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Response Kinetics of Radiation-induced Micronucleated Reticulocytes in Human Bone Marrow Culture

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Abstract

The frequency of micronucleated reticulocytes (MN-RETs) in the bone marrow or peripheral blood is a sensitive indicator of cytogenetic damage. While the kinetics of MN-RET induction in rodent models following irradiation have been investigated and reported, information about MN-RET induction of human bone marrow after radiation exposure is sparse. In this report, we describe a human long-term bone marrow culture (LTBMC), established in three-dimensional (3D) bioreactors, which sustains long-term erythropoiesis. Using this system, we measured the kinetics of human bone marrow red blood cell (RBC) and reticulocyte (RET) production, as well as the kinetics of human MN-RET induction following radiation exposure up to 6 Gy. Human bone marrow established in the 3D bioreactor demonstrated an average percentage of RBCs among total viable cells peaking at 21% on day 21. The average percentage of RETs among total viable cells reached a maximum of 11% on day 14, and remained above 5% by day 28, suggesting that terminal erythroid differentiation was still active. Time- and dose-dependent induction of MN-RET by gamma radiation was observed in the human 3D LTBMC, with peak values occurring at approximately 3 days following 1 Gy irradiation. A trend towards delayed peak to 3–5 days post-radiation was observed with radiation doses ≥ 2 Gy. Our data reveal valuable information on the kinetics of radiation-induced MN-RET of human bone marrow cultured in the 3D bioreactor, a synthetic bioculture system, and suggest that this model may serve as a promising tool for studying MN-RET formation in human bone marrow, thereby providing opportunities to study bone marrow genotoxicity testing, mitigating agent effects, and other conditions that are not ordinarily feasible to experimental manipulation in vivo.

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Keywords

radiation; micronuclei; genotoxicity; micronucleated reticulocytes; 3D bone marrow culture

1. INTRODUCTION

Micronucleus (MN) formation in reticulocytes (RETs) reflects cytogenetic damage induced by clastogenic agents, such as ionizing radiation and alkylating agents, or by aneugenic agents, such as compounds that interfere with the mitotic spindle apparatus [1–3]. The rodent-based MN-RET analysis is widely used to assess the genotoxic potential of chemicals [4,5], and to support the registration of new pharmaceutical agents. Analysis of MN-RET frequency in humans is useful for evaluating cytogenetic damage resulting from chemotherapy, radiotherapy, medicine, diet, and lifestyle choices [6–13].

Radiotherapy is one of the most common treatment modalities for cancer, due to the DNA-damaging effects of ionizing radiation. Radiation damage to the bone marrow compartment results in a dose-dependent acute depletion of stem cells, progenitor cells, and precursor cells of all cell lineages, as well as genotoxicity for cells surviving the direct cytotoxic effect [14–16]. Continued investigation of the human bone marrow compartment is essential to the understanding of the bone marrow's response to radiation injury and of the genotoxic effect, which may confer carcinogenic potential. Cells from different hematopoietic lineages and differentiation status have varying sensitivities to radiation. Thus, radiation significantly affects the kinetics of differentiation and proliferation [17]. How ionizing radiation may affect the kinetics and magnitude of human MN-RET formation remains largely unknown.

To our knowledge, thorough response kinetics of radiation-induced MN-RET of human bone marrow has not been reported. Investigation of radiation response kinetics is limited by the inability to design studies of normal human bone marrow exposed to radiation. Studies conducted in cancer patients receiving radiation as part of the cancer therapy are confounded by many clinical factors, such as radiation dose-volume to localized skeletal regions, cancer effects on the normal bone marrow compartment, and effects of anti-neoplastic agents (such as chemotherapy or immunotherapy) on the bone marrow.

An in vitro radiation model makes studying human bone marrow response to radiation more amenable to experimental manipulation. The 3D long-term bone marrow culture (LTBMC) system described herein has been optimized for erythropoiesis in vitro, an important requirement when studying cytogenetic damage in the form of MN in RETs. These studies are an extension of previous work with a murine 3D LTBMC system [18]. In the current report, we describe the kinetics of human red blood cell (RBC) and RET production, as well as the dose response and kinetics of MN-RET formation, following radiation doses up to 6 Gy.

