Published in final edited form as:

Exp Hematol. 2011 April ; 39(4): 434–445. doi:10.1016/j.exphem.2011.01.010.

Sublethal radiation injury uncovers a functional transition during erythroid maturation

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Abstract

Objective—Clastogenic injury of the erythroid lineage results in anemia, reticulocytopenia, and transient appearance of micronucleated reticulocytes (MN-RET). However, the MN-RET dose-response in murine models is only linear to 2 Gy total body irradiation (TBI) and paradoxically decreases at higher exposures, suggesting complex radiation effects on erythroid intermediates. To better understand this phenomenon, we investigated the kinetics and apoptotic response of the erythron to sublethal radiation injury.

Materials and Methods—We analyzed the response to 1 and 4 Gy TBI of erythroid progenitors and precursors using colony assays and imaging flow cytometry (IFC), respectively. We also investigated cell cycling and apoptotic gene expression of the steady-state erythron.

Results—Following 1 Gy TBI, erythroid progenitors and precursors were partially depleted. In contrast, essentially all bone marrow erythroid progenitors and precursors were lost within two days following 4 Gy irradiation. IFC analysis revealed preferential loss of phenotypic erythroid colony-forming units (CFU-E) and proerythroblasts immediately following sublethal irradiation. Furthermore, these populations underwent radiation-induced apoptosis, without changes in steady-state cellular proliferation, at much higher frequencies than later-stage erythroid precursors. Primary erythroid precursor maturation is associated with marked Bcl-xL upregulation and Bax and Bid down-regulation.

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Category: Erythropoiesis

Conflict of Interest Disclosure:

J.C.B. and S.D.D. are employees of Litron Laboratories, which is pursuing patent protection for methods and products to assess hematotoxicity resulting from exposure to clastogenic injury, via enumeration of blood cells and platelets using flow cytometry. The remaining authors have no conflicts of interest.

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Conclusions—MN-RET loss following higher sublethal radiation exposures results from rapid depletion of erythroid progenitors and precursors. This injury reveals that CFU-E and proerythroblasts constitute a particularly proapoptotic compartment within the erythron. We conclude that the functional transition of primary proerythroblasts to later-stage erythroid precursors is characterized by a shift from a pro-apoptotic to an anti-apoptotic phenotype.

Keywords

Erythropoiesis; Erythroid Precursor Cells; Radiation; Apoptosis; Micronuclei

Introduction

Erythropoiesis has classically been characterized as the sequential movement of lineage-committed cells through three compartments. The first compartment, erythroid progenitors, is comprised of cells capable of colony formation *in vitro*, specifically erythroid burst-forming units (BFU-E) that mature to form erythroid colony-forming units (CFU-E) [1–3]. The second compartment, erythroblast precursors, is divided into sequentially maturing erythroid intermediates, specifically proerythroblasts (ProE), basophilic erythroblasts (BasoE), polychromatophilic erythroblasts (PolyE), and orthochromatic erythroblasts (OrthoE). These morphologically identifiable cells undergo a limited number of maturational cell divisions as they accumulate hemoglobin and condense their nuclei. Following enucleation, reticulocytes enter the bloodstream and complete maturation to contribute to the third compartment, comprised of circulating erythrocytes.

Reticulocytes and red blood cells (RBC) are highly radioresistant due to their lack of DNA and apoptotic machinery [4]. However, sublethal total body irradiation (TBI) results in the rapid decrease of total circulating reticulocytes [5–8] and an increase in reticulocytes containing DNA fragments or lagging chromosomes, so called micronucleated reticulocytes (MN-RET) [9–13]. MN-RET emerge as a cohort into the bloodstream of mice 35–55 hours after radiation exposure [14]. The MN-RET assay has served as a widely used and extremely sensitive marker of clastogenesis, capable of detecting exposure to doses as low as 0.1 Gy [15–18]. Although MN-RET frequency has long been thought to be directly proportional to the amount of radiation exposure, we recently have shown that the MN-RET response is only linear to 1–2 Gy TBI in the mouse model. As exposure is increased above 2 Gy, the frequency of MN-RET paradoxically decreases [19]. Since MN-RET are the result of clastogenic injury, or chromosomal disruption and damage, to erythroid progenitors and precursors in the bone marrow, we hypothesized that the downturn is due to the inability of erythroblasts to repair and survive higher doses of radiation.

