Severe telomere shortening affects both GPI- and GPI+ hematopoiesis in patients with paroxysmal nocturnal hemoglobinuria

Anastasios Karadimitris¹,², David J. Araten¹, Lucio Luzzatto¹,³ and Rosario Notaro³

1, Memorial Sloan-Kettering Cancer Center, Department of Human Genetics and Department of Medicine, New York, N.Y, U.S.A;
2, Imperial College School of Medicine, Hammersmith Hospital, Department of Hematology, London, U.K; Laboratory of Human Genetics,
3, IST, Istituto Nazionale per la Ricerca sul Cancro, Laboratorio di Genetica Umana, Dipartimento di Eziologia ed Epidemiologia, Genova, Italy

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Corresponding author: Lucio Luzzatto, MD, Scientific Director,
IST, Istituto Nazionale per la Ricerca sul Cancro, Largo Rosanna Benzi 10,16132 Genova, ITALY. Phone: +39 010 352776. Fax: +39 010 355573.
e-mail: lucio.luzzatto@istge.it

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Abstract

A most distinctive feature of paroxysmal nocturnal hemoglobinuria (PNH) is that in each patient GPI- and GPI+ hematopoietic stem cells (HSC) co-exist, and both contribute to hematopoiesis. Telomere size correlates inversely with the cell division history of HSC. In 10 patients with hemolytic PNH the telomeres in sorted GPI- granulocytes were shorter than in sorted GPI+ granulocytes in 4 cases, comparable in 2 cases, and longer in the remaining 4 cases. Furthermore, the telomeres of both GPI- and GPI+ hematopoietic cells were markedly shortened compared to age-matched controls. The short telomeres in the GPI- cells probably reflect the large number of cell divisions required for the progeny of a single cell to contribute a large proportion of hematopoiesis. The short telomeres of the GPI+ cells indicate that the residual hematopoiesis contributed by these cells is not normal. This epigenetic change is an additional feature shared by PNH and aplastic anemia.
Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disorder of the hematopoietic stem cell (HSC) characterized by intravascular hemolysis, venous thrombosis, and variable degrees of bone marrow failure. The exquisite susceptibility of PNH red cells to complement-dependent hemolysis is due to a somatic mutation of the X-linked PIG-A gene in HSC, resulting in complete or partial deficiency of several glycosylphosphatidylinositol (GPI)-linked proteins (including CD59) from the surface of the blood cells that are the progeny of the mutated HSCs. Because the mutation is somatic, normal and mutant cells co-exist in the blood of PNH patients. Very rare GPI-cells are present in normal subjects: but only patients with PNH have a GPI-cell population so expanded that contributes substantially to hematopoiesis. Therefore, in the pathogenesis of PNH this expansion is a necessary component: it has been surmised that it results from negative selection against GPI+ (i.e. normal) HSC by an auto immune process, akin to what is widely believed to be the pathogenetic basis for aplastic anemia (AA). One implication of this model is that the residual GPI+ cells in PNH are not in fact normal: recently Chen et al. have reported their impaired growth and over-expression of FAS-receptor.

Telomeres of somatic cells become shorter with each cell division and therefore their size provides information on the mitotic history of cells: accordingly, telomeres of hematopoietic cells become shorter with age, during in vitro cultures, and during the in vivo expansion that reconstitutes hematopoiesis after bone marrow transplantation. Ball et al. have reported that total leucocytes from 68 AA patients (17 of whom had small PNH clones) and from 3 PNH patients had shortened telomeres. More recently Brümmendorf et al. did not find any difference in telomere length between granulocytes from 6 PNH patients and from normal controls. However, neither of these studies have analyzed separately the GPI- and GPI+ blood cells that co-exist in PNH patients.
Materials and Methods

Subjects. Blood samples from 10 patients with hemolytic PNH (median age 32 years, range 24-60) and from 45 normal individuals (median age 34 years, range 16-73) were obtained under IRB-approved protocols. We included only patients with primary classical PNH who had a large PNH population (GPI- granulocytes over 30%), florid hemoglobinuria, and no severe cytopenias (table 1).

Immunomagnetic separation of GPI- from GPI+ granulocytes. Granulocytes, isolated as previously described\(^9\),\(^{20}\), were incubated with 2 µg per 10^6 cells of the IgM mAb anti-CD16 (Leu-1b, Becton-Dickinson) and then with rat anti-mouse-IgM microbeads according to manufacturer instructions (Miltenyi). After 30 minutes on ice, GPI-(CD16-) cells were separated from GPI+(CD16+) cells by a column in a strong magnetic field (MACS, Miltenyi). All separation steps were carried out strictly at 4ºC.

