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Reticulocyte and micronucleated reticulocyte responses to gamma irradiation: dose-response and time-course profiles measured by flow cytometry

Stephen D. Dertinger^{1,*}, Ying Tsai², Irena Nowak², Ollivier Hyrien³, Hongliang Sun², Jeffrey C. Bemis¹, Dorothea K. Torous¹, Peter Keng², James Palis⁴, and Yuhchyan Chen²

¹ Litron Laboratories, Rochester, NY

² University of Rochester Medical Center, Dept. Radiation Oncology

³ University of Rochester Medical Center, Dept. Biostatistics and Computational Biology

⁴ University of Rochester Medical Center, Dept. Pediatrics

Abstract

A flow cytometric, anti-CD71-based method was used to measure peripheral blood reticulocyte and micronucleated reticulocyte frequencies in response to ¹³⁷Cs total body irradiation (TBI). In three independent experiments, groups of five female C57BL/6N mice were irradiated at graded doses up to 3 Gy, and peripheral blood specimens were collected at 43 hrs post-irradiation. Whereas the frequency of reticulocytes declined over the range of doses studied, micronucleated reticulocyte incidence was observed to increase in a dose-dependent manner up to 1 Gy. At doses greater than approximately 1 Gy, micronucleated reticulocyte frequencies declined with increasing exposure. These responses were highly reproducible, with significant effects on reticulocyte and micronucleated reticulocyte frequencies observed for the lowest dose studied (0.125 Gy). A time-course experiment was performed to test whether radiation-induced cell cycle delay may explain saturation of the micronucleated reticulocyte endpoint at doses > 1 Gy. For this experiment, groups of four female C57BL/6N mice were exposed to 1, 1.5, or 2 Gy TBI, and blood collection occurred at 12 hr intervals from 43 to 115 hrs post-exposure. Reduced reticulocyte frequencies were observed for each dose studied, and the recovery of reticulocytes was increasingly delayed with higher radiation doses. Maximal micronucleated reticulocyte frequencies were observed at 43 or 55 hrs, with progressively lower values at later time points. At no time did micronucleated reticulocyte frequencies induced by 1.5 or 2 Gy significantly exceed that observed for 1 Gy at 43 hrs. These time-course data suggest that radiation-induced cell cycle delay cannot account for the micronucleated reticulocyte downturn phenomenon observed at doses greater than 1 Gy. An alternate hypothesis is discussed whereby apoptotic elimination of severely damaged bone marrow erythroid precursors plays a dominant role in saturating the radiation-induced micronucleated reticulocyte response observed for C57BL/6N mice.

Keywords

Micronuclei; Radiation; Automation; Flow cytometry; Chromosomal damage

CORRESPONDING AUTHOR *S.D.D., Litron Laboratories, 200 Canal View Blvd., Rochester, NY 14623 Tele: 585-442-0930; fax: 585-442-0934; email: sdertinger@litronlabs.com.

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1. Introduction

Micronuclei are pieces of extranuclear chromatin that arise when chromosome fragments or lagging whole chromosomes fail to be incorporated into daughter nuclei as cells divide. The occurrence of micronuclei is increased following exposure to clastogenic agents that cause double-strand DNA breaks and also by aneugens that disrupt chromosomal segregation [1–2]. The *in vivo* rodent micronucleus (MN) test is widely used to identify or otherwise study chemicals with genotoxic potential [3–5].

As conventionally practiced, the rodent MN test involves microscopy-based scoring of peripheral blood or bone marrow specimens. The cell population most commonly studied in this assay is newly formed erythrocytes (reticulocytes, or RETs). There are two main advantages to RET-based analyses. First, these cells have recently undergone division, and hence were recently at risk of MN formation. This makes RETs well suited to reflect increased DNA damage associated with a recent exposure, without the need for *ex vivo* cell culture as is necessary for lymphocyte-based analyses [6]. Secondly, as RETs extrude their nuclei, simple staining techniques make MN readily observable in this otherwise DNA-deficient cell population. Although micronucleated reticulocytes (MN-RETs) are readily apparent with brightfield or fluorescence microscopy, their rarity makes this scoring method a tedious and time-consuming endeavor.

This laboratory has developed a three-color flow cytometric method for scoring MN-RETs [7]. In this system, whole blood is fixed and then incubated with anti-CD71-FITC and anti-CD61-PE. These reagents serve to differentially label RETs from mature erythrocytes and platelets. In conjunction with RNase, propidium iodide fluorescence provides differential staining of MN-RETs, RETs and nucleated cells. Aside from the more objective nature of flow cytometric scoring relative to microscopy, another important advantage of this technology is that vastly greater numbers of cells can be interrogated for MN.

