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Gamma Radiation Induces Micronucleated Reticulocytes in 3-D Bone Marrow Bioreactors in Vitro

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Abstract

Radiation injury to the bone marrow is potentially lethal due to the potent DNA-damaging effects on cells of the hematopoietic system, including bone marrow stem cell, progenitor, and the precursor cell populations. Investigation of radiation genotoxic effects on bone marrow progenitor/precursor cells has been challenged by the lack of optimal in vitro surrogate organ culture systems, and the overall difficulty to sustain lineage-specific proliferation and differentiation of hematopoiesis in vitro. We report the investigation of radiation genotoxic effects in bone marrow cultures of C57Bl/6 mice established in 3-D bioreactors, which sustain long-term bone marrow cultures. For these studies, genotoxicity is measured by the induction of micronucleated reticulocytes (MN-RET). The kinetics and dose-response relationship of MN-RET induction in response to gamma-radiation of bioreactor-maintained bone marrow cultures are presented. Our data showed that 3-D long-term bone marrow cultures had sustained erythropoiesis capable of generating reticulocytes up to 8 weeks. The peak time-interval of viable cell output and percentage of reticulocytes increased steadily and reached the initial peak between the 14th to 21st days after inoculations. This was followed by a rebound or staying relatively constant until week 8. The percentage of MN-RET reached the maximum between 24 and 32 hours post 1 Gy gamma-ray. There was a near linear MN-RET induction by gamma radiation from 0 Gy to 1.0 Gy, followed by an attenuated increase to 1.5 – 2.0 Gy. The MN-RET response showed a downtrend beyond 2 Gy. Our data suggest that bone marrow culture in the 3-D bioreactor may be a useful organ culture system for the investigation of radiation genotoxic effect in vitro.

Keywords

micronuclei; radiation; genotoxicity; reticulocytes; bone marrow; 3-D bioreactor

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INTRODUCTION

Bone marrow is one of the most sensitive organs to radiation injury. Radiation-induced DNA injury to the bone marrow compartment invariably results in both cytotoxicity and genomic damage to hematopoietic cells of all subpopulations, including stem cells, progenitor cells, and precursor cells of all lineages. While cytotoxic effects of direct radiation may lead to acute depletion of bone marrow reserve, the genotoxic effect may lead to long-term carcinogenic potential, as radiation is one of the most potent clastogenic agents [1–3]. The investigation of radiation genotoxic effects on bone marrow cells thus is essential to the research of long-term effects after acute radiation exposure.

Micronuclei (MN) are pieces of extranuclear chromatin caused by genotoxic agents. Micronuclei represent chromosome fragments or lagging whole chromosome(s) failing to incorporate into the daughter cell nuclei during mitotic divisions after genotoxic insults [4,5]. The frequency of MN is increased following exposure to either DNA damaging agents (clastogens) or agents interfering with microtubule function at the time of cell division (aneugens) [5–8]. Micronucleated reticulocytes (MN-RET) in the peripheral blood or in the bone marrow have been recognized as a sensitive biomarker of cytogenetic damage, and are useful in assessing carcinogenic potential of chemicals [8,9]. The rodent-based MN-RET analysis has been standardized commercially [10,11], and the assay fulfills FDA and international regulatory agencies' requirements for in vivo cytogenetic damage assessments [8–10]. Approximately 70% of known human carcinogens are detected by in vivo MN tests, which are also useful in measuring occupational and environmental exposures to genotoxic agents in humans [12–14].