Little is known about the response of erythroid progenitors and precursors to radiation injury. 2 Gy TBI in rodents leads to an overall decrease in marrow cellularity and loss of erythroblast precursors from erythroblast islands within 2 days [20, 21]. Furthermore, BFU-E and CFU-E are rapidly lost following sublethal TBI [22, 23]. In addition, pioneering *in vitro* tritiated thymidine studies showed that rapidly cycling CFU-E were highly radiosensitive in contrast to more slowly cycling BFU-E [24–27]. Subsequent *in vivo* studies illustrated that BFU-E and CFU-E are lost in a dose-responsive and cell-cycle dependent manner following tritiated thymidine exposure [28]. Beyond these studies, the erythroid progenitor and erythroblast precursor compartments have not been further investigated following sublethal radiation injury.

In this study, we examined the progenitor, precursor, and peripheral blood compartments of the erythron following 1 and 4 Gy TBI in C57BL/6J mice and found that sublethal (4 Gy) TBI led to massive erythroblast cell death. These results support the hypothesis that fewer

MN-RET are formed at higher radiation doses because severely damaged erythroblasts do not repair and survive to the reticulocyte stage. Our laboratory has developed an imaging flow cytometry (IFC) approach utilizing morphological and phenotypic features to quantitatively analyze large numbers of primary erythroblast precursors [29]. Here we use the recent characterization of the cell surface immunophenotype of CFU-E [30, 31], in combination with our IFC approach, to directly analyze the response of “phenotypic” CFU-E and erythroblast precursors to radiation injury. Overall, these studies reveal an important functional transition as proerythroblasts mature into later erythroblast populations.

Materials and Methods

Animals and irradiation

7–9 week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used for all experiments. A Shepherd Irradiator with a 6000 Ci ^{137}Cs source and collimating equipment was used for irradiation. Unanaesthetized mice were irradiated while held in a Plexiglas restraint and exposed to external beam TBI doses of 1 or 4 Gy at a dose rate of 1.6 Gy/min.

Blood and marrow extraction

Mice were euthanized by CO_2 narcosis and peripheral blood obtained. Bone marrow was obtained by flushing of mouse femurs with PB2 (DPBS, Invitrogen, Carlsbad, CA; 0.3% BSA, Gemini Bio- Products, Sacramento, CA; 0.68 mM CaCl_2 , Sigma-Aldrich, St. Louis, MO; 0.1% glucose) in 12.5 $\mu\text{g}/\text{mL}$ heparin and single cell suspensions made by trituration. Marrow cell counts were obtained by hemocytometer and cell viability determined by trypan blue exclusion.

Erythron analysis

Colony assays were performed to quantify BFU-E and CFU-E by plating single cell suspensions of whole bone marrow at 2×10^5 cells/mL for BFU-E and 1×10^5 cells/mL for CFU-E into methylcellulose media consisting of IMDM (Invitrogen), 10% PDS (Animal Technologies, Tyler, TX), 20% BIT 9500 (StemCell Technologies, Vancouver, Canada), 5% PFHM-II, 2 mM glutamine, and 55 nM 2-mercaptoethanol (Invitrogen) in 1% methylcellulose (StemCell Technologies). CFU-E media contained 0.3 U/mL rhEPO (Amgen, Thousand Oaks, CA), while BFU-E media was supplemented with 2 U/mL rhEPO, 0.02 $\mu\text{g}/\text{mL}$ IL-3 and IL-6, and 0.12 $\mu\text{g}/\text{mL}$ SCF (Peprotech, Rocky Hill, NJ). CFU-E and BFU-E were quantified at 2 and 7 days after plating, respectively.

Analysis of erythroblast precursor subpopulations was performed as recently described [29]. Bone marrow cells were blocked in 12.5% rat whole serum (Invitrogen) and stained with thiazole orange (Sigma-Aldrich) at 2 $\mu\text{g}/\text{mL}$ and PE-ckit and biotin-Ter119 (eBioscience, San Diego, CA) at 1:100 dilution. Secondary staining with PE-Texas Red (PE-TR) streptavidin (BD Biosciences, San Jose, CA) was performed at 1:500 dilution. Cells were stained with 20 μM DRAQ5 (Biostatus, Shepshed, United Kingdom) and analyzed on the ImageStream IS100 (Amnis, Seattle, WA). Phenotypic CFU-E were analyzed on the ImageStream X by staining bone marrow with FITC-ckit, PE-endoglin, PE-Cy5-Sca1, PE-Cy7-Ter119, and APC-CD150 (eBioscience) at 1:100 dilution and 5 $\mu\text{g}/\text{mL}$ DAPI (Invitrogen).

