Factor H-Mediated Cell Surface Protection from Complement is Critical for the Survival of PNH Erythrocytes

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Running Title: Factor H-mediated protection of PNH erythrocytes

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Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) cells are partially (type II) or completely (type III) deficient in GPI-linked complement regulatory proteins CD59 and CD55. PNH III erythrocytes circulate 6-60 days *in vivo*. Why these cells are not lysed as rapidly by complement as unprotected foreign cells, which normally lyse within minutes, remains undetermined. Factor H plays a key role in the homeostasis of complement in fluid-phase and on cell surfaces. We have recently shown that a recombinant protein encompassing the C-terminus of factor H (rH19-20) specifically blocks cell-surface complement regulatory functions of factor H without affecting fluid-phase control of complement. Here we show that PNH II and III cells become highly susceptible to complement-mediated lysis by non-acidified normal human serum *in vitro*, when the cell surface complement-regulatory functions of factor H are blocked. The results indicate that cells deficient in surface-bound regulators are protected for extended periods of time by factor H.
Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired stem cell disorder of clonal nature. PNH stem cells that have an acquired PIG-A mutation generate little to no glycosylphosphatidylinositol (GPI) resulting in partial (type II) or complete (type III) deficiency of GPI-linked membrane proteins including the complement regulatory molecules decay-accelerating factor (DAF; CD55) and CD59. The in vivo lifespan of PNH III erythrocytes is 6 days or more and that of PNH II erythrocytes can be close to that of normal erythrocytes (120 days). Unprotected foreign cells such as rabbit erythrocytes, which lack regulators of the human alternative pathway convertase as well as CD59, lyse in less than 5 minutes when exposed to normal human serum (NHS). Similarly unprotected PNH erythrocytes are not, as would be expected, rapidly lysed by complement.

Factor H (fH), a serum protein composed of 20 CCP domains, plays a key role in the homeostasis of the complement system on host cell surfaces and in plasma. It controls activation of the alternative pathway through its four N-terminal domains, limiting formation of C3b by acting as a cofactor for factor I in the inactivation of C3b and by accelerating the decay of alternative pathway C3/C5 convertase (C3b,Bb). The sites on CCP domains 19-20 are essential for fH-mediated interaction with host cells through the binding of both surface-bound polyanions and C3b, iC3b, or C3d. We have shown that a recombinant form of these C-terminal domains (rH19-20) competes with full length fH, inhibiting its binding to C3b and host polyanions on cells. This leads
to impaired fH complement-regulatory functions and increased complement activation on host surfaces, without affecting complement control in plasma.\textsuperscript{6}

Since PNH III cells survive longer than expected for cells that are devoid of GPI-linked membrane-bound regulatory proteins, we examined the contribution of fH to their extended half-life by specifically inhibiting fH-mediated cell surface protection with rH19-20.
Patients, materials, and methods

Human erythrocytes

Blood from 4 patients with PNH and 2 healthy adults was collected by venipuncture and the erythrocytes were frozen at -80°C by standard methods. All PNH patients had positive acidified serum tests for PNH. The University of Texas Health Science Center Institutional Review Board approved protocols, and written informed consent was obtained from all donors. It should be noted that all samples used in this study were collected prior to 1986 and have been stored frozen since then.

Proteins and buffers

C-terminal domains 19 and 20 of human fH (rH19-20) were cloned, expressed in yeast and purified as described. Human fH was purified from NHS. Buffers: VBS, 5 mM veronal, 145 mM NaCl, 0.02% NaN3, pH 7.3; GVB, VBS containing 0.1% gelatin; GVBE, GVB containing 10 mM EDTA (ethylenediaminetetraacetic acid); MgEGTA, 0.1 M MgCl2, 0.1 M EGTA (ethyleneglycoltetraacetic acid), pH 7.3.

