

Expression and function of hematopoiesis-stimulating factor receptors on the GPI⁻ and GPI⁺ hematopoietic stem cells of patients with paroxysmal nocturnal hemoglobinuria/aplastic anemia syndrome

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Received October 13, 2014; Accepted September 28, 2015

DOI: 10.3892/etm.2016.3163

Abstract. Paroxysmal nocturnal hemoglobinuria/aplastic anemia (PNH/AA) syndrome presents a markedly increased population of cells deficient in glycoposphatidylinositol (GPI⁻ cells) and signs of bone marrow failure, which requires treatment with hematopoiesis-stimulating factors, such as granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF). However, little is known about the effects of these stimulating factors on GPI⁻ cells. In order to explore the effects of stimulating factors in PNH/AA, G-CSF receptor (CD114) and SCF receptor (CD117) expression levels on GPI⁺ and GPI⁻ hematopoietic stem cells (HSCs) were measured by flow cytometry (FCM). The mean fluorescence intensity (MFI) values of signal transducer and activator of transcription 5 (STAT5) and phosphorylated (P)-STAT5 were measured in GPI⁺ and GPI⁻ HSCs by FCM following stimulation with G-CSF or SCF *in vitro*. The expression levels of CD114 and CD117 on GPI⁻ HSCs were significantly lower ($P < 0.01$) than those on GPI⁺ HSCs in PNH/AA patients and normal controls. The MFI values of STAT5 in the GPI⁻ and GPI⁺ HSCs of PNH/AA patients and normal controls were not significantly different. However, the MFI values of P-STAT5 in the GPI⁻ HSCs of PNH/AA patients were significantly lower than those in the GPI⁺ HSCs of PNH/AA patients and normal controls prior to and following stimulation with G-CSF or SCF ($P < 0.01$). The GPI⁻ HSCs of PNH/AA patients responded poorly to stimulation by hematopoiesis-stimulating factors, which indicates that these factors can be used safely in patients with PNH/AA.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematopoietic disorder arising from a mutation in the X chromosome-linked phosphatidylinositol glycan anchor biosynthesis, class A (PIGA), leading to a deficiency of proteins linked to the cell membrane via glycoposphatidylinositol (GPI) anchors. Abnormal and normal clones co-exist in the bone marrow of patients and the disease is characterized by bone marrow failure, intravascular hemolysis and thrombosis (1,2). The clinical association between PNH and aplastic anemia (AA) has long been recognized. PNH frequently occurs in association with suppressed hematopoiesis, including bone marrow failure syndrome. Hemolytic PNH can occur in the setting of AA, and conversely AA can be a late complication of PNH (3).

In a previous study, we confirmed that the expression levels of the erythropoietin receptor (EPOR) and thrombopoietin receptor (TPOR) on bone marrow (BM) GPI⁻ hematopoietic stem cells [HSCs; cluster of differentiation (CD)34⁺CD59⁻] in patients with PNH are significantly lower than those on the BM GPI⁺ HSCs (CD34⁺CD59⁺) in these patients (4). Furthermore, *in vitro*, following stimulation with erythropoietin (EPO) and thrombopoietin (TPO), the signal transducer and activator of transcription 5 (STAT5) phosphorylation levels of the EPOR and TPOR in the GPI⁺ clone of patients with PNH were clearly superior to those in the GPI⁻ clone (5).

The aim of the present study was to further investigate the GPI⁻ HSC response to granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) in PNH/AA syndrome. The expression of their respective receptors, CD114 and CD117, on GPI⁻ and GPI⁺ HSCs were explored, and the mean fluorescence intensity (MFI) of the intracellular signaling pathway proteins STAT5 and phosphorylated (P)-STAT5 were measured before and after stimulation with G-CSF or SCF.

