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THE PIG-A GENE SOMATIC MUTATION RESPONSIBLE FOR PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria is the first example of a non neoplastic human disease caused by the somatic mutation of an X-linked gene. The PIG-A gene maps to Xp22.1 and is required for the transfer of N-acetyl glucosamine to phosphoinositol, an early step in the production of the GPI anchor. A deficiency of GPI-linked proteins on the cell surface is responsible for the PNH cell defect, which can be detected by flow cytometry not only on red cells, but also on myeloid cells and in some patients even on lymphoid cells. Its location on the X-chromosome explains how a single recessive mutation can cause the appearance of the abnormal clone. A number of patients may have more than one PNH clone, suggesting that the expansion of GPI-deficient clones occurs under the pressure of a selection mechanism.

Key words: PNH, PIG-A gene, GPI anchor

Twenty-six years ago Lucio Luzzatto and his group in Ibadan, through the study of two African women suffering from paroxysmal nocturnal hemoglobinuria (PNH) and heterozygous for G-6-PD, produced the first evidence that PNH is a clonal disorder of hematopoiesis. Only one type of G-6-PD was found in the lysate after the Ham test, while both types were present in untreated red cells.[1] Later, *in vitro* studies showed that hematopoietic progenitor cells from PNH patients could give rise to two different types of erythroid colonies, normal and PNH,[2] thus confirming that the PNH defect was transmitted from a cell to its progeny. Only recently the molecular basis of the PNH defect has been fully elucidated, and one might have thought that the problems posed by this rare disease had finally been resolved; however, some of the new findings raise other intriguing questions.

Too many markers

For several decades PNH was identified on the basis of a typical clinical picture and a few tests devoted to detecting *in vivo* or *in vitro* red cell fragility. However, starting in the late 50's, an increasing number of molecules were found defective on the PNH cell surface: acetylcholinesterase first,[3] alkaline phosphatase next,[4] then the decay accelerating factor (DAF),[5] and soon after that many others, amounting now to more than a dozen different proteins.[6] It did not take long to realize that the primary defect in PNH could not directly cause reduced production of so many different molecules. Since it was known that DAF is bound on the outer cell surface through a special structure, the glycosylphosphatidyl-inositol (GPI) anchor,[7] it was obviously important to verify the type of binding of the other proteins, and it turned out that all of them had the same anchoring structure. A failure of the anchoring mechanism could well explain the multiplicity of defective molecules. Two important biological and diagnostic implications derived from this finding. First of all, red cell hypersensitivity to activated complement was only one, albeit the most impressive, aspect of the PNH defect. Second, surface

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marker detection by monoclonal antibodies and cytofluorimetric analysis could be a rapid, specific and sensitive tool for diagnosing PNH and at the same time measuring the size of the PNH population and the lineages involved.[6]

The GPI anchor

The possibility of having a number of molecules bound on the outer membrane surface without a transmembrane portion must have very important biological implications, since GPI-linked proteins are widely present in eukaryotic cells and highly conserved during evolution.[8] The best known examples are the *Trypanosoma* species, whose surface antigens are often GPI-linked. It is thought that this is a useful trick for the parasite in order to rapidly get rid of antigenic determinants against which the host has produced specific antibodies.[9,10] With minor changes, the GPI anchor has the same structure in all species (Figure 1); it is used to bind on the cell surface a large number of molecules having different structures and functions. A number of functional properties have been associated with this peculiar type of link: lateral mobility, capping, ease of assemblage and removal, endocytosis, exocytosis, potocytosis.[8,11-13] Some of these, or other still unknown properties, must be essential for cell biology.

Anchor biosynthesis

GPI assemblage is a multistep process occurring in the endoplasmic reticulum. Once completed, the anchor is covalently attached to the preformed protein by truncation of a 17-31-aminoacid residue on its C terminal end.[14-15] Two molecular forms are produced for some proteins, xwz of gene duplication or by alternative splicing of a single gene product: a transmembrane form and an isoform that will acquire the GPI anchor in the endoplasmic reticulum. The biosynthetic pathway is depicted in Figure 2. Most of the information on it has been obtained through the study of murine or human GPI-deficient cell lines produced by experimental mutagenesis followed by immunoselection. Having a number of defective cell lines, cell fusion experiments made it possible to characterize different complementation groups; the biochemical defect was then identified by metabolic studies, even though some of the pertinent enzymes are not fully characterized. Cells from three different complementation classes (A,C,H) fail to synthesize an early intermediate (N-acetylglucosaminyl-phosphatidyl inositol), indicating that this biosynthetic stage can be impaired by numerous defects. Other dasses, such as B and F, have defects in a late reaction step.[16-24]