Telomere length measurement. Telomere length (TRF) measurement was carried out by a method we have previously developed\(^{20}\) based on a probe, TelBam8, that is unique for the subtelomeric region of the long arm of chromosome 7\(^{23}\).

Statistical analysis. All data are expressed as mean±SD. The expected-for-age telomere length (TRFE) has been estimated by linear regression of TRF against age of 45 normal individuals: TRFE = 18352 bp – 53 bp x age (years) (R^2=0.12; P=0.02). The 53bp/year TRF loss is in agreement with previous reports\(^{17,18,24}\). Wilcoxon rank sum test on paired samples, Kendall correlation and Fisher’s Exact test have been used when appropriate. Statistical significance was accepted for P<0.05.
Results and Discussion

In order to study the dynamic relationship between the clonal GPI- hematopoiesis and the co-existing residual GPI+ hematopoiesis in each PNH patient, we compared the telomere length of both these populations from 10 patients with classical hemolytic PNH (Table 1). We measured side by side in each patient the telomere length of purified GPI- and GPI+ peripheral blood granulocytes (Fig.1A): being terminally differentiated cells, these are likely to reflect the replicative history of HSCs. This internally controlled comparison showed that, overall, the average telomere length was similar in GPI- and GPI+ granulocytes: 13.9±0.9 Kb vs. 13.8±0.9 Kb; P=0.64 (Fig.1B,C). Nevertheless, we found different patterns in individual patients: the telomeres of GPI- granulocytes, compared to those of GPI+ granulocytes, were longer in 4 patients, similar in two patients, and shorter in the remaining 4 patients. We have investigated how these patterns relates to the patients’ hematological and biological characteristics (Table 1). We have found that a difference in telomere length in favor of GPI- granulocytes correlates directly with the size of the PNH cell population (P=0.032).

Next, we compared the telomere length of granulocytes from PNH patients with that from normal individuals. As telomere length is extremely variable in the population\(^{18,24}\) we resorted to calculating the difference between the telomere length observed (TRF\(^\text{O}\)) in each individual and the expected-for-age telomere length (TRF\(^\text{E}\)): TRF\(^\text{O-E}\) (as defined by Ball et al.\(^{21}\)). In 40 age-matched normal individuals (median age, 34y; range: 21-63y) the values of the TRF\(^\text{O-E}\) were, as expected, quite variable (TRF\(^\text{O-E}\): 140±1935 bp) and symmetrically distributed around the Zero point (Fig.1D). By contrast, in both GPI- and GPI+ granulocytes from PNH patients all but one TRF\(^\text{O-E}\) values were below Zero (TRF\(^\text{O-E}\): GPI-, -2563±1306 bp, GPI+, -2730±1256 bp: Fig.1D); and both TRF\(^\text{O-E}\) distributions were significantly different from that in normal individuals (Normal vs. GPI-: P=0.011; Normal vs. GPI+: P=0.001).

Thus, we have found in this study that both the GPI- and the GPI+ blood cells from patients with classical hemolytic PNH have shorter telomeres than blood cells from age-matched normal individuals. Assuming that telomeres lose ~100 bp per cell division\(^{17}\), the telomere shortening observed in PNH patients would be equivalent to approximately 25 extra cell divisions. The extreme telomere shortening in the GPI-
blood cells is in keeping with the fact that in hemolytic PNH patients one (or few) GPI-HSC clone(s) support a substantial proportion of hematopoiesis. It stands to reason that in order to achieve this task, which entails substantial expansion, these HSC must perform a considerable number of extra mitotic divisions.

As for the shortening of telomeres found in the GPI+ blood cells, it is reminiscent of that reported in AA patients (it may be in fact even greater): indicating that in PNH patients the residual GPI+ hematopoiesis is not normal. A hypothetical explanation is that selective destruction of normal (GPI+) HSCs has left very few survivors: these must then once again expand to support the residual GPI+ hematopoiesis in PNH patients. This hypothesis could also explain why different patterns are seen in different patients: this may depend on how far the PNH clone(s) have expanded at a particular point time; on whether damage to GPI+ cells is still on-going; etc. Indeed, telomeres being shorter in GPI+ than in GPI- granulocytes in patients with larger PNH cell population may suggest that, because of an on-going damage, GPI+ HSCs are less able to contribute to hematopoiesis. Thus, telomere shortening could be regarded as an epigenetic marker of disease: and one more feature that is shared by PNH and AA patients.