Materials and methods

Patients. A total of 23 patients with PNH/AA syndrome were enrolled in the study. These patients were hospitalized in the

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Key words: hemoglobinuria, paroxysmal, hematopoietic stem cell, anemia, aplastic, CD114, CD117, STAT5

Department of Hematology of Tianjin Medical University General Hospital (Tianjin, China) from March 2012 to May 2013 and diagnosed according to international criteria (6). Characteristics of the patients are listed in Table I. In addition, 15 healthy volunteers with a median age of 36 years (range, 22 to 65 years) were included as healthy controls. The study was approved by the Ethics Committee of Tianjin Medical University. Informed written consent was obtained from all patients or their parents in accordance with Declaration of Helsinki.

Determination of CD114 and CD117 expression by flow cytometry (FCM). Samples (2 ml) of fresh bone marrow (BM) were obtained from the patients and healthy controls. After filtration, 300 µl BM samples were divided into one control and two test tubes. An antibody against monoclonal mouse IgG1-PE (cat no. 555749; 1:5) was added as a negative control and the mouse monoclonal antibodies CD114-PE (cat no., 554538; 1:5) and CD117-PE (cat no., 340529; 1:5) were added to each test tube, respectively. Mouse monoclonal antibodies CD34-PerCP (cat no., 340430; 1:5) and CD59-FITC (cat no., 555763; 1:5) were stained in all tubes as markers of stem cells and the PNH clone. All antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Following incubation at 4°C for 30 min, red blood cells (RBCs) in the sample were lysed with 2 ml RBC lysing solution (BD Biosciences). Next, the bone marrow hemopoietic cells were washed twice with phosphate-buffered saline (PBS) and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) with CellQuest software, version 3.1. At least 100,000 cells were acquired for each sample.

STAT5 and P-STAT5 analysis. BM mononuclear cells (BMMNCs) were isolated by Ficoll-Hypaque density gradient centrifugation (750 g x for 5 min at 20°C; density, 1.077; Tianjin Hao Yang Biological Products Technology Co., Ltd., Tianjin, China). The samples were stimulated with BMMNCs ($2-3 \times 10^6$ cells) G-CSF (100 ng/ml) or SCF (100 ng/ml) to induce the phosphorylation of STAT5 and fixed with crosslinking reagent (2% paraformaldehyde; Sigma-Aldrich, St. Louis, MO, USA). To stabilize the phosphorylation, the cells were fixed using 2% paraformaldehyde in PBS for 10 min at 4°C. The monoclonal antibodies CD34-PerCP and CD59-FITC were then added to the samples. After incubating at 4°C for 30 min, RBCs in the samples were lysed with 2 ml RBC lysing solution. The cells were mixed with 1.0 ml of FACS™ permeabilizing solution (BD Biosciences) for 10 min in the dark, and then incubated with STAT5-PE (cat no., 562077; 1:100 dilution) and p-STAT5-PE (cat no., 129010; 1:100 dilution) mouse monoclonal antibodies (BD Biosciences) at 4°C for 30 min. The cells were then washed twice with PBS. Information about $\geq 100,000$ cells was acquired for each sample by FCM.

Statistical analysis. SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) was used. Measurement data are displayed as the mean \pm standard deviation. Analysis of variance was used to evaluate differences between groups, followed by Student-Newman-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Table I. Baseline characteristics of the patients.

| Patient no. | Age (years) | Gender | Disease phenotype | PNH clone (%) |
|-------------|-------------|--------|-------------------|---------------|
| 1 | 33 | F | PNH/AA | 31.2 |
| 2 | 24 | M | PNH/AA | 44.4 |
| 3 | 29 | F | PNH/AA | 41.4 |
| 4 | 77 | M | PNH/AA | 37.7 |
| 5 | 25 | M | PNH/AA | 39.6 |
| 6 | 72 | F | PNH/AA | 20.5 |
| 7 | 56 | F | PNH/AA | 45.3 |
| 8 | 40 | F | PNH/AA | 50.5 |
| 9 | 63 | F | PNH/AA | 26.8 |
| 10 | 28 | M | PNH/AA | 32.2 |
| 11 | 32 | M | PNH/AA | 49.7 |
| 12 | 60 | F | PNH/AA | 42.0 |
| 13 | 55 | F | PNH/AA | 37.9 |
| 14 | 30 | M | PNH/AA | 35.0 |
| 15 | 14 | F | PNH/AA | 32.1 |
| 16 | 39 | F | PNH/AA | 22.9 |
| 17 | 11 | M | PNH/AA | 47.8 |
| 18 | 42 | M | PNH/AA | 61.9 |
| 19 | 51 | F | PNH/AA | 29.8 |
| 20 | 36 | M | PNH/AA | 44.7 |
| 21 | 18 | F | PNH/AA | 31.0 |
| 22 | 44 | M | PNH/AA | 40.1 |
| 23 | 35 | M | PNH/AA | 28.4 |

F, female; M, male; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia.

