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Induction of *Pig-a* mutant erythrocytes in male and female rats exposed to 1,3-propane sultone, ethyl carbamate, or thiotepa

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Abstract

Validation of the *Pig-a* gene mutation assay has been based mainly on studies in male rodents. To determine if the mutagen-induced responses of the X-linked *Pig-a* gene differ in females compared to males, groups of five male and female Sprague Dawley rats were exposed to the mutagens 1,3-propane sultone (80 mg/kg/day), ethyl carbamate (600 mg/kg/day), or thiotepa (7.5 mg/kg/day) for three consecutive days (study Days 1-3). *Pig-a* mutant phenotype reticulocyte (RET^{CD59-}) and mutant phenotype erythrocyte (RBC^{CD59-}) frequencies were determined on study Days -4, 15, 30 and 46 using immunomagnetic separation in conjunction with flow cytometric analysis (*In Vivo* MutaFlow®). While the percentage of reticulocytes (%RET) was markedly higher for pre-treatment blood samples from males compared to females (6.6% vs. 3.5%), this sex effect was slight or nonexistent at later time points. Treatment-related effects to %RET were generally modest owing to the 12-day interval between last exposure and blood sampling. Mean RET^{CD59-} and RBC^{CD59-} frequencies were consistently low in vehicle control animals of both sexes, with 77% of samples exhibiting mutant cell frequencies $< 1 \times 10^{-6}$ over study Days 15-46. Treatment with each mutagen caused significant increases to mean RET^{CD59-} and RBC^{CD59-} frequencies. Whereas genotoxicant-induced RET^{CD59-} values were maximal on Day 15, induced RBC^{CD59-} frequencies were highest at the last sampling time. Sex did not affect 1,3-propane sultone- or thiotepa-induced mutant cell frequencies. While ethyl carbamate-exposed females exhibited higher mean mutant cell frequencies compared to like-treated males, statistical significance was achieved only for RBC^{CD59-} at one time point ($7.6 \pm 1.0 \times 10^{-6}$ compared to $4.7 \pm 0.6 \times 10^{-6}$ on Day 30). Thus, while some quantitative differences were evident, there were no

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Disclosure Statement

C.L., K.C., S.L.A., A.B., J.C.B. and S.D.D. are employees of Litron Laboratories, and J.T.M. serves as a consultant to Litron. Litron holds patents covering flow cytometric methods for scoring GPI anchor-deficient erythrocytes and sells kits based on this technology (*In Vivo* MutaFlow®).

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qualitative differences in how males and females responded to three diverse mutagens. These data support the use of both sexes for *Pig-a* gene mutation studies.

Keywords

In vivo; mutation assay; CD59; flow cytometry; peripheral blood; sex effect

Introduction

The phosphatidylinositol glycan-class A (*Pig-a*) gene has been used as a reporter for somatic cell mutation. In this system flow cytometric analysis is used to score the frequency of cells that express the mutant phenotype associated with ablated *Pig-a* expression—that is, lack of cell surface glycosylphosphatidylinositol (GPI) anchored protein(s) (1-4). Rodent studies have primarily focused on erythrocytes that can be collected in abundance in low volume blood samples. Interest in *Pig-a* assays for safety assessment has been high, in large part due to the ease with which the blood-based endpoint can be integrated into other studies with common laboratory rodent models (5-7).

An important feature of this mutagenesis assay is that the *Pig-a* gene is on the X chromosome, and because in males there is only one copy of the X chromosome per cell only a single inactivating mutation is required to cause expression of the mutant phenotype. The process of lyonization in the female leaves just one functional copy of the X chromosome, a phenomenon that is expected to make female rodents compatible with the assay. However, most of the validation work conducted to date has used male rats and data demonstrating that the mutagenic response in females is similar to that in males are scant. This led the 2013 International Workshops on Genotoxicity Testing (IWGT) *Pig-a* Workgroup to suggest that the scarcity of female-associated results is an important data gap that should be addressed. This Workgroup stated that: "...there is still a question of how test agent treatments, animal age, diet, etc. might affect *Pig-a* mutant frequencies detected in females vs. males. The Workgroup suggests conducting studies to evaluate any sex-related differences (8)." More recently two reports have appeared that begin to address this issue, both of which focused on the reference mutagen *N*-ethyl-*N*-nitrosourea (ENU). Chikura and colleagues showed that one administration of 10 or 40 mg ENU/kg induced similar mutant phenotype cell frequencies in both sexes (9). Labash *et al.* reported that while spontaneous frequencies were similar between the sexes, three consecutive days of exposure at 25 mg ENU/kg/day caused modest quantitative differences in young male versus female Sprague Dawley rats, possibly related to differences in hematopoietic activity (10).

