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molecular basis of the disease

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The molecular basis of paroxysmal nocturnal hemoglobinuria

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disease characterized by chronic intravascular hemolysis, cytopenia due to bone marrow failure and increased tendency to thrombosis. All patients with PNH studied so far have a somatic mutation in an X-linked gene, called PIG-A (phosphatidyl inositol glycan complementation group A), which encodes for a protein involved in the biosynthesis of the glycosyl phosphatidylinositol (GPI) molecule, that serves as an anchor for many cell surface proteins. The mutation occurs in a hematopoietic stem cell and leads to a partial or total deficiency of the PIG-A protein with consequent impaired synthesis of the GPI anchor: as a result, a proportion of blood cells is deficient in all GPI-linked proteins. The mutations are spread all over the gene and in some patients more than one mutated clone have been identified. The absence of GPI-anchored proteins on PNH cells explains some of the clinical symptoms of the disease but not the mechanism that enables the PNH clone to expand in the bone marrow of patients. Both *in vitro* and *in vivo* experiments have shown that PIG-A inactivation *per se* does not confer a proliferative advantage to the mutated hematopoietic stem cell. Clinical observations have shown a close relationship between PNH and aplastic anemia. Taken together, these findings corroborate the hypothesis that one or more additional factors are needed for the expansion of the mutant clone. Selective damage to normal hematopoiesis could be the cause which enables the PNH clone(s) to proliferate.

Key words: paroxysmal nocturnal hemoglobinuria, PIG-A, GPI

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired chronic hemolytic anemia, caused by a somatic mutation in a hematopoietic stem cell. Chronic intravascular hemolysis, variable degrees of cytopenia and recurrent thrombotic events are the most common symptoms of the disease; the last is also the main cause of morbidity and, together with progressive pancytopenia, of mortality.[1,2] Whereas the cause of the high frequency of thrombotic events is unknown, hemolysis is recognized to be the result of an increased sensitivity to complement-mediated lysis of affected red cells.[3] However, not all red cells have this increased sensitivity, since a variable proportion of them is normal; at the same time a proportion of the other blood cells (neutrophils, monocytes, platelets and sometimes lymphocytes) displays the same defect of the abnormal red cells, namely deficiency of all the proteins bound to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor.[4] The residual normal blood cell population is often reduced in number, suggesting that it is derived from failing bone marrow. In fact, PNH has been described in patients affected by aplastic anemia and, conversely, patients with PNH can develop aplastic anemia.[5,6] Few diseases have been investigated over the last years like PNH and even fewer of them have had the molecular mechanisms underlying their pathogenesis clarified as they have been for PNH. However, despite great advances having been achieved in the understanding of the molecular basis of this disease, some aspects of its pathogenesis are still unclear. Here we report the current knowledge on the pathogenesis of PNH and discuss the most recent interpretations of the mechanism determining the expansion of the PNH clone.

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The GPI-linked proteins

The unique feature of PNH blood cells is the deficiency on their surface of all the proteins linked to the membrane by a GPI molecule.[4] As a consequence of the absence of their anchor, the surface proteins are degraded within the cell. GPI-linked proteins belong to different groups such as enzymes, receptors, complement regulators, adhesion molecules, and other proteins without a defined functions (Table 1). Deficiency can be complete (giving rise to the so called *PNH type III cells*) or partial (*PNH type II cells*). Type I cells have normal expression of the GPI-linked proteins on their surface. This variability in the severity of the deficiency as well as in the proportion of the affected cell population is relevant to the clinical manifestations of the disease.[3] Most importantly, flow cytometric analysis of granulocytes with monoclonal antibodies directed towards GPI-linked proteins is currently the most reliable test for the diagnosis of PNH.[7]

A schematic representation of the structure of the GPI-anchor and of its biosynthetic pathway is reported in Figure 1. Both the structure and the biosynthetic pathway have been highly conserved during evolution.[8] The reason why a protein is GPI-linked is not actually well understood: in fact, some proteins exist both in a transmembrane form and in a GPI-linked form. This can occur either by alternative splicing[9] (as for CD58) or through synthesis by two dif-

Table 1. GPI-linked proteins on blood cells.*

Adhesion molecules	***Enzymes***
CD48	Acetylcholinesterase
CD58 (LFA-3)	Neutrophil alkaline phosphatase
CD66	CD73
CD67	ADP-ribosyl transferase
Blood group antigens	***Receptors***
Comer antigen (DAF)	CD14
JMH antigen	CD16 (FcγRIII)
Holley Gregory antigen	CD87 (u-PAR)
Yt antigens	
Dombrock residue	***Others***
	CD16
Complement regulators	CD24
CD55 (DAF)	CDw52 (CAMPATH-1)
CD59 (MIRL)	GP175
C8bp (HRF)	GP500

