The Pathophysiology of Disease in Patients with Paroxysmal Nocturnal Hemoglobinuria

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia caused by the expansion of a hematopoietic progenitor cell that has acquired a mutation in the X-linked *PIGA* gene. PNH occurs on the background of bone marrow failure. Bone marrow failure and the presence of the abnormal cells account for the clinical phenotype of patients with PNH including hemolysis, cytopenia, and thrombophilia. PIGA is essential for the synthesis of glycosyl phosphatidylinositol (GPI) anchor molecules. PNH blood cells are therefore deficient in all proteins that use such an anchor molecule for attachment to the cell membrane. Two of these proteins regulate complement activation on the cell surface. Their deficiency therefore explains the exquisite sensitivity of PNH red blood cells to complement-mediated lysis. Complement-mediated lysis of red blood cells is intravascular, and intravascular hemolysis contributes significantly to the morbidity and mortality in patients with this condition. PNH is an outstanding example of how an increased understanding of pathophysiology may directly improve the diagnosis, care, and treatment of disease.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia caused by the clonal expansion of a hematopoietic progenitor cell that has acquired a mutation in the X-linked *PIGA* gene. The name of the disease refers to the occurrence of hemoglobinuria, the passage of red or dark brown urine.[1] Hemoglobinuria in patients with PNH is due to intravascular lysis of red blood cells that are abnormally sensitive to complement attack[2] (reviewed in Bessler[3]). Patients with PNH suffer from thrombosis and have complications of bone marrow failure.[4] The median survival has been estimated to be about 10 to 15 years.[5-7] Thrombosis is the most frequent cause of death. Apart from bone marrow transplantation, there is currently no cure for the disease. Interestingly, however, about 10% to 15% of patients show spontaneous remission, which may occur even after many years of disease. In the past, therapy was often restricted to the treatment and prevention of complications, for example, red blood cell transfusions for the treatment of anemia, anticoagulation for the prevention of thrombosis, or immunosuppression for the treatment of bone marrow failure (for review see Parker et al 2005[8]). The recent introduction of complement inhibitors for the treatment of PNH[9,10] makes it necessary to revise our traditional approach of treating patients with PNH. The purpose of this review is to summarize our current knowledge of the pathophysiology of PNH and highlight how this relates to a better understanding of many of its clinical symptoms, improves our approach to diagnosis, and leads to more specific and appropriate treatment of patients with this disease.

Clinical Manifestations

The natural history of PNH is that of a chronic disorder. The diagnosis of PNH is most frequently made in young adults; however, PNH occurs also in the elderly and in children. The disease affects both genders equally, is encountered in all parts of the world, and occurs in individuals of every socioeconomic status. The clinical manifestations of disease in patients with PNH are those of a hemolytic anemia, thrombophilia, and bone marrow failure. The degree to which each contributes to the clinical presentation varies between patients and during the course of the disease. The variability of clinical manifestations has led to the classification of PNH into clinical subgroups including hemolytic PNH and thrombotic PNH, also referred to as classic PNH, and PNH in the setting of other bone marrow failure conditions, such as aplastic anemia/PNH (AA/PNH) or myelodysplastic syndrome/PNH (MDS/PNH). Subclinical PNH or laboratory PNH refers to patients with a very small PNH clone and no clinical or laboratory signs of hemolysis.[8] **Table 1** summarizes clinical manifestations that may be seen in patients with PNH.

Pathophysiology of Disease in Patients with PNH

The diagram in **Figure 1** (see Color Figures, page 492) summarizes our current understanding of the pathophysiology of PNH. The two central components of PNH pathophysiology are bone marrow failure and the occurrence of blood cells that are deficient in all proteins linked to the membrane by a glycosyl phosphatidylinositol molecule (GPI-anchored proteins, PNH blood cells). Bone marrow failure is present in all patients with PNH, even when peripheral blood counts are normal and the bone marrow is hyper-

Table 1. Clinical manifestations of paroxysmal nocturnal hemoglobinuria (PNH).