Results

Expression of CD114 and CD117 on GPI⁺ and GPI⁻ HSCs isolated from BM. The expression levels of CD114 and CD117 on the GPI⁻ HSCs of patients with PNH/AA (44.23 ± 19.77 and $49.20 \pm 26.80\%$, respectively) were significantly lower than those on the GPI⁺ HSCs of patients with PNH/AA (73.72 ± 17.42 and $67.62 \pm 17.41\%$, respectively; $P < 0.01$) or normal controls (65.91 ± 13.70 and $70.21 \pm 12.68\%$, respectively; $P < 0.01$). No significant difference was observed between the latter two groups ($P > 0.05$; Fig. 1).

Expression of STAT5 on GPI⁺ and GPI⁻ HSCs isolated from BM. The MFI values for STAT5 in the GPI⁻ and GPI⁺ HSCs of patients with PNH/AA and the GPI⁺ HSCs of the normal controls were 270.01 ± 181.26 , 205.05 ± 146.16 and 227.39 ± 156.65 , respectively. No significant difference was observed among the three groups ($P > 0.05$; Fig. 2).

Expression of P-STAT5 on GPI⁺ and GPI⁻ HSCs isolated from BM. Prior to stimulation and following stimulation with G-CSF or SCF, the MFI values for P-STAT5 in the GPI⁻ HSCs of PNH/AA patients were 23.44 ± 17.90 , 35.73 ± 33.93 and 33.19 ± 26.45 , respectively, which were significantly lower than