To expand on these *Pig-a*/rodent sex efforts, we exposed male and female rats to the diverse mutagenic agents 1,3-propane sultone, ethyl carbamate, or thiotepa. *Pig-a* data were collected from serial blood draws in order to evaluate whether sex influences the responsiveness and/or the kinetics of appearance of circulating mutant phenotype cells. The results are discussed in terms of the suitability of using each sex when conducting *Pig-a* gene mutation studies.

Materials and Methods

Reagents

1,3-Propane sultone (CAS No. 1120-71-4), thiotepa (CAS No. 52-24-4), and ethyl carbamate (CAS No. 51-79-6) were purchased from Sigma-Aldrich, St. Louis, MO. Reagents used for flow cytometric scoring of *Pig-a* mutant phenotype cells (Anticoagulant Solution, Buffered Salt Solution, Nucleic Acid Dye Solution, Anti-CD59-PE, and Anti-CD61-PE) were from Rat MutaFlow® Kits, Litron Laboratories. Additional supplies included Lympholyte®-Mammal cell separation reagent from CedarLane, Burlington, NC; Anti-PE MicroBeads, LS Columns, and a QuadroMACS™ Separator from Miltenyi Biotec, Bergisch Gladbach, Germany; and CountBright™ Absolute Count Beads and fetal bovine serum from Invitrogen, Carlsbad, CA.

Animals, Treatments, Blood Harvests

Experiments were conducted with the oversight of the University of Rochester's Institutional Animal Care and Use Committee. Male and female Sprague Dawley rats (Crl:SD) were purchased from Charles River Laboratories, Wilmington, MA. Rodents were allowed to acclimate for approximately one week before use. Water and food were available *ad libitum* throughout the acclimation and experimental periods. 1,3-Propane sultone, thiotepa, and ethyl carbamate solutions were prepared fresh each day of use in deionized water. At the start of treatment animals were 8 weeks old, and exposure was for three consecutive days at approximately 24 hr intervals *via* oral gavage in a volume of 10 mL per kg body weight.

Rats were treated with vehicle, 80 mg 1,3-propane sultone/mg/kg/day, 600 mg ethyl carbamate/kg/day, or 7.5 mg thiotepa/kg/day ($n = 5$ per dose level per sex). Treatments were based on previous studies (11-13) with male rats that showed these dose levels are tolerated and significant increases in *Pig-a* mutant phenotype cells are induced.

Peripheral blood samples were collected on Days -4, 15, 30, and 46 by nicking a lateral tail vein with a surgical blade after animals were warmed briefly under a heat lamp. Approximately 200 μ L of free-flowing blood were collected directly into heparinized capillary tubes (Fisher Scientific, CAT no. 22-260-950) per time point. 175 μ L blood were immediately transferred to K₂-EDTA tubes (Greiner Bio-One International, CAT no. 450480) that contained 35 μ L kit-supplied Anticoagulant Solution for storage at 4 °C until further sample processing occurred (within 2 days).

As described previously, pre-treatment *Pig-a* mutant phenotype cell frequencies were used as study inclusion criteria (14). Briefly, pre-treatment frequencies were compared to the distribution of 756 historical controls animals, and rats that exceeded a one-sided 95% upper tolerance interval, $\alpha 0.05$, were considered outliers and excluded from the study (15). Animals that did not exceed these threshold values (*i.e.*, $2.86 \text{ RBC}^{\text{CD59-}} \times 10^{-6}$ total RBC and/or $2.97 \text{ RET}^{\text{CD59-}} \times 10^{-6}$ total RET) were randomized across treatment groups.