Radiation-induced MN formation in reticulocytes (RET) reflects the kinetics of progenitor/precursor cells of erythroid lineage in the bone marrow in the response to radiation genotoxicity. While most MN-RET genotoxicity studies have been reported in experimental animals, there is a great advantage in developing in vitro bone marrow cultures as surrogate bone marrow model in radiation research, when irradiation of the entire animal may not be feasible or ethically acceptable. The potential of an in vitro radiation model makes studying human bone marrow in the response to radiation more amenable to experimental maneuvers, especially when direct investigation of humans after radiation exposure is often not plausible. There are limited human data available to validate the application of the human bone marrow cultures in studying the induction of MN-RET after irradiation. We are the first to test radiation-induced MN-RET in the murine bone marrow in the 3-D culture system and to reference our recently published work in the mouse in vivo model after irradiation. Here we report investigation of gamma- radiation-induced MN-RET of bone marrow precursor cells involved in erythropoiesis in C57Bl/6 mouse bone marrow cultures established in 3-D bioreactors. The three-dimensional bone marrow bioreactor is different from the 2-D flask culture system in that the architectural scaffolding may support a more conducive microenvironment for bone marrow homeostasis. Our bone marrow cultures in the 3-D bioreactors have previously shown sustained long-term bone marrow hematopoietic proliferation and differentiation, and facilitate the full-spectrum of erythropoiesis [15–17]. It is noteworthy that the conventional 2-D flask, Dexter-style long-term bone marrow culture system has been able to stimulate the growth of more primitive burst-forming unit – erythroid (BFU-E) and the colony-forming unit – erythroid (CFU-E) [18–21], but the effective differentiation into the late precursors and the terminally differentiated red blood cells has not been reported. It is also noteworthy that the suspension cultures of human CD34+ or mouse Lin- progenitors can produce reticulocytes, but only in the final stage lasting for only several days [22–25]. In contrast, the 3-D long-term bone marrow culture (3-D LTBM) system can potentially support the long-term growth and differentiation of erythroid progenitors and precursors to generate erythroblasts, reticulocytes, and mature

erythrocytes, thus amenable to the investigation of MN-RET induction of bone marrows maintained in the in vitro condition.

METHODS AND MATERIALS

Murine bone marrow cells

Four- to nine-week-old mice were purchased from the National Cancer Institute (Frederick, MD) and 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 animal studies were conducted according to an animal experimental protocol approved by the University of Rochester's Committee on Animal Resources.

Murine bone marrow cells were flushed from femurs and tibias of C57BL/6 mice into ice cold RPMI 1640 containing 10% FBS and 0.8% penicillin/streptomycin. The cells were pooled to provide uniform inoculums. Red blood cells (RBCs) were hemolysed by ACK buffer (0.83% NH_4 , 0.1% KHCO_3 solution). The bone marrow mononuclear cells were suspended in the culture media and kept on ice until seeding.

3-D long-term bone marrow culture

Ten million mononuclear cells from mouse bone marrow were seeded into a 3-D scaffolding of a bioreactor. Each bioreactor was packed with porous microspheres to provide a 3-D growth configuration mimicking the bone marrow in vivo as previously described [15,17,26]. The culture medium consisted of RPMI 1640 plus 10mM Hepes, 5mM L-glutamine, 0.8% penicillin/streptomycin, 10^{-4}M 2-mercaptoethanol, 10^{-7}M hydrocortisone (Sigma, St. Louis, MO), 0.2U/ml recombinant human erythropoietin (EPO) (Amgen, Thousand Oaks, CA), 0.5mg/ml iron-saturated human holo-transferrin (Chemicon International, Temecula, CA) and 15% charcoal-treated FBS (Hyclone, Logan UT). The media (0.6ml/well) were changed daily. Cells in the lower chamber were partially harvested weekly by suctioning away the upper chamber media and by gently mixing the cells in the lower chamber. 50 μl of cells was harvested from a well in the first week and 100 μl in the following weeks. The harvested cells were mainly floating mature cells and loosely adherent cells and were regarded as the "weekly output".

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 with cytopspin of 6×10^4 cells in 200 μl Dulbecco's Phosphate Buffered Saline (DPBS) onto slides at room temperature. The slides were then air-dried and Wright-Giemsa stain (Sigma, St. Louis, MO) was applied. The percentage of enucleated erythrocytes among total viable cells was obtained from the slides. To score the percentage of reticulocytes among enucleated erythrocytes, 6×10^4 cells in 100 μl DPBS were mixed with 100 μl new methylene blue "N" staining solution (RICCA Chemical, Arlington, Texas) and incubated at room temperature for 15 min. The mixture was spun down with cytopspin onto slides and Wright-Giemsa stain was applied to increase the readability. The percentage of reticulocytes among total viable cells was calculated by multiplying the percentage of enucleated erythrocytes among the viable cells with the percentage of reticulocytes among enucleated erythrocytes.