Hemolytic assays

CD59 or CD55 were inhibited on normal human erythrocytes (E_H) with monoclonal antibodies (clone MEM43 or BRIC216, respectively; Chemicon, Temecula, CA), followed by incubation at 37°C with NHS, 5 mM MgEGTA and rH19-20. The percent lysis was determined as described. In a separate experiment, the extent of lysis of PNH and normal erythrocytes in the presence or absence of rH19-20, but without the CD59 or
CD55 inhibitory antibodies, was assessed as above. The remaining unlysed cells were analyzed by flow cytometry.

**Flow cytometry**

The CD59 profile of the PNH and normal erythrocytes before and after treatment with NHS, with or without rH19-20, was determined by incubating the cells with the anti-CD59 antibody, followed by fluorescein isothyocyanate-conjugated rabbit anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). The cells were analyzed in a FACScan (Becton Dickinson, San Jose, CA) using CellQuest Pro software. The acquired events were presented as populations with normal, intermediate, or complete CD59 deficiency (PNH I, PNH II, and PNH III, respectively). The percent lysis of PNH II+III cells was calculated as described.
Results and Discussion

To study the effect of inhibiting fH cell surface protection on cells with varying degrees of CD59 and CD55 deficiency, each regulator was inhibited individually on normal E$_H$ (Fig. 1A-B). A maximum of 23% lysis by NHS+MgEGTA was observed when CD59 alone was blocked (Fig. 1A). No lysis was detected when CD55 alone was blocked (Fig. 1B). Addition of rH19-20 to these reactions (Fig. 1A-B) at 14 µM, a concentration sufficient to inhibit 93% of fH surface activity, resulted in 82% lysis of E$_H$ when CD59 was blocked (Fig. 1A) and 68% lysis when CD55 was blocked (Fig. 1B). Inhibition of fH alone resulted in 19% lysis (zero input of antibody Figs. 1A and 1B). Thus, inhibiting fH-mediated cell surface protection functions renders normal cells partially susceptible to complement-mediated lysis in 20 min in 40% serum, while cells also lacking CD59 or DAF function become aggressively lysed. PNH erythrocytes survive many days in vivo whether they are partially or completely deficient in GPI-linked complement regulatory proteins. Our results suggest that fH provides a significant portion of the protection for normal erythrocytes and may be critical to the survival of PNH erythrocytes.

To test this hypothesis, PNH erythrocytes were treated with NHS+MgEGTA in the presence or absence of rH19-20. The remaining cells were analyzed for CD59 expression by flow cytometry (Fig. 1C). The PNH erythrocytes treated with unacidified NHS (Fig. 1C, Cells Post-NHS Treatment) showed CD59 levels similar to untreated cells and typical of normal, type II and type III PNH cells. It has been shown that PNH cells are minimally lysed by NHS+MgEGTA unless it is acidified to pH 6.5, which is the optimal pH for initiation and amplification of the alternative pathway. However, when PNH and normal erythrocytes were incubated with unacidified NHS+MgEGTA in the
presence of rH19-20 (Fig. 1C, Cells Post-NHS+rH19-20 Treatment), the remaining unlysed cells were mainly of the normal PNH I type. An average 86% of the PNH II and III cells were lysed. Even the distribution of normal E₉ slightly shifted to the right, suggesting that the cells with lower CD59 levels, older cells,¹⁹ were eliminated preferentially. In addition, Table 1 shows a direct correlation between the percentage of erythrocytes lysed by unacidified NHS+rH19-20 and the initial percentage of PNH type II and III cells before NHS treatment. These data indicate that PNH type II and III cells become highly susceptible to hemolysis by the alternative pathway of complement when fH-mediated cell surface protection is inhibited.

The critical role of fH in cellular homeostasis has been demonstrated here using PNH erythrocytes. This goes against the previous concept that complement activation on cell surfaces is primarily controlled by membrane-bound regulators. FH polymorphisms and mutations have been linked to human diseases that often lead to severe complement-mediated tissue damage such as atypical hemolytic uremic syndrome, age-related macular degeneration, and MPGN.²⁰ Studies of the functional properties of these variants²⁰ support the conclusions of the present study.

