Mutation Research 657 (2008) 122–126

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Erythrocyte-based *Pig-a* gene mutation assay: Demonstration of cross-species potential

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ARTICLE INFO

Article history:
Received 15 July 2008
Received in revised form 8 August 2008
Accepted 11 August 2008
Available online 26 August 2008

Keywords:
Pig-a
Mutation
GPI anchor
Flow cytometry
CD24
Erythrocytes

ABSTRACT

Glycosylphosphatidylinositol (GPI) anchors attach specific proteins to the cell surface of hematopoietic cells. Of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Prior work with rats suggests that the GPI anchor deficient phenotype is a reliable indicator of *Pig-a* mutation [Bryce et al., Environ. Mol. Mutagen., 49 (2008) 256–264]. The current report extends this line of investigation by describing simplified blood handling procedures, and by testing the assay principle in a second species, *Mus musculus*. With this method, erythrocytes are isolated, incubated with anti-CD24-PE, and stained with SYTO 13. Flow cytometric analyses quantify GPI anchor-deficient erythrocytes and reticulocytes. After reconstruction experiments with mutant-mimicking cells demonstrated that the analytical performance of the method is high, CD-1 mice were treated on three occasions with 7,12-dimethyl-1,2-benz[a]anthracene (DMBA, 75 mg/kg/day) or ethyl-*N*-nitrosourea (ENU, 40 mg/kg/day). Two weeks after the final treatment, DMBA-treated mice were found to exhibit markedly elevated frequencies of GPI anchor deficient erythrocytes and reticulocytes. For the ENU experiment, blood specimens were collected at weekly intervals over a 5-week period. Whereas the frequencies of mutant reticulocytes were significantly elevated 1 week after the last administration, the erythrocyte population was unchanged until the second week. Thereafter, both populations exhibited persistently elevated frequencies for the duration of the experiment (mean frequency at termination = 310×10^{-6} and 523×10^{-6} for erythrocyte and reticulocyte populations, respectively). These data provide evidence that *Pig-a* mutation does not convey an appreciable positive or negative cell survival advantage to affected erythroid progenitors, although they do suggest that affected erythrocytes have a reduced lifespan in circulation. Collectively, accumulated data support the hypothesis that flow cytometric enumeration of GPI anchor deficient erythrocytes and/or reticulocytes represents an effective *in vivo* mutation assay that is applicable across species of toxicological interest.

1. Introduction

This laboratory has described a flow-cytometry-based approach for quantifying *in vivo* mutation [1]. The method is based on *Pig-a*, a gene whose product is essential for the synthesis of glycosylphosphatidylinositol (GPI) anchors [2]. Hematopoietic cells require GPI anchors to attach a host of proteins to their cell surface, for instance CD24, CD59, CD55, and CD48 [3]. Importantly, of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Together, this information has led us to hypothesize that GPI anchor deficiency should represent a reliable phenotypic marker of *Pig-a* mutation.

In addition to the proof-of-principle data reported by Bryce et al., other important feasibility data have been generated by Miura and coworkers, who demonstrated that both rat blood erythrocytes and splenic lymphocytes can be readily evaluated for the GPI anchor deficient phenotype [4]. Furthermore, through DNA sequencing experiments, these investigators reported that cells that exhibit the GPI anchor deficient phenotype are in fact bona fide *Pig-a* mutants [5].

Beyond these rat-based investigations, a wealth of information regarding the underlying biology of GPI anchor deficiency has been developed through the study of a human disease caused by *Pig-a* mutation, paroxysmal nocturnal hemoglobinuria (PNH). Investigations into PNH etiology have shown that the GPI anchor deficient phenotype is invariably due to *Pig-a* mutation [6], each important class of mutation can ablate GPI anchors (base-pair substitutions, frame-shifts, small and large insertions/deletions) [7,8], and that

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doi:10.1016/j.mrgentox.2008.08.011

Pig-a mutation is neutral, meaning it does not convey a cell survival advantage or disadvantage [9,10]. Based on these characteristics, combined with their own extensive work in this field, Araten et al. [11] speculated, "...quite apart from its relevance to the pathogenesis of PNH, the analysis of the *PIG-A* gene may serve for the detection and quantitation of somatic mutations in hematopoietic cells in general."