Radiation of 3-D LTBM

3-D LTBM cultures were irradiated on the 18th or 19th day after inoculums using a Cs^{137} irradiator at 2.8 Gy/min dose rate. Independent bone marrow bioreactors were irradiated separately to a dose range of radiation: sham radiation (0 Gy), 0.125 Gy, 0.25 Gy, 0.5 Gy, 0.75 Gy, 1Gy, 1.5 Gy, 2.0 Gy, 2.5 Gy and 3.0 Gy.

RET and MN-RET scoring

RET and MN-RET scoring was accomplished with Acridine Orange (AO) staining [27]. Cells were harvested at 24 hours post-radiation or at the serial time points to measure the kinetics of induction of MN-RET by washing microspheres with DPBS containing 2mM EDTA four times. Slides were made after cytopspin, air-dried, and fixed in methanol for 10 min. Slides were kept at 4°C until the MN-RETs were stained 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 8 min, and then transferred to fresh DPBS to be washed for one additional min. Additional washing with fresh DPBS might be added if the DNA color was orange instead of yellowish-green. One drop of DPBS was then added to the slides and a cover slip was placed before microscopic scoring. RETs were stained red without any green DNA stain, while the MN-RETs were stained red and contained one or two small green DNA fragments. The frequency of reticulocytes among all red blood cells and RETs cells were scored for % RETs, while more than 2000 RETs were counted in each sample for the percentage of MN-RETs among all RETs.

Statistical methods

One and two-way analyses of variance (ANOVA) were conducted using the nonparametric Friedman's test [28]. Fisher's method was used to combine p-values. The paired t- tests were performed to compare the difference between two observations. All tests were two-sided.

RESULTS

Erythropoiesis in the 3-D LT BMC

Fig. 1A and B shows the morphology of cells 2 weeks after inoculation of mononuclear cells in the 3-D LT BMC, and harvested for staining. Fig. 1A shows the cell morphology after the Wright-Giemsa stain. The figure shows erythroid lineages at different stages of differentiation including proerythroblasts, normoblasts, erythrocytes, and enucleated nuclei (small dark particles without cytoplasm). Fig. 1B shows the cell morphology after the methylene blue "N" stain. The figure shows the presence of reticulocytes in the 3-D LT BMC with blue residual RNA content in the cells. Fig. 1C shows a micronucleated reticulocyte induced by radiation, stained with Acridine Orange and observed under the fluorescent microscopy. Three of the four nucleated cells in Fig. 1C are likely mononuclear cells of non-erythroid origin, such as hematopoietic progenitors/precursors and mature cells of granulocytes, monocytes, and macrophage. One of the four nucleated cells is likely an erythroid progenitor.

The kinetics of erythropoiesis in 3-D LT BMC is shown in Fig. 2. Fig 2A shows the absolute weekly viable cell output from the culture wells, while Fig 2B shows the percentage of viable cells from each weekly samples. The weekly viable cell output (Fig. 2A) and percentage of reticulocytes (Fig. 2D) increased and reached the initial peak between the 14th to 21st days after inoculations. This was followed a rebound or staying relatively constant until week 6~8. The terminal erythropoiesis reached a peak between week 2 and 3 (Fig. 2C). The average percentage of RBCs among total viable cells in the weekly output reached 57%, 79% and 70% on days 14, 21, and 28, respectively (Fig. 2C). The average percentage of RETs among total viable cells was 35%, 38% and 24% in the same period (Fig. 2D). The lineage distribution in the mononuclear cells (MNCs) was measured in the two cultures. The erythroid precursors in the MNCs were averagely 22%, 69%, 48% and 65% at days 7, 14, 28 and 42. The myeloid lineage, mainly neutrophil and monocytes, were 64%, 19%, 33% and 27% in the same time. The macrophages were 11%, 9%, 13% and 4%. The other lineages and early hematopoietic progenitors consisted of the remaining small fraction. When a culture well was washed with DPBS, the number of harvested cells increased several fold to 6~12 million, with a slight decrease in the percentages of RBCs and RETs.