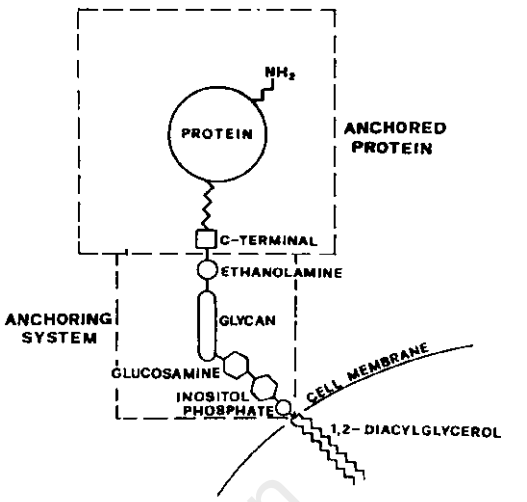

Figure 1. The GPI anchor structure.

PNH cell lines

The observation coming from cytofluorimetric analysis that in some PNH patients even B lymphocytes might belong to the PNH clone led to the idea of generating PNH cell lines by immortalizing them through EBV infection.[25,26] Next, T lymphoblastoid PNH cell lines were also generated by HTLV-1 transformation.[27,28] Since only a portion of lymphoid cells in a PNH patient carries the PNH defect, it was possible to obtain both normal and PNH lines from a single patient. A greater number of PNH cell lines were obtained by negative immunoselection for lymphocytes expressing GPI-linked molecules.[26] Within a few months several PNH cell lines were established in London and in Japan, and new data of paramount importance

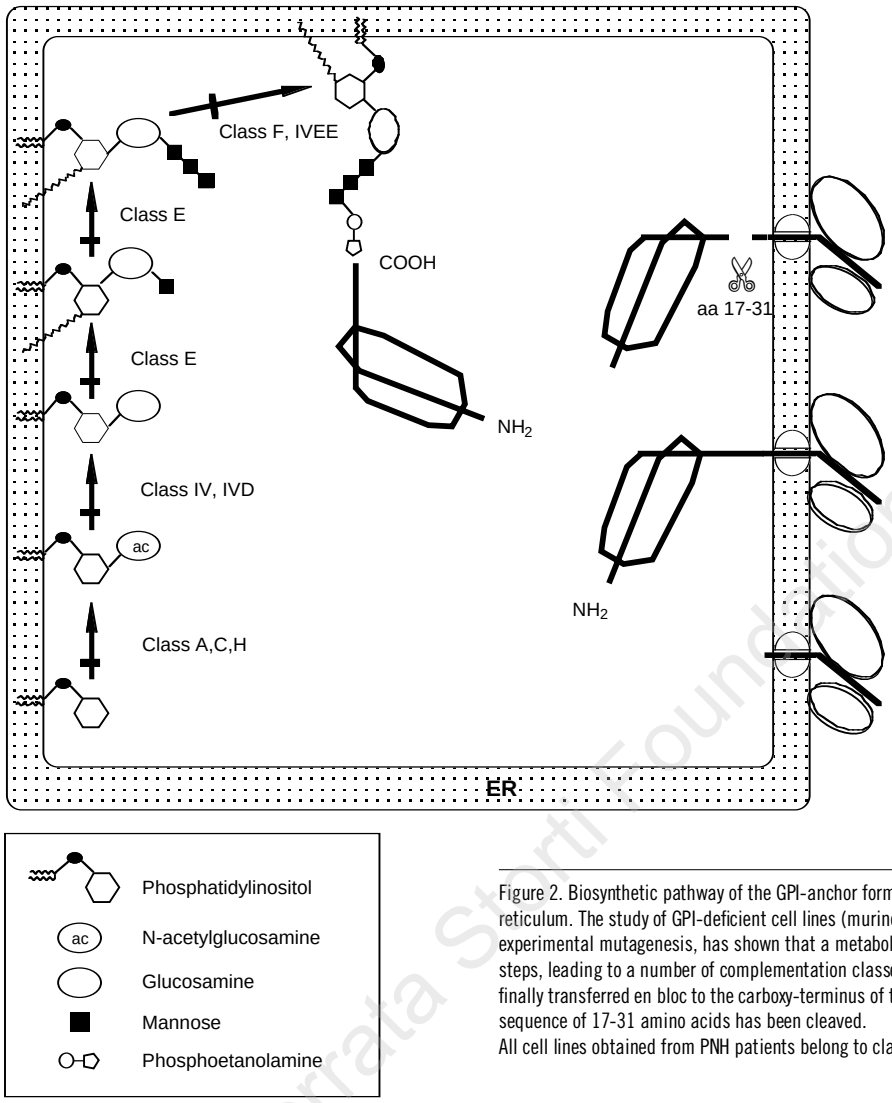

Figure 2. Biosynthetic pathway of the GPI-anchor formation in the endoplasmic reticulum. The study of GPI-deficient cell lines (murine and K562), obtained by experimental mutagenesis, has shown that a metabolic block may occur at various steps, leading to a number of complementation classes. The complete GPI-core is finally transferred en bloc to the carboxy-terminus of the protein, from which a sequence of 17-31 amino acids has been cleaved.
All cell lines obtained from PNH patients belong to class A.

