bottom). These results show that the mutant protein is expressed, secreted, and present in plasma in a truncated form.

Purification of Factor H20 from Plasma

Heparin affinity chromatography was used to separate factor H20 and factor Hwt proteins. The mutant factor H20 eluted in one peak, at a NaCl concentration of approximately 230 mM and factor Hwt in a second peak at approximately 300 mM NaCl (data not shown). The individual fractions were separated by SDS-PAGE and analyzed by Western blotting. The factor H20 protein was present in fractions 18 to 26, fraction 28 contained both proteins, and factions 30 to 36 contained the factor Hwt protein. Thus, factor H20 binds with reduced affinity to heparin.

Fractions that contained either factor H20 or factor Hwt protein were combined, and the protein was purified to homogeneity. Purified proteins were free of contamination bands as determined by SDS-PAGE in combination with Western blotting (Figure 1B, top) or revealed silver staining (Figure 1B, bottom). The 145-kD band did not react with the mAb D24, confirming that this band represented the purified mutant protein (Figure 1B, middle, lane 2).

Binding of Purified Mutant Factor H20 to C3b and C3d

Surface plasmon resonance was used to assay how the truncation of SCR20 affects binding to the central complement component C3b. C3b was coupled covalently to the surface of the sensor chip, and factor H20 or factor Hwt was added in equal amounts to the fluid phase. Factor H20 bound weakly to immobilized C3b, as indicated by the low and weak association and dissociation profile (Figure 2). Thus, truncation of the most C-terminal domain strongly reduced binding to C3b. In contrast, factor Hwt bound with high affinity to immobilized C3b, as indicated by the strong association and dissociation profile. Similar binding profiles were observed for C3d, the cleavage product of C3b, that is recognized by the C-terminal domain 20 of factor H. In contrast to factor Hwt, the mutant factor H20 did not bind to C3d (Figure 2). These results show that deletion of the C-terminal domain in the factor H20 protein severely affects binding to C3b and to C3d.

Binding of Purified Mutant Factor H20 to Endothelial Cells

Flow cytometry was used to assay binding of purified factor H20 or factor Hwt to HUVEC. Mutant factor H20 bound weakly to HUVEC (mean fluorescence 4.1), and factor Hwt was stronger (mean fluorescence 77.0; data not shown).
In a biochemical assay, show reduced cell binding of mutant factor H. Thus, three approaches, confocal microscopy, flow cytometry, and surface plasmon resonance, were used to analyze the binding of mutant factor H to the endothelial cell surface. Nuclei were stained with DAPI (blue color) and cell surfaces with Alexa 633–conjugated wheat germ agglutinin (red color). Upon merging of the channels, the fluorescence of factor H was visualized by confocal microscopy. Binding of either mutant factor H$_{A20}$ or factor H$_{wt}$ was detected with an antiserum that resulted in green fluorescence. Factor H$_{A20}$ bound weakly and factor H$_{wt}$ strongly to the endothelial cell surface. Nuclei were stained with DAPI (blue color) and cell surfaces with Alexa 633–conjugated wheat germ agglutinin (red color). Upon merging of the channels, the yellow signal reveals surface localization of factor H (Figure 3A). Thus, three approaches, confocal microscopy, flow cytometry, and a biochemical assay, show reduced cell binding of mutant factor H$_{A20}$ and strong binding of factor H$_{wt}$. This identifies SCR20 as the major cell attachment domain of factor H.

### Assaying Cell Surface–Bound Proteins by Western Blotting

Binding of factor H$_{A20}$ or factor H$_{wt}$ to endothelial cell surface was examined further with a biochemical method. After incubation with purified proteins, cells were lysed and surface-attached proteins were assayed by Western blotting after SDS-PAGE separation. Reduced binding of the mutant factor H$_{A20}$ to the cell surface as compared with factor H$_{wt}$ is indicated by the different intensities of the bands (Figure 3B, lanes 2 and 3). Cells that were treated in medium alone showed no surface-attached factor H (Figure 3B, lane 1).