Kinetics of MN-RET and RET in response to gamma-radiation in 3-D LTBM

An important aspect of applying the in vitro model to MN-RET investigation was to first establish the kinetics of RET changes and MN-RET induction after radiation in the 3-D LTBM. Reticulocytes have a very short lifespan and are continuously generated in the bone marrow from erythroblasts. In vivo, RETs remain in the bone marrow compartment for approximately 10 hours before entering into peripheral blood [29]. Thus, radiation-induced MN-RETs in the bone marrow and the circulating blood represent a dynamic process between the bone marrow and peripheral circulation. In contrast to the in vivo organ, the reticulocytes generated in the 3-D LTBM remain in the culture chamber until cells are harvested. Therefore, the kinetics of MN-RET induction is expected to be different from the in vivo rodent model.

The kinetics of induction of MN-RET in the 3-D LTBM after radiation exposure was measured in three independent experiments as shown in Fig. 3A. The 18th or 19th day after inoculation was chosen as the time to irradiate the bone marrow cultures, because of relatively high percentage of reticulocytes at day 18th and 19th (Fig. 2D). In the control cultures of sham irradiation (0 Gy), the percentage of MN-RET, i.e. the background, was at an average of 1.3% (range 0.9 – 1.5%) in the two experiments (open diamonds and triangles) and around 2.6% (range 2.4 – 2.7%) in the third experiment (open squares) conducted with different lots of reagents, indicating a stable background level of MN-RET in the 3-D LTBM in each experiment. The MN-RET induction after radiation exposure was significantly above the background level at as early as 12 hours after 1 Gy irradiation ($p = 0.043$; two-sided paired t-test), and peaked at approximately 24–32 hours post irradiation to 6%–7% among reticulocytes (Fig. 3A). The percentages of MN-RET following 1 Gy exposure were significantly affected by time ($p = 0.012$; Friedman's non-parametric 2-way ANOVA) and gamma-radiation ($p = 0.0073$; Friedman's non-parametric 2-way ANOVA and Fisher's combination method). In contrast, the effect of time on the percentage of MN-RET was not significant after sham irradiation ($p = 0.25$; Friedman's non-parametric 2-way ANOVA).

The kinetic data of RETs was quite different from the MN-RETs (Fig. 3B). At each time point, the percentage of RET [% RET/(RET + mature erythrocytes)] was normalized by the value in the control culture of sham irradiation to derive relative percentage of RET. Compared with the values at 0 hour, the average relative RET percentage of RET after 1 Gy irradiation decreased significantly to 0.91 at 12 hours post radiation ($p=0.043$, two-sided paired t-test), 0.82 at 40 hours ($p = 0.049$; two-sided paired t-test) and 0.87 at 48 hours ($p = 0.02$; two-sided paired t-test), reflecting the cytotoxic impact of radiation to the precursor cells. The relative RET percentage was significantly affected by the time post 1Gy exposure ($p = 0.035$; Friedman's non-parametric one way ANOVA).

MN-RET radiation dose response

Fig. 4 shows radiation-induced MN-RET responses of the 3-D LTBM in three independent experiments. The 3-D LTBM was sensitive to gamma-radiation with the percentage of MN-RET at all doses above the level of MN-RET expression at 0 Gy of sham irradiation ($p<0.02$, two-sided paired t-test). This is evident even at very low doses of irradiation at 0.125 Gy, with the MN-RET level significantly higher than the background at 0 Gy ($p=0.01$, two-sided paired t-test). The average percentage of MN-RET, shown by the thick line, increased linearly with increasing radiation doses from 0 to 1 Gy in the culture (linear coefficient $r=0.99$, $p<0.001$), followed by an attenuated increase to 1.5 – 2.0 Gy. The MN-RET response showed a downtrend beyond 2 Gy.