Hematocrit values were obtained using the HESKA HemaTrue automated analyzer (Heska, Loveland, CO). Reticulocyte and total RBC values were obtained by staining 5 μL of blood with 10 nM SYTO13 (Molecular Probes, Eugene, OR) diluted in HBSS (Mediatech, Herndon, VA). Reticulocytes were defined as SYTO13 low/intermediate cells, while RBC were defined as SYTO13-negative cells. A known number of CountBright™ Absolute

Counting Beads (Invitrogen) were added to each sample to determine the number of reticulocytes and RBC.

Apoptosis staining

Bone marrow cells were stained with Alexa Fluor 488-Annexin V (Invitrogen) at 1:20 dilution in Annexin binding buffer (10 mM HEPES, Sigma-Aldrich; 140 mM NaCl, Mallinckrodt Baker, Phillipsburg, NJ; 2.5 mM CaCl₂) and fixed in 2% formaldehyde, followed by blocking and staining with PE- ckit, biotin-Ter119, and PE-TR streptavidin as above. Cells were stained with 8 μ M DRAQ5 and analyzed on the ImageStream IS100.

Cell cycle analysis

C57BL/6J mice were injected intraperitoneally with BrdU (100 μ L of 10 mg/mL stock) 45 minutes prior to bone marrow harvest. BrdU incorporation in erythroid subpopulations was detected on the ImageStream X using a FITC BrdU flow kit (BD Biosciences) in combination with PE-ckit, biotin- Ter119, PE-TR streptavidin, and DRAQ5 as described above.

Erythroid cell sorting

For depletion of non-erythroid lineages, bone marrow cells were blocked, stained with biotinated-B220, CD3, Gr1, and Mac1 (eBioscience), and incubated with IMag streptavidin magnetic particles (BD Biosciences). Lineage-positive cells were magnetically separated using the BD iMagnet system. Depleted cells were stained with PE-CD71, APC-Ter119, and PE-Cy7 ckit (eBioscience) at 1:100 dilution. Following addition of DAPI at 5 μ g/mL to exclude dead cells, ProE were sorted as ckit⁺/Ter119⁺ large cells, and late erythroblasts were sorted as ckit⁻/Ter119⁺/CD71⁺ medium-sized cells on a FACSaria cell sorter (BD Biosciences).

RNA isolation and quantitative PCR analysis

RNA from sorted erythroblast populations was prepared with RNeasy (Qiagen, Valencia, CA). cDNA was prepared and qPCR performed as previously described [32]. Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA) were used to determine expression levels of Bcl-xL (Mm00437783_m1), Bax (Mm00432051_m1), and Bid (Mm00432073_m1). 18s Taqman expression assays (Hs99999901_s1) were used to control for cDNA quantity.

Results

The erythroid lineage is severely injured in a dose-dependent manner following TBI

Functional analysis of the erythroid progenitor compartment using colony assays revealed a 55% and 42% loss of BFU-E and CFU-E, respectively, 2 days after 1 Gy irradiation (Figure 1A, left panel). In marked contrast, 4 Gy TBI induced a rapid loss of BFU-E and CFU-E progenitors by 2 days post-irradiation (Figure 1A, right panel). IFC analysis of the erythroblast precursor compartment following 1 Gy TBI revealed a moderate loss of precursor subcompartments, with some recovery evident in BasoE and PolyE by 2 days post-irradiation (Figure 1B, left panel). In contrast, 4 Gy TBI induced a virtually complete depletion of erythroblast precursors within 2 days (Figure 1B, right panel). Taken together (Figure 1A–1B), these data indicate that 4 Gy TBI results in damage that is lethal to almost all nucleated erythroid cells in the murine bone marrow.

