

Molecular Pathogenesis of Paroxysmal Nocturnal Haemoglobinuria

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Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired anaemia caused by complement-mediated haemolysis. Patients with PNH have abnormal red blood cells that are sensitive to complement due to the deficiency of cell surface complement regulatory proteins CD59 and decay-accelerating factor (DAF, CD55). The CD59/DAF-deficient red blood cells are lysed by complement slowly during physiologic low-level activation and rapidly during elevated activation caused by infections and other events. The affected red cells are of clonal origin and coexist with normal cells at a ratio which varies among patients. The CD59/DAF-deficient cell population appears also in various other haematopoietic lineages but not in nonhaematopoietic lineages, indicating that PNH is a haematopoietic stem cell disorder.

CD59 and DAF are glycosylphosphatidylinositol (GPI)-anchored proteins whose cell surface expression is dependent on posttranslational attachment of the GPI glycolipid anchor (reviewed in 1,2). The deficiency of their surface expressions seen in PNH is caused by defective biosynthesis of the GPI anchor (reviewed in 3,4). At least seventeen GPI-anchored proteins, such as alkaline phosphatase and acetylcholinesterase, are known to be deficient on various haematopoietic cells.^(3,4) A gene responsible for the deficiency of the GPI-anchor in PNH was cloned four years ago through a somatic genetic approach.

Somatic Genetic Approach Towards a Gene Responsible for GPI-Anchor Deficiency in PNH

Hyman and colleagues established many GPI anchor-deficient mutant cell lines from murine thymoma cells and grouped them into several complementation classes by somatic cell hybridization technique.⁽⁵⁾ Biochemical analysis of glycolipids in these mutant cells of different complementation classes elucidated respective defective steps in the GPI biosynthesis pathway, in which at least eight steps are involved (reviewed in 2). We took advantage of this successful somatic genetic approach to determine a defective biosynthesis step in affected blood cells from patients.

We established affected B lymphoblastoid cell lines from two patients that are deficient in the surface expressions of CD59 and DAF. We hybridized them by cell fusion to the classified mutant thymoma cells and found that PNH cell lines from both patients belong to complementation class A.⁽⁶⁾ Cells of class A are defective in the first step of the biosynthesis of GPI, which is a transfer of N-acetylglucosamine (GlcNAc) to phosphatidylinositol (reviewed in 1,2). The PNH cell lines were also defective in the first step.⁽⁶⁾ These results, confirmed by other groups (reviewed in 3,4), established that a class A mutant gene that participates in the first step of GPI biosynthesis is defective in the affected cells from many patients with PNH.

We further took a genetic approach to clone the gene responsible for the class A mutation and hence for GPI-anchor deficiency in PNH. We transfected a HeLa expression cDNA library into human B lymphoblastoid mutant cells of class A complementation group, concentrated cells that regained the surface expressions of CD59 and DAF by means of a cell-sorter, and recovered plasmids from them. We obtained a cDNA that restored synthesis of GPI anchor precursors in, and the surface expression of GPI-anchored proteins on, class A mutant cells upon transfection, and termed the gene PIG-A (phosphatidylinositol glycan-class A).⁽⁷⁾

As expected, PIG-A cDNA restored the surface expressions of CD59 and DAF on the affected B lymphoblastoid cells from the two patients.⁽⁸⁾ The cells from one of the patients, female, transcribed only abnormal PIG-A mRNA, which lacked the entire exon 5. We found a responsible mutation, a T deletion within the 5' splice site of intron 5, in one of the alleles of PIG-A. This was a somatic mutation presumably in a hematopoietic stem cell because wild-type B lymphoblastoid cells derived from the same patient did not have it and because affected granulocytes also had it.⁽⁸⁾ To understand the selective transcription of the mutant allele, we determined the chromosomal location of the PIG-A gene and found that it resides on the short arm of the X-chromosome at Xp22.1.⁽⁸⁾ Since one of the X-chromosomes in female somatic cells is inactivated (X-chromosome inactivation or lyonization), the selective transcription of the mutant allele should be due to the presence of the mutant allele on the active and the normal allele on the inactive chromosomes. The X-chromosomal location of PIG-A, therefore, accounts for the phenotypic expression of a recessive somatic mutation. These results indicated that somatic mutation in PIG-A is responsible for GPI-anchor deficiency of affected cells from patients with PNH.⁽⁸⁾

Structure and Function of the PIG-A Gene and Protein

The PIG-A cDNA consists of 3589 bp and contains an open reading frame of 1452 bp that encodes a protein of 484 amino acids.⁽⁷⁾ The PIG-A gene consists of six exons that span about 17 kbp.^(9,10) Two alternative splice sites within exon 2 generate two shorter transcripts without a functional activity of PIG-A.^(11,12)

PIG-A is one of the three genes—PIG-A, -H and -C—that are necessary for synthesis of the first intermediate, N-acetylglucosaminyl phosphatidylinositol, in the endoplasmic reticulum (ER) (reviewed in 1,2). PIG-A protein is an ER transmembrane protein with its large amino-terminal portion on the cytoplasmic side and its small carboxy-terminal portion on the luminal side of the ER membrane (R. Watanabe, unpublished data). A region within the cytoplasmic portion has homology to a bacterial GlcNAc transferase, termed RfaK, that is involved in synthesis of lipopolysaccharide, suggesting that PIG-A is a catalytic subunit of the GlcNAc transferase.⁽¹³⁾ We recently demonstrated that PIG-H is also an ER membrane protein facing the cytoplasm and that it forms a complex with PIG-A (R. Watanabe, unpublished data). Although the function of PIG-H is not known, these results are in agreement with the idea that the first intermediate is formed on the cytoplasmic side of the ER. We obtained PIG-C cDNA recently; however, its cellular localization has not been determined (N. Inoue, unpublished data).