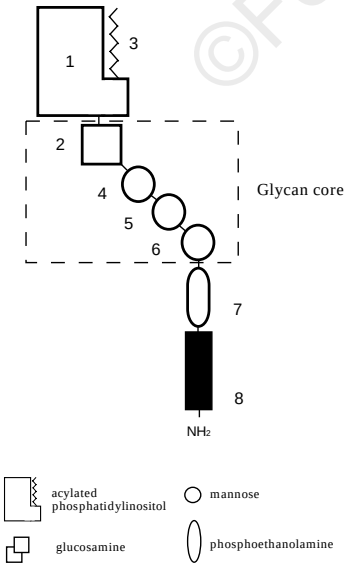
**When possible the CD classification has been used for the description of the proteins. Other common names or acronyms of the proteins are reported in brackets.*

ferent genes[10] (as for CD16). An advantage in being GPI-linked could be that the protein can be easily and rapidly cleaved from the cell surface by GPI-specific phospholipases (in a way similar to that which happens in trypanosome where such a mechanism enables the parasite to escape the host immune response[11]). In addition, GPI-linking confers to a protein a higher degree of lateral mobility on the cell surface. As to the specific function of the GPI molecule, there is so far no definitive interpretation. A specific role in a particular form of endocytosis called *potocytosis* has been reported,[12] as has a role in signal transduction.[13] Finally, it has been recently shown, using an animal model, that the absence of the GPI-anchor in the basal cells of the skin is associated with a defect in the transport of ceramides.[14]

The functions of many of the GPI-linked proteins are presently unknown; however the roles of two of them, CD55 or DAF (decay accelerating factor) and CD59 or MIRL (membrane inhibitor of reactive lysis), are important in the regulation of complement activity. In particular, CD55 is an inhibitor of C3 covertase[15] whereas CD59 protects the cell membrane from the attack of the C5-C9 complex.[16] Inherited absence of CD59 has been described,[17,18] and results in intravascular hemolysis and hemoglobinuria, whereas inherited deficiencies of CD55[19,20] have never been associated with typical symptoms of PNH, such as hemolysis and/or recurrent thrombotic events, with the exception of one patient who developed hepatic vein thrombosis.[21] Thus, it seems that it is the deficiency of CD59 which is mainly responsible for the increased sensitivity to complement and the consequent intravascular hemolysis observed in PNH patients. No relationship between thrombotic events and absence of GPI-linked proteins has been so far demonstrated.

The fact that so many proteins were absent from PNH blood cells and that all of them had in common the GPI-anchor led quickly to the hypothesis that the defect had to be found in one of the steps of the synthesis of the anchor itself. By using lymphoblastoid cell lines derived from patients with PNH, which were obtained by cellular cloning of EBV transformed B-lymphocytes, a block at an early step of the GPI-anchor biosynthesis was identified, namely at the first step of the pathway, when a N-acetylglucosamine is transferred from UDP-N-acetylglucosamine to phos-

Structure of the GPI anchor



Biosynthetic pathway

1) molecule of phosphatidylinositol
2) **N-acetylglucosamine is transferred to inositol*** and the product is deacetylated
3) acylation of inositol
4) a first mannose is donated from dolichol-phosphate-mannose
5) a second mannose is added
6) a third mannose is added
7) phosphoethanolamine is added
8) the COOH terminus of a protein is then attached to the ethanolamine

* this step requires the presence of the product of PIG-A and it is blocked in PNH cells.

Figure 1. Structure of the essential core of the GPI-anchor and its biosynthetic pathway. The biosynthesis takes place in the endoplasmic reticulum. It is believed that the first steps occur at the cytoplasmic side of the endoplasmic reticulum and the following at the luminal side; however, the precise step at which the GPI-anchor is flipped to the cisterna is unknown. The transfer of N-acetyl-glucosamine from UDP-N-acetylglucosamine to phosphatidylinositol is the step blocked in PNH cells. This reaction is catalyzed by α1,6-N-acetyl-glucosamine transferase. The preformed GPI molecule is then covalently linked to the carboxy-terminus of the protein by transpeptidation, after removal of amino acids 17-31 from the carboxy-terminus itself.

phatidylinositol.[22,23] A further step toward the comprehension of the molecular defect underlying the block in the synthesis of the GPI molecule was made when a cDNA able to restore the expression of GPI-linked proteins was isolated in an *in vitro* mutagenized cell line previously characterized by complementation analysis as belonging to group A: the gene was called PIG-A, which stands for phosphatidyl inositol glycan A.[24,25] Definitive demonstration of the involvement of PIG-A was obtained when it was shown that PIG-A cDNA was able to complement PNH cell lines from patients with the disease and that these cells had a mutation(s) in their own PIG-A gene.[25,26] Since then, all patients with PNH tested have been found to harbor a mutation in the PIG-A gene.[27,28,29]