Peripheral blood analysis of the erythron following sublethal TBI indicated a progressive loss of reticulocytes that was more severe at 4 Gy TBI (Figure 1C), consistent with the dose-

dependent loss of upstream marrow erythroblasts following irradiation. Hematocrit levels remained relatively constant during the first two days following both 1 Gy and 4 Gy TBI (Figure 1C). These reticulocyte and total RBC kinetics are consistent with their known levels of radioresistance and *in vivo* short and long half-lives, respectively.

Ter119 expression initiates in CFU-E, a Ter119^{very low} ckit^{high} population of the erythron

We had previously defined ProE, using IFC, as large cells expressing low levels of Ter119 and detectable levels of ckit on their cell surface [29]. More recently, we detected a small population of marrow cells with high brightfield area and extremely low levels of Ter119 on their surface (Figure 2A, blue wedge on left panel). These cells also expressed very high levels of ckit (Figure 2A, right panel). The high brightfield area, Ter119^{very low}/ckit^{high} characteristics suggested that this population might represent CFU-E, cells that lie immediately upstream of ProE. As the ImageStream cannot sort cellular populations to test the colony-forming ability of IFC-defined CFU-E, we compared this Ter119^{very low}/ckit^{high} population with the recently described cell surface staining characteristics of CFU-E [30, 31]. These ckit⁺/Endoglin⁺/Sca1⁻/CD150⁻/Ter119^{low} phenotypic CFU-E, when sorted by traditional FACS methods, form colonies in methylcellulose culture at a plating efficiency of approximately 40% (data not shown). Furthermore, 85% of this ckit⁺/Endoglin⁺/Sca1⁻/CD150⁻/Ter119^{low} population backgated into our previously defined high brightfield area, Ter119^{very low} cell population (Figure 2B), indicating our IFC population is highly enriched for colony-forming CFU-E. These results provide evidence that IFC-defined CFU-E can be distinguished from ProE by their very low, but detectable, levels of Ter119 and by higher levels of ckit (Figure 2C).

CFU-E and ProE are lost more rapidly than late-stage erythroblast precursors at 1–6 hours post-irradiation

Phenotypic CFU-E and erythroblast precursors were analyzed by IFC during the first 12 hours following 1 Gy or 4 Gy irradiation. Following 1 Gy TBI, CFU-E and ProE were rapidly depleted by 2 hours post-irradiation, decreasing to 40% of unirradiated control levels by 6 hours (Figure 3A). In contrast, the more mature BasoE, PolyE, and OrthoE precursors revealed a markedly different initial response, remaining at normal levels through 4 hours and only decreasing to 70% of control levels by 6 hours after 1 Gy TBI (Figure 3A). Similarly, phenotypic CFU-E and ProE populations were rapidly lost by 2 hours post-4 Gy TBI and decreased to approximately 40% of unirradiated controls by 6 hours post-irradiation (Figure 3B). These differential population kinetics indicate that CFU-E and ProE initially respond similarly to each other but in a manner that is different from late-stage erythroblast precursors at both 1 Gy and 4 Gy radiation exposures.

By 12 hours following 1 Gy TBI, all erythroblast precursors decreased to 40% of unirradiated controls. Interestingly, erythroid recovery begins by 12 hours post-1 Gy TBI in the CFU-E population, with a doubling of CFU-E numbers between 6 and 12 hours post-irradiation (Figure 3A). Taken together with 1 Gy data as shown in Figure 1A–B, CFU-E recovery continues at 1 day post-irradiation, followed by ProE, BasoE, and PolyE recovery by 2 days. In contrast, by 12 hours following 4 Gy TBI, ProE to OrthoE are depleted to less than 20% of unirradiated controls, with CFU-E exhibiting more delayed loss (Figure 3B). By 1 day following 4 Gy irradiation, all populations are depleted and continue to nadir at 2 days post-irradiation (Figure 1A–B). These data indicate that 4 Gy TBI severely depletes the erythroid lineage and, unlike 1 Gy exposure, does not lead to recovery of the erythron during the first two days following irradiation.