When setting out to test the feasibility of a *Pig-a* based assay, our laboratory initially focused on rat erythrocytes using a 3-fluorochrome method. Two populations of erythrocytes were studied: circulating red blood cells without regard to maturity (RNA-negative as well as RNA-positive, abbreviated RBCs), and the immature fraction (RNA-positive, or reticulocytes, abbreviated RETs). The current investigation also focuses on these two populations, and was initiated in an effort to (1) reduce the technical requirements for conducting the assay by developing a 2-fluorochrome method, and (2) test the cross-species potential of the assay system. A detailed description of the simplified staining/analysis methods, as well as mouse data generated from 7,12-dimethyl-1,2-benz[a]anthracene and ethyl-*N*-nitrosourea treatments, are reported herein.

2. Materials and methods

2.1. Reagents

Sesame oil, ethyl-*N*-nitrosourea (ENU; cas no. 759-73-9), and 7,12-dimethyl-1,2-benz[a]anthracene (DMBA; cas no. 57-97-6) were purchased from Sigma–Aldrich, St. Louis, MO. Phosphate buffered saline (PBS; phenol red and Ca^{2+}/Mg^{2+} free) was from Mediatech, Inc., Herndon, VA. A solution of 0.9% NaCl for injection was obtained from Ricca Chemical Co., Arlington, TX. Disodium-EDTA (cas no. 6381-92-6) was from JT-Baker, Phillipsburg, NJ. Purified unlabeled and PE-conjugated anti-mouse CD24 (clone number M1/69) were from BD Biosciences, San Jose, CA. SYTO 13 was obtained from Invitrogen, Carlsbad, CA.

2.2. Animals, treatments, blood draws

Female CD-1 mice were purchased from Charles River Laboratories, Wilmington, MA. Mice were allowed to acclimate for at least 1 week before treatment. Water and food were available *ad libitum* throughout the acclimation and experimental periods. Treatment with mutagens occurred three times at 2-day intervals when animals were between 7 and 8 weeks of age. ENU was administered at 40 mg/kg/day and DMBA at 75 mg/kg/day. All treatments occurred via intraperitoneal injection. Solvents were 0.9% saline and sesame oil for ENU and DMBA, respectively, and vehicle exposed mice served as negative controls.

For the DMBA experiment, three mice were randomly selected for the 0 and 75 mg/kg/day groups. Blood collection occurred 2 weeks after the final administration. For the ENU experiment, 20 mice were treated with genotoxicant and blood was collected from four of these mice on each of five occasions: 1, 2, 3, 4 and 5 weeks after the final administration (i.e., no serial draws so that erythropoiesis function was not disturbed). Five mice were assigned to the vehicle control group, and for each of the five time-points, one of these mice supplied a blood specimen.

Blood harvests occurred via heart puncture using a 26 gauge needle and syringe following CO_2 overdose. To prevent coagulation, the syringes contained approximately 90 μL of PBS with heparin at 500 units/mL. Approximately 500 μL of blood per mouse was obtained and stored on ice or in a refrigerator until staining occurred (same day). (Note that while 500 μL was collected per mouse; we estimated that only 30–50 μL was needed to provide 2×10^8 cells for staining (Section 2.3).)

2.3. Cell staining

Each heparinized whole blood specimen was diluted 1:1 with PBS. One part diluted blood (typically 1000 μL) was then layered on top of eight parts room temperature Lympholyte Mammalian Cell Separation Reagent (CedarLane, Burlington, NC; cat. no. CL5110) in a 15 mL conical polypropylene tube. To enrich for erythrocytes, specimens were centrifuged at 800 × *g* for 20 min. The resulting supernatants were carefully aspirated and 1 mL of PBS was gently added to the side of each tube with the goal of minimally disturbing the erythrocyte pellets. The PBS rinse solution was removed and a second addition of PBS (1 mL/tube) was added as before. After removing the second rinse volume, a Pipetman was used to transfer each erythrocyte pellet to a new 15 mL polypropylene tube containing 10 mL PBS. The specimens were washed twice with 10 mL PBS, the first for 10 min and the second time for 5 min at 300 × *g*. Cells were finally resuspended with 5 mL PBS and placed on ice.

