Mutation Research 789 (2016) 26–32

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

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

Moderate malnutrition in rats induces somatic gene mutations

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 7 January 2016
Accepted 30 January 2016
Available online 8 March 2016

Keywords:
Malnutrition
Mutagenesis
Reticulocytes
Erythrocytes
Pig-a assay

ABSTRACT

The relationship between malnutrition and genetic damage has been widely studied in human and animal models, leading to the observation that interactions between genotoxic exposure and micronutrient status appear to affect genomic stability. A new assay has been developed that uses the phosphatidylinositol glycan class A gene (*Pig-a*) as a reporter for measuring *in vivo* gene mutation. The *Pig-a* assay can be employed to evaluate mutant frequencies (MFs) in peripheral blood reticulocytes (RETs) and erythrocytes (RBCs) using flow cytometry. In the present study, we assessed the effects of malnutrition on mutagenic susceptibility by exposing undernourished (UN) and well-nourished (WN) rats to *N*-ethyl-*N*-nitrosourea (ENU) and measuring *Pig-a* MFs. Two week-old UN and WN male Han-Wistar rats were treated daily with 0, 20, or 40 mg/kg ENU for 3 consecutive days. Blood was collected from the tail vein one day before ENU treatment (Day-1) and after ENU administration on Days 7, 14, 21, 28, 35, 42, 49, 56 and 63. *Pig-a* MFs were measured in RETs and RBCs as the RET^{CD59-} and RBC^{CD59-} frequencies. In the vehicle control groups, the frequencies of mutant RETs and RBCs were significantly higher in UN rats compared with WN rats at all sampling times. The ENU treatments increased RET and RBC MFs starting at Day 7. Although ENU-induced *Pig-a* MFs were consistently lower in UN rats than in WN rats, these differences were not significant. To understand these responses, further studies should use other mutagens and nucleated surrogate cells and examine the types of mutations induced in UN and WN rats.

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1. Introduction

Poor intake of nutrients by an organism may result in an active pathological condition known as malnutrition, with different degrees and manifestations; malnutrition is defined as an inadequate intake of nutrients [1,2]. Malnutrition is a leading cause worldwide of mortality among children less than 5 years old [3,4].

There are a large number of in vivo and in vitro genetic toxicology assays designed to monitor the various effects that physical and chemical agents have on the genetic integrity of cells and the possible risks they pose for an organism [5–8]. These procedures include measurements of DNA-associated proteins and gene-specific mutations (i.e., heritable alterations in the DNA sequence) [5].

Studies have detected increased genetic damage in malnourished humans and animal models, as measured based on sister chromatid exchange, chromosomal aberrations, DNA damage determined through single-cell electrophoresis (i.e., comet assay), and the percentage of peripheral blood reticulocytes (RETs) with micronuclei [9–12]. For instance, we have observed significant increases in the micronucleus (MN) frequency in RETs from the peripheral blood of malnourished (UN) rats compared with well-nourished (WN) rats [10]. Increases in DNA damage have also been detected in the peripheral blood lymphocytes of malnourished children [13–15]. Although these previous experiments have focused on the measurement of DNA damage and cytogenetic endpoints, these studies indicate that malnourishment produces DNA damage in vivo and that this damage can have negative effects on the developing organism [16].

For the evaluation and monitoring of *in vivo* gene mutations in experimental animals, the *Pig-a* assay is an effective tool for identifying compounds with a potential health risk [17–20]. The mutation reporter employed in this assay is the endogenous X-linked phosphatidyl inositol glycan class A gene (*Pig-a*). The product of the *Pig-a*

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http://dx.doi.org/10.1016/j.mrfmmm.2016.01.008
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gene is implicated in an early step in the biosynthesis of glycosyl phosphatidyl inositol (GPI), an anchor that tethers a number of tissue-specific protein markers to the mammalian cell surface [21]. An inactivating mutation in the single functional copy of the *Pig-a* gene results in a cell that is deficient in GPI anchors and, consequently, deficient in GPI-anchored surface markers; these mutants can be enumerated using flow cytometry. Several versions of the rodent *Pig-a* assay had been described [17–20]. In one version, the frequency of mutant cells among total red blood cells (RBCs) and RETs is determined. The RET population is the youngest cohort of RBCs, and following treatment with a test agent, the frequency of mutant RETs relays mutagenicity information earlier than mature RBCs [19]. In another version of the *Pig-a* assay, mutants are enumerated among total RBCs, but with positive identification of target cells using a relatively simple protocol. This version of the assay requires no gradient centrifugation of whole blood to remove leukocytes and no fluorescence compensation adjustments for flow cytometry [20]. Another high-throughput version of the assay was developed involving immunomagnetic enrichment of mutant RBCs and RETs [18].

In this study, we experimentally induced malnutrition in rats via food competition and measured the spontaneous mutant frequency (MF) as well as the MFs induced by the potent mutagen *N*-ethyl-*N*-nitrosourea (ENU) using a standard version of the *Pig-a* assay (no immunomagnetic enrichment) [19].