Sublethal radiation exposure preferentially induces CFU-E and ProE to undergo apoptosis

We used an IFC-based approach that combined Annexin V positivity with nuclear fragmentation to quantify the percentage of apoptotic cells in progressive erythroid subpopulations following sublethal TBI (Figure 4A) [33]. As shown in Figure 4B, 1 Gy TBI induced CFU-E and ProE populations to undergo apoptosis, with 7% of CFU-E and 12% of ProE becoming apoptotic by 2 hours. 4 Gy TBI induced a markedly higher frequency of apoptosis in the CFU-E and ProE populations compared with 1 Gy exposure. Specifically, levels of apoptotic ProE approached 35% by 6 hours post-irradiation and exceeded 50% by 12 hours post-irradiation (Figure 4C). CFU-E showed a somewhat more delayed apoptotic induction at 4 Gy exposure (Figure 4C), which directly corresponds with the delayed kinetic loss of CFU-E at 12 hours following 4 Gy TBI (Figure 3B). In addition, ProE were found to be more susceptible to apoptosis than CFU-E following 4 Gy, indicating that ProE are the most proapoptotic of all bone marrow erythroblasts (Figure 4C). Overall, these data suggest that apoptosis is a major underlying cause of CFU-E and ProE loss following radiation injury.

In contrast, significantly lower levels of apoptosis were induced by 12 hours in late-stage erythroblasts at both 1 and 4 Gy TBI (Figure 4B–C), illustrating a dramatic alteration in apoptotic sensitivity at the ProE to BasoE transition. These data provide evidence that proapoptotic ProE are more closely related in their apoptotic sensitivity to CFU-E than to the more mature, apoptosis-resistant BasoE, PolyE, and OrthoE. Nevertheless, cellular loss of late-stage erythroblast precursors, especially following 4 Gy TBI, also occurs at later time-points despite their resistance to induction of apoptosis (Figures 1B, 3). This loss of late-stage erythroid cells may occur by necrosis, mitotic cell cycle checkpoint arrest, or other non-apoptotic means of cell death. Alternatively, some erythroblasts could complete maturation without replacement by upstream intermediates.

Erythroid loss and apoptotic induction following TBI do not correlate with cell cycle status

Rapidly cycling cells that undergo radiation-induced DNA damage are more likely to encounter cell cycle damage checkpoints that engage p53/ATM pathways to either repair DNA or, if cellular repair fails, induce apoptosis [34–37]. To determine whether proliferation rates were maturationally correlated with our observed apoptotic loss, we utilized BrdU incorporation to specifically determine the steady-state cycling status of IFC CFU-E and erythroblast precursors (Figure 5A). Importantly, since erythroid subpopulations for cell cycle analysis were also defined by IFC, cycling rates and kinetic/apoptotic data were measured on the same cell populations. CFU-E, ProE, and BasoE, and PolyE all cycle at rates greater than 75%, with proliferation peaking at the ProE and BasoE stages. Cycling rates drop sharply at the OrthoE stage of maturation to approximately 30% (Figure 5A–B). These data correlate closely with previously published proliferation rates of bone marrow CFU-E as determined by tritiated thymidine suicide assay [24–27] as well as more recent BrdU-based proliferation studies of the fetal liver erythron [38]. Thus, the sensitivity to rapid apoptotic cell loss following sublethal TBI does not correlate with downregulation of cellular proliferation during erythroid maturation, since sensitivity to erythroid loss and apoptotic induction changes dramatically at the ProE to BasoE transition (Figures 3,4) but cycling rates do not change significantly until the PolyE to OrthoE transition (Figure 5).

The anti-apoptotic gene Bcl-xL and the pro-apoptotic genes Bax and Bid are differentially regulated during maturation of marrow erythroblasts

Anti-apoptotic genes, such as Bcl-xL, must be upregulated for erythroblast precursors to mature successfully [39–42]. Since cell cycle is not correlated with erythroid apoptotic loss following sublethal TBI, we asked whether steady-state changes in apoptotic gene expression underlie the change in sensitivity to apoptotic induction at the ProE to BasoE

transition. The expression levels of Bcl-xL and the pro-apoptotic genes Bax and Bid were determined by qPCR in primary ProE and late erythroblast populations purified from normal bone marrow. Bcl-xL was expressed at very low levels in ProE but, as expected, was upregulated 6-fold in late-stage erythroblasts (Figure 6). In contrast, the pro-apoptotic genes Bax and Bid were downregulated 17-fold and 100-fold, respectively, during erythroblast maturation (Figure 6). These results are consistent with the notion that erythroid maturation is characterized by a shift from a pro-apoptotic to an anti-apoptotic phenotype as ProE mature into BasoE/PolyE/OrthoE.