In summary, we have shown that severe telomere shortening affects roughly to the same extent GPI- and GPI+ hematopoiesis in patients with PNH. However, it is likely that this apparently similar epigenetic changes are caused by somewhat different mechanisms: clonal expansion in the GPI- HSC; oligoclonal regeneration after selective destruction in the GPI+ HSC.
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Table 1. Clinical and hematological features of PNH patients

| Patient | Sex/Age | Years from diagnosis | Hb (g/dl) | PMN (X10^9/µl) | Plts (X10^9/µl) | Retics (%) | Size of PNH population # (%) | Absolute counts GPI+ PMN (X10^9/µl) | GPI- PMN (X10^9/µl) | Hemoglobinuria | Thrombosis | Treatment | Transfusions |
|---------|---------|---------------------|----------|----------------|----------------|------------|-----------------------------|-------------------------------|----------------|--------------|------------|------------|-----------|-------------|
| A       | F/24    | 6                   | 10.1     | 2.3            | 130            | 4.5        | 39                         | 0.46                         | 1.84           | Yes         | no         | ATG        | occasional |
| B       | F/27    | 7                   | 10.8     | 4.2            | 115            | 3.9        | 30                         | 0.42                         | 3.78           | Yes         | no         | ATG        | occasional |
| C       | M/40    | 2                   | 11.8     | 3.2            | 137            | 3.4        | 13                         | 2.18                         | 1.02           | Yes         | cerebral   | supportive | occasional |
| D       | M/30    | 3                   | 9.0      | 2.6            | 180            | 14.9       | 43                         | 0.16                         | 2.44           | Yes         | splenic    | ATG        | occasional |
| E       | F/37    | 7                   | 8.2      | 1.9            | 115            | 8.2        | 12                         | 0.78                         | 1.12           | Yes         | no         | supportive | never      |
| F       | F/24    | 5                   | 10.9     | 3.1            | 103            | 3.0        | 60                         | 0.62                         | 2.48           | Yes         | mesenteric | ATG        | occasional |
| G       | F/34    | 11                  | 12.4     | 2.3            | 74             | 2.1        | 23                         | 1.38                         | 0.92           | Yes         | Budd-Chiari| supportive | never      |
| H       | F/45    | 7                   | 9.7      | 1.3            | 160            | 5.2        | 18                         | 0.30                         | 1.00           | Yes         | Pulmonar   | supportive | occasional |
| I       | F/28    | 10                  | 10.6     | 8.4            | 62             | 5.4        | 72                         | 0.34                         | 8.06           | Yes         | Budd-Chiari| supportive | never      |
| J       | F/60    | 9                   | 7.0      | 3.0            | 229            | 46         | 78                         | 0.66                         | 2.34           | Yes         | no         | supportive | occasional |

Hb, hemoglobin; PMN, granulocytes; Plts, platelets; Retics, reticulocytes; RBC, Red blood cells; Yes, recurrent episodes of macroscopic hemoglobinuria throughout several years of clinical history; ATG, Anti-thymocyte globulin.

#: The percentage of PNH PMN reflects the relative size of the PNH cell population more accurately than the percentage of PNH RBC, because the latter will be grossly underestimated as a consequence of selective hemolysis; this may be further complicated by blood transfusion.
Figure 1. Telomere dynamics of GPI- and GPI+ granulocytes from patients with PNH.

A. Immunomagnetic separation of GPI- and GPI+ granulocytes from a patient with PNH (left-hand panel). By staining with anti-CD59 one sees the efficient separation of GPI- (middle panel) and GPI+ (right-hand panel) granulocytes. This technique enabled us to recover both the GPI- and the GPI+ granulocytes with a purity higher than 90%, as assessed by flow-cytometry after staining with anti-CD59.

B. Southern blot analysis of the telomere length of GPI- and GPI+ granulocytes from 3 patients with PNH (see table 1). 10 µg of high molecular weight genomic DNA, digested to completion with BamHI, was resolved on 1% agarose gel by field-inversion gel electrophoresis. After blotting, the filter was hybridized with TelBam8, a probe that is unique for the subtelomeric region of the long arm of chromosome 723. The length of the BamHI telomeric fragment was calculated from the densitometric profile. We have previously shown that by this method, in a side-by-side comparison, differences greater than 320 bp are significant20.

C. Telomere length in GPI+ and in GPI- granulocytes from individual patients. The differences in telomere length between paired DNA samples of GPI- and GPI+ granulocytes from each PNH patient are shown by individual straight lines. Each patient is indicated by a capital letter (Table 1).

D. Scattergrams of the TRF\textsuperscript{O-E} (difference between the observed telomere length – TRF\textsuperscript{O–} and the expected-for-age telomere length –TRF\textsuperscript{E–}) in GPI- and GPI+ granulocytes from PNH patients, and in 40 age-matched normal individuals (controls). The straight lines across each set of data points are the median values.
References.


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