The responses in the two experiments using the same batch of reagents (diamonds and triangles) were very similar. The third experiment (squares) conducted with a different batch of reagents

showed slightly higher MN-RET percentage in each dose below 1 Gy and an earlier saturation at 1.5 Gy, instead of 2 Gy observed in other two experiments (diamonds and triangles).

DISCUSSION

In this study, we applied a bone marrow culture system established in the 3-D bioreactors to the investigation of radiation-induced MN-RET, a marker of genotoxicity as a result of DNA damage by clastogens or aneugens. Though the traditional Dexter-style bone marrow cultures in flasks or dishes support the growth of early erythroid progenitors BFU-E and CFU-E, and the suspension cultures show potentials in generating erythrocytes [18–25], long-term effective generation of reticulocytes up to 8 weeks has not been previously reported. Our 3-D Long-Term Bone Marrow Culture (3-D LTBM) is an improvement of Dexter's culture (2-D culture) that is established in the flat surface of flasks/dishes. The 3-D LTBM established in the bioreactor is packed with porous microspheres to provide the three dimensional matrix to mimic in vivo bone architectural marrow framework. The cultures are supplemented with hydrocortisone, recombinant human erythropoietin, transferrin, and charcoal-treated FBS to enrich lineage-specific proliferation and differentiation of red blood cells. Such combination of nutrients and cytokines appears to mimic the essential chemical microenvironment for erythropoiesis in bone marrow in vivo.

Our optimized culture condition in the 3-D bioreactor has enriched terminal erythropoiesis for up to 8 weeks, while still maintaining the multiple lineage differentiation. The terminal erythropoiesis reached a peak between week 2 and 3, following by a rebound or staying relatively constant until week 6–8. The expression of % RBC and % RET did vary after 28 days. The exact cause of this observation is not clear. One of the putative mechanisms is that the performance of the culture after Day 28 depends mainly on how many hematopoietic primitive progenitors have survived after 28 days. This process is prone to the random disturbance. Of note that in the traditional 2-D Dexter's cultures, the cultures have to be recharged with fresh bone marrow cells after 3 weeks to maintain the longevity of culture, because of the heavy loss of hematopoietic primitive progenitors in the first 3 weeks [30]. In our 3-D culture, "recharge" is not necessary for maintain the culture beyond Day 28. The cell population has varied due to random loss of progenitor cells from weekly sampling for data analyses. Nonetheless, the common feature we wanted to show with our data from the 3-D culture was that the reticulocytes were generated in the significant level in all cultures for up to 6–8 weeks and the activity didn't seem to die at the end of the cultures.

While comparatively little work in MN-RET has been accomplished with radiation research, we have recently published the work on gamma radiation-induced MN-RET in C57Bl/6 mouse after total body irradiation in vivo [31]. Because the generation of MN-RET requires the dynamic process of erythroid progenitor and precursor cell proliferation, differentiation, and terminal maturation, almost all MN-RET investigations were conducted in the rodent in vivo testing systems with the exception of a more recent publication using a bone marrow suspension culture system [25]. Data from this suspension bone marrow culture supports the validation in testing several drugs but did not test radiation effect. In contrast to this reported two-stage suspension culture system that has supported the generation of reticulocytes in the last stage for 2 days, our 3-D LTBM supports the continuous erythropoiesis and the generation of reticulocytes and mature erythrocytes for as long as 8 weeks. Our observation suggests that the primitive progenitors for erythropoiesis actively proliferated and differentiated in the 3-D LTBM, and the entire erythroid development chain was expressed in yielding the mature erythrocytes. Therefore, this culture model provides a potentially physiological testing environment, making it possible to investigate bone marrow genotoxic effect of radiation on erythroid lineage in vitro.

