Chromosomal Instability in Unirradiated Hemopoietic Cells Resulting from a Delayed *In vivo* Bystander Effect of γ Radiation

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Abstract

Untargeted effects of ionizing radiation (*de novo* effects in the unirradiated descendants or neighbors of irradiated cells) challenge widely held views about the mechanisms of radiation-induced DNA damage with implications for the health consequences of radiation exposures particularly in the context of the induction of malignancy. To investigate *in vivo* untargeted effects of sparsely ionizing (low linear energy transfer) radiation, a congenic sex-mismatch bone marrow transplantation protocol has been used to repopulate the hemopoietic system from a mixture of γ-irradiated and nonirradiated hemopoietic stem cells such that host-, irradiated donor– and unirradiated donor–derived cells can be distinguished. Chromosomal instability in the progeny of irradiated hemopoietic stem cells accompanied by a reduction in their contribution to the repopulated hemopoietic system is consistent with a delayed genomic instability phenotype being expressed *in vivo*. However, chromosomal instability was also shown in the progeny of the nonirradiated hemopoietic stem cells implicating a bystander mechanism. Studies of the influence of irradiated recipient stromal microenvironment and experiments replacing irradiated cells with irradiated cell–conditioned medium reveal the source of the *in vivo* bystander effect to be the descendants of irradiated cells, rather than irradiated cell themselves. Thus, it is possible that a radiation-induced genomic instability phenotype *in vivo* need not necessarily be a reflection of intrinsically unstable cells but the responses to ongoing production of inflammatory-type damaging signals as a long-term unexpected consequence of the initial single radiation exposure. (Cancer Res 2005; 65(13): 5668-73)

Introduction

The major adverse effects of exposures to ionizing irradiation are genetic lesions and cell death, and these are conventionally attributed to unrepaired or misrepaired DNA damage as a consequence of energy deposition in the cell nucleus. Recently, however, the view that DNA damage is restricted to directly irradiated cells has been challenged by radiation-induced effects being observed in nonirradiated cells. These so-called untargeted effects are broadly grouped into two categories: effects in the descendants of irradiated cells known as radiation-induced genomic instability and effects in cells that are in close proximity to, or have received damaging signals from, irradiated cells known as radiation-induced bystander effects (1–8).

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Radiation-induced genomic instability and bystander effects have been shown *in vitro* and high linear energy (LET) transfer (densely ionizing) radiation such as α particles or neutrons tends to be a more effective inducer than low LET sparsely ionizing radiation such as γ- or X-rays (9). Currently, there is limited evidence for bystander effects in whole animals and tissues *in vivo* and such effects have been shown primarily after high LET α-particle radiation (10). However, for the foreseeable future, there will be important low-LET radiation exposures from medical applications, waste cleanup, and other industrial/environmental exposures and potentially from terrorism events. Accordingly, it is important to investigate the potential of low-LET radiation to produce untargeted effects *in vivo* and because the responses of the hemopoietic system are major determinants of outcome following therapeutic, occupational, and accidental radiation exposures, effects in hemopoietic cells are of particular interest.

Previously, chromosomal instability shown *in vitro* in the progeny of hemopoietic stem cells after their exposure to low fluences of α particles (11) was shown to persist for many months *in vivo* by transplantation of irradiated mouse bone marrow into syngeneic recipients (12). A feature of the *in vitro* findings was that more colonies exhibited instability than the number of clonogenic cells traversed by the Poisson distribution of α particles. That a bystander mechanism contributed to such instability was subsequently confirmed by direct experiment (13). Because of the Poisson distribution of α particles, the transplantation studies (12) were complicated by nonirradiated cells inevitably and unavoidably being transferred with irradiated survivors, and it was therefore unclear to what extent chromosomal instability shown *in vivo* could be attributed to bystander mechanism(s). In an attempt to model the *in vivo* mixture of irradiated and nonirradiated cells in the α-irradiation experiments, we previously transplanted mixtures of nonirradiated bone marrow with bone marrow exposed to neutrons (a densely ionizing radiation like α-particle irradiation) using a sex-mismatch congenic transplantation protocol to provide a three-way cytogenetic marker system that allowed us to distinguish not only host-derived cells from donor-derived cells but also cells derived from irradiated or nonirradiated donor stem cells. Using this system, we confirmed that high-LET–induced radiation-induced chromosomal instability *in vivo* could be associated with a bystander mechanism (14). In the present investigations, using low-LET radiation we have used this congenic system to investigate untargeted damage in hemopoietic cells, contributions to hemopoiesis from irradiated versus unirradiated stem cells, and potential cellular sources of damaging bystander signals.