2. Methods and Materials

2.1 3D bioreactor

The 3D bioreactor was fabricated using polycarbonate plates as described previously [19]. Briefly, there were six independent culture wells in a 3D bioreactor. A 3D culture well consisted of two center-vertically-aligned chambers: the large upper medium chamber and the small lower culture chamber (Figure 1). The lower culture chamber was packed with 10 mg CellsnowTM-EX, type L (low ion-charged), macroporous cellulose microcarriers (Kirin, Japan; 1–2 mm diameter; 100–200 µm pore size; 95% porosity), which formed the 3D artificial scaffolding for human bone marrow cells. The upper medium chamber contained

most of the medium. A Teflon™ membrane (50 µm thickness) was fabricated into the bottom of the culture chamber to facilitate gas exchange. After the bioreactor was autoclaved with Dulbecco's Phosphate Buffered Saline (DPBS) in the medium chamber, the microcarriers were balanced overnight with the culture medium. Before seeding cells, the medium in medium chamber and culture chamber was removed.

2.2 Human bone marrow cells and human LTBMC

Fresh human bone marrow mononuclear cells were purchased from Lonza (Walkersville, MD). Cells were shipped from the vendor on an ice bag overnight. Upon arrival, cells were spun down and then re-suspended in the culture medium. Mononuclear cells (3.5×10^6 cells in 100 µl medium) were seeded into the packed bed of microcarriers in the lower chamber of a 3D culture well, and incubated at 37°C for 3 h. To ensure sufficient cells for scoring MN-RET and RET, two culture wells were set up for each experimental data point. Medium (0.6 ml) was then added into the upper chamber to overlay the microcarriers. The culture was incubated at 37°C in humidified air with 5% CO₂. Medium in the upper chamber was replaced daily with 0.6 ml/well of fresh pre-warmed medium. Cells in the lower chamber were partially harvested weekly by suctioning away medium in the upper chamber and by gently mixing the cells in the lower chamber. An aliquot (50 µl) of cells was harvested from a well in the first week and 100 µl aliquots in the following weeks. The culture medium was Iscove's Modified Dulbecco's Medium (IMDM) plus hydrocortisone (Sigma, St. Louis, MO; 1 µM); iron-saturated human holo-transferrin (Chemicon International, Temecula, CA; 0.5 mg/ml); 500-fold diluted chemically-defined iron supplement (Sigma, St. Louis, MO), GM-CSF (R&D Systems, Minneapolis, MN; 1 pg/ml); recombinant human EPO (Amgen, Thousand Oaks, CA; 0.2 U/ml); L-glutamine, 2 mM; 0.8% penicillin/streptomycin; and 15% charcoal-treated FBS (Hyclone, Logan, UT).

2.3 Slide preparation and staining for differentiation count

The viable cells were distinguished by Trypan blue and counted using a hemacytometer (Hausser Scientific, Horsham, PA). Slides were prepared by spinning 4×10^4 cells in 200 µl Dulbecco's Phosphate-Buffered Saline (DPBS) onto the slides using a cytospin at room temperature. Cells from duplicate culture wells were used to generate slides for the staining and scoring. The slides were air-dried, and then benzidine staining or Wright staining was applied. The procedure for benzidine staining was modified from the method as described [20]. Benzidine specifically reacts with hemoglobin, and thus stains late erythroid precursors and erythrocytes. It was used to identify hemoglobin+ cells such as nucleated erythroid precursors and enucleated erythrocytes. Slides were first fixed in methanol for 5 min, incubated in 1% 3', 3'-dimethoxy-benzidine (Sigma, St. Louis, MO), then in methanol for 2 min and 1% H₂O₂ solution (in 50% methanol: 50% water) for 2 min, washed in water for 1 min, counter-stained with diluted Wright-Giemsa stain, and again washed in water for 1 min. The percentage of enucleated erythrocytes among total viable cells was obtained from the slides.