In mice, reticulocytes generated in the bone marrow enter the peripheral blood after residing in the bone marrow for 10 hours, and fully mature to erythrocytes in peripheral blood after approximately 15 hours [29]. The peak time for MN-RET induction after irradiation in the mouse *in vivo* model has been previously reported. Published work on the kinetics of MN-RET induction in mice irradiated *in vivo* shows that MN-RET induction is affected by the radiation doses and the types of radiation. In general the peak of MN-RET expression in the bone marrow is between 24 to 28 hours in the bone marrow *in vivo* [29,32–34], and it is between 30 to 44 hours in the peripheral blood [29,32,35]. In our 3-D LTBM, the peak time for radiation-induced MN-RET expression occurs at approximately 24 to 32 hours post-irradiation, which falls between what is observed in the bone marrow and the peripheral blood compartment in mice irradiated *in vivo*. This timeframe seems appropriate because reticulocytes generated in the 3-D LTBM are retained in the culture at all times without a relevant peripheral blood compartment. Thus the peak time of MN-RET expression in the 3-D LTBM (24–32 hours) is expected to occur sometime between the peak time in bone marrow (24–28 hours) and the peripheral blood (30–44 hours) in the *in vivo* model after total body irradiation of mice. The percentage of RETs in the 3-D LTBM first declined in a small level at 12 hours, and then big drop took place after 24 hours post irradiation (Fig. 3B). The similar phenomena has also been observed in bone marrow *in vivo* [32].

The difference between our 3-D culture model and the *in vivo* mouse model in the %RET was the intermediate rebound at 24 hours post radiation in our culture model. The putative mechanism behind this was that the cell cycle of most erythroblasts in our 3-D culture might have been synchronized by the daily media change. Thus, the development of erythroblasts was in the pattern of waves. At 0–12 hour post radiation, in the non-synchronized erythroid development, the erythroblasts at the last mitosis that were beyond S phase at the time of radiation would enucleate. In our 3-D culture, the major portion of these erythroblasts would have been synchronized to the previous or next developmental wave so that there were fewer enucleation events, leading to the greater decrease of %RET than that in the non-synchronized erythroid development. Radiation would have caused the delay of the cell cycle and thus increased the interval from the S phase to enucleation in the last mitosis of erythroid development from 9–12 to 24–27 hours [36]. At 24 hours post radiation, the %RET would rebound to some extent when the delayed enucleation of synchronized erythroblasts was finished, probably before the next enucleation wave in the control culture without irradiation. We have planned to test this hypothesis in the future. The comparable kinetics in the 3-D LTBM culture and in mice after *in vivo* irradiation suggests that the time-course for cell cycle progression and enucleation in the culture are similar between the 3-D LTBM and bone marrow *in vivo*.

Similar to our finding of MN-RET induction after irradiation, the *in vivo* MN-RET assay reported in the animal models also showed a sensitivity to gamma radiation. It has been reported that the MN-RET assay is able to detect 6–10 cGy X-ray [37,38] and 2.5–3 cGy gamma-ray irradiation [34,39]. In our 3-D LTBM system, radiation doses as low as 12.5 cGy (0.125 Gy) gamma-radiation induced significantly higher MN-RET than 0 Gy ($p=0.01$, two-side paired *t*-test). Thus, the sensitivity of the 3-D LTBM was similar to that of *in vivo* mice model, although we did not test doses lower than 12.5 cGy.

In the 3-D LTBM system, we observed a near linear increase of MN-RET from 0 Gy to 1 Gy after radiation exposure, with a maximum reached at 1.5–2.0 Gy. This was similar to our previous report of *in vivo* MN-RET induction in the peripheral blood of C57Bl/6 mice after total body irradiation *in vivo* [31]. The previously reported *in vivo* investigation also showed a lack of increase of MN-RET beyond 1.5 Gy, suggesting that competing radiation cytotoxic effect may be working on the bone marrow precursors, i.e., erythroblasts. By depleting the erythroblasts in the bone marrow through radiation-induced apoptosis, it would be difficult to

induce more MN-RET at doses higher than 1.5 Gy in mice. Our finding of the lack of further induction of MN-RET beyond 1.5 – 2.0 Gy in the 3-D LT BMC was similar to our published *in vivo* data, lending further support that 3-D LT BMC may serve as an *in vitro* model for MN-RET assay for genotoxicity testing after irradiation.