While this CD71-based flow cytometric technique has been applied extensively to the investigation of clastogenic and aneugenic chemicals [8–13], comparatively little work has been accomplished with radiation [14–15]. Given the significance that is attached to ionizing radiation's clastogenic activity, it was of interest to apply this flow cytometric method to the study of irradiated rodents. Dose-response and time-course data are presented herein from mice externally exposed to gamma radiation. These data should be useful to investigators interested in designing experiments that involve low linear energy transfer (LET) radiation and the peripheral blood MN-RET endpoint. Additionally, a biological model is offered which attempts to describe the unexpected dose-response relationship that is described herein.

2. Materials and Methods

2.1 Reagents

Reagents required for blood collection, fixation, and staining included: a heparin-based anticoagulant solution, buffer solution, methanol fixative, anti-CD71-FITC, anti-CD61-PE, RNase, and propidium iodide. These reagents are all components of the commercially available kit: Mouse MicroFlow^{PLUS} Kit (Litron Laboratories, Rochester, NY). The instrument calibration standard, fixed *Plasmodium berghei*-infected mouse erythrocytes ("malaria biostandard"), was also provided in these kits.

2.2 Animals

Female C57BL/6N mice were purchased from the National Cancer Institute. Mice were allowed to acclimate for at least one week prior to treatment. Mice were handled in accordance with the standards established by the U.S. Animal Welfare Acts set forth in the National

Institutes of Health (NIH) guidelines. All procedures were reviewed and approved by the University of Rochester's Committee on Animal Resources. Purina Mills Rodent Chow 5001 and water were available *ad libitum*.

2.3 Irradiation

The age of mice at the time of treatment was between 8 and 9 weeks. For three independent dose-response experiments, treatments were performed with groups of 5 mice, and doses ranged from 0.125 to 3.0 Gy. For the time-course experiment, each dose and each time point consisted of separate groups of 4 mice, and doses ranged from 1.0 to 2.0 Gy. Total body irradiation was performed with a single dose of ^{137}Cs gamma-rays. The dose rate was approximately 2 Gy per minute. Control animals were sham-irradiated. After irradiation, the mice were maintained in groups of 5 or fewer animals per cage in pathogen-free rooms.

2.4 Blood Collection and Fixation

Blood collection occurred via an incision to the tail vein. To facilitate bleeding, mice were warmed briefly under a heat lamp. Approximately 100 μl of free-flowing blood was collected into tubes containing 350 μl heparin solution. Heparinized blood was maintained at room temperature until fixation occurred (within 4 hours). For each of three dose-response experiments, blood was collected 43 hrs post-exposure. For the time-course experiment, blood was collected at 12 hr intervals between 43 and 115 hrs post-exposure.

Heparinized blood samples were fixed with ultracold methanol according to Mouse MicroFlowPLUS Kit instructions. These fixed blood specimens were returned to the ultracold freezer for storage until flow cytometric analyses were performed as described below.

2.5 Staining and Flow Cytometric Analyses

Methanol-fixed blood samples were washed and labeled for flow cytometric analysis according to procedures described in the Mouse MicroFlowPLUS Kit manual (vP4.3M). Samples were analyzed by a flow cytometer equipped with a 488-nm laser (FACSCalibur, Becton Dickinson, San Jose, CA). Anti-CD71-FITC, anti-CD61-PE, and propidium iodide fluorescence signals were detected in the FL1, FL2, and FL3 channels, respectively (stock filter sets). The gating logic used to ensure that quantitative analyses of erythrocyte subpopulations were not contaminated by other cellular or noncellular events has been described previously [7].

Calibration of the flow cytometer was accomplished by staining malaria-infected erythrocytes in parallel with test samples on each day of analysis [12,16–17]. By adjusting voltages applied to the photomultiplier tube, it was possible to standardize the propidium iodide-associated fluorescence intensity exhibited by erythrocytes housing a single parasite (i.e., an MN-like event). In this manner, analysis regions were consistent between experiments.

Data were acquired with CellQuest software (v3.3, BD-Immunocytometry Systems, San Jose, CA). The stop mode was set so that 20,000 CD71-expressing RETs were analyzed per blood sample. As shown in Fig. 1, four erythrocyte subpopulations were enumerated, thereby providing the means to calculate %RET, an index of erythropoiesis function, and %MN-RET, an index of recent chromosomal damage.

2.6 Statistics

All statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC, USA). All tests were two-sided and conducted at the 5% level of significance.