Due to intravascular hemolysis:
- Anemia, hemoglobinuria, fatigue, acute / chronic renal failure, recurrent urinary tract infection,
- Abdominal pain, bloating, back pain, headache,
- Esophagospasms, erectile dysfunction
- Cholelithiasis
- *Rare:*
- Choledocho dyskinesia, acute pancreatitis
- Ischemia and ulceration of duodenum or colon

Due to thrombosis:
- Venous thrombosis:
- Abdominal vein thrombosis: Budd-Chiari, splenic, mesenteric, renal vein thrombosis
- Portal hypertension, esophageal varices, caput medusae (dilated abdominal veins)
- Cerebral vein thrombosis: headache, hemorrhagic infarct
- Retinal vein thrombosis, loss of vision
- Deep vein thrombosis, pulmonary emboli
- *Rare:*
- Cutaneous vein thrombosis, pyoderma gangrenosum
- Arterial thrombosis (less common):
- Stroke, myocardial infarction

Due to bone marrow failure:
- Anemia, infections, bleeding
- Myelodysplastic syndrome
- Bone pain
- *Rare:*
- Transformation to acute myeloid leukemia (AML)

cellular. The degree of bone marrow failure may vary; some may present with the clinical and laboratory picture consistent with severe aplastic anemia while others may have a reduced number of stem cells as the only sign of bone marrow failure. The causes of bone marrow failure underlying PNH are still poorly understood but most likely are heterogeneous.

The Cellular Phenotype of PNH Cells

The hallmark of PNH blood cells is that they are deficient in all surface proteins that use a GPI-anchor molecule.[11] Today, at least 27 different GPI-linked proteins are known to be expressed on blood cells, as summarized in **Figure 2** (see Color Figures, page 492).

GPI-linked proteins on hematopoietic cells carry out a multitude of functions. They serve as ecto-enzymes, accessory molecules for growth receptors, complement inhibitors, or adhesion molecules. In patients with PNH, all blood cell lineages and their progenitors are affected, although lymphoid lineages usually to a lesser extent. The deficiency of at least two of these GPI-linked molecules on circulating blood cells is sufficient for the diagnosis of PNH.[12] **Table 2** summarizes the laboratory tests used for the diagnosis of PNH.

Figure 3A (see Color Figures, page XX) shows the hemolysis of PNH red blood cells exposed to acidified serum. The acidified serum lysis test, also known as the Ham test, has been used for the diagnosis of PNH for the last 75 years. Today flow cytometric analysis of peripheral blood cells is preferred for the diagnosis of PNH, as it directly determines the percentage of blood cells that are deficient in the expression of GPI-anchored proteins (**Figure 3C**; see Color Figures, page 493). Typically, normal and PNH blood cells coexist in patients with PNH, and the percentage of myeloid cells deficient in GPI-linked proteins is used to indicate the size of the PNH clone. PNH red blood cells, due to their increased sensitivity to complement, have a reduced half-life in circulation. The percentage of PNH red blood cells therefore usually does not reflect the size of the PNH clone in the bone marrow. However, the flow cytometric analysis of red blood cells is used to determine the degree of GPI-anchor deficiency. PNH type III cells are defined as cells that completely lack the expression of all GPI-linked proteins, whereas PNH type II cells show some residual expression, and PNH type I cells express the proteins at normal levels (see **Figure 3D**; see Color Figures, page 493). This distinction is usually only used for red blood cells, as residual expression of GPI-linked proteins on circulating white blood cells is often more difficult to determine, and detection greatly depends on the antigen targeted for analysis. The diagnosis of PNH using flow cytometry is highly sensitive and specific for the diagnosis of PNH. Nonetheless, the diagnosis of PNH is delayed on average by 2 to 3 years. The major causes of this delay are that the correct patient population is not tested for PNH, the correct cell population is not tested, and that testing is only performed once.

Table 3 includes recommendations of whom should be tested for PNH, and for whom the analysis should be performed repeatedly.

The GPI-Anchor

GPI-anchored proteins are widely distributed among cell surface proteins in eukaryotic organisms and are highly conserved in all eukaryotic cells. In all species, the GPI-anchor shares a common core region consisting of ethanolamine (EthN) phosphate (P), three mannose residues (Man), glucosamine (GlcN), and inositol (I) (**Figure 4**; see Color Figures, page 494).[13]

The biological role of the GPI-anchor is not fully understood. GPI-linked proteins can be released by specific phospholipases (GPI-PLC and GPI-PLD (for review see Low[14]). In trypanosomes, GPI-PLC controlled release of the variant specific glycoprotein (VSG), the main coat protein, enables the parasite to evade the host immune response. The functional role of GPI-PLC and GPI-PLD in human tissues remains elusive. Good lateral mobility, which is another characteristic of the GPI-anchor, is likely relevant for many GPI-linked proteins that require the clustering of

Table 2. Laboratory texts for the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH).