To score the percentage of RETs among enucleated erythrocytes, 4×10^4 cells in 100 µl DPBS were mixed with fresh methylene blue "N" staining solution (RICCA Chemical, Arlington, TX; 100 µl) and incubated at room temperature for 12 min. The mixture was spun down with cytospin onto slides and Wright-Giemsa stain was applied to increase the readability. The percentage of RETs among total viable cells was calculated by multiplying the percentage of enucleated erythrocytes among the viable cells with the percentage of RETs among enucleated erythrocytes.

2.4 Radiation of 3D LT BMCs

3D LT BMCs were irradiated on day 18 or 19 after inoculation, using a Cs¹³⁷ irradiator at dose rate 2.8 Gy/min. Independent bone marrow bioreactors were irradiated separately to a dose range of radiation: sham radiation (0 Gy), 1, 2, 3, 4, 5, or 6 Gy. We chose the 18–19 day time point to avoid a period when RET counts were rapidly changing, and because the number of RETs during this period was sufficient to facilitate slide counting.

2.5 RET and MN-RET scoring

RET and MN-RET scoring was accomplished after staining with Acridine Orange (AO) [21]. Cells were harvested at serial time points post-irradiation, to measure the induction of MN-RET, by washing microspheres once with culture medium (200 μ l). The cells from two wells were used to prepare 8–10 smear slides. Smear slides were made, air-dried, and fixed in methanol for 9 min. They were then stained before counting by immersion in solution containing 12.5 mg Acridine Orange (Sigma, St. Louis, MO) in 100 ml DPBS (pH6.8) for 1 min, washed in DPBS (pH6.8) for 7 min, and transferred to fresh DPBS to be washed for 2 min. One drop of DPBS was added to the slides and a cover slip was placed before microscopic scoring. Additional washing with fresh DPBS was applied if the DNA color was orange instead of yellowish-green. RETs were scored as cells that had a well-defined border and a distinct red fluorescence signal, while the MN-RETs were scored as RETs that contained one or two small green DNA micronuclei. To minimize biases of scoring, more than 2000 RETs from more than three slides (up to ten slides in the high-radiation-dose groups) were scored for MN-RETs and RETs for each data point. The percentage of MN-RETs among all RETs is defined as MN-RETs/(MN-RETs+RETs).

2.6 Statistical methods

Analyses of variance (ANOVA) were conducted using the nonparametric Friedman's test. Paired t-tests were performed to compare differences between two observations. All tests were two-sided.

3. Results

3.1 Terminal erythropoiesis in the human 3D LT BMC

Fig. 2 shows the microscopic cell morphology revealed by the staining protocols applied to human LT BMC established in 3D bioreactors. Fig. 2A–B shows the presence of white blood cells (WBC) and cells at different stages of erythropoiesis, including nucleated and enucleated RBCs. Fig. 2C shows a slide made from an irradiated LT BMC, illustrating the staining characteristics of many small mononucleated cells, large nucleated cells of WBC lineage, mature RBCs, RETs, and a MN-RET.

For the investigation of RETs, cell cultures were maintained for 4–5 weeks. The average cell counts and viability of cultures seeded with bone marrow from three different healthy donors are shown in Fig. 3A–D. Data up to 28 days are shown here, as culture duration up to 10 days was sufficient to observe the induction of MN-RET (Fig. 4 and 5). Data values represent mean $\pm$ standard deviation. The weekly viable cell output, obtained by partly harvesting a well, reached a peak on day 14 and remained at about 0.5×10^6 cells/well at day 28 (Figure 3A). The viability was above 85% in the first three weeks (Figure 3B). In these non-irradiated cultures, the average percentage of RBCs among total viable cells increased to 16% by day 14, peaked at day 21 (21%), and decreased to 17% by day 28 (Figure 3C). The average percentage of RETs among total viable cells reached a maximum of 11% on day 14 and was 5% by day 28, suggesting the terminal erythroid differentiation was still active (Figure 3D).