2.6.1 Dose-Response Experiments—Five mice were randomly allocated to each experimental block—one block being defined by a dose of irradiation and an independent

experiment. A single flow cytometric analysis was performed per mouse, and observations were considered independent across mice. Our primary endpoints were %MN-RETs and %RETs. Basic descriptive statistics, including the means, medians and standard deviations, were computed for each block. The significance of the dose-response curve was assessed for each endpoint using the nonparametric Kruskal-and-Wallis test. The results were confirmed by (parametric) analyses of variance (ANOVA), where formal hypotheses testing was conducted using *F*-tests. Nonparametric Wilcoxon rank-sum tests were used for pairwise comparisons of dose groups. Linear regression analyses including dose as a continuous covariate were conducted to assess the decrease in %MN-RETs observed for doses > 1 Gy. Our hypothesis was tested using a *F*-test. Wherever needed, the results of independent analyses were combined using Fisher's method for combining *p*-values [17].

2.6.2 Time-Course Experiments—Four mice were randomly allocated to each of the 21 experimental blocks (each block being defined by a dose and a time point). The primary endpoints were also %MN-RETs and %RETs. The nonparametric Kruskal-and-Wallis test was used to assess the existence of differences between the medians of %RETs across time points. One such test was conducted for each dose of irradiation. The effect of dose on %RETs was also assessed using the same test. This was done for each time point separately. Similarly analyses were carried out to investigate %MN-RETs, and its relationship with time post-irradiation and with the dose of gamma-rays. The results of these primary statistical analyses were confirmed using parametric ANOVA, where formal hypotheses testing was carried out using *F*-tests.

3. Results

3.1 Flow Cytometric Analyses

Representative bivariate graphs illustrate the fluorescent resolution achieved for mouse blood specimens stained with MicroFlow kit reagents (Fig. 1). The erythrocyte subpopulations exhibited very consistent fluorescence profiles across these experiments that were conducted over a period of approximately 11 months. This can be attributed in large part to the use of the malaria biostandard, a kit-supplied reagent that facilitated careful calibration of photomultiplier tube voltages and compensation settings each day before experimental samples were evaluated. With this analytical system in place, the processing of experimental samples occurred very efficiently, with data acquisition times occurring in the range of 3 to 5 minutes per blood specimen.

3.2 Dose-Response Experiments

Fig 2 shows the effect of graded doses of radiation on RET frequencies 43 hrs after exposure. As expected, mean %RETs was reduced with increasing dose ($p < 0.0001$). Compared with the sham group, the lowest dose tested, 0.125 Gy, had a reproducible and marked effect on this endpoint ($p = 0.0028$).

MN-RET frequencies were observed to increase in a dose-dependent manner up to 1 Gy ($p < 0.0001$) (Fig 3). These responses were highly reproducible, with a significant difference in mean %MN-RET observed for the lowest dose tested, 0.125 Gy ($p = 0.0008$). At doses greater than 1 Gy, %MN-RET declined significantly with increasing dose ($p < 0.0001$). These results were confirmed by ANOVA analyses.

3.3 Time-Course Experiment

Results for %RETs versus time are shown in Fig. 4. Each of the three doses studied resulted in significant changes to %RET over time ($p < 0.0001$). These responses followed the same general pattern—reduction followed by a rebound effect that brought the values to higher than

normal levels. These distributions of %RET were affected by the dose of irradiation ($p < 0.0001$). Specific group comparisons suggested that differences in %RET were significant at the following time points: 43, 55, 67, 79, and 91 hrs ($p < 0.05$ in each case). ANOVA confirmed these results. The most striking feature of these data is the increasingly delayed rebound at higher doses.

MN-RET data from 43 to 115 hrs post-exposure blood samples are presented in Fig 5. Each of the three doses studied was observed to significantly affect %MN-RET over time ($p < 0.0001$). These distributions were also influenced by dose of irradiation ($p < 0.0001$). Regarding the 1 Gy dose, the peak incidence of MN-RET was observed at the 43 hr time point. This frequency was maintained at 55 hrs, but dropped quickly thereafter. The peak mean MN-RET response for the 1.5 and 2 Gy doses was not realized until the 55 hr time point. Even so, these values were not statistically different than those observed for the 1 Gy treatment group at the 43 hr time point. Rather, specific group comparisons suggested that differences in %MN-RET were only statistically significant at the following time points: 67, 79, 91, 103 and 115 hrs post-exposure ($p < 0.05$ in each case). ANOVA confirmed these results. While a temporal shift was evident, it does not appear to be of sufficient magnitude to explain the downturn phenomena noted above. More specifically, at no time did %MN-RET resulting from doses of 1.5 or 2 Gy significantly exceed the frequencies observed for 1 Gy at 43 hrs.

