Mutation Research 680 (2009) 49–55

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Gamma-radiation induces micronucleated reticulocytes in 3D bone marrow bioreactors in vitro

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ARTICLE INFO

Article history:
Received 16 June 2009
Received in revised form
12 September 2009
Accepted 16 September 2009
Available online 26 September 2009

Keywords:
Micronuclei
Radiation
Genotoxicity
Reticulocytes
Bone marrow
3D bioreactor

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 3D bioreactors, which sustain long-term bone marrow cultures. For these studies, genotoxicity is measured by the induction of micronucleated reticulocytes (MN-RETs). 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 3D 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 and 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 h and 32 h 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 3D bioreactor may be a useful organ culture system for the investigation of radiation genotoxic effect in vitro.

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1. 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-RETs) 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

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doi:10.1016/j.mrgentox.2009.09.006

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 3D 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 3D bioreactors. The three-dimensional bone marrow bioreactor is different from the 2D flask culture system in that the architectural scaffolding may support a more conducive microenvironment for bone marrow homeostasis. Our bone marrow cultures in the 3D 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 2D 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 3D long-term bone marrow culture (3D LTBMC) 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.

2. Methods and materials

2.1. 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% $NHCl_4$ and 0.1% $KHCO_3$ solution). The bone marrow mononuclear cells were suspended in the culture media and kept on ice until seeding.

2.2. 3D long-term bone marrow culture

Ten million mononuclear cells from mouse bone marrow were seeded into a 3D scaffolding of a bioreactor. Each bioreactor was packed with porous microspheres to provide a 3D growth configuration mimicking the bone marrow in vivo as previously described [15,17,26]. The culture medium consisted of RPMI 1640 plus 10 mM Hepes, 5 mM L-glutamine, 0.8% penicillin/streptomycin, 10^{-4} M 2-mercaptoethanol, 10^{-7} M hydrocortisone (Sigma, St. Louis, MO), 0.2 U/ml recombinant human erythropoietin (EPO) (Amgen, Thousand Oaks, CA), 0.5 mg/ml iron-saturated human holo-transferrin (Chemicon International, Temecula, CA) and 15% charcoal-treated FBS (Hyclone, Logan UT). The media (0.6 ml/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. Fifty microliters of cells were 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".

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 with cytospin 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, TX) and incubated at room temperature for 15 min. The mixture was spun down with cytospin 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.

2.4. Radiation of 3D LTBMC

3D LTBMC cultures were irradiated on the 18th or 19th day after inoculations 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.

2.5. RET and MN-RET scoring

RET and MN-RET scoring was accomplished with Acridine Orange (AO) staining [27]. Cells were harvested at 24 h post-radiation or at the serial time points to measure the kinetics of induction of MN-RET by washing microspheres with DPBS containing 2 mM EDTA four times. Slides were made after cytospin, 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 (pH 6.8) for 1 min, washed in DPBS (pH 6.8) for 8 min, and then transferred to fresh DPBS to be washed for one additional minute. 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 RET cells were scored for %RETs as described in 2.3, while more than 2000 RETs were counted in each sample for the percentage of MN-RETs among all RETs.

2.6. Statistical methods

One- and two-way analyses of variance (ANOVA) were conducted using the non-parametric 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.

3. Results

3.1. Erythropoiesis in the 3D LTBMC

Fig. 1A and B shows the morphology of cells 2 weeks after innoculation of mononuclear cells in the 3D LTMBC, and harvested for staining. Fig. 1A shows the cell morphology after the Wright–Giemsa stain. The figure shows erythoid 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 3D LTBMC 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 3D LTBMC 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 and 21st days after inoculations. This

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