Sample Preparation, Data Acquisition

Pig-a analyses were in accordance with MutaFlow kit manual v1404003 instructions, and have been described previously (12). Each analysis was based on 80 μ L of anticoagulant-treated blood that was processed according to the so-called 96 well plate-based method, whereby microtiter plates facilitated multisample/parallel labeling and washing procedures, as well as walk-away data acquisition through the use of a BD HTS instrument that automatically delivered well contents to the flow cytometer. As described previously, “pre-column” samples consisted of a small aliquot of each fully labeled and stained sample which was analyzed for 1 minute in order to provide %RET measurements as well as RBC to Counting Bead and RET to Counting Bead ratios. The majority of each sample was then used for an immunomagnetic separation procedure, and the resulting “post-column” eluates were analyzed for 3 minutes to provide mutant phenotype RBC to Counting Bead and mutant phenotype RET to Counting Bead ratios. As described previously, pre- and post-column data were used to calculate mutant phenotype erythrocyte (RBC^{CD59-}) and mutant phenotype reticulocyte (RET^{CD59-}) frequencies (12). For the current studies $> 1.5 \times 10^6$ RET and $> 150 \times 10^6$ RBC equivalents per rat per time point were scored.

An Instrument Calibration Standard was generated on each day of data acquisition. As approximately one half of these erythrocytes were not incubated with anti-CD59-PE, these samples contained a high prevalence of mutant-mimic cells and provided a means to define the location of GPI anchor-deficient erythrocytes (16). A BD FACSCanto II flow cytometer running Diva v6.1.2 software was used for data acquisition and analysis.

Calculations, Statistical Analyses

The incidence of RETs is expressed as frequency percent, while the incidence of mutant phenotype cells is expressed as number of CD59-negative cells per 10^6 cells. All %RET, mutant phenotype cell frequencies, averages, and standard error calculations were performed with Excel Office X for Mac[®] (Microsoft, Seattle, WA).

Prior to conducting statistical analyses RBC^{CD59-} and RET^{CD59-} frequencies (number of mutant phenotype cells per 10^6 total cells) were log(10) transformed to ensure a normal distribution. Since zero RET^{CD59-} values were occasionally observed, a 0.1 offset was added to each RET^{CD59-} value prior to log transformation: $\text{Log}_{10}(0.1 + \text{number of mutant phenotype cells per } 10^6 \text{ total cells})$.

Statistical tests were performed in JMP[®] software for Mac, v8.01 (SAS Institute Inc., Cary, NC). All analyses utilized a two-tailed Student's t-test, and $p < 0.05$ was considered to indicate a significant effect. For pre-treatment blood samples, %RET, RET^{CD59-} and RBC^{CD59-} frequencies were evaluated for sex-related differences. For each post-treatment time point, the effect of sex on %RET, RET^{CD59-} and RBC^{CD59-} frequencies was evaluated for each like-treated group. Post-treatment samples were also evaluated on a per sex basis for treatment-related differences in %RET, RET^{CD59-} and RBC^{CD59-} frequencies.

Results

1,3-Propane sultone

Prior to dosing, males exhibited a 1.9-fold higher mean %RET frequency compared to females (Figure 1, Day -4). Whereas a pronounced age-related effect on %RET was evident, especially between Day -4 and 15, only slight sex or treatment-related effects were observed. Of the two instances of treatment-related differences in %RET, both were slight 1,3-propane sultone treatment-associated increases in %RET. This is likely attributable to the relatively lengthy time frame between final treatment and first post-treatment blood sampling. In this case, increases are evidence of a rebound effect whereby a presumed initial reduction to erythropoiesis function is followed by a sustained compensatory effect.