4. Discussion

While the MN-RET endpoint has been studied extensively by radiation researchers, there has been relatively limited utilization of flow cytometric procedures for enumerating these events [14–15,19–21]. There is reason to believe that automated systems will become more widely utilized, as they are more objective by nature, and have the ability to score many times more cells compared with microscopy-based methods. Thus, the current experiments were designed to provide flow cytometry-based RET and MN-RET data that describe the dose-response relationships and kinetics associated with total body gamma-ray exposure. The experimental data reported herein should be useful to investigators that wish to design micronucleus-centric experiments for purposes as varied as studying radio-protectants/sensitizers, or characterizing the effect that targeted gene deletions have on the incidence of unrepaired double-strand breaks.

Regarding our dose-response data, we were somewhat surprised to observe a saturation of the MN-RET response at doses of approximately 1 Gy TBI in C57BL/6N mice. The third report from the International Workshop on Genotoxicity Testing (IWGT) contains the following statement: “Theoretically, the ‘downturn phenomenon’ should not occur when immature erythrocytes were targeted for analysis and the monotonic dose–response may be obtained when the sample time was optimized for each dose-level” [3]. As radiation-induced cell cycle delay is well documented [22–24], this was a reasonable explanation for the downturn we observed. This prompted our time-course experiment that consisted of blood sample collection from 43 to 115 hrs post-irradiation.

However, the results of the time-course experiment do not support cell cycle delay and sub-optimal blood harvest time as major causes of the MN-RET saturating effect. We propose an alternate model, illustrated by Fig 6, that invokes selection against highly damaged erythroid precursors and progenitors. We hypothesize that relatively less damaged erythroid precursors (with or without micronuclei) are able to proceed through the process of terminal maturation. Conversely, those that are more severely affected, and thus more likely to develop micronuclei, are disproportionately eliminated. Preliminary experiments with bone marrow specimens collected 24 hrs post-irradiation support this model, as late-stage erythroid precursors appear to be extremely sensitive targets of irradiation, particularly at doses > 1 Gy (data not shown). Further experiments are planned to test this model, as an explanation for the downturn

phenomenon could be important to diverse radiation researchers, as well as for groups that rely on the *in vivo* erythrocyte-based assay to serve as a hazard identification tool [3–5].

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Figure 1.

Representative 0, 0.5 and 1.0 Gy specimens (panels A, B, and C, respectively) illustrate fluorescent staining characteristics of four erythrocyte subpopulations: lower left quadrant = mature erythrocytes; lower right = micronucleated mature erythrocytes; upper left = CD71-positive reticulocytes (RETs); and upper right = micronucleated CD71-positive reticulocytes (MN-RETs). Note that only gated events are shown (i.e., platelets and nucleated cells have been excluded based on their high anti-CD61-PE and propidium iodide-associated fluorescence signals, respectively).

Figure 2. Mean percentage peripheral blood reticulocyte frequencies (%RET) are graphed as a function of gamma radiation dose. All blood samples were collected 43 hrs post-total body irradiation.

Figure 3.

Mean percentage peripheral blood micronucleated reticulocyte frequencies (%MN-RET) are graphed as a function of gamma radiation dose. All blood samples were collected 43 hrs post-total body irradiation.

Figure 4.

Mean percentage peripheral blood reticulocyte frequencies (%RET) are graphed as a function of time. Blood samples were collected between 43 and 115 hrs post gamma irradiation at 12 hrs intervals.

Figure 5.

Mean percentage peripheral blood micronucleated reticulocyte frequencies (%MN-RET) are graphed as a function of time. Blood samples were collected between 43 and 115 hrs post gamma irradiation at 12 hrs intervals.

Figure 6.

The kinetics by which radiation-induced MN-RET enter the blood stream does not appear to explain the downturn that is evident at doses $> 1 \text{ Gy}$. The alternate model depicted here invokes selection against highly damaged precursors and progenitors. As illustrated above, relatively less damaged cells are seen to proceed through the process of division and differentiation. Conversely, those that have been more severely damaged and therefore more likely to express micronuclei are disproportionately eliminated via apoptosis. Preliminary experiments with bone marrow specimens collected 24 hrs post-irradiation support this model, as erythroid precursor and progenitor numbers are both severely reduced at doses $> 1 \text{ Gy}$ (data not shown).