Materials and Methods

Irradiation of cells. CBA/Ca mice were used in this study, which was carried out in compliance with the guidance issued by the Medical Research Council and Home Office Project Licences PPL 30/1272 and 60/2841. Femoral bone marrow suspensions were obtained from 12-week-old male

mice and γ irradiated at a dose rate of 0.45 Gy/min using a CIS Bio International 637 Cesium irradiator to a total dose of 4 Gy, a potentially leukaemogenic dose for CBA strains of mice (15).

Bone marrow transplantation. A previously described protocol (14) was used in which nonirradiated, irradiated, or a mixture of irradiated and nonirradiated male bone marrow cells was transplanted into female recipients. Irradiated and nonirradiated cells were distinguished by using marrow from CBA/Ca mice (40XY cells) and a congenic CBA strain (40XYT6T6 cells) that is homozygous for the stable T6 reciprocal translocation between chromosomes 14 and 15, resulting in two distinctive small marker chromosomes (Fig. 1). Previous studies in our laboratory had shown that transplantation of cell suspensions containing 200 short-term repopulating stem cells assayed *in vivo* as day 12 spleen colony-forming units (CFU-S) correlated with long-term survival and donor repopulation in this mouse strain (12). Therefore, to standardize the transplantation procedure, the total number of cells injected for each treatment was adjusted accordingly. In normal marrow the incidence of CFU-S is $\sim 200/10^6$ cells and in 4 Gy γ-irradiated bone marrow, $10/10^6$ (5% surviving fraction). Irradiated and sham-irradiated cells were placed on ice immediately after irradiation and mixed *in vitro* within 10 minutes, diluted appropriately, and 0.2 mL aliquots injected i.v. within 1 hour of completion of irradiation into female recipients that had received 9.5 Gy γ-irradiation less than 2 hours before transplantation. The cell mixture of 40% irradiated and 60% nonirradiated stem cells was chosen to model the Poisson distribution of hit and nonhit stem cells in previous studies of α-irradiated marrow (11, 12) that had shown chromosomal instability in a manner compatible with expression in the descendants of nonirradiated cells (13). An additional transplantation study was conducted in which unirradiated cells for transplantation were incubated at 5×10^6 cells/mL for 4 hours before injection with control cell–conditioned medium or irradiated cell–conditioned medium obtained by centrifugation of cell suspensions obtained from total body–irradiated mice.

Cytogenetic and immunocytochemical analyses of repopulated bone marrow. At 10, 30, and 100 days posttransplantation, femoral bone marrow was obtained from three recipient mice per sample time and direct chromosome preparations were obtained from each animal. Metaphases were accumulated for 1 hour by adding 0.02 μg mL^{-1} colcemid to the disaggregated marrow cells, suspended in α-MEM supplemented with 15% FCS. These cells were then suspended in 5 mL hypotonic (0.55% w/v) potassium chloride (KCl) for 30 minutes, at which time 2 to 3 mL of KCl in sodium citrate were added (0.28 g KCl and 0.5 g sodium citrate in 100 mL of distilled water). The suspension was gently mixed and incubated for a further 8 minutes. The cells were fixed in suspension by adding 1 to 2 mL of a 3:1 methanol/acetic acid mixture to a final volume of 10 mL. After 10 to 15 minutes, the cells were resuspended in at least two additional changes of the fixative mixture. Air-dried preparations were aged for 10 to 14 days before Giemsa staining.