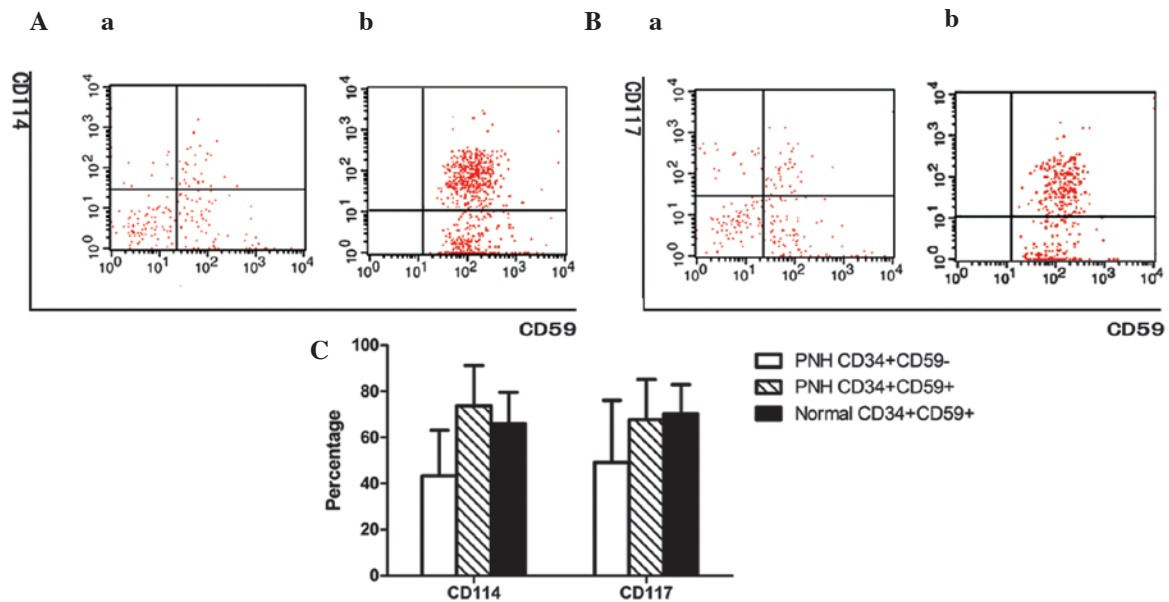


Figure 1. (A) Expression of CD114 on the (Aa) GPI⁺ and GPI⁻ cells of patients with PNH and (Ab) GPI⁺ cells of normal controls. (B) Expression of C117 on the GPI⁺ and GPI⁻ cells of (Ba) patients with PNH and (Bb) normal controls. (C) Percentage of CD114 or C117 expression on GPI⁺ and GPI⁻ cells isolated from the bone marrow. CD, cluster of differentiation; CD114, granulocyte colony-stimulating factor receptor; CD117, stem cell factor receptor; GPI, glycosphosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria.

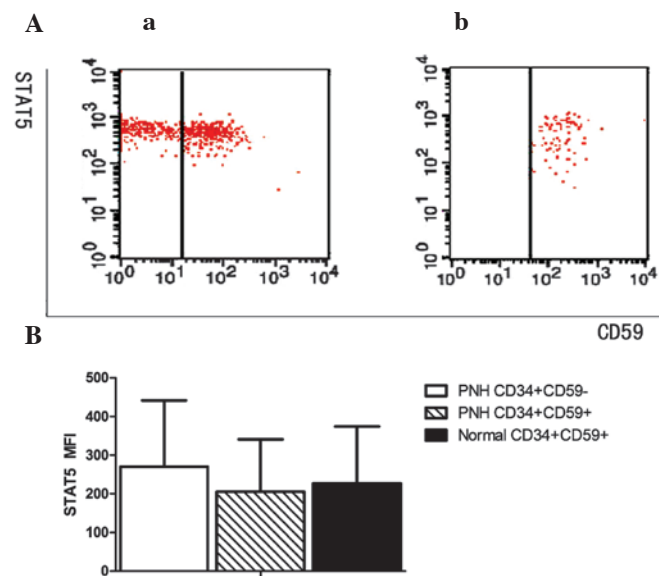


Figure 2. (A) MFI of STAT5 in the (Aa) GPI⁺ and GPI⁻ cells of PNH patients and (Ab) GPI⁺ cells of normal controls. (B) Percentage of STAT5 in GPI⁺ cells and GPI⁻ cells of the bone marrow. MFI, mean fluorescence intensity; STAT5, signal transducer and activator of transcription 5; GPI, glycosphosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; CD, cluster of differentiation.

those in the GPI⁺ HSCs of PNH/AA patients (63.42 ± 47.63 , 123.45 ± 80.40 and 123.41 ± 93.40 , respectively; $P < 0.01$) or normal controls (61.30 ± 32.13 , 116.00 ± 56.52 and 126.86 ± 60.20 , respectively; $P < 0.01$). No statistically significant difference was identified in the P-STAT5 MFI values between the latter two groups ($P > 0.05$; Fig. 3).

Increases in P-STAT5 expression in GPI⁺ and GPI⁻ HSCs following stimulation with G-CSF or SCF. The increased MFI values for P-STAT5 in G-CSF- or SCF-stimulated GPI⁻ HSCs

of PNH/AA patients were 3 and 5, respectively, which were significantly lower than those in the G-CSF- or SCF-stimulated GPI⁺ HSCs of PNH/AA patients (47 and 31, respectively) and normal controls (44 and 46, respectively) ($P < 0.01$; Fig. 3).

