Review

The *In Vivo* *Pig-a* Gene Mutation Assay

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The phosphatidylinositol glycan anchor biosynthesis, Class A gene (*Pig-a* in rodents, *PIG-A* in humans) codes for a catalytic subunit of the *N*-acetylglucosamine transferase complex that is involved in an early step of glycosylphosphatidyl inositol (GPI) anchor synthesis, and GPI anchors tether specific protein markers to the surface of various types of cells. Two distinct strategies for *Pig-a* gene mutation assay used peripheral blood have been developed, one using flow cytometry and the other using limiting-dilution cloning. The limiting-dilution cloning assay using bacterial protoxin, proaerolysin, as a selective agent is resource intensive. For routine analysis of mutant frequency, the flow cytometric procedures employing fluorescently labeled antibodies against GPI-anchored markers (e.g., anti-CD59 for rat red blood cells, anti-CD24 for mouse red blood cells) are preferred for routine analysis of mutant frequency. The advantage of the cloning assay, however, is in-depth analyses of the mutant genotype (mutational spectra analysis). The characteristics of the flow cytometric *Pig-a* gene mutation assays, i.e., induced *Pig-a* mutant frequencies were persistence and the effect of split doses of chemicals were additive, make the assay on reticulocytes and total red blood cells an attractive possibility for developing detailed mutagenicity data *in vivo*. Although a large amount of information must be gathered on assay performance, progress toward the goal in the last few years has been rapid, and multi-laboratory trial has been initiated and ongoing.

Key words: phosphatidylinositol glycan anchor biosynthesis Class A gene, glycosylphosphatidyl inositol-anchor, flow cytometry

Introduction

The identification of human genotoxic hazard is an important step in the development of pharmaceuticals. To this end, genotoxicity testing is performed to ensure safety during clinical trials and during the treatment of general patient populations (1). The core test battery for hazard identification, agreed upon by regulatory agencies and pharmaceutical companies from the United States, the European Union, and Japan, consists of a series of *in vitro* and *in vivo* genotoxicity assays. It is

generally agreed, however, that the ability of the battery to predict carcinogenicity (a principal application of the battery) is less than perfect (2,3). One possible reason for this is that the gene mutation assays that are part of the battery, i.e., the bacterial reverse mutation and the mouse lymphoma assays, are *in vitro* tests. *In vitro* assays do not replicate the metabolism and pharmacodynamics that occur *in vivo*, and this deficiency may negatively impact the predictive ability of the battery.

The last decade has seen the emergence of a new *in vivo* gene mutation assay based on the phosphatidylinositol glycan anchor biosynthesis, Class A gene (*Pig-a* in rodents, *PIG-A* in humans). The Japanese collaborative research group in the Japanese Environmental Mutagen Society (JEMS) has played an important role in the development of this assay, and it seems fitting that a collection of papers to familiarize readers with recent advances by the collaborative research group would appear in a special issue of the JEMS journal, *Genes and Environment*.

The *Pig-a* Gene

The *Pig-a* codes for a catalytic subunit of the *N*-acetylglucosamine transferase complex that is involved in an early step of glycosylphosphatidyl inositol (GPI) anchor synthesis (4–7), and GPI anchors tether specific protein markers to the surface of various types of cells (e.g., hematopoietic cells) in several animal species, including rodents and humans (8,9). Much of what is known about *Pig-a* mutation has been derived from research on a rare, acquired genetic disease in human, paroxysmal nocturnal hemoglobinuria (PNH). Although several genes besides *PIG-A* are required for GPI synthesis (e.g., *PIG-B* and *PIG-C*, Fig. 1) and silencing of genes in the anchor synthesis pathway has

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Fig. 1. Structure and synthesis of GPI-anchored protein.

been known to occur (10), a GPI-deficient phenotype occurs in patients with PNH and its basis is mutation of the *PIG-A* (6,7). Only the *PIG-A*, as well as *Pig-a* in rodents, is located on the X chromosome (4,11), and being on the X-chromosome means that a single mutation can inactivate the enzymatic function of the *PIG-A* or *Pig-a* protein and disrupt anchor synthesis. Thus, the GPI anchor-deficient phenotype appears to be virtually equivalent to *PIG-A* mutation, and by analogy, *Pig-a* mutation. The preferred method for diagnosing PNH is currently the staining of peripheral blood with fluorescently labeled antibodies against GPI-anchored markers, e.g., CD55 and CD59, followed by measuring the fraction of marker-deficient cells by flow cytometry (12).