Diagnostic tests	
Traditionally:	
• Ham test (acidified serum lysis) • Sucrose lysis test • Thrombin lysis test	The lysis of PNH red blood cells exposed to activated complement tests for the deficiency of CD59 and CD55 on red blood cells. The tests vary in the pathways activating complement. Advantage: Cheap and simple to perform Disadvantage: Labor intensive, decreased sesitivity due to the short half-life of circulating PNH red blood cells.
Today:	
• Flow cytometric analysis:*	
- CD59 and/or CD55 on peripheral blood red cells	Advantage: Useful to determine the degree of GPI anchor deficiency (PNH type I, type II, type III). Disadvantage: Decreased sensitivity due to the short half-life of circulating PNH red blood cells.
- CD59, CD24, CD16, or any other GPI-linked proteins expressed on peripheral blood granulocytes	Advantage: The deficiency of at least 2 GPI-linked proteins is sensitive and specific for the diagnosis of PNH. Disadvantage: Might be difficult to perform in severe aplastic anemia when the number of circulating granulocytes is very low.
- FLAER (fluorescently labeled inactive toxin aerolysin) binding of peripheral blood granulocytes	FLAER binds the GPI anchor. Advantage: The lack of FLAER binding on granulocytes is sufficient for the diagnosis of PNH. Disadvantage: Cannot be used for the analysis of red blood cells or platelets. Might be difficult to perform in severe aplastic anemia when the number of circulating granulocytes is very low.
- *PIGA* gene mutation analysis	Although very specific is *NOT* used for diagnosing PNH.
Supportive laboratory tests	
• Increased lactate dehydrogenase (LDH) • Low haptoglobin • Increased unconjugated bilirubin • Hemoglobinuria • Hemosiderinuria • Reticulocytosis • Erythroid hyperplasia in the bone marrow	Are parameters of intravascular hemolysis, they are supportive but not diagnostic.

* The flow cytometric analysis of bone marrow cells or the expression of GPI-linked proteins is of *NO* diagnostic value.

Table 2. Who should be tested for paroxysmal nocturnal hemoglobinuria (PNH) and how often?

Once	Repeatedly*
• All patients with hemoglobinuria	• All patients with PNH
• All patients with unexplained hemolysis (increased LDH)	• All patients who have aplastic anemia
• All patients with abdominal and cerebral vein thrombosis	• All patients who have had aplastic anemia (except after bone marrow transplantation
• All patients with thrombocytopenia and macrocytosis or signs of hemolysis	• All patients with myelodysplastic syndrome (MDS)

* Initially once every 6 months; then annually

proteins for optimal function. GPI-anchor molecules are also associated with microdomains, or "rafts," enriched in glycosphingolipids and cholesterol. The association with microdomains contributes to the specific surface distribution of GPI-linked proteins and aids in the recruitment of accessory molecules for cell signaling.

Figure 4 (see Color Figures, page 494) is a schematic illustration of GPI-anchor synthesis (reviewed in Maeda et al[15]). In all PNH cells examined, the defect has always occurred in the very first step of anchor synthesis when *N*-acetylglucosamine is transferred onto phosphatidylinositol.

Genetics of PNH

PNH is an acquired disease. No inherited form has been described. Patients with PNH have somatic mutations in the X-linked *PIGA* gene. *PIGA* stands for phosphatidylinositol glycan complementation class A. *PIGA* encodes a protein subunit essential for the activity of *N*-acetylglucosaminyltransferase (UDP-GlcNAc:PI-α-1,6-GlcNAc–transferase), an enzyme required for the synthesis of GPI-anchor molecules.[16] *PIGA* maps to the short arm of the X chromosome, is approximately 17 kb long, and has 6 exons.[17,18] Due to its localization on the X chromosome, and due to X inactivation in female somatic cells, only one mutation is required in either males or females to abolish the expression GPI-linked proteins. To date over 180 mutations have been identified in the *PIGA* gene in blood cells from patients with PNH (**Figure 5**; see Color Figures, page 494).[19] The mutations are spread over the entire coding region with no obvious clustering. The majority of *PIGA*

gene mutations are frame shift mutations that predict an inactive PIGA protein and a loss of glycosyltransferase activity, which explains the lack of GPI-anchored molecules on blood cells from patients with PNH. Type II PNH cells derive from point mutations in the *PIGA* gene that encode a protein with some residual function, which explains their low-level expression of GPI-linked proteins. Because all blood cell lineages carry the same mutation, the *PIGA* gene mutation must occur in an early hematopoietic progenitor cell, possibly a hematopoietic stem cell.[20,21]