The PIG-A gene

The PIG-A gene comprises 6 exons, spanning 17 kb on the X chromosome (Xp22.1). The complementary DNA has an open reading frame of 1455 bp (Figure 2). A pseudo PIG-A gene has been mapped to 12q21 and has the structure of a processed pseudo gene.[25,30,31] PIG-A encodes a putative protein of 484 amino acids, whose molecular weight is approximately 60 kD; the protein is located in the endoplasmic reticulum, with the amino terminus on the cytoplasmic side. It is a part of the α-1,6-N-acetylglucosaminyl transferase activity.[32] Mutations in the PIG-A gene are acquired (congenital forms of PNH do not exist nor is there an increased incidence within families): this also accounts for the equal frequency of the disease in males and females. In fact, for mutations taking place after X-chromosome inactivation, female cells are haploid like their male counterparts with respect to almost all the X-linked genes. Hence, a single mutational event is sufficient to cause the PNH phenotype.

Up to now more than 100 different mutations of PIG-A have been described in patients affected by PNH.[25,27-29,33-40] Mutations are spread all over the gene, with the highest number occurring in exon 2, probably because this is the largest of the exons of PIG-A. Most of the mutations are small deletions or insertions, resulting in an early stop codon or a frame shift; large deletions have been reported in a few cases.[35,40] More common are nonsense mutations and mutations causing altered splicing: both of these result in a non-functional protein. Of the missense mutations, some may cause a total loss of function of the protein while others may leave the product of PIG-A with residual activity.[29,33,34] This residual activity would be the situation of those patients showing a partial deficiency of GPI-linked proteins on the membranes of their blood cells (PNH type II cells). Differently from the other type of mutations, missense mutations are almost completely clustered on exon 2, suggesting that this exon contains important domains for the formation of the enzyme complex. All mutations described so far in PNH patients are somatic with the exception of one, which has been found in four different families, has a Mendelian inheritance and entails the replacement of an arginine with a tryptophan residue in codon 19.[29,41] However, this mutation does not cause PNH, possibly because the first codons of the gene are not critical for the function of the protein.

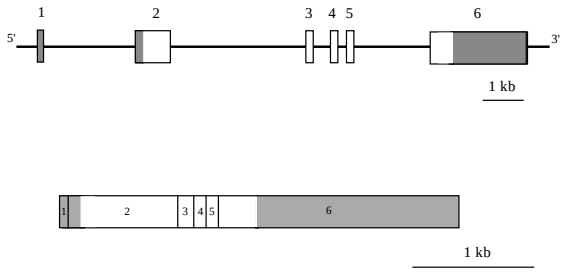


Figure 2. Schematic representation of the human PIG-A gene and its cDNA. The top part of the figure shows the structure of the PIG-A gene encoding for the PIG-A protein. Exons are represented by boxes, with gray areas indicating non-coding regions. The initiation codon lies in exon 2 and the termination codon in exon 6. The bottom part of the figure shows the structure of the PIG-A cDNA. Corresponding exon numbers are displayed. The 5' untranslated region (exon 1 and 5' end of exon 2) and the 3' untranslated region (3' end of exon 6) are represented in gray.

The fact that the somatic mutation arises in a hematopoietic stem cell is indicated by the fact that multiple cell lineages are involved. Many other pieces of evidence support the concept that PNH cells belong to the same clone. The first piece of evidence came from the demonstration that, in two female patients heterozygous for two different G6PD variants, the same allele was present in the blood cells lysed by an acidified serum test, whereas in their normal red cells both the alleles were expressed.[42] Then, Rotoli *et al.* found a dual population of BFU-E in the blood of patients with PNH. One of these populations was normal and the other sensitive to complement-mediated lysis and devoid of acetylcholinesterase, which is GPI-linked.[43] Finally, the conclusive evidence was given by the finding that the same PIG-A mutation was found in different blood cell lineages; in this regard it should be noted that more than one PNH clone (and mutation) have been described in the same patient.[35,38,44,45] This last observation explains why both type II and type III red cells can be found in some patients.