3.2 Kinetics of induction of MN-RET in the human 3D LTBM

The kinetics of the radiation-induced MN-RET were measured in the cultures of three healthy bone marrow donors (Fig. 4). (These samples represent different donors from those in Fig. 3 due to the limited number of cells obtained from each donor.) We noted consistently low baseline MN-RET frequencies, defined by MN-RETs/(MN-RETs + RETs) in the sham-irradiated group, with mean = 0.89% (range 0.73% ~ 1.1%), 0.64% (range 0.55% ~ 0.90%), and 0.78% (range 0.58% ~ 0.97%), for the three donors. The background MN-RET frequency did not change with time ($p > 0.6$, Friedman's non-parametric ANOVA). We observed no significant change in MN-RET frequency on day 1 when compared with the sham-irradiated group. The MN-RET frequency significantly increased on day 2 for both 1 Gy and 4 Gy radiation exposures ($p \leq 0.02$, two-sided paired t-test). The percentage of MN-RET following exposure to 1 Gy was significantly elevated on days 3 and 4 ($p < 0.02$, two-sided paired t-test) and reached the maximum of $13\% \pm 3\%$ (mean $\pm$ std) on day 3. There was a significantly higher percentage of MN-RET above the background after 4 Gy irradiation, an effect that persisted from days 2 to 6 ($p < 0.04$, two-sided paired t-test). The mean percentage was $25\% \pm 8\%$ on day 3 and $24\% \pm 6\%$ on day 4. Compared with 1 Gy radiation, 4 Gy induced higher MN-RET percentages on days 4 to 6 ($p < 0.05$, two-sided paired t-test). The percentage of MN-RET post 1 Gy and 4 Gy radiation was significantly influenced by time ($p < 0.01$, Friedman's non-parametric ANOVA).

To build upon the observation from the three donor bone marrow cultures, the kinetics of MN-RET induction was further measured in four additional donors' marrows after irradiation with a wider dose range (1–6 Gy), and data collected at four different time points from days 2 to 5. Figure 5 shows a compilation of the data from four additional donors. Again, a stable and low level of baseline MN-RET expression was observed in the sham-treated cultures. While donor-to-donor variations were noted for the peak level of MN-RET expression, maximum MN-RET induction was observed on day 3 after 1 Gy treatment in all cultures. With the exception of one donor, maximal responses were observed between days 3 and 4 following 2 Gy radiation, while maximal responses following radiation doses ≥ 3 Gy were observed between days 4 and 5 (Fig. 5).

4. Discussion

MN-RET induction in the bone marrow cultures in vitro requires terminal erythropoiesis through the continuum of the proliferation and differentiation processes of bone marrow cells. When cultures are studied over prolonged periods of time, this involves critical steps of differentiation from stem cells to progenitor erythroblasts, precursor cells, and RETs. With increasing understanding of the regulation of erythropoiesis, human CD34+ or murine Lin- bone marrow cells are able to be expanded and differentiated along the erythroid lineage to generate RETs and RBCs, in the presence of cytokine cocktails [22–25]. At least one report shows that suspension cultures of murine Lin- bone marrow culture can induce MN-RET after genotoxic drug treatment, but with a limited time window of approximately 2–3 days for generating RETs [25]. This window does not adequately reflect in vivo conditions. Suspension cultures thus may not be most suitable for studying the kinetics of MN-RET induction.

LTBM has been improved from the traditional Dexter's bone marrow culture in flasks [26]. These improved cultures support long-term growth and differentiation of erythroid progenitors and precursors [27–32]. Wu and colleagues have further improved the performance by maintaining the 3D organization of bone marrow cells in the 3D bioreactor [19,33–35], where the configuration mimics bone marrow structure in vivo and erythropoiesis is sustained up to several weeks [33,34].