Discussion

We investigated the acute response of the erythron to radiation injury and found that a wide spectrum of the maturing erythron in the bone marrow is extremely sensitive in a dose-dependent manner to radiation injury. Recent studies have found similar depletions of hematopoietic stem cell (HSC) populations following sublethal TBI, indicating HSC are also very radiosensitive [43]. Dose-response studies of the bone marrow indicate that granulocyte-macrophage progenitors and stromal cell precursors are most radioresistant, followed by multilineage hematopoietic progenitors and HSC-enriched populations (CAFC-28), and finally by highly radiosensitive erythroid progenitors [22, 23, 44]. Our findings confirm the marked radiosensitivity of erythroid progenitors and provide new data indicating that maturing erythroid precursors are also very rapidly injured and depleted.

These findings provide an explanation of the downturn in MN-RET frequency seen at higher sublethal doses of radiation [14, 19]. Despite the significant loss of maturing erythroblasts 24 hours after 1 Gy TBI, there is persistence and recovery of erythroblast numbers by 2 days post-irradiation (Figure 1A–B, 1 Gy). These cells, which are moderately damaged but still able to undergo terminal erythroid maturation, are the likely source of MN-RET that appear as a cohort in the bloodstream 43–50 hours post-irradiation [14, 19]. However, higher sublethal irradiation doses lead to chromosomal damage and possibly other injuries that preclude effective repair, thereby inducing extensive erythroblast cell death (Figure 1A–B, 4 Gy). Although the proportion of erythroblast precursors that form micronuclei following 4 Gy TBI are likely even higher than at 1 Gy TBI, the vast majority of these severely damaged cells are lost before terminal erythroid maturation can occur, leading to a marked decrease in the frequency of MN-RET at higher doses of sublethal irradiation.

Our investigation using IFC allows for a new perspective of erythroid maturation by quantitative analysis of highly specific and previously difficult-to-access erythroid subpopulations. CFU-E were defined more than 30 years ago by their ability to give rise to small colonies of mature erythroid cells *in vitro* [1, 3]. Although this methodology provides quantitative, functional data, the actual colony-forming cell cannot be directly investigated by this approach. Building on recent studies describing the immunophenotype of CFU-E [30, 31], we identified CFU-E using a novel three-stain IFC panel. This was possible because IFC can detect Ter119 antibody binding at levels below standard flow cytometry detection by restricting measurements to cell areas and decreasing background. One benefit of this novel phenotypic accessibility is that despite the presence of phenotypic CFU-E at 1 hour following 4 Gy TBI (Figure 3B), colony assay analysis at this same time-point showed no evidence of CFU-E (data not shown). This lack of functional CFU-E is likely due to the severe injury and apoptotic induction of CFU-E following 4 Gy TBI (Figure 4C), preventing damaged CFU-E from forming colonies *in vitro*. As a result, these injured CFU-E can only be analyzed by phenotypic classification methods. Thus, our IFC gating method allows, for the first time, access to CFU-E along with all of the downstream erythroid precursor subpopulations.

The rapid kinetics of cell loss and the marked induction of apoptosis in response to radiation injury indicate that CFU-E and ProE behave similarly to each other and very differently from later-stage erythroblast precursors. In addition, steady-state gene regulation underlies a switch from a pro-apoptotic phenotype in ProE to an anti-apoptotic phenotype in later erythroblasts. This erythroid compartmentalization does not correlate with changes in cell cycle during erythroid maturation, as significant downregulation of cellular proliferation does not occur until the final OrthoE stage of erythroblast maturation. Although cellular proliferation rates contribute to radiosensitivity following sublethal TBI [34, 35], our cell cycle data demonstrate that observed differences in erythroid loss and apoptosis at the ProE to BasoE transition are not primarily due to cell cycle-related induction of checkpoint repair and apoptosis pathways. Thus, erythroid maturation can be viewed not only as a progression of colony-forming progenitors to phenotypic precursors, but also as a progression of immature, pro-apoptotic erythroblasts (CFU-E/ProE) to maturing, anti-apoptotic erythroblasts (BasoE/PolyE/OrthoE) based on the functional response of these cell types to radiation injury (Figure 7).