Females' pre-treatment RET^{CD59-} frequencies were similar to those exhibited by males (Figure 2). Genotoxicant-exposed males and females exhibited similarly elevated RET^{CD59-} frequencies, with maximal or near maximal induction evident on Day 15. Although later time points also showed elevated mean RET^{CD59-} frequencies, the responses were somewhat more variable. In one case (vehicle- versus 1,3-propane sultone-exposed males, Day 30), this led to a lack of statistical significance. Even so, no evidence of a statistically significant sex-related effect was found.

RBC^{CD59-} data are shown in Figure 3. As with RET^{CD59-}, both sexes exhibited similar mean RBC^{CD59-} frequencies over the study duration. 1,3-Propane sultone-induced RBC^{CD59-} frequencies rose more slowly than RET^{CD59-}, an expected result given the slower turnover rate of the total RBC pool. Rats of both sexes exhibited similar 1,3-propane sultone-induced RBC^{CD59-} kinetics, with maximal values evident at Day 46. No evidence of a statistically significant sex-related effect was observed.

Ethyl carbamate

Longitudinal %RET frequencies are shown in Figure 4. Aside from the marked age-related effect noted above, only slight sex or treatment-related effects were observed.

Ethyl carbamate exposure was associated with significant induction of RET^{CD59-} in both sexes (Figure 5). Maximal frequencies were observed on Day 15, with similar values observed at the later two time points. While females exhibited somewhat higher RET^{CD59-} values compared to males at each post-treatment time point, these sex differences did not achieve statistical significance.

RBC^{CD59-} data are shown in Figure 6. As with RET^{CD59-}, both sexes exhibited similar RBC^{CD59-} kinetics, with maximal frequencies occurring on Day 46. As anticipated given the somewhat higher RET^{CD59-} frequencies in exposed females compared to males, there were also higher mean RBC^{CD59-} frequencies in females. However, a statistically significant effect of sex on treatment-induced RBC^{CD59-} frequency was only observed on Day 30.

Beland and colleagues have shown a greater increase in hepatocellular tumors in exposed female compared to male mice (17), which is consistent with our observation of higher mean

RET^{CD59-} and RBC^{CD59-} frequencies. In the Beland *et al.* study the observation of increased hepatocellular proliferation in females and not males may explain their results.

Thiotepa

Longitudinal %RET frequencies are shown in Figure 7. In addition to the marked age-related effect noted above, thiotepa was observed to cause decreased %RET on Day 15 in both sexes. Females also showed evidence of compensatory erythropoiesis, as elevated %RET frequencies were observed on Days 30 and 46.

Thiotepa exposure was associated with significant induction of RET^{CD59-} in both sexes (Figure 8). Maximal frequencies were exhibited on Day 15, with somewhat lower values observed at the later two time points. No evidence of a statistically significant sex-related effect was observed.

RBC^{CD59-} data are provided in Figure 9. Rats of both sexes exhibited similar thiotepa-induced RBC^{CD59-} kinetics, with maximal values evident at Day 46. No evidence of a sex-related effect was observed.

Discussion

It is standard toxicological practice to evaluate potential adverse health effects in both sexes. However, unlike most other toxicological endpoints, evaluation of genetic damage has often been conducted only in males. This is because until recently it has been necessary to conduct separate animal studies to evaluate genetic damage and the tests employed have been relatively expensive and laborious to score due to the low frequency of observed events and the manual methods of scoring employed. Rationale for using males only is that they generally have more active oxidative metabolic activation systems, and are therefore usually more sensitive to those mutagens that require metabolic activation (18). Even so, sex-related differences that include higher responsiveness in females compared to males have been reported (18-20).

Because the *Pig-a* assay can be easily integrated into routine toxicology assays, it is feasible to obtain highly sensitive genotoxicity information as a part of routine testing in both sexes and at low cost. It is therefore important to verify that the lyonization process in females does not introduce any unexpected problems with the conduct of the *Pig-a* assay. Our present data and that of previous reports (9-10) demonstrate that female rats are similarly responsive to genotoxic agents and show the same kinetic behavior as male rats. Thus, we believe that integration of the *Pig-a* assay with routine toxicology testing has the major advantages of eliminating the need for any additional animals for genotoxicity evaluation while at the same time providing sensitive genotoxicity information in both sexes, and in the context of relevant information on toxicity, pharmacokinetics, and metabolism that is routinely assessed as part of toxicity studies.