With improved 3D bioreactor and cytokine combinations, we have previously optimized a murine 3D LTBM capable of generating high percentages of RETs for up to 8 weeks. We have demonstrated the system's utility in a study of MN-RET induction following irradiation of mouse bone marrow [18]. We observed parallel MN-RET induction in vivo after total body irradiation in C57Bl/6 mice when compared with MN-RET induction of bone marrow cultures of C57Bl/6 in 3D bioreactors with very similar radiation dose responses, as well as comparable patterns of kinetics of MN-RET induction after radiation [18,36]. The investigation of human bone marrow in 3D LTBM has required additional modifications of the culture condition, as the optimized culture condition for mouse bone marrow does not sustain the culture for human bone marrow.

Although enucleated erythrocytes have been observed in the human LTBM by other investigators, the percentage of RETs and the kinetics have not been reported [32,34,37]. We have optimized serum, cytokines, and iron sources to establish a working system for generating RETs up to 4 weeks (Figure 3), although full optimization may not have been achieved. The average RET percentage in the output from three cultures reached 11% on day 14 and was about 5% by day 28, suggesting that terminal erythroid differentiation was still active in the end of the culture. This condition has been tested in cultures from different healthy human donors, and appears robust despite the variation among different healthy donors. All cultures were able to generate sufficient RETs and radiation-induced MN-RET after irradiation with a wide range of doses from 1–6 Gy.

Gamma radiation caused robust MN-RET formation in the human 3D LTBM. The kinetics of MN-RET induction from each individual were observed in our experimental design and shared common features. First, the background percentage of MN-RET post sham (0 Gy) radiation was consistently low and stable throughout the culture period. The percentage of MN-RET started to rise only “after” a latent period of approximately one-day post-radiation, with a significant increase by day 2. Maximum MN-RET induction was observed on day 3 after 1 Gy treatment in all cultures. With the exception of one donor, delayed peak to 3–4 days post-radiation was observed with radiation doses of 2 Gy, and to 4–5 days post-radiation with radiation doses ≥ 3 Gy (Fig. 5). A trend was evident whereby the peak of %MN-RET was delayed as radiation exposure levels increased in each culture. We would have preferred to include triplicate experimental data points for the presentations of Fig. 4 and 5, but, given the limited yield of cells from each bone marrow donor and the need for multiple experimental data points of the 3D culture system, we were only able to include duplicate cultures. To minimize scoring biases, each data point was generated with the scoring of a minimum of 2000 RETs.

It is likely that higher doses of radiation led to longer cell-cycle delay [38,39] and thereby to an increase of MN-RETs at later time points and, consequently, a wider peak as well. While our experimental design has covered radiation doses as high as 6 Gy, the peak value may not have reached the maximum for radiation doses beyond 6 Gy. There were fewer and fewer cells for scoring, as apoptotic effects dominated at higher radiation doses. We noted some variation of the peak time interval in higher dose radiation than in low dose radiation among different donors. For example, after 4 Gy exposure, the MN-RET percentage peaked on day 3 in three donor cultures, on day 4 in three other donor cultures, and on day 5 in another culture (Fig. 4 and 5). This result contrasts with the peak MN-RET level on day 3 in all cultures after low dose radiation exposure (1 Gy). Apart from the potential cell-cycle delay, the underlying mechanisms of such variables can be confounded by differences in radiation sensitivity, DNA repair capacity, and nutrients involved in erythropoiesis among human population.

The increase of MN-RET from day 2 did not appear to be random, but instead was part of a pattern observed in most bone marrow samples we have examined. At least one cell division-cycle was required for the formation of micronuclei after radiation. The minimum time for the detection of MN-RET post radiation, i.e. the latent period, would have equaled the sum of time required by a cell division cycle and the enucleation event. While not much is known about the human MN-RET kinetics after radiation, MN-RET formation in mouse bone marrow and in the peripheral blood has been observed to peak at about 45 hours post-irradiation at 1 Gy [40].

Our group has previously reported MN-RET induction in the human peripheral blood from cancer patients receiving partial body irradiation. We noted a latent period of 2–3 d in peripheral blood post radiotherapy in most cancer patients [6]. We anticipate differences in the kinetics of human and mouse bone marrow. Likewise, we also expect differences in the kinetics of MN-RET in bone marrow and in the peripheral blood, as the spleen constantly removes MN-RET from the circulation. Others have estimated that a minimum of 2 d is needed for MN-RET to appear in the peripheral blood [41,42]. Grawe et al. observed an approximate 3 d latent time from patients with normal erythropoiesis and argued that the shorter latent time in some patients was caused by iron-deficient erythropoiesis and the anemia suffered by them [12].