The *In Vivo* *Pig-a* Gene Mutation Assay

Pig-a was first suggested as a possible reporter of *in vivo* gene mutation by Araten *et al.* (13). Two distinct strategies for *Pig-a* gene mutation assay used peripheral blood have been developed, one using flow cytometry and the other using limiting-dilution cloning. Flow cytometry-based methods, which are derived from techniques used to diagnose PNH, employ fluorescently labeled antibodies against GPI-anchored markers (e.g., anti-CD59 for rat red blood cells (RBCs), anti-CD24 for mouse RBCs) and/or flow cytometry strategies for focusing the analysis on the cells of interest. In earlier work, for instance, light scattering was used to distinguish cells from platelets and an antibody specific for white blood cells was used to enrich the cell population for RBCs (14), or a nucleic acid stain was used to distin-

guish reticulocytes (RETs) from normochromatic erythrocytes (NCEs) (15). Kimoto *et al.* developed the method that facilitates measuring mutant frequencies in early arising populations of blood cells, bone marrow erythroids (BMEs) and RETs (PIGRET assay) (16,17). BMEs were identified using an antibody against rat erythroid-specific marker, HIS49, and RETs were selectively enriched from peripheral blood using magnetic separation of cells positive for CD71, a transferrin receptor expressed on the surface of RETs, but not on the surface of mature RBCs. All the *Pig-a* gene mutation assays described to date have used either peripheral blood cells or bone marrow cells. The main reason for this is that it is difficult to process solid tissues to produce sufficient numbers of single cells with intact cell surfaces to adopt flow cytometry strategies. Protocols for the dissociation of tissues into individual cells typically call for digestion of intercellular linkages, and such treatments may damage either the GPI anchors or GPI-anchored markers used in the detection of *Pig-a* gene mutation, artificially inflating the fraction of mutant cells.

An alternative approach for measuring *Pig-a* gene mutation used limiting-dilution clonal growth of *Pig-a* mutant GPI-deficient T-cells in the presence of the bacterial protoxin, proaerolysin (ProAER) (18). T-cells convert ProAER to a toxin (aerolysin) that binds directly to GPI anchors, resulting in a disruption of membrane integrity and cell death (19,20). *Pig-a* mutants, lacking GPI anchors, expand in the presence of the toxin to form clones in 96-well plates. The method for con-

ducting limiting-dilution cloning assay using ProAER as a selective agent are analogous to how the *Hprt* lymphocyte mutation assay is performed using 6-thioguanine selection. The cloning assay for *Pig-a* gene mutation, like the similar *Hprt* lymphocyte assay, is resource intensive. For routine analysis of mutant frequency and experiments requiring the analysis of large numbers of animals, therefore, the flow cytometric procedures are preferred. The main advantage of the cloning assay, however, is that it is a ready source of growing, nucleated *Pig-a* mutant cells on which in-depth analyses of the mutant genotype, i.e., mutational spectra analysis, and phenotype can be performed. Miura *et al.* showed that ProAER-resistant T-cells from *N*-ethyl-*N*-nitrosourea (ENU)-treated rats had a mutation spectrum characteristic of the DNA damage produced by ENU and very similar to the mutation spectrum produced by ENU in the rat T-cell *Hprt* assay (Table 1) (21). These data established the mutational basis (i.e., alteration to DNA sequence) for the ProAER-resistant phenotype in rat T-cells, and by analogy, the CD48-negative phenotype in the rat flow cytometric T-cell assay (18) and the CD59-negative phenotype in the flow cytometric rat RBC assay (14). As for more convincing support for the *Pig-a* mutational basis of the flow cytometric RBC assay, Kimoto *et al.* reported an ENU-specific mutation spectrum in nucleated erythroid cells sorted by flow cytometry from the bone marrow of ENU-treated mice (16).

Table 1. Comparison of independent *Pig-a* and *Hprt* mutations found in ProAER-resistant and 6-thioguanine-resistant clones from ENU-treated Fisher 344 rats (21)

Sequence alteration	<i>Pig-a</i> mutations		<i>Hprt</i> mutations [§]	
	Number*	Percentage [†]	Number*	Percentage
Basepair substitution				
G : C→A : T	5	10%	7	10%
A : T→G : C	9	18%	17	25%
G : C→T : A	3	6%	3	4%
G : C→C : G	1	2%		
A : T→T : A	22	45%	33	48%
A : T→C : G	7	14%	9	13%
Frameshifts				
- 1 bp	2	4%		
Others (possibly splicing mutations) [‡]				
- 339 bp	1			
+ 309 bp	1			
- 134 bp	1			
+ 90 bp	1			
+ 31 bp	2			
Total	55		69	

*Number of "independent" mutations. Mutations found in more than one clone from a single rat were not considered to be "independent". [†]Expressed as the percentage among basepair substitutions and frameshifts. [‡]These types of mutations were not detected in the analysis used for *Hprt* mutations. [§]*Hprt* mutation data are from Ref. 37.