In conclusion, integrated study designs would benefit from *Pig-a* measurements in both sexes when they are available in the underlying study. This would represent a convenient and cost-effective way to identify potential sex differences in the mutagenic activation,

internal exposure to the active form, or repair of DNA lesions, as well as providing improvements to statistical power. While further comparative studies of male versus female responses to different classes of mutagenic agents are warranted, data accumulated to date with four diverse mutagens suggest that most studies would benefit from the inclusion of both sexes.

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Highlights

- To date *Pig-a* gene mutation assays have overwhelmingly involved male rats.
- Rats of both sexes were exposed to propane sultone, ethyl carbamate, or thiotepa.
- The frequency of mutant phenotype reticulocytes and erythrocytes were measured.
- No qualitative differences in *Pig-a* responses were observed for male and female rats.
- These data support the use of both sexes for *Pig-a* gene mutation studies.

Figure 1.

Mean percent reticulocyte (%RET) frequencies are graphed for vehicle and 1,3-propane sultone-treated rats for each of four time points according to sex (males = black bars, females = white bars; $n = 5$ mice per treatment group per sex). All error bars are SEM. P-values reflect two-tailed Student t-test results comparing mean %RET frequencies for sex-related differences in like-treated animals, and also for treatment-related differences with males and females analyzed separately.

Figure 2.

Mean mutant phenotype reticulocyte (RET^{CD59}) frequencies are graphed for vehicle and 1,3-propane sultone-treated rats for each of four time points according to sex (males = black bars, females = white bars; n = 5 mice per treatment group per sex). All error bars are SEM. P-values reflect two-tailed Student t-test results comparing mean RET^{CD59} frequencies for sex-related differences in like-treated animals, and also for treatment-related differences with males and females analyzed separately. Note that while RET^{CD59} values are displayed as number of mutant cells per 10⁶ total reticulocytes, the statistical tests and associated p-values are from log-transformed data.

Figure 3.

Mean mutant phenotype erythrocyte (RBC^{CD59-}) frequencies are graphed for vehicle and 1,3-propane sultone-treated rats for each of four time points according to sex (males = black bars, females = white bars; n = 5 mice per treatment group per sex). All error bars are SEM. P-values reflect two-tailed Student t-test results comparing mean RBC^{CD59-} frequencies for sex-related differences in like-treated animals, and also for treatment-related differences with males and females analyzed separately. Note that while RBC^{CD59-} values are displayed as number of mutant cells per 10⁶ total erythrocytes, the statistical tests and associated p-values are from log-transformed data.

Figure 4.

Mean percent reticulocyte (%RET) frequencies are graphed for vehicle and ethyl carbamate-treated rats for each of four time points according to sex. Same format as Figure 1.

Figure 5.

Mean mutant phenotype reticulocyte (RET^{CD59-}) frequencies are graphed for vehicle and ethyl carbamate-treated rats for each of four time points according to sex (males = black bars, females = white bars). Same format as Figure 2.

Figure 6.

Mean mutant phenotype erythrocyte (RBC^{CD59-}) frequencies are graphed for vehicle and ethyl carbamate-treated rats for each of four time points according to sex. Same format as Figure 3.

Figure 7.

Mean percent reticulocyte (%RET) frequencies are graphed for vehicle and thiotepa-treated rats for each of four time points according to sex. Same format as Figure 1.

Figure 8.

Mean mutant phenotype reticulocyte (RET^{CD59-}) frequencies are graphed for vehicle and thiotepa-treated rats for each of four time points according to sex (males = black bars, females = white bars). Same format as Figure 2.

Figure 9.

Mean mutant phenotype erythrocyte (RBC^{CD59}-) frequencies are graphed for vehicle and thiotepa-treated rats for each of four time points according to sex. Same format as Figure 3.