### Binding of Serum-Derived Factor H of Patient R043 to Endothelial Cells

Patient R043 is heterozygous for the E1172Stop mutation; therefore, in plasma, both mutant factor H$_{A20}$ and factor H$_{wt}$ are present (Figure 1A, top, lane 2). When serum of patient R043 and that of a healthy control subject were used for cell binding, a dosage-dependent binding of factor H to HUVEC was observed. In the patient’s serum, binding was weaker than with the control serum. A direct comparison of the median fluorescence confirmed the reduced binding of the patient’s plasma (Figure 3C).

### Dimerization of Factor H

The reduced binding profile that was observed with plasma of the heterozygous patient suggested an interaction of mutant factor H$_{A20}$ and factor H$_{wt}$. Therefore, dimer formation was tested by chemical cross-linking. First, purified factor H$_{wt}$ was treated with the cross-linker DMA and analyzed by SDS-PAGE together with Western blotting. This approach identified two prominent bands of 150 and 300 kD, representing the monomeric form and a dimeric complex (Figure 4, lane 2). Upon treatment of human plasma, the same monomeric and dimeric complexes were identified (Figure 4, lanes 4 and 5). However, when plasma of patient R043 was treated, the pattern was more complex. In addition to the monomeric 145- and 150-kD bands, representing mutant factor H$_{A20}$ and factor H$_{wt}$, additional bands of approximately 290 and 300 kD were detected (Figure 4, lanes 7 and 8). This pattern suggests dimer formation of both the mutant factor H$_{A20}$ and factor H$_{wt}$ proteins. On the basis of the intensity of the 145-kD mutant and the 150-kD wild-type bands, it seems that the wild-type protein forms dimers more easily. Matrix-assisted laser desorption ionization–time of flight analysis yielded for the purified factor H$_{wt}$ and factor H$_{A20}$ monomer masses of 151,126.617 and 145,116.287 Da. The masses of the dimeric forms of factor H$_{wt}$ and factor H$_{A20}$ were 301,065.401 and 290,979.657 Da, respectively.

### Co-Factor Activity of factor H$_{A20}$ and factor H$_{wt}$

To assay how the factor H mutation relates to disease, we compared the regulatory activity of the factor H$_{A20}$ and factor H$_{wt}$ proteins. In fluid phase, mutant factor H$_{A20}$ and factor H$_{wt}$ showed comparable co-factor activity as evidenced by the appearance and the intensity of the α’46- and α’43-kD bands of C3b (Figure 5A, factor H$_{wt}$ versus factor H$_{A20}$). C3b cleavage products were detected already after 20 s, and their intensity increased with time. Thus, in fluid phase, the mutant factor H$_{A20}$ and factor H$_{wt}$ proteins have comparable regulatory activities, and SCR20 is dispensable for this activity.

### Co-Factor Activity on the Cell Surface

HUS is associated with endothelial cell damage. To link defective cell binding with surface control, we analyzed co-factor activity of factor H directly on the cell surface.

The endothelial cells that were used here express MCP (CD46), DAF (CD55), and CD59 but not CR1 (CD35; data not shown). First, the endogenous co-factor activity of the integral membrane complement regulators was assayed. Endothelial cells were incubated with C3b and factor I, and C3b degradation was followed upon separation of the cell lysate by SDS-PAGE in combination with Western blotting. Degradation products in the form of α’68-, α’46-, and α’43-kD fragments of the C3b α’ chain were detectable after 45 min, and their intensity increased with time (Figure 5B, top). Therefore, the endog-
enous, integral membrane regulators MCP and DAF control C3 activation at the surface of HUVEC.

To demonstrate a role of surface-attached factor H, we incubated HUVEC with factor Hwt. In the presence of factor Hwt, degradation products were detected earlier, already after 2.5 min, and after 120 min, their intensity was prominent, thereby demonstrating that factor H displays co-factor activity on the cell surface and that a combination of endogenous and attached complement regulators controls C3b on the cell surface. On the basis of the intensities of the cleavage products of factor H–treated versus untreated cells, factor Hwt enhances the regulatory activity approximately 10-fold. Therefore, surface-attached factor H contributes to C3b inactivation and mediates complement control (Figure 5B, middle).