2. Materials and methods

2.1. Reagents

ENU (CAS no. 759-73-9) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) was obtained from Microlab (Mexico City, Mexico). A mouse anti-rat CD59-PE antibody (cat. no. 562106) was procured from BD Biosciences (San Jose, CA, USA), and the nucleic acid stain SYTO 13 (cat. no. S-7575) was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Animals and diets

All experiments were performed according to the guidelines of the Universidad Autonoma Metropolitana (UAM), Official Mexican Guidelines (Norma Oficial Mexicana NOM-062-ZOO-1999) and the National Institutes of Health (NIH). Han-Wistar rats were raised at the Bioterio of the Universidad Autonoma Metropolitana Campus Iztapalapa in Mexico City, Mexico. All animals were housed in conventional animal rooms and maintained under a 12-h light/dark cycle. Nursing mothers were fed with a balanced diet for rodents (TEKLAD GLOBAL, Brentwood, MO, USA) and provided with filtered water *ad libitum*.

2.3. Experimental postnatal malnutrition

Experimental postnatal malnutrition was induced via food competition [22]. One-day-old rats from different litters were randomly assigned to either the experimental malnourished group (UN) or the control WN group. In the WN group, 6–8 female and male pups were fed by each nursing mother, whereas in the UN group, 16 pups were placed with each nursing mother. WN and UN pups were selected from different litters. Categories of malnutrition were established based on the deficit in the weight of the UN rats compared with the weight of the WN rats. Moderate malnutrition or second-degree malnutrition was defined as a weight deficit of 25–40%, and moderately malnourished pups were selected for use in the study. The pups were maintained with nursing mothers for 21 days after birth (weaning) [23].

2.4. Experimental post-weaning malnutrition

To maintain moderate malnutrition after weaning, rats assigned to the WN and UN groups received a diet of either normoproteic food (18% protein, TEKLAD GLOBAL) or hypoproteic food through 50% food restriction. We determined the limit of food to be consumed by the animals by previously by tracking the food consumed by these rats; based on the food consumed by well-nourished animals, we weighed out the food to be malnourished rats according to 50% restriction of the amount of food consumed by well-nourished animals. Nutritional status was assessed by comparing variations in body mass [24] and was evaluated for ten weeks.

2.5. Animal treatment and blood collection

On each day of dosing, ENU was prepared fresh in PBS adjusted to pH 6.1. The animals were weighed before dosing and were divided into treatment groups, with 6 animals/group. Two-week-old (postnatal day (PND) 14) rats were treated with 0, 20 or 40 mg/kg of ENU for 3 consecutive days (PNDs 14–16), with a dosage volume of 10 mL/kg body weight.

On Days−1, 7, 14, 21, 28, 35, 42, 49, 56, and 63, blood samples of 30 μL were collected through tail vein bleeding and mixed with 100 μL of heparin solution (500 U/mL PBS). The blood collected at each time point was used to conduct the RET/RBC *Pig-a* assay. On each sampling day, an additional 30 μL of blood was collected from one of the vehicle control animals and used to prepare an instrument calibration standard (ICS).

2.6. Sample processing and cell staining

The samples were processed according to the procedure described by Phonethepswath et al. [25], except for the leukodepletion step because we used FL1-H vs FSC-H gating to restrict the analyses to erythrocytes. When this strategy was reported by these authors, they noted that this gating procedure excludes any platelets and leukocytes that may remain after the leukodepletion step. The *Pig-a* assay was conducted on the same day that blood was collected. Each diluted blood sample (130 μL) was added to 155 μL of PBS, then re-suspended via gentle pipetting and centrifuged at 800 *g* for 5 min; the supernatant was subsequently removed, and another 155 μL of PBS was added with gentle mixing.

Then, 150 μL of this suspension was added to a polypropylene tube containing 100 μL of the following mixture: 3 parts anti-CD59-PE (antibody against CD59 conjugated to phycoerythrin (PE), used to detect mutations based on deficiency in the expression of this protein) and 17 parts PBS +2% v/v fetal bovine serum.

The samples were subsequently incubated on ice for 30 min, followed by two 5 min washes with 1 mL of PBS at 800 *g*. Next, the supernatant was removed, and the pellet was resuspended in 1 mL of PBS containing 150 nM SYTO 13, followed by incubation at 37 °C for 30 min. The SYTO-labeled samples were kept on ice until flow cytometric analysis. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) running CellQuest™ Pro version 3.0.1 software for data acquisition and analysis (BD Biosciences). Data were acquired scoring 1.5×10^6 RBCs/sample. For the analysis of RETs, 0.5 mL of SYTO 13 was added before the acquisition of each sample; we analyzed 0.3×10^6 RETs and calculated the mutants per million RETs.

On each experimental day, an instrument calibration standard (ICS) was prepared by mixing approximately 50% cells from control rats that were not exposed to ENU (labeled with CD59-PE and SYTO 13) and 50% mutant-mimic cells (cells labeled with SYTO 13 only) Fig. 1. The ICS was used to optimize the photo-multiplier tube voltage, to set fluorescence compensation and to define the vertical

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