were rapidly obtained: (i) it was shown by hybrid cell generation that a single copy of the putative gene (unknown at that time) was sufficient to restore a normal pattern of GPI-linked molecules,[29] raising the question of how an apparently recessive disease could be caused by a single somatic mutation; (ii) in cell fusion experiments uncovered the surprising fact that all PNH lymphoblastoid cell lines were able to correct any complementation class of GPI-deficient cell lines except group A, i.e. all the PNH lines obtained from different patients belonged to complementation group A; (iii) finally, having the PNH cell lines was the key to identifying the PNH gene.

The PIG-A gene

Using expression cloning methods, the group of Taroh Kinoshita in Japan was able to identify in 1993 the genes whose mutations were responsible for the complementation class A and F mutant cell lines, which they called PIG-A and PIG-F, respectively.[30,31] The transfection of PIG-A into a GPI-deficient lymphoblastoid cell line obtained from a PNH patient restored surface expression of the GPI-anchored proteins, thus giving formal proof that the PNH gene had been pinpointed.[32,33] Chromosomal assignment was then rapidly performed using a variety of techniques, including FISH;[32,34] the gene was mapped on the short arm of chromo-

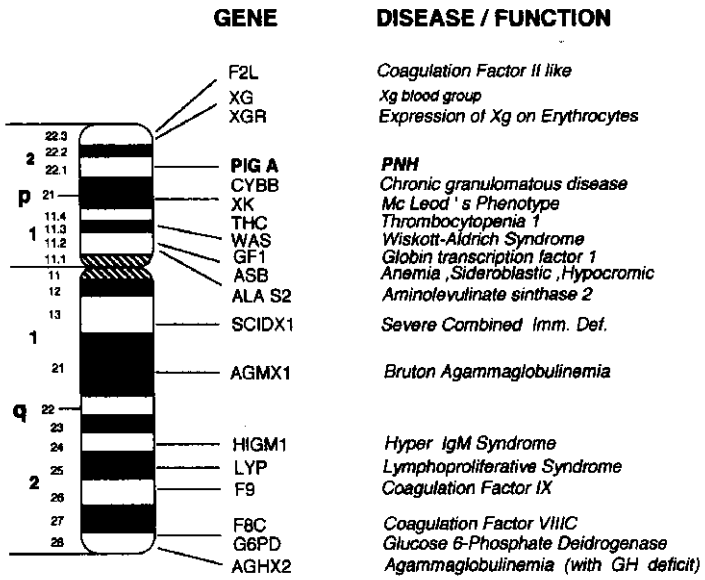

Figure 3. List of the numerous genes involved in hematological disorders that are located on the X-chromosome, to which PIG-A has been added.

some X (Figure 3). This finding explains the apparent paradox of a recessive disease caused by a single somatic mutation. Indeed the PIG-A gene is functionally present in a single copy in every human cell (males are hemizygotes, females have only one active X), thus a single mutation is sufficient for the cell to acquire the PNH defect. PIG-A is probably the only GPI-related gene located on the X chromosome, and this explains why of all the genes involved in GPI biosynthesis, PIG-A is the only one responsible for the naturally occurring human disease.

The gene was then extensively investigated.[35,36] It was found to consist of 6 exons spanning 17 kb (Figure 4); the first exon and most of the last one are non coding areas. Three different mRNA transcripts were identified in normal cells, probably arising from alternative splicing in the area of exon 2.[36] Bessler discovered a PIG-A pseudogene which has been mapped on chromosome 12q12 and found to lack intronic sequences.[36] Little information is available on the protein encoded by the PIG-A gene; it is still not known whether the protein itself is the lacking enzyme or if it is indirectly connected with enzyme production or function. However, the functional role of the PIG-A protein is certainly intracellular, and therefore even a large

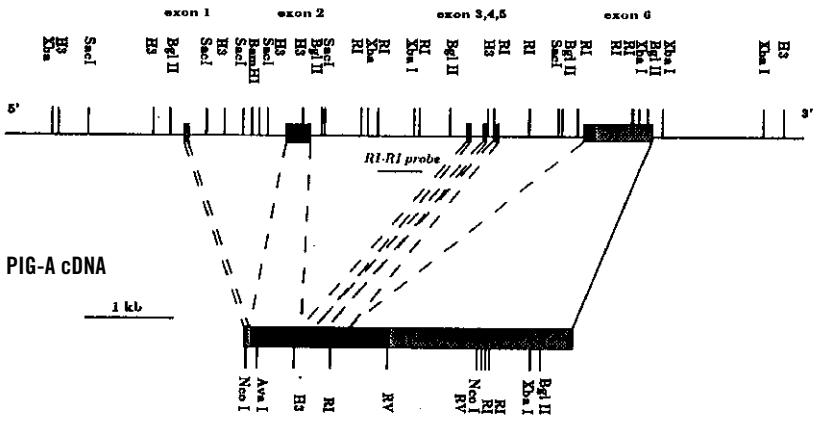

Figure 4. Genomic structure of the PIG-A gene (from Bessler *et al.*[36] slightly modified). Solid areas correspond to translated sequences, shaded areas are untranslated.

