Brief report

Factor H–mediated cell surface protection from complement is critical for the survival of PNH erythrocytes

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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 to 60 days in vivo. Why these cells are not lysed as rapidly by complement as unprotected foreign cells, which normally lyse within minutes, remains underdetermined. 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 nonacidi- fied 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 more3,4 and that of PNH II erythrocytes can be close to that of normal erythrocytes (120 days).4 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). 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 4 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 and 20 of human fH (rH19-20) were cloned, expressed in yeast, and purified as described.14 Human fH was purified from serum (NHS).15 The following buffers were used: VBS, 5 mM veronal, 145 mM NaCl, 0.02% NaN3, pH 7.3; GVB, VBS containing 0.1% gelatin; GVB, 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 was inhibited on normal human erythrocytes (EHS) with monoclonal antibodies (clone MEM43 or BRIC216, respectively; Chemicon, Temecula, CA), followed by incubation at 37°C with NHS. The following buffers were used: VBS, 5 mM veronal, 145 mM NaCl, 0.02% NaN3, pH 7.3; GVB, VBS containing 0.1% gelatin; GVB, GVB containing 10 mM EDTA (ethylenediaminetetraacetic acid); MgEGTA, 0.1 M MgCl2, 0.1 M EGTA (ethyleneglycoltetraacetic acid), pH 7.3.

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 flow cytometry.
incubating the cells with the anti-CD59 antibody, followed by fluorescein isothiocyanate–conjugated rabbit anti–mouse IgG (Sigma-Aldrich, St Louis, MO). The cells were analyzed in a FACScan (BD Biosciences, San Jose, CA) using CellQuest Pro software (BD Biosciences). 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.17

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 EHs (Figure 1A,B). A maximum of 23% lysis by NHS + MgEGTA was observed when CD59 alone was blocked (Figure 1A). No lysis was detected when CD55 alone was blocked (Figure 1B). Addition of rH19-20 to these reactions (Figure 1A,B) at 14 μM, a concentration sufficient to inhibit 93% of fH surface activity,6 resulted in 82% lysis of EHs when CD59 was blocked (Figure 1A) and 68% lysis when CD55 was blocked (Figure 1B). Inhibition of fH alone resulted in 19% lysis (zero input of antibody Figure 1A,B). Thus, inhibiting fH-mediated cell surface protection functions renders normal cells partially susceptible to complement-mediated lysis in 20 minutes 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.3,4 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 (Figure 1C). The PNH erythrocytes treated with unacidified NHS (Figure 1C, Cells After 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.18 However, when PNH and normal erythrocytes were incubated with unacidified NHS + MgEGTA in the presence of rH19-20 (Figure 1C, Cells After NHS + rH19-20 Treatment), the remaining unlysed cells

Figure 1. Blocking fH-mediated cell surface protection of human red blood cells that have normal or decreased CD59 and CD59 function increases their susceptibility to autologous complement-mediated lysis. (A) EHs (5 × 10^6) in GVB were preincubated with a neutralizing anti-CD59 monoclonal antibody (0-7.5 μg/mL) for 20 minutes 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 minutes at 37°C. Cold GVB (200 μL) was then added to stop the reaction, and lysis was subsequently measured by hemoglobin release (A540) after centrifugation to remove unlysed cells. (B) Same as panel A, but the EHs were preincubated with anti-CD59 monoclonal antibody. Averages and standard deviations of 3 separate experiments are graphed in panels A and B. (C) CD59 profile analysis of PNH and normal erythrocytes after in vitro exposure to NHS (in the presence or absence of rH19-20). The PNH erythrocytes (patients 1-4) and normal EHs (one representative sample shown) were treated with 40% NHS (first column titled “Cells After NHS Treatment”) or with NHS in the presence of 17 μM rH19-20 (second column titled “Cells After NHS + rH19-20 Treatment”), for 20 minutes at 37°C. The CD59 profile of the remaining unlysed cells was determined by incubation with an anti-CD59 monoclonal antibody (5 μg/mL) for 45 minutes at 0°C, followed by incubation with fluorescein isothiocyanate–conjugated rabbit anti–mouse IgG antibodies for 45 minutes at 0°C, and analyzed by fluorescence-activated cell sorting (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 After NHS + rH19-20 Treatment,” the percent lysis of PNH II + III cells is indicated and was calculated as described17: 100 − [(% PNH II + III Cells After NHS + rH19-20–% PNH I Cells After NHS + rH19-20)/(% PNH II + III Cells After NHS(% PNH I Cells After NHS)) × 100]. At least 10 000 events were acquired per sample. The results shown are representative of 3 separate experiments. The samples that were treated with NHS (panels A–C) contained 5 mM MgEGTA to prevent classical pathway activation.
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 E$_{Nh}$ slightly shifted to the right, suggesting that the cells with lower CD59 levels, older cells, 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 F$_H$-mediated cell surface protection is inhibited.

The critical role of F$_H$ in cellular homeostasis has been demonstrated here using PNH erythrocytes. This goes against the previous concept that complement activation on cell surfaces is controlled primarily by membrane-bound regulators. F$_H$ 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 membranoproliferative glomerulonephritis (MPGN). Studies of the functional properties of these variants support the conclusions of the present study.

Although the average F$_H$ plasma concentration is 500 μg/mL, a 5-fold range in F$_H$ plasma levels has been described. Our data show that F$_H$ protection is necessary for the survival of PNH II, PNH III, and even normal erythrocytes. Therefore, the possibility exists that PNH patients with F$_H$ levels in the lower normal range may have the shortest erythrocyte lifespan (ie, 6 vs 60 days), and may manifest more severe hemolytic symptoms.

In summary, our results highlight the essential cooperation between F$_H$ 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.

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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.

Conflict-of-interest disclosure: One of the authors (M.K.P.) is an officer of and has a financial interest in Complement Technology, Inc. (www.ComplementTech.com), a supplier of complement reagents. The other authors declare no competing financial interests.

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**References**


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