Somatic Mutations of PIG-A in Patients with PNH

The PIG-A gene has been analyzed in affected cell lines and/or granulocytes from more than 100 patients with PNH from American, European and Asian countries.^(8,11,12,14-23) Somatic mutations were found in most of them. In several patients, abnormality of PIG-A was demonstrated only at the mRNA level, presumably because responsible mutations reside within yet unidentified regulatory regions in PIG-A. It seems, therefore, that PIG-A is responsible for GPI-anchor deficiency in most if not all patients with PNH worldwide. In a few cases, attempts to determine the mutations have been unsuccessful due to the small fraction of affected cells.

The X-chromosome location of PIG-A would account for this uniformity of the responsible gene among ten or so genes involved in biosynthesis of the GPI-anchor. As discussed above, one mutation in PIG-A would cause GPI-anchor deficiency for both males and females. In contrast, mutations should occur in both alleles in a single cell for autosomal genes. Since this event would be very rare, the X-chromosomal location of PIG-A would be the basis of its consistent involvement if all other GPI-synthesis genes are autosomal. In fact, the three other genes—PIG-F, -H and -B—localized to date are on autosomes.⁽²⁴⁻²⁶⁾

Somatic mutations varied among patients and are small, 83 % involving only one or two bases. Of 122 mutations determined, 40 were single-base deletions and 41 were base substitutions. There were ten single-base insertions, sixteen 2-14 base deletions, eight 2-19 base insertions and one 4 kb deletion. Six cases were deletion/insertion. They were distributed widely in the coding regions and splice sites. There was no mutation clustering region, suggesting that mutations occur at random sites.

Some patients had two to four mutant clones. Affected granulocytes usually contained a dominant clone, so only one mutation was identified in each of most patients by analysis of DNA and/or RNA from total granulocytes. In exceptional cases, two major clones of granulocytes coexist and both mutations were identified.⁽¹⁷⁾ Mutations in minor clones have been successfully determined by analyzing lymphoid cell lines or colonies/bursts of CFU-GM or BFU-E. According to data so far reported, two or more clones have been identified in about 10% of patients. It is known that a quarter to a third of patients have a partially deficient red blood cell population and a completely deficient population. A mutation that causes partial deficiency must be different from that which causes complete deficiency, suggesting that those patients may have two mutant clones.⁽¹⁵⁾ The real percentage of patients bearing multiple clones, therefore, would be much higher.

Is the PIG-A Mutation Sufficient for PNH?

There are two key events that lead to PNH: 1) Generation of a GPI-anchor deficient haematopoietic stem cell due to a somatic mutation of PIG-A, and 2) clonal expansion of that mutant and subsequent appearance of a GPI-anchor deficient red blood cell population. It is not clear whether mutation of PIG-A alone causes both GPI-anchor deficiency and clonal expansion or whether other factors are involved in clonal

expansion. We addressed this issue by making GPI-anchor deficient mice.⁽²⁶⁾ We disrupted a mouse homologue of PIG-A, termed *Pig-a*,⁽¹³⁾ in a male embryonic stem (ES) cell. This resulted in a loss of the surface expression of GPI-anchored proteins, showing that biosynthesis of the GPI-anchor was abolished due to the disruption of *Pig-a*.⁽²⁷⁾ We made chimaeric mice with this GPI-anchor deficient ES cell. Because of a lethal effect of GPI-anchor deficiency, high chimaerism was not achieved. Six of the chimaeras had 0.2 to 4% GPI-anchored protein-negative red blood cells three weeks after birth. If the loss of *Pig-a* function alone confers an ability to expand on haematopoietic stem cells, proportions of the GPI-anchored protein-negative red blood cells would increase with time. So we monitored them for five to seventeen months. They were constant or decreased until ten months, indicating that *Pig-a* mutation does not cause immediate expansion of the haematopoietic stem cells.⁽²⁷⁾ Therefore, other pathological or physiological factors may be necessary for the clonal expansion of GPI-anchor deficient cells.

Interestingly, a proportion of the GPI-anchored protein-negative red blood cells in one of the six mice began to increase at twelve months of age from 4-5% and reached 30% at seventeen months of age when the mouse died for an unknown reason.⁽²⁷⁾ This mouse may have experienced other factors. If this is true, mice that harbor an inactivating mutation of *Pig-a* in haematopoietic stem cells may have a good chance to experience other factors leading to clonal expansion of the mutant.

Conclusions and Perspectives

Somatic mutation of PIG-A is responsible for the GPI-anchor deficiency in most if not all patients with PNH worldwide. Results with *Pig-a*-deficient mice, however, suggested that other factor(s) may also be involved in the clonal expansion of the mutant haematopoietic cells. Identification of these factors is essential for a better understanding of the molecular basis of PNH. A mouse model would be useful for this. To obtain mice developing clonal expansion, many chimaeric mice should be generated. To circumvent a lethality caused by GPI-anchor deficiency in various tissues, use of conditional or tissue-specific knock out of *Pig-a* by means of Cre-loxP system should be effective.

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