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Short communication

Reticulocyte and micronucleated reticulocyte responses to gamma irradiation: Effect of age

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ABSTRACT

The effect of age on the formation of radiation-induced micronucleated reticulocytes (MN-RETs) and reticulocytes (RETs) was investigated by exposing female C57BL/6J mice to graded doses of gamma rays from a ^{137}Cs source. Age at time of irradiation was 6, 16, or 32 weeks, and doses ranged from 0.5 to 3 Gy. A flow cytometric technique based on anti-CD71 labeling was used to measure RET and MN-RET frequencies in blood specimens collected 43 h post-irradiation. Mean RET frequencies declined in a dose-dependent manner for each age group. There was only one significant difference among the ages, that is, %RETs were not significantly reduced in the oldest animals at 0.5 Gy, whereas this dose did have a significant effect on the other age groups. MN-RET data were more complex. Age was observed to influence the baseline frequency of MN-RET, with the oldest mice exhibiting a significantly higher mean value. Each group's %MN-RETs values increased up to 1 Gy, but past this dose the frequencies plateaued or decreased. Age was observed to influence micronucleus frequency, with older mice exhibiting higher mean MN-RET values, especially at the high doses where the response was saturated (2–3 Gy). We hypothesize that these dissimilar responses can largely be explained by an age-related down-regulation of apoptosis whereby younger animals eliminate damaged bone marrow erythroid precursors with a greater efficiency compared with aged mice.

1. Introduction

The *in vivo* erythrocyte-based micronucleus assay is extensively used to evaluate the potential of chemicals to induce cytogenetic damage. Indeed, regulatory agencies throughout the world base their assessments of chemicals' genotoxic risk on data from this assay [1–3]. Although the endpoint has been applied to the study of physical agents on a more limited basis, it is nonetheless well appreciated to be a valuable tool for studying radiation's clastogenic activity [4–8].

This laboratory has recently contributed to the radiobiology literature by reporting dose-responses and kinetic data for peripheral blood reticulocytes (RETs) and micronuclated reticulocytes (MN-RETs) in mice exposed to gamma rays from a ^{137}Cs source [9]. That study demonstrated the sensitivity of the MN-RET endpoint, as marked increases were observed at the lowest dose evaluated (0.125 Gy). We also reported an interesting phenomenon whereby a dose-related increase in the percentage of MN-RET was followed by a downturn once exposures exceeded approximately 1 Gy. Cell cycle delay leading to a suboptimal blood harvesting time was excluded as a possible reason for this observation, since varying harvest time did not provide specimens with MN-RET frequencies that exceeded those observed at 43 h for the 1 Gy exposed mice.

The current study was designed to extend our previous work with gamma irradiation in two respects. First, we wished to evaluate the effect of age on radiation-induced MN-RET yield. Second, to explore the above-noted downturn phenomenon further, we included a range of doses that were expected to saturate and even diminish the MN-RET response. The resulting data should be of interest to radiation biologists as well as investigators who utilize the micronucleus assay to study the genotoxic potential of chemicals.

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,

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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, USA). The instrument calibration standard, fixed *Plasmodium berghei*-infected erythrocytes ("malaria biostandard"), was also provided in these kits.

2.2. Animals

Female C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were allowed to acclimate for at least 1 week prior to treatment. Rodents 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. Treatment

At the time of treatment mice were approximately 6-, 16-, or 32-week-old. Doses were 0.5, 1, 2, and 3 Gy, and sham irradiation was used as the negative control. Five mice were randomly selected for each group, and total body irradiation was performed with a single dose of ^{137}Cs gamma rays. The dose rate was approximately 2 Gy/min. After irradiation, the mice were maintained in groups of five per cage in a pathogen-free room.

2.4. Blood collection and fixation

Blood was collected 43 h post-exposure via an incision to the tail vein after mice were warmed briefly under a heat lamp. Approximately 50 μL of free-flowing blood was collected into tubes containing 200 μL heparin solution. Heparinized blood was maintained at room temperature until fixation occurred (within 4 h).

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, USA). 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 [10]. Calibration of the flow cytometer was accomplished by staining malaria-infected erythrocytes in parallel with test samples on each day of analysis [11–12].