Our data showed approximately 2–3 d latent time for MN-RET induction in the human 3D LTBMCM after radiation, which suggested that the cell proliferative rate and cell cycle time of bone marrow progenitor and precursor cells for erythropoiesis are comparable between the human 3D LTBMCM and human bone marrow in vivo [6]. However, our data represents findings of the kinetics of MN-RET and RET production of bone marrow established in the 3D bioreactor, a synthetic bioculture system that sustains the generation of reticulocytes and erythrocytes from the upstream stem cells, progenitor cells, and precursor cells. The 3D bioculture system is not intended to simulate both the bone marrow and the peripheral blood compartments of humans, nor is it suitable to address the complexity of the interaction between the bone marrow and peripheral blood; the 3D culture system resembles only one compartment in its production of RETs and radiation-induced MN-RETs. It lacks the two-compartment steady-state of erythropoiesis of the in vivo condition, as there is constant relocation of RETs and MN-RETs to peripheral blood after a certain residence time in the bone marrow in vivo. To complicate the in vivo kinetics further, the spleen serves as a filter and constantly clears RETs and MN-RETs from the peripheral blood circulation. Thus the data generated from the 3D bioreactor at most resembles the condition in the bone marrow only and not the kinetics in the peripheral blood.

In summary, we report a 3D bone marrow culture system that has sustained long-term in vitro erythropoiesis. There is time and dose-dependent induction of MN-RET by gamma radiation in the human 3D LTBMCM. A trend was evident whereby the peak of % MN-RET was delayed as radiation exposure levels increased in most donor cultures. Our data from human 3D LTBMCM reveal novel findings of kinetics of radiation-induced MN-RET in a time and dose-dependent manner. Our data also support that the human 3D LTBMCM model may offer great potential for investigating the induction of MN-RETs of human bone marrow by other genotoxic agents.

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Figure 1.
A schematic presentation of the 3D culture well in the bioreactor.

Figure 2.

A) Microscopic view of cells from the human 3D LT BMC that were stained with benzidine. RBC: red blood cell. B+ MNC: Benzidine+ mononuclear cell. B- MNC: Benzidine- mononuclear cell. Magnification: 1000X. B) Microscopic view of cells from the human 3D LT BMC that were stained with New Methylene Blue and counter-stained with Wright staining. Magnification: 1000X. C) Microscopic view of MN-RET induced by gamma-radiation in the human 3D LT BMC. Cells were stained with acridine orange. Only a typical MN-RET (mn-r), reticulocyte (ret), and mature erythrocytes (me) are respectively labeled with mn-r, ret, and me. Magnification: 400X.

Figure 3.

The kinetics of terminal erythropoiesis in human 3D LTBM. A) Weekly viable cell output per well in the human 3D LTBM from three independent cultures seeded with bone marrow from healthy donors. B) Viability. C) The percent erythrocytes (RBCs) among total viable cells including RETs in the weekly output, and D) the percentage of RETs among total viable cells in the weekly output. Data are shown as mean $\pm$ standard deviation (n=3).

Figure 4.

MN-RET induction by 0, 1, 4 Gy gamma radiation (solid square: sham radiation, solid circle: low-dose radiation at 1 Gy, solid triangle: higher dose radiation at 4 Gy) in 3D LTBM as a function of time from bone marrow cultures of three different healthy donors. The donor information is listed below each panel. Percent MN-RET is defined as $\text{MN-RETs}/(\text{MN-RETs} + \text{RETs})$.

Figure 5. MN-RET induction by 0~6 Gy gamma-radiation from four different healthy donors in 3D LTBM as a function of time. Percent MN-RET is defined as $\text{MN-RETs}/(\text{MN-RETs} + \text{RETs})$. The donor information is listed under each panel.