Having demonstrated a regulatory role of factor H on the surface, we assayed the mutant protein. Mutant factor H 20 protein showed a reduced regulatory activity; cleavage products were identified after 35 and 45 min (Figure 5B, bottom). The protective activity of the mutant protein was lower than that of the wild-type protein. A densitometric analysis of the α46-kD band showed that the activity of the mutant protein is approximately one third that of the wild-type protein.

Hemolysis of Sheep Erythrocytes

In addition, a hemolysis assay with factor H–depleted plasma was used to analyze how the E1172Stop mutation affects the protective function of the protein. Sheep erythrocytes were incubated in factor H–depleted human plasma, and increasing amounts of either purified factor Hwt or factor H 20 were added. Factor Hwt has a protective effect, as demonstrated by the concentration-dependent decrease of hemolysis (Figure 6). In contrast, addition of the same concentrations of factor H 20 resulted in an increase in lysis; no protective effect was observed (Figure 6). Therefore, the loss of SCR20 in mutant factor H 20 results in a reduction of the protective activity on sheep erythrocytes from complement-mediated lysis.

Discussion

Factor H gene mutations are associated with HUS; however, it is unclear how exactly such mutations cause endothelial cell
damage. Here, we demonstrate for a mutant factor H protein that was derived from a patient with HUS severely reduced regulatory activity on the cell surface. The mutant factor H protein was purified from plasma of a heterozygous patient with HUS, with a G3587T exchange, that introduces a premature stop codon at position 1172. The mutant 145-kD factor H mutant protein shows defective binding to heparin, to C3b, to C3d, and to the surface of endothelial cells. The complement regulatory activity of the mutant protein was comparable to factor Hwt in fluid phase but was strongly reduced on the cell surfaces (HUVEC and sheep erythrocytes). These results demonstrate a protective role of the plasma regulator factor H for complement control on the surface of endothelial cells and erythrocytes.

Factor H is the central fluid-phase complement regulator and, as shown here, also regulates complement activation on the surface of endothelial cells and erythrocytes. The mutant factor H mutant protein shows defective recognition functions (reduced binding to C3b, C3d, heparin, and endothelial cells; Figures 2 and 3). Defective cell binding of factor H was observed both with purified mutant protein and with plasma of the heterozygous patient. In the patient’s plasma, which contains a mixture of mutant factor H mutant and factor Hwt proteins, factor H levels were increased (908 μg/ml factor H), and both proteins are present at very similar levels (Figure 1, top, lane 2). The reduced cell binding that was observed with patient plasma suggests an interaction of the mutant factor H mutant and factor Hwt proteins, which, however, seems to affect the function of intact factor Hwt (Figure 3C). Factor H dimerization adds a new facet to the pathomechanism of HUS in heterozygous patients.

With a cross-linking approach, dimer formation was demonstrated for both factor Hwt and factor H mutant. Factor H dimerization was reported earlier, and recently the C-terminus was suggested as the domain that mediates dimer formation (29,30). The 290-kD band that was observed in the patient’s plasma after cross-linking shows that the mutant protein, which lacks domain 20, forms dimers. Oligomer formation under native conditions was confirmed further by size exclusion chromatography and by mass spectrometry (data not shown).

When assayed in fluid phase, the complement regulatory activity of mutant factor H was normal and comparable to the wild-type protein. We recently proposed a conformational model for native factor H. In this model, factor H forms an omega-type structure and has the C-terminal binding sites accessible, which then form the first and initial contact with surface-bound C3b and heparin (31). This model explains the reduced C3b binding that was observed in surface plasmon resonance (Figure 2) and the reduced regulatory activity, as demonstrated by Figure 4B. Upon surface contact, the condensed factor H molecule unfolds and has the additional binding sites accessible. Apparently the recognition domain 20 of factor H, which makes the first and initial contact, includes a C3b- and heparin-binding site (32). In fluid phase, interaction of factor H with C3b seems to be different from the interaction with surface-bound C3b and is explained by the greater flexibility of the two proteins in solution or different conformations of C3b. In the hemolysis assay, factor Hwt but not factor H mutant showed a dosage-dependent, protective activity (Figure 6).