Discussion

PNH is an acquired clonal disease characterized by complement-mediated hemolysis, bone marrow failure and thrombosis. Some patients present with markedly increased levels of GPI⁻ HSCs and signs of bone marrow failure, which lead to a diagnosis of PNH/AA syndrome (7). The conventional methods for the treatment of PNH/AA syndrome include glucocorticoids, androgens, cyclosporine, anti-T-lymphocyte globulin (ATG) and hemopoietic stem cell transplantation (8-10). A new targeted and disease-modifying treatment strategy is the inhibition of the terminal complement cascade with the humanized monoclonal anti-C5 antibody, eculizumab, which can effectively control hemolysis and thrombosis (11-13). Economic reasons cause many patients with PNH/AA syndrome to undergo immunosuppressive treatment and treatment with hemopoietic stimulating factors, such as G-CSF and EPO. However, at present, little is known about the effects of these hemopoietic stimulating factors on GPI⁻ HSCs. It has been demonstrated that adding EPO and TPO to chemotherapy may promote the proliferation and differentiation of normal clone cells, and eliminate the abnormal PNH clone fundamentally (4,5). In the present study, the aim was to explore the effects of G-CSF and SCF in PNH/AA syndrome.

The cytokine G-CSF promotes the proliferation, differentiation, survival and functional maturation of cells within the neutrophilic granulocyte lineage (14). Its cell-surface receptor (G-CSFR; CD114) plays an important role in the production, survival and activation of neutrophilic granulocytes during normal and emergency hematopoiesis (14). This has led to

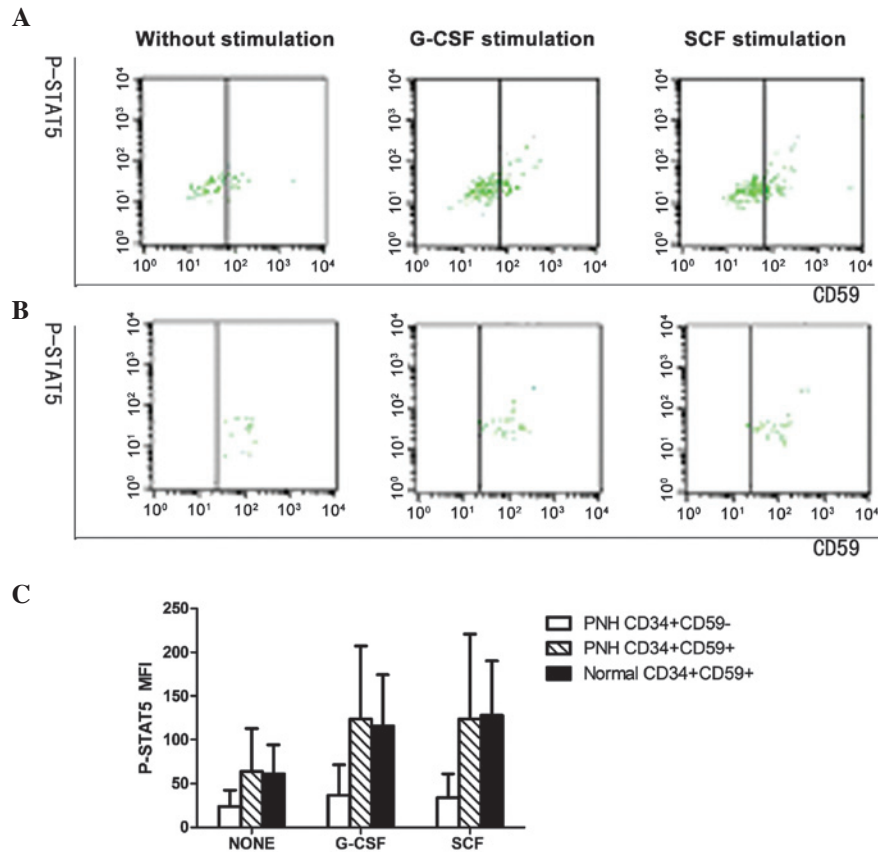


Figure 3. The P-STAT5 MFI in the (A) GPI⁺ cells and GPI⁻ cells of PNH/AA patients and (B) GPI⁺ cells of normal controls with or without stimulation by G-CSF or SCF. (C) The MFI of P-STAT5 in GPI⁺ and GPI⁻ cells of the bone marrow before and after G-CSF or SCF stimulation. G-CSF, granulocyte colony-stimulating factor; SCF, stem cell factor; P-STAT5, phosphorylated signal transducer and activator of transcription 5; MFI, mean fluorescence intensity; GPI, glycosylphosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; CD, cluster of differentiation.

several important clinical applications for its ligand, G-CSF, which binds to the G-CSFR causing activation via homodimerization and subsequent phosphorylation on four tyrosine residues of the receptor intracellular domain (15). This initiates a range of intracellular signaling events including the activation of Janus kinase (JAK)/STAT pathways (15,16). Ward *et al* (17) further highlighted the importance of STAT5 in the mediation of proliferative responses to G-CSF after studying the association between STAT5 and severe congenital neutropenia.