Two inherited genetic conditions have been described that share some of the clinical features characteristic of patients with PNH. In one case a patient was described with a complete deficiency of CD59 due to homozygosity for a frame shift mutation in the *CD59* gene.[22,23] The other case concerns two families with an inherited deficiency of GPI anchor synthesis due to homozygosity for a hypomorphic mutation in the promoter region of the *PIGM* gene, another enzyme essential in GPI anchor synthesis (**Figure 4**; see Color Figures, page 494), resulting in decreased expression of GPI-linked proteins on the cell surface.[24] The individual with the inherited CD59 deficiency suffers from attacks of complement-mediated hemolysis and arterial thrombosis. Individuals who inherited the *PIGM* promoter mutations have only very mild hemolysis, but mainly suffer from venous thrombosis and a severe seizure disorder.[24] Bone marrow failure, which is a central component of PNH, is absent in individuals with either inherited condition.[22-24] Both inherited conditions are exceedingly rare.

Complement Activation and Intravascular Hemolysis in PNH

In humans, the complement system consists of more than 30 plasma and cell-surface proteins. Complement activation occurs along a chain of reactions involving proteolysis and assembly, and results in the deposition of C3b clusters on a target cell (**Figure 6**; see Color Figures, page 495). This initiating or activating pathway is followed by the lytic pathway, during which the membrane-attack complex (MAC) is assembled, forming a hole in the membrane and causing lysis of the target cell. **Figure 6** (see Color Figures, page 495) schematically summarizes the pathway of complement activation. A number of proteins regulate complement activation on the surface of human cells. Most human cells, including platelets and white blood cells, express two GPI-linked inhibitors of complement activation (CD55 and CD59) as well as membrane cofactor protein (MCP or CD46),[25] another inhibitor of complement activation that is localized at the plasma membrane via a transmembrane domain. Human red blood cells lack MCP and express only CD55 and CD59 to guard against inappropriate complement activation on their cell surface. CD55 and CD59 are GPI-linked and consequently are deficient on PNH cells. PNH red blood cells are therefore much more sensitive to lysis by complement than PNH platelets or white blood cells.

In the past the clinical effects of intravascular hemolysis in patients with PNH have been largely undervalued. Only with the availability of complement-blocking drugs, which also efficiently block intravascular hemolysis, have the clinical symptoms associated with hemolysis became increasingly appreciated. The lysis of red blood cells leads to the release of hemoglobin into the circulation. Hemoglobin is bound by haptoglobin before being efficiently cleared from the circulation. However, after the binding capacity of haptoglobin is saturated, free hemoglobin is found in the plasma of patients with PNH. Cell-free hemoglobin rapidly and irreversibly binds nitric oxide, leading to a rapid drop of nitric oxide levels in peripheral blood. Nitric oxide is a regulator of smooth muscle tone. Nitric oxide scavenging by free hemoglobin leads to smooth muscle contraction with consequent vasoconstriction, constriction of the gut, and pulmonary hypertension. These effects could explain many of the clinical symptoms that are seen in PNH patients in conjunction with a hemolytic attack. These symptoms significantly improve after treatment with complement inhibitors and include abdominal pain, bloating, back pain, headache, esophageal spasms, erectile dysfunction, and fatigue.[26]

PNH Hemolysis and Renal Failure

Renal vein thrombosis, acute tubulonecrosis due to pigment nephropathy, and recurrent urinary tract infection are the major causes of renal failure in patients with PNH. Renal vein thrombosis, in contrast to pigment nephropathy, is associated with flank pain and macroscopic hematuria. Acute tubulonecrosis and acute renal failure usually occur after a major hemolytic attack and are due to hemoglobinuria and the toxicity of heme and iron, decreased renal perfusion, and tubular obstruction with pigment casts. Siderosis of the kidney is found by magnetic resonance imaging (MRI).[27] Progressive chronic renal failure occurs after years of hemoglobinuria and is associated with significant glomerulonecrosis, tubular atrophy, and interstitial fibrosis. Hemoglobinuria is also associated with recurrent urinary tract infections, particularly in female patients with PNH.