It appeared that the background of MN-RET is affected by reagents used in the culture. Among three experiments for MN-RET kinetics (Fig. 3), the background percentage of MN-RET in the control cultures of sham irradiation (0 Gy) was at an average of 1.3% (range 0.9 – 1.5%), which was not significantly different ($p=0.41$; Friedman's non-parametric 2-way ANOVA), in the two experiments using the same lots of reagents (open diamonds and triangles). It was, however, significantly higher at 2.6% (range 2.4 – 2.7%) ($P = 0.003$; Friedman's non-parametric 2-way ANOVA) in the third experiment conducted with different lots of reagents (open squares). The MN-RET expression has long been known to be sensitive to the stimulation by various chemicals [9,36]. Thus the higher level of background MN-RET in the 3-D LT BMC may be affected by the sensitivity to the chemicals in different lots of culture reagents. In Fig. 4, the higher background in the third experiment (squares) might be contributing to the higher levels of MN-RET induction below 1 Gy of radiation and the earlier saturation in 1.5 Gy, vs. 2 Gy observed in other two experiments. Our finding supports using similar batches of reagents with adequate internal controls for all experiments using 3-D LT BMC to minimize the influence from chemicals in the culture media.

In summary, a 3-D LT BMC system capable of continuously generating reticulocytes from the progenitor and precursor cells of erythropoiesis for mouse bone marrow culture has been successfully applied to the research in radiation genotoxicity testing, yielding a MN-RET increase in a dose-dependent manner. Our study also reveals the similarities of the dose responses of MN-RET induction after radiation exposure between the mouse *in vivo* model [31] and the 3-D LT BMC. The peak time of MN-RET expression in 3-D LT BMC appears to fall between the peak time in the bone marrow *in vivo* and the peripheral blood of mice after total body irradiation, likely due to the differences in the kinetics of reticulocytes by the physical presence of the bone marrow compartment of experimental animals. Our 3-D LT BMC condition offers the potential for the research of erythropoiesis and bone marrow radiation injury in an *in vitro* model established in the bone marrow bioreactor.

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Fig 1.

A) Cells from the 3-D LT BMC in the 2nd week of culture stained with Wright-Giemsa stain, showing proerythroblasts (p), normoblasts (n), erythrocytes (e), and enucleated nuclei (en). Magnification: 1000X. B) Cells from the 3-D LT BMC in the 2nd week of culture first stained with New methylene blue and then counter-stained with Wright-Giemsa stain, showing reticulocytes (r) with blue residual RNA content in the cells. Magnification 1000X. C) Gamma-radiation induced MN-RET. Micronucleated reticulocyte, MN-RET (mn) and reticulocyte, RET (r) stained with Acridine Orange. There are larger nucleated cells, which are mononuclear cells with large yellowish-green fluorescence in the nucleus. The RETs are small red cells, and the MN-RET cell is the small the red cell with a small yellowish-green focus inside the cell. Magnification 400X.

Fig 2.

The kinetics of erythropoiesis in 3-D LTBM. A) Weekly viable cell output per well of 3-D LTBM from four independent experiments (squares, up triangles, diamonds and down triangles, respectively). B) Weekly % viability. C) The percent erythrocytes (RBCs) among total viable cells including reticulocytes in the weekly output, and C) the percentage of reticulocytes (RETs) among total viable cells in the weekly output.

Fig 3.

The kinetics of radiation induced MN-RET and the changes of RET after irradiation of cells maintained in 3-D LT BMC. Three independent experiments are shown with the symbols of diamonds, triangles and squares, respectively. A) MN-RET induction by gamma-radiation in 3-D LT BMC as a function of time. The solid symbols and solid lines show the groups irradiated with 1 Gy gamma-radiation while the open symbols and dashed lines show the groups with sham irradiation. The thick lines represent the averages from three independent experiments. B) Relative changes of percentage of RETs among erythrocytes in the 3-D LT BMC after 1 Gy radiation, normalized by the values in control culture with sham irradiation.

Fig 4. MN-RET induction in the murine 3-D LTBM cells after exposure to a gradient of gamma radiation doses. Results of three independent experiments are shown with symbols of diamonds, triangles and squares, respectively. The solid line represents the averages of the three independent experiments.