Although the average fH plasma concentration is 500 µg/ml,²¹ a 5-fold range in fH plasma levels has been described.²² Our data shows that fH protection is necessary for the survival of PNH II, III, and even normal erythrocytes. Therefore, the possibility exists that PNH patients with fH levels in the lower normal range may have the shortest erythrocyte lifespan (i.e. 6 vs 60 days), and may manifest more severe hemolytic symptoms.
In summary, our results highlight the essential cooperation between fH and membrane-bound regulators for inhibiting complement activation on autologous cell surfaces and help explain how PNH cells that are partially or completely deficient in CD59 and CD55 survive for days or weeks in vivo.
Acknowledgments

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Authorship contribution: V.P.F. designed and performed the research and wrote the paper; M.K.P. provided key reagents, discussed the results and supervised the project.
References


Table 1. The initial percentage of PNH erythrocytes correlates with the percentage of cells lysed by NHS containing the factor H inhibitor rH19-20.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% PNH II + III cells (FACS)*</th>
<th>% Hemolysis† (NHS+rH19-20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNH 1</td>
<td>33 ± 5</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>PNH 2</td>
<td>46 ± 5</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>PNH 3</td>
<td>48 ± 9</td>
<td>48 ± 0</td>
</tr>
<tr>
<td>PNH 4</td>
<td>72 ± 11</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

Correlation coefficient = 0.96

*PNH and normal EH incubated with anti-CD59 antibody, followed by specific fluorescein-conjugated secondary antibody (see Fig. 1C).

†rH19-20 used at 17 µM in 40% NHS+MgEGTA (final concentration). A414 was measured in the supernatant after 20 min. Hemolysis included lysis of PNH II and III cells as well as some lysis of normal cells with low CD59 (see Figs. 1A and 1C) due to fH inhibition.
Legend

Figure 1. Blocking fH-mediated cell surface protection of human red blood cells that have normal or decreased CD55 and CD59 function increases their susceptibility to autologous complement-mediated lysis. (A) EH (5x10^6) in GVB were pre-incubated with a neutralizing anti-CD59 monoclonal antibody (0-7.5 µg/ml) for 20 min at 4°C. NHS (40% final) in the presence or absence of 14 µM rH19-20 (as an inhibitor of fH cell-surface protection) was added and the mix (24 µl total containing 5 mM MgEGTA) was incubated for 20 min at 37°C. Cold GVBE (200 µl) was then added to stop the reaction and lysis was subsequently measured by hemoglobin release (A414) after centrifugation to remove unlysed cells. (B) Same as panel A, but the EH were pre-incubated with anti-CD55 monoclonal antibody. Averages and standard deviations of three separate experiments are graphed in panels A and B. (C) CD59 profile analysis of PNH and normal erythrocytes after in vitro exposure to NHS+MgEGTA in the presence or absence of rH19-20. The PNH erythrocytes (Patients 1-4) and normal EH (one representative sample shown) were treated with 40% NHS (first column titled Cells Post-NHS Treatment) or with NHS in the presence of 17 µM rH19-20 (second column titled Cells Post-NHS+rH19-20 Treatment), for 20 min at 37°C. The CD59 profile of the remaining unlysed cells was determined by incubation with anti-CD59 monoclonal antibody (5 µg/ml) for 45 min at 0°C, followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibodies for 45 min at 0°C, and analyzed by FACs. The markers I, II, and III indicate the populations of CD59 normal, CD59 partially positive, and CD59 negative cells, respectively. In the histograms titled Cells Post-NHS+rH19-20 Treatment the percent lysis of PNH II+III cells are indicated.
and was calculated as described\textsuperscript{17}: 100 – \(((\dfrac{\%\text{PNH II+III cells post-NHS+rH19-20}}{\%\text{PNH I cells post-NHS+rH19-20}} \div \dfrac{\%\text{PNH II+III cells post-NHS}}{\%\text{PNH I cells post-NHS}}) \times 100)\). At least 10,000 events were acquired per sample. The results shown are representative of three separate experiments. The samples that were treated with NHS (panels A-C) contained 5 mM MgEGTA to prevent classical pathway activation.
Figure 1
Factor H-mediated cell surface protection from complement is critical for the survival of PNH erythrocytes

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