Thrombosis in PNH

Patients with PNH have an increased propensity for the development of life-threatening venous thrombosis, particularly in cerebral, hepatic, portal, mesenteric, splenic, and renal veins. About 40% of patients with PNH experience a thrombotic event during the course of the disease.[5-7] Venous thrombosis is the major cause of death in patients with PNH.[6] While it had been thought that the frequency of venous, but not arterial thrombosis, is increased in patients with PNH, more recent clinical data, obtained during the international trials of complement inhibitors in 195 pa-

tients with PNH, suggested that the frequency of arterial thrombosis is also increased.[28] The pathophysiology of thrombosis in patients with PNH is not fully understood. Patients with a large PNH clone are at a higher risk of developing thrombosis than patients with a small PNH clone.[28-30] This suggests that there is a direct relationship between the number of circulating PNH blood cells and the risk of thrombosis. Numerous studies have been performed to explain the thrombophilia seen in patients with PNH.[31-35] Increased procoagulant and fibrinolytic activity, suggesting increased fibrin generation and turnover,[33] have been identified. In addition, several failures of the fibrinolytic system have been identified, including a deficiency of urokinase-type plasminogen activator receptor on PNH granulocytes, and increased plasma levels of soluble urokinase-type plasminogen activator receptor.[35] Platelets from PNH patients are deficient in GPI-linked proteins[36] (**Figure 1**, Color Figures, page 492), and, although to a lesser degree than PNH red blood cells, they are hypersensitive to complement.[31,32] However, none of these identified platelet or coagulation abnormalities can fully explain the hypercoagulable state in patients with PNH.

Interestingly, thrombosis in PNH patients frequently follows episodes of acute hemolysis, suggesting that inadequate complement inhibition or hemolysis might be an important underlying factor contributing to the thrombophilia seen in these patients. Indeed, recent results from the international trial using complement inhibitors for treatment of hemolysis in patients demonstrated that inhibition of complement activation and inhibition of intravascular hemolysis drastically reduce thrombosis in patients with PNH[28] (see also Dr. Hillmen's contribution in this volume[37]). The pathway by which intravascular hemolysis feeds into the pathway of thrombogenesis in PNH is not fully understood. However, nitric oxide scavenging through cell-free plasma hemoglobin (reviewed in Lancaster 1997[38]), and alterations of platelet and red cell membranes, including the formation of microparticles,[39] are thought to play important roles in the pathogenesis of thrombosis in this disorder (reviewed in Cappellini[40]).

Lessons from the Mouse Model of PNH

The inactivation of the *Piga* gene in mouse embryonic stem cells (ES cells) is lethal in early development. However, despite the lack of all GPI-linked proteins, *Piga*-deficient ES cells are competent for hematopoietic differentiation.[41,42] By restricting the inactivation of the mouse *Piga* gene specifically to hematopoietic cells, mice have been generated that have a proportion of blood cells deficient in GPI-linked proteins, thus faithfully mimicking the cellular phenotype of blood cells from patients with PNH.[43-45] Although these mice perfectly reproduce the cellular phenotype of PNH blood cells, including increased sensitivity to complement, they do not develop spontaneous hemolysis or thrombosis. One of the major reasons is that in addition to CD55 and CD59, mice also express the transmembrane protein CRRY on their red blood cells, which is a potent inhibitor of complement activation.[46] The study of mice with blood cells deficient in GPI-linked proteins demonstrated that lack of GPI-linked proteins is not sufficient to cause hematopoietic progenitor cells to expand[43,44] (see also Dr. Brodsky's discussion in this volume[47]). These studies also showed altered T-cell survival and response, and further indicated that in addition to complement-mediated lysis there is also extravascular hemolysis mediated by macrophages.[48] Indeed, subsequent studies of patients on complement inhibitors demonstrates a previously unappreciated extravascular hemolysis that becomes unmasked, and may become clinically significant in patients treated with complement component C5-inhibiting antibodies (personal observation). The pathways of extravasuclar hemolysis in mouse and man remain to be determined.

In summary, PNH is an outstanding example of how an increased understanding of pathophysiology may directly improve diagnosis, care, and treatment of disease. However, several crucial questions remain that need to be answered in order to fully understand the secrets of this disease and to cure patients of PNH.

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