The functional transition from ProE to late-stage erythroblasts is supported by the response of the erythron to other proapoptotic stimuli. For instance, interaction of Fas on immature erythroblasts with FasL on later erythroblasts has been shown to be crucial for erythropoietic regulation by inducing apoptosis in early Fas-expressing erythroid precursors [45, 46]. A similar negative feedback of erythropoiesis occurs through the apoptotic TRAIL pathway [45, 47, 48]. Furthermore, significant evidence indicates that steady-state erythropoiesis is regulated by erythropoietin (EPO)-mediated survival of EPO-responsive cells, which encompass CFU-E and ProE [49–52]. These data, taken together, support the concept that EPO mediates survival and proliferation of pro-apoptotic, EPO-responsive cells until the switch in apoptotic phenotype occurs during later erythroid maturation. Our findings of the response of the erythron to sublethal radiation injury, in combination with steady-state apoptotic gene expression studies, uncover a critical transition as proerythroblasts progress to later-stage erythroid cells.

Acknowledgments

This work was supported by funding from the Center for Medical Countermeasures against Radiation Program (U19 AI067733 pilot program [JP] and U19 AI091036-1 [YC, JP, JPW]), National Institute of Allergy and Infectious Diseases; R01 AI080401 (JP), National Institute of Allergy and Infectious Diseases; F30 DK085706 (SAP), National Institute of Diabetes and Digestive and Kidney Diseases; and the Michael Napoleone Memorial Foundation. Scott A. Peslak is a trainee in the Medical Scientist Training Program funded by NIH T32 GM07356. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or NIH.

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Figure 1. Response of erythroid progenitor, erythroblast precursor, and peripheral blood cell compartments to sublethal TBI

A. Erythroid progenitor (BFU-E, CFU-E) kinetics in the bone marrow following 1 Gy and 4 Gy TBI. Erythroid progenitors are depleted in a dose-dependent manner, with partial loss following 1 Gy TBI and near-complete loss by 2 days following 4 Gy TBI. **B.** Erythroblast precursor (ProE, BasoE, PolyE, OrthoE) kinetics in the bone marrow following 1 and 4 Gy TBI. All precursors are moderately depleted at 1 day following 1 Gy TBI, but begin to increase in numbers at 2 days post-radiation. In contrast, all erythroblast precursors are profoundly depleted following 4 Gy TBI. Progenitors and precursors are normalized per femur and expressed as a percent of unirradiated control marrow. **C.** Hematocrit (HCT) and reticulocyte (Retic) levels in the peripheral blood following 1 and 4 Gy TBI. Reticulocytes are calculated as absolute reticulocyte index (percent reticulocytes $\times$ total RBC and reticulocyte number $\times$ percent hematocrit) and expressed as a percent of unirradiated control reticulocytes. Dotted lines represent unirradiated control levels in all graphs. Error bars represent standard error of the mean of at least 3 experiments. Statistical analyses were performed using Student's t-test. * $p < 0.05$; ** $p < 0.01$; significantly different from unirradiated 0 Gy control.

Figure 2. Analysis of phenotypic CFU-E by imaging flow cytometry (IFC)

A. IFC gating strategy for identifying phenotypic CFU-E. Phenotypic CFU-E were defined as large, Ter119^{very low}/ckit^{high} cells. **B.** CFU-E gating method modified from Pronk et al [31] compared to IFC CFU-E gating as in Figure 2A. Briefly, 85% of ckit⁺/Endoglin⁺/Ter119^{very low}/Sca1⁻/CD150⁻/DAPI⁻ cells backgated as IFC-defined CFU-E. **C.** Representative morphology of phenotypic CFU-E and ProE (Ter119⁺/ckit⁺ cells) as analyzed by IFC. Phenotypic CFU-E display higher levels of ckit and lower levels of Ter119 compared with ProE. Cells are visualized in brightfield (BF, 40x objective), FITC-ckit, PE-endoglin, PE-Cy7 Ter119, PE-Cy5.5 Sca1, and APC-CD150.