In theory, treatment-induced *Pig-a* mutant RBCs could originate in any of the nucleated precursors of RBCs, from pluripotent stem cells to nucleated erythroid precursor cells in the last stages of differentiation. In experiments measuring mutant manifestation in peripheral blood after treatment of rats with the five mutagens (ENU, 7,12-dimethylbenz[*a*]anthracene, *N*-methyl-*N*-nitrosourea, 4-nitroquinoline-*N*-oxide, benzo[*a*]pyrene), all mutagens increased the frequencies of both CD59-deficient (*Pig-a* mutant) RBCs and RETs, but the frequency of mutant RETs was always leading (achieving maximum responses at around 2 weeks post-treatment), and the frequency of mutant RBCs was always trailing (achieving maximum responses at around 2 months post-treatment) (22). These kinetics are expected of mutagenized cells that transit from the bone marrow (mainly as RETs) to populate the large pool of circulating RBCs (mainly in the form of mature NCEs that initially have a relatively low background mutant frequency). The rate of mutant manifestation in the total RBC pool is probably influenced heavily by the turnover time of mature cells in rat peripheral blood, about 8–9 weeks (23,24).

Further observations regarding induced *Pig-a* mutant frequencies is their persistence. Rats exposed acutely to ENU exhibit high mutant RET and total RBC frequencies over prolonged periods (22,25). These observations on the persistence of *Pig-a* mutants in peripheral blood are at least consistent with the incidence of GPI anchor-deficient RBCs reflecting the mutant frequencies in very long-lived committed progenitor and/or pluripotent stem cells. This persistence of the induced mutant yield is similar to what occurs in transgenic systems, whose mutational targets are regarded as having a neutral (or no) phenotype *in vivo* (26). The conclusion that the *Pig-a* gene is functionally neutral, at least in hematopoietic cells, is supported by results using *Pig-a* conditional knockout mice. In these studies, mice mosaic for *Pig-a* mutation displayed relatively stable frequencies of *Pig-a* mutant and wild-type cells (27,28).

In addition, Miura *et al.* reported that the effects of split doses of ENU were additive (25). That is, a single administration of ENU (e.g., 35.6 mg/kg) produced approximately the same maximum frequency of CD59-deficient RBCs as dividing the total dose into 4 smaller weekly treatments (e.g., 4 doses of 8.9 mg/kg). An additive effect may be advantageous when integrating the *Pig-a* gene mutation assay into the conventional general toxicology study, e.g., 28-day or 90-day repeat-dose toxicity study. In contrast to the micronucleus assay or the comet assay, which generally are sensitive only to damage produced in a relatively small window of time before the sampling time, the *Pig-a* gene mutation assay potentially can take advantage of the cumulative damage produced during an extended animal exposure.

Validation of the Methodology

Before the *Pig-a* gene mutation assay is adopted as a gene mutation assay that is part of the test battery for genotoxic hazard identification, much more information must be generated about how best to conduct it. Basic information on the elements making up a standard protocol and guidelines must be developed, including the optimum number of animals, dosing and sampling schedule, the number of cells interrogated, data analysis, criteria for positive and negative responses, and the limits of toxicity in the assay. Also, more information on the responses of various classes of agents in the assay must be generated and mutant cells identified by marker-deficient phenotype should be confirmed by the analysis of genetic material (i.e., sequencing the *Pig-a* gene). Eventually, a formal validation of the assay will have to be organized. To address some of these issues, an international multi-laboratory trial has been initiated (29–35). This inter-laboratory trial placed an initial focus on evaluating the portability of flow cytometric methodology (i.e., mutant RET and mutant total RBC scoring). The early work involved acute treatment of rats with predefined doses of ENU and collection of serial blood specimens at predetermined time-points. By expanding the number of expert laboratories that are proficient with this assay, this international trial is still ongoing and it should be possible to rapidly acquire information on experimental variables and additional chemicals that require further study.

In Japan, collaborative work on the *Pig-a* gene mutation assay was conducted as a project supported by the Japan Health Sciences Foundation. The participating laboratories conducted the PIGRET assay to examine its transferability and reproducibility and to compare the response in this assay with those of the RBC *Pig-a* assay (36). The finding suggested that the PIGRET assay may detect the *in vivo* mutagenicity of test chemicals earlier than the RBC *Pig-a* assay. By expanding its participants, the Japanese multi-laboratory research of the PIGRET assay is currently ongoing by the collaborative research group in the JEMS, and recent advances by the group would appear in this special issue of Genes and Environment.

Conclusion

As regulatory testing schemes move beyond simple hazard identification and put more emphasis on *in vivo* responses as a better measure of human risk, an *in vivo* gene mutation assay becomes more valuable. Two distinct strategies for the *Pig-a* gene mutation assay used peripheral blood have been developed, one using flow cytometry and the other using limiting-dilution cloning. The relatively fast, cost-effective way that data can be produced, and the ability to perform multiple samplings and mutation assays on individual animals, make the

flow cytometry strategy for the *Pig-a* assay on RETs and total RBCs an attractive possibility for developing detailed mutagenicity data. Although a large amount of information must be gathered on assay performance, progress toward the goal in the last few years has been rapid, and we can remain optimistic that the *Pig-a* gene mutation assay will become a useful assay for measuring *in vivo* gene mutation. The cloning assay is resource intensive, therefore, the flow cytometric procedures are preferred for routine analysis of mutant frequency. The advantage of the cloning assay, however, is in-depth analyses of the mutant genotype (mutational spectra analysis).

Conflicts of Interest: The author declares that there are no conflicts of interests.

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