A Coulter Counter (model ZM) was used to determine the density of the fractionated, washed erythrocytes. 2×10^8 cells were then transferred to a tube containing 100 μL antibody solution (10 μL stock anti-CD24-PE for every 90 μL PBS plus 2% v/v fetal bovine serum). After incubating for 30 min on ice, cells were washed with 10 mL PBS per tube. After centrifugation and aspiration, pellets were resuspended with 1 mL SYTO 13 solution (156 nM, prepared in PBS). After incubating for 30 min at room temperature, samples were moved to ice until flow cytometric analysis occurred (within approximately 2 h).

2.4. Instrument calibration standards

The extreme rarity of *Pig-a* mutant cells represents a challenge to flow cytometer operators who ideally set PMT and compensation settings based on fluorescence profiles of both positive and negative events. This was addressed by generating mutant mimicking cells in parallel with the experimental samples each day that flow cytometric analysis occurred. The mutant-like cells consisted of RBCs that lacked anti-CD24-PE fluorescence. These specimens were generated from mouse blood that was fractionated and stained with SYTO 13 as described above. However, instead of being incubated with PE-conjugated anti-CD24, the same amount of unlabeled anti-CD24 was used. This substitution generated RBCs that exhibited the mutant (GPI-anchor deficient) phenotype. Before experimental samples were analyzed, equal volumes of these mutant-like cells were added to a tube that contained vehicle control RBCs that were stained according to the standard staining protocol. The resulting instrument calibration specimen provided sufficient numbers of RBCs that exhibited a full range of PE and SYTO 13 fluorescence intensities that were useful for optimizing PMT voltages and fluorescence compensation settings on a daily basis. The position of the CD24-negative cells also provided a rational approach for defining the vertical demarcation line that was used to distinguish CD24-negative from CD24-positive events.

2.5. Reconstruction experiments

The analytical performance of the staining and data acquisition procedures were evaluated in several independent experiments by staining a naïve mouse blood specimen as described above, and then adding various, known numbers of mutant mimicking RBCs. As with the biological standards for instrument set up purposes, these cells exhibited a GPI-anchor deficient phenotype, and their frequencies were determined via flow cytometric analysis as described below.

2.6. Data acquisition

A FACSCalibur providing 488 nm excitation and running CellQuest Pro v5.2 software was used for these studies (BD Biosciences). For analyses that sought to measure the frequency of CD24-negative RBCs, data acquisition was triggered on the forward scatter parameter, and the sample rate was set to low. This facilitated efficient collection of 1×10^6 RBCs per specimen (typically ≤ 5 min).

For analyses that sought to measure the frequency of RETs that were CD24-negative, the sample rate was medium and the data acquisition trigger was set on SYTO-13-associated fluorescence. This SYTO 13 threshold value was set sufficiently high such that the majority of blood cells (i.e., mature erythrocytes which lack RNA and therefore express low SYTO-13-associated fluorescence) was eliminated from consideration. This strategy provided greater rates of RET analysis, making it feasible to evaluate 1×10^6 RETs per specimen (typically ≤ 10 min).

2.7. Calculations

The incidence of *Pig-a* mutation is expressed as the number of CD24-negative RBCs per one million RBCs, and as the number of CD24-negative RETs per one million RETs. All frequency, average, and standard error calculations were performed with Excel Office × for Mac® (Microsoft, Seattle, WA). In order to evaluate the longitudinal ENU data, a non-parametric Kruskall–Wallis test was performed, and a p value ≤ 0.05 was used to indicate significance.

3. Results

3.1. Method optimization

Initial experiments focused on the development of simple and reproducible methods for staining and analyzing peripheral blood cells for the GPI anchor-deficient phenotype. As reported elsewhere, we found that the GPI-anchored protein CD24 is highly and stably expressed on erythrocytes throughout their lifespan in circulation [9]. A second fluorescent reagent was chosen to differentially stain mature erythrocytes, RETs, and leukocytes. Whereas we had previously used thiazole orange for this purpose [1], more recent work indicated that SYTO 13 is superior in at least two respects. Firstly, it provided a more stable fluorescence signal