Endothelial cells that were used in this study bind plasma regulators to their surface and express endogenous membrane-bound complement regulators such as MCP, DAF, and CD59 but not CR1 (data not shown). The integral membrane regulators mediate complement control at the cell surface and inactivate C3b as evidenced by the appearance of the α’46 and α’43-kD fragments (Figure 5B, top). Here, we demonstrate that attached factor H contributes to the protective activity and cooperates together with the membrane-bound regulators. Under the experimental conditions used here, the regulatory activity of surface-attached factor H was pronounced and increased degradation of C3b approximately 10-fold. This finding is consistent with previous reports (31–34). However, the mutant factor H protein showed reduced regulatory activity. During a local inflammatory response, when complement is activated, endothelial cells require maximum protection. In this situation, a reduction of factor H attachment to cell surfaces and regulatory activity causes inefficient complement control and leads to cell damage. The defective complement control described here for a mutant factor H protein that lacks the recognition module, has implications for the other
C-terminal factor H mutations that frequently are observed in HUS. It is likely that all mutations in SCR19 and 20 show a defective recognition function.

Atypical HUS is associated with endothelial cell damage and defective complement control as evidenced by low C3 plasma levels and increased levels of C3b degradation products. Apparently, the alternative pathway amplification convertase C3bBb is essential for this reaction. Consequently, a defective regulation of this enzyme, by increasing either its stability or its activity, enhances complement activation and results in tissue damage and HUS. This explains why in HUS, mutations of several complement regulators occur (e.g., factor H, MCP [CD46], factor I) and why autoantibodies to factor H have the same effect (5–10). So far, more than 75 patients with factor H gene defects have been reported, and the vast majority (75%) of the mutations are clustered in the C-terminus (SCR19 and 20) and make the C-terminal recognition domain a hotspot for disease-associated mutations in HUS. Although the initial trigger of HUS that causes endothelial cell damage still is unclear, the pathomechanism of the disease—how endothelial cells are damaged as a result of improper control of the alternative pathway convertase C3bBb—becomes evident.

Figure 5. Co-factor activity of purified factor H_{wt} and mutant factor H_{Δ20}. (A) Fluid phase. (Top) Co-factor activity of purified factor H_{wt} was assayed in fluid phase. C3b was incubated with factor I, and factor H_{wt} was added. Aliquots were removed at the indicated time points, inactivated, separated by SDS-PAGE, and transferred to a membrane, and the appearance of C3 fragments was analyzed by Western blotting. Co-factor activity is evidenced by disappearance of the α' chain and the appearance of the cleavage products of α'68- and α'43-kD (arrows). (Bottom) Co-factor activity of purified mutant factor H_{Δ20} in fluid phase. (B) Co-factor activity on the surface of endothelial cells. (Top) Endogenous co-factor activity on HUVEC in the absence of factor H. Cells were incubated with C3b and factor I at 37°C and, at the indicated time points, inactivated, separated by SDS-PAGE, and transferred to a membrane, and the appearance of C3 fragments was analyzed by Western blotting. (Middle) Co-factor activity of purified factor H_{wt} on endothelial HUVEC. Cells were incubated with C3b, factor I, and purified factor H_{wt}, and analyzed in the same manner. (Bottom) Co-factor activity of purified mutant factor H_{Δ20} on the surface of endothelial cells. HUVEC were incubated with C3b and factor I, and mutant factor H_{Δ20} was added as a co-factor.

Figure 6. Lysis of sheep erythrocytes. Sheep erythrocytes were incubated with factor H–depleted human plasma. Increasing concentrations of purified factor H_{wt} or factor H_{Δ20} were added. Erythrocyte lysis was measured by determination of the absorbance of released hemoglobin at 414 nm.

C-terminal factor H mutations that frequently are observed in HUS. It is likely that all mutations in SCR19 and 20 show a defective recognition function.
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Disclosures

None.

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