Expansion of PNH clone(s)

The elucidation of the molecular defect affords a clear explanation of why PNH cells are devoid of all the GPI-linked proteins and in turn their increased sensitivity to complement and subsequent intravascular hemolysis. What is in some way surprising is that a clone which lacks all GPI-linked proteins and, therefore, presumably at a remarkable disadvantage with respect to all the other normal hemopoietic clones can expand and displace normal hematopoiesis. The first hypothesis which can be made is that the deficiency of GPI-linked proteins confers an intrinsic growth advantage to the PNH clone. However, there is evidence that disproves this hypothesis:

first of all, in many patients the PNH clone, after its initial expansion, coexists for a long time with normal hematopoiesis. Second, spontaneous recover from PNH has been described and in some of these patients the clone was still present – at a low level – many years after the remission.[1] Finally, in mice chimeric for a non-functional PIG-A gene, red blood cells devoid of GPI-linked proteins did not have any growth advantage compared to their normal counterpart but, rather, they tended to decline rapidly over time.[46-48] A second hypothesis stems from the observation that a close relationship exists between PNH and aplastic anemia. Indeed, patients with PNH can develop aplastic anemia and conversely, PNH cells have been found in a significant proportion of patients affected by aplastic anemia. This second hypothesis, which has been defined as the relative growth advantage theory[49] or the escape theory,[50] is based on the concept that the rate of expansion of a PNH clone depends on the existence of one or more additional external environmental factors which exert a selective pressure in favor of the PNH clone. One of these factors could be an injury to normal hematopoietic cells which spares abnormal PNH cells. It is obviously tempting to imagine that the injury to hematopoiesis is mediated through a GPI-linked mechanism (for an extensive treatment of this matter see *refs. #60* and *61*). Other evidence indirectly supports the hypothesis of a relative growth advantage. As reported above two (or more) PNH clones can be present in the blood of the same patient: in this case the two mutations must have arisen independently, suggesting that a positive selection for PNH cells has occurred.[35] Moreover, neither *in vitro* culture of bone marrow derived progenitor cells (both at the CFC and at the LTC-IC level)[51,52] nor *in vivo* experiments have shown that the PNH clone has an intrinsic growth advantage. As regards the latter, in addition to the above mentioned *knock out* mice models,[46-48] experiments performed by transplanting SCID mice with normal and PNH hematopoietic progenitor cells have shown a preferential engraftment of PNH cells compared to normal cells.[53] However, since the engraftment of normal cells in these animals was impaired one could speculate that this provided the relative advantage to the PNH clone necessary for the latter's expansion. Further indirect proof in favor of the relative growth advantage theory came from the observation that patients affected by non-Hodgkin's lymphoma treated with the monoclonal antibody Campath 1H developed GPI-deficient T-cells.[54-57] This antibody specifically recognizes the CD52 antigen which is expressed on lymphocytes and monocytes and is GPI-linked. In the GPI-negative T-cells of some of these patients, mutations of PIG-A were documented. When the treatment with the antibody was withdrawn, GPI-negative T-cells gradually disappeared. Interestingly, it was also shown that in one of these patients, PNH cells were present before the treatment.[57] Thus, the treatment with Campath 1H acted as a positive selective pressure which created the relative growth advantage allowing the expansion of GPI-negative cells. These observations, beside strengthening the relative growth advantage theory, also brought with them the question of how frequent PNH clones are in the normal population. It could well be that the frequency of such clones is much higher than the frequency of the disease; however, in the absence of the second conditional event, the clone does not expand and remains difficult to detect. In keeping with this hypothesis is the recent report by Araten *et al.*[58] who dected, by means of flow cytometry, GPI-negative granulocytes, albeit in a very low frequency in 9 normal individuals. Using flow sorting, these cells were isolated and the PIG-A mutations identified by PCR in six of the nine subjects. Thus, small clones of PNH cells bearing a PIG-A mutations exist in normal people but do not become clearly evident until a second conditional event favors their expansion.

Conclusions

Two events seem to be necessary to give the clinical phenotype of PNH: one is a somatic mutation of the PIG-A gene in a hematopoietic stem cell; the other is a condition of cellular selection which is reasonable to identify in a mechanism of failure of normal bone marrow. The first alone will result in the production of a PNH clone which will not be associated with any clinical syndrome; the second alone will result in a condition of aplastic anemia. When both the mutation and the bone marrow failure occur in the same individual, the clinical picture of PNH becomes evident (the so-called *dual pathogenesis of PNH*[59-61]). The balance between the degree of bone marrow failure and the expansion of the PNH clone will direct the clinical symptoms towards either hemolytic or hypoplastic PNH.

Although great progress has been made in the last years, we are still far from a complete understanding of the pathogenesis of PNH. Many questions remain to be clarified and among them one of the most important is certainly the identification of the specific GPI-linked proteins responsible for the escape of PNH cells from the injury determining the bone marrow failure.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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