Figure 3. Kinetics of primary late-stage erythroid progenitor (phenotypic CFU-E) and erythroblast precursor (ProE/BasoE/PolyE/OrthoE) loss at 1–6 hours post-TBI
 CFU-E and erythroblast precursor kinetics determined by IFC analysis of bone marrow cells following 1 Gy TBI (A) and 4 Gy TBI (B). CFU-E and ProE are extremely sensitive to radiation-induced damage and loss at all dose levels. Following 1 Gy TBI, recovery begins in the CFU-E population by 12 hours post-radiation, while 4 Gy TBI leads to further erythroid loss. Progenitor and precursor numbers are normalized per femur and expressed as a percent of unirradiated control marrow. Error bars represent standard error of the mean of at least 3 experiments. Statistical analyses were performed using Student's t-test. * $p < 0.05$; ** $p < 0.01$; significantly different from unirradiated 0 Gy control.

Figure 4. Apoptosis of phenotypic CFU-E and erythroblast precursors following TBI

IFC was used to quantify AnV⁺ and morphologically apoptotic erythroblasts following 1 and 4 Gy TBI. **A.** Representative brightfield (BF, 40x objective), AF488-AnV (green), and DRAQ5 nuclear stain (red) morphology of non-apoptotic and apoptotic (AnV⁺ and morphologically apoptotic) ProE. Apoptotic cells are highly AnV⁺ and display membrane blebbing and fragmented nuclei, hallmarks of apoptosis. In order to be considered apoptotic, erythroblasts were required to be both highly AnV-positive and morphologically apoptotic. As compared to 0 Gy ProE, the 4 Gy ProE population undergoes a significant shift to apoptotic nuclear morphology. **B.** Apoptotic CFU-E and erythroblast precursors expressed as a percent of each total parent subcompartment following 1 Gy TBI. Radiation injury rapidly induces apoptosis of CFU-E and ProE. In contrast, BasoE, PolyE and OrthoE populations are more resistant to apoptosis. **C.** 4 Gy TBI induces high levels of apoptosis of

CFU-E and ProE by 6–12 hours post-TBI, in contrast to significantly lower levels of apoptosis in late-stage erythroblast precursors. Comparison of 1 and 4 Gy exposures revealed a dose-dependent increase in apoptosis of phenotypic CFU-E and ProE. Error bars represent standard error of the mean of at least 3 experiments. Statistical analyses were performed using Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; significantly different from unirradiated 0 Gy control.

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Figure 5. Cell cycle status of steady-state IFC CFU-E and erythroblast precursors

A. BrdU assay for cell cycle was combined with IFC classification to determine the proliferation rates of CFU-E and erythroblast precursors. Representative BrdU/DNA plots are shown for each erythroid subpopulation and cell cycle phases are labeled. **B.** Average cell cycle profiles are shown for each erythroid subpopulation. Proliferation rates remain high from CFU-E to PolyE and dramatically decrease at the OrthoE stage of erythroid maturation. Error bars represent standard error of the mean of at least 3 experiments. Statistical analyses were performed using Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** significantly different from ProE cell cycle rates.

Figure 6. Expression of the anti-apoptotic gene Bcl-xL is upregulated and expression of the pro-apoptotic genes Bax and Bid are downregulated during erythroblast maturation

Quantitative PCR (qPCR) transcript levels were normalized to 18s and expressed as late erythroblast (LateE) to ProE ratios. Bcl-xL transcripts are upregulated 6-fold while Bax and Bid transcript levels are downregulated 17- and 100-fold, respectively, during erythroid maturation. qPCR was performed on three independently sorted primary cell populations. Error bars represent standard error of the mean of at least 3 experiments. Statistical analyses were performed using Student's t-test. * $p < 0.05$; ** $p < 0.01$; significantly different from unchanged gene expression (LateE:ProE ratio of 1).

Figure 7. Functional compartmentalization model of erythropoiesis

The response of the erythron to sublethal radiation and steady-state expression of apoptosis-related genes uncover an important functional transition at the ProE to BasoE stage of maturation. Based on the functional response of erythroid cells to radiation injury, erythroid maturation can be viewed not only as a progression of colony-forming progenitors to morphologically-defined precursors, but also as a progression of immature, pro-apoptotic erythroblasts (CFU-E/ProE) to maturing, anti-apoptotic erythroblasts (BasoE/PolyE/OrthoE).