Data were acquired with CellQuest Pro software (v5.2, BD-Immunocytometry Systems, San Jose, CA, USA). The stop mode was set so that 20,000 CD71-expressing RETs per blood sample were evaluated for micronuclei. The following measurements were calculated from the resulting flow cytometric data: percent reticulocytes (%RETs, an index of erythropoiesis function), and percent micronucleated reticulocytes (%MN-RETs, an index of recent chromosomal damage).

2.6. Statistics

Each animal's %RETs and %MN-RETs measurements were used to calculate group mean and standard error values. The nonparametric Wilcoxon rank sum and Kruskal and Wallis tests were used for one-way ANOVA. The effect of age across multiple dose groups was assessed using classical two-way ANOVA, including dose and age as factors, as well as their interaction. All tests were two-sided at the 5% level of significance. All statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Reticulocyte responses

The effect of total body gamma irradiation on mean %RETs is shown in Fig. 1. Each group exhibited dose-related reductions of RET. At the highest dose level tested, mean values were reduced by approximately 90% relative to sham-exposed mice. Age was not observed to significantly affect the RET endpoint, except in the case of 0.5 Gy. At this dose, %RETs values for the 32-week-old mice were indistinguishable from those exhibited by the sham controls ($P = 0.60$), whereas younger mice exhibited marked reductions ($P = 0.016$ for both 6- and 16-week-old mice).

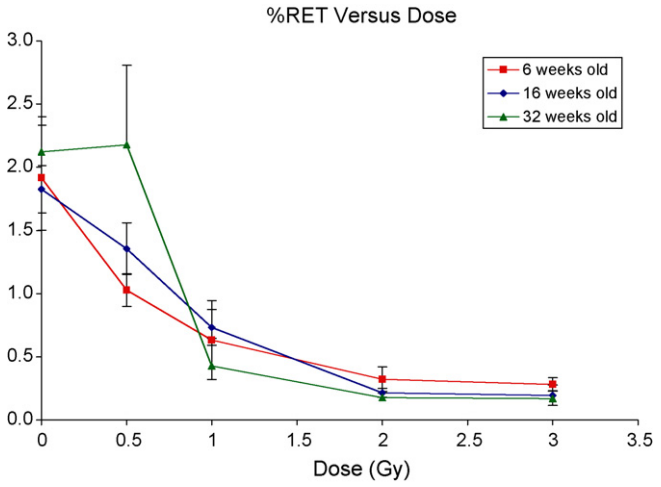

Fig. 1. Mean percentage peripheral blood reticulocyte frequencies (%RETs) with standard error bars are graphed as a function of gamma radiation dose. Blood samples from each of the three age groups were collected 43 h post-total body irradiation.

3.2. Micronucleated reticulocyte responses

Mean MN-RET frequencies for sham-exposed animals were 0.18, 0.24, and 0.53% for 6-, 16-, and 32-week-old mice, respectively. The mean value for the oldest group of mice is significantly higher than the other two when compared by the Wilcoxon test ($P = 0.001$).

The induction of MN-RET by gamma rays is shown in Fig. 2. Irrespective of age, MN-RET values were observed to rise with increasing dose up to 1 Gy. Whereas the frequencies for 6- and 16-week-old mice were equivalent across these moderate dose levels, significantly higher values were observed for the oldest group of animals ($P < 0.0001$).

Consistent with earlier findings, at doses greater than 1 Gy, we observed MN-RET frequencies to plateau or actually decline with increasing dose. In this region of the dose-response curve, MN-RET frequencies were significantly different among each of the three age groups, with successively older mice yielding higher frequencies ($P < 0.0001$).