number of normal cells in a PNH patient are of no help in correcting the metabolic abnormality of the mutant clone.

PIG-A mutations in PNH patients

The structural gene abnormality causing the PNH defect was investigated by RT-PCR and nucleotide sequencing initially in PNH lymphoblastoid cell lines, then directly on granulocytes from PNH patients.[32,33,37,38] A variety of molecular lesions were found, such as bp insertions, short deletions, or single point mutations, involving either exonic or intronic sequences and causing a frameshift, a stop codon, a new site for alternative splicing, or other mechanisms leading to a non functional gene. In a series of 26 patients Luzzatto's group has identified by sequence analysis 34 PIG-A gene mutations so far[39] (and personal communication); molecular lesions were different in each patient and spread out along the entire gene, i.e. no specific mutable sites were found inside the gene. The type of molecular defect may dictate the PNH phenotype. Complete absence of a functional transcript (which we may call PIG-A°, by analogy to the thalassemia terminology) is expected to produce a clone totally deprived of GPI-linked surface molecules. Cells so affected will match the isotypic negative control in cytofluorimetric analysis, and they are called PNH III in terms of red cell serological studies. The presence of a residual amount of functional transcript (PIG-A$^+$), as can be found mainly with missense mutations, will be associated with a PNH population that appears positive by flow cytometry analysis for GPI-linked molecules, but clearly shifted to the left with respect to the positive control cell profile, and corresponds to the PNH II population in red cell serological studies. From a clinical point of view, we may expect that a PIG-A° PNH clone will cause a more severe disease than a PIG-A$^+$ PNH clone, at least as far as hyperhemolysis is concerned. Thus, there is now a molecular basis to account for the existence of PNH clones with different degrees of defect severity,[40] a fact that had already been described long ago.[41] However, it was also observed that a few patients may simultaneously possess PNH III and PNH II cell populations in circulation; in the past this had been a strong argument in favor of those who objected to the clonal origin of the PNH cells.

A PNH patient may have more than one PNH done

The first piece of evidence that PNH patients may harbor more than a single PNH clone came from the establishment of lymphoblastoid cell lines: different cell lines obtained from the same patient may have different PNH characteristics.[25] Later it was definitely proven that different cell lines obtained from the same PNH patient may carry different molecular defects.[32,33] Some patients may have two PIG-A° or two PIG-A$^+$ PNH clones which are indistinguishable from each other by cytofluorimetric analysis or red cell functional tests. Others may have a PIG-A° clone associated with a PIG-A$^+$ clone. In the latter case, flow cytometry analysis will show two PNH cell populations[42] (amounting to three cell populations if residual normal cells are present), and both PNH III and PNH II populations will be found by red cell functional tests. The coexistence of more than one PNH clone in a single PNH patient does not mean that the disease is not clonal (clonal does not necessarily means monoclonal); rather it may help to elucidate the pathogenesis of the disease.

The PIG-A knock-out mouse

Kinoshita has recently presented preliminary data on a mouse whose PIG-A gene had been knocked out (ASH Meeting, Nashville 1994, oral presentation). The mouse was born with about 15% PNH cells in its blood. Sequential measurements for several months did not show increase in the PNH cell population, excluding any proliferative advantage of the PNH clone over normal hematopoiesis. Thus, a PIG-A gene alteration seems to be sufficient for the appearance of a PNH clone but not for its expansion. Mice with a more extended PIG-A gene deficiency have not yet been reported. A complete absence of the gene in all cells may well be lethal.

The selective pressure hypothesis

Both recent findings: the absence of an

absolute proliferative advantage for the PNH population and the presence of multiple PNH clones in a single patient, clearly indicate that some kind of selection is operating in PNH patients whereby GPI-deficient cells are allowed to grow while normal cells are inhibited. This hypothesis had already been formulated some years ago on a purely speculative basis[43,44] and it will require more direct confirmation. It implies that a PNH patient is actually suffering from two diseases, the first (inhibition of normal hematopoiesis) giving rise to the second (escape of GPI-deficient clones). If confirmed, it may have important clinical implications for PNH treatment; moreover, it could well be a model for the interpretation of other non neoplastic clonal hematological disorders.

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