SCF is a critical cytokine during hematopoiesis, which regulates stem and progenitor cell survival and proliferation. The receptor for SCF, c-Kit (CD117), is a member of the tyrosine kinase family of receptors and undergoes autophosphorylation upon binding with SCF, resulting in the activation of multiple signaling proteins such as JAK/STAT, phosphoinositide 3-kinase, Src kinases, Shc and Ras (18,19). Brizzi *et al* (20) found that STAT1 α , STAT5A and STAT5B participate in the signaling transduction of SCF.

In the present study, the expression levels of CD114 and CD117 on HSCs from the BM of PNH/AA patients were detected, and it was found that the expression levels on GPI⁻ HSCs were significantly lower than those on the GPI⁺ HSCs of PNH/AA patients or normal controls. Therefore, this characteristic can be utilized in the treatment of PNH/AA to promote the proliferation of the normal clone instead of the PNH clone.

In order to further investigate the functions of CD114 and CD117, the signaling pathway protein STAT5 was measured

in the HSCs by FCM. No significant difference in the STAT5 MFI was observed among the three groups, that is, among the GPI⁻ and GPI⁺ HSCs of patients with PNH/AA and the GPI⁺ HSCs of normal controls. This indicates that there were no abnormal quantities of intracellular pathway proteins among the three groups. Furthermore, the expression levels of P-STAT5 were measured in the BMMNCs of PNH/AA patients and normal controls prior to and following stimulation with G-CSF or SCF *in vitro*. It was found that the MFI of P-STAT5 in the unstimulated or G-CSF- or SCF-stimulated PNH (GPI⁺) clone cells was lower than that in normal clone cells, and no significant difference was observed between the GPI⁺ HSCs of the PNA patients and normal controls. This indicates that G-CSF or SCF can significantly increase the proliferation and differentiation of normal clones, while having little effect on abnormal PNH clones. In a previous study (21), it was found that *in vitro*, the BMMNCs of normal controls had better proliferative capacity and gave a stronger response to G-CSF than those of patients with PNH, which is consistent with the results of the present study. The present study also found that the expression level of P-STAT5 in PNH clone cells was lower than that in normal clone cells prior to stimulation with G-CSF or SCF. However, normal clone cells did not acquire a proliferative advantage. By contrast, PNH clone cells were amplified, which would eventually lead to a series of clinical manifestations. This may be due to a complex mechanism, leading to PNH clones evading immune attacks,

undergoing a reduction in apoptosis decrease and gaining a proliferative advantage (22,23). Through the application of hematopoiesis-stimulating factors in PNH/AA patients, the degree of phosphorylation of normal clone cells can be significantly increased, which may overcome the various factors that lead to proliferation of the abnormal clone, leading to gradual proliferation of the normal clone and the restoration of normal hematopoiesis.

In conclusion, PNH clone cells responded poorly to stimulation by the hematopoiesis-stimulating factors G-CSF and SCF. The findings of the present study may facilitate the deeper development of hematopoiesis-stimulating factors in PNH/AA patients. However, further studies are required in order to investigate the mechanism in more detail.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grants nos. 30971285, 30971286 and 81170472), Tianjin Municipal Natural Science Foundation (grant nos. 14JCYBJC25400 and 12JCZDJC21500), Tianjin Medical University Foundation (grant nos. 2011ky07 and 2010ky20), Science and Technology Foundation of Tianjin Municipal Health Bureau (grant nos. 2011kz115 and 2010kz105), Tianjin Municipal Health Industry Key Project (grant no. 11KG135) and Tianjin Municipal Anticancer Major Project (grant nos. 122ZCDSY18000 and 12ZCDZSY17900).

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