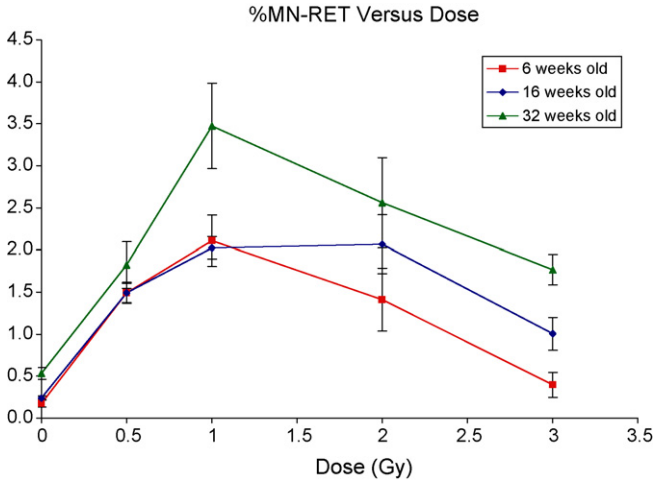

Fig. 2. Mean percentage peripheral blood micronucleated reticulocyte frequencies (%MN-RETs) with standard error bars are graphed as a function of gamma radiation dose. Blood samples from each of the three age groups were collected 43 h post-total body irradiation.

4. Discussion

The reproducibility of flow cytometry-based MN-RET measurements is well documented [13–16]. Furthermore, our previous report on gamma ray-exposed mice demonstrated that inter-experimental reproducibility is high [9]. Thus, with this backdrop, we are confident that the differences described herein are attributable to age-related effects as opposed to inter-experimental variation.

The relationship that we observed between baseline MN-RET frequency and age is consistent with other reports that have shown a direct correlation. For instance, it is well established in the cytokinesis block lymphocyte literature that micronucleus frequency is age-dependent. For example, Peace and Succop [17] noted that in 10 out of the 11 studies they surveyed, age was positively correlated with micronucleus frequency. As to the mechanism responsible for age-related increases in spontaneous micronucleus frequencies, there is evidence that whole chromosome loss is a major factor [18,19].

Regarding radiation-induced MN-RET, the findings presented here confirmed our earlier observations that the frequency of MN-RET becomes saturated at approximately 1 Gy in C57BL mice. Cell cycle delay is not a compelling explanation for this result, since delayed blood harvest times did not lead to higher MN-RET values [9]. Rather, we attribute the downturn phenomenon primarily to apoptosis. That is, we speculate that relatively less damaged erythroblasts (with or without micronuclei) are able to proceed through the process of terminal differentiation. Conversely, those that are more severely affected, and thus more likely to develop micronuclei, are disproportionally eliminated. Support for this hypothesis comes from work by Shimura et al. [20] who reported that *p53* null allele mice are hypersensitive to X-rays for induction of peripheral blood MN-RETs.

Interestingly, we observed the greatest age-related differences as MN-RET responses became saturated. Thus, if apoptosis is indeed a major factor, one is led to conclude that age-related differences in MN-RET frequency are related to aged animals' reduced capacity to apoptose radiation-damaged erythroblasts. Support for this hypothesis comes from Suh et al. [21], who treated rats with a direct-acting clastogen, methyl methanesulfonate. Whereas aged rats exhibited a modest increase in apoptotic hepatocytes, young rats showed a robust response. Also, Polyak and colleagues [22] observed a reduced apoptotic response of mouse lymphocytes to 5 Gy radiation as age increased.

The present data set cannot rule out alternative explanations for dissimilar MN-RET yields. These include age-related changes to the kinetics by which MN-RETs appear in circulation, and/or altered efficiencies of double-strand break repair. However, these mechanisms do not explain the downturn phenomenon as readily apoptosis does. First, our previous report, wherein blood harvest times were varied, does not lend support to the kinetics explanation [9]. Second, the notion of age-related differences in efficiency of repair is not consistent with our observations that radiation-induced frequencies of micronucleated lymphocytes are similar across the 6–32 weeks age range (unpublished observations in this same mouse strain, C57BL/6J).

In conclusion, data presented herein confirm our observations that the MN-RET endpoint becomes saturated in female C57BL mice at a total body dose of approximately 1 Gy. Age is observed to affect MN-RET frequencies, especially at doses $\geq$1 Gy. We hypothesize that these response profiles can largely be explained by an age-related down-regulation of apoptosis whereby younger animals eliminate damaged bone marrow erythroid precursors with a greater efficiency compared with aged mice. Direct measures of apoptosis in the bone marrow-derived cells, especially erythroblasts, are needed to test this hypothesis further.

Conflicts of interest

Several of the authors are employed by Litron Laboratories, a company that owns patents regarding flow cytometry-based scoring of micronucleated erythrocytes as described herein. Litron sells kits related to these patented methods.

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