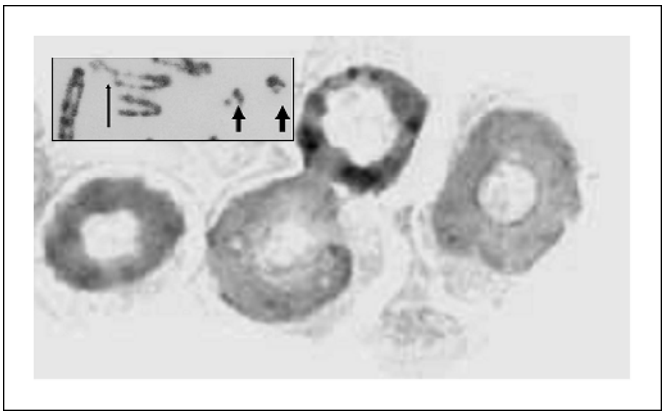

Figure 1. A cytocentrifuge preparation of bone marrow cells obtained 100 days postirradiation shows phosphorylated H2AX foci in a bone marrow granulocytic cell alongside cells with no evidence of foci. *Inset,* partial metaphase showing a chromatid break (*long arrow*) in a preparation of 40XYT6T6 cells readily distinguished from 40XY cells by the two small marker chromosomes (*heavy arrowheads*) resulting from the reciprocal translocation.

Coded slides were examined and the donor origin of cells for analysis confirmed by the presence of the Y chromosome. For each metaphase, chromosomal aberrations were recorded along with the origin of the cell from irradiated (40XY) or nonirradiated (40XYT6T6) stem cells—distinguished by the two small marker chromosomes resulting from the stable reciprocal translocation (14). Data were pooled from three replicates from three independent transplantations and differences between the proportions of aberrant cells in the decoded preparations were analyzed by the Fisher's exact test. To investigate further the contribution from irradiated and nonirradiated stem cells, an *in vitro* clonogenic assay, operationally defined as the CFU-A assay, was used to obtain clones of cells derived from members of the hemopoietic stem cell compartment as previously described (16). Cytogenetic preparations were obtained from individual colonies (11) and the T6 status of the metaphases determined to identify the clonogenic cell as derived from irradiated or nonirradiated stem cells. Differences between the observed and expected ratios of 40XY/40XYT6T6 cells or clonogenic stem cells were analyzed by the Fisher's exact test. For the transplantation study in which cells were preincubated with control cell–conditioned medium or irradiated cell–conditioned medium, data were pooled from triplicate recipients from two independent transplantations and differences between the proportions of aberrant cells in the decoded preparations were analyzed by the Fisher's exact test. Samples of the bone marrow used for cytogenetic analyses were collected onto glass slides using a cytocentrifuge for immunocytochemical detection of γH2AX foci. Cells were dried at room temperature before fixing in −20°C methanol/acetone. Cells were subsequently dried and stored at −70°C. Phosphorylated H2AX was detected with an affinity-purified rabbit antiserum (Upstate Ltd., Milton Keynes, United Kingdom) diluted 1:20,000 and incubated overnight at 4°C. Positive foci were identified by an ABC-peroxidase technique (ABC Elite, Vector Laboratories) according to the recommendations of the manufacturer and using diaminobenzidine as chromogen. Cells were lightly counterstained with hematoxylin, dehydrated, mounted in DPX resin, and viewed by light microscopy. Differences between proportions of cells with foci in the decoded preparations were analyzed by the Wilcoxon sum of ranks Mann-Whitney test.

Results and Discussion

After bone marrow transplantation (Table 1), the frequency of cells with chromosome aberrations in the recipients on nonirradiated donor cells (1.26%) was not significantly different from the spontaneous level of aberrations (1.7%) in nonirradiated control mice ($P = 0.5640$). After transplantation of γ-irradiated cells, at all three time points (Table 1), there was a significantly greater frequency of cytogenetic aberrations characteristic of chromosomal instability in the recipient bone marrow than in controls (overall, 8.46% compared with 1.26%; $P < 10^{-7}$). After transplantation of a mixture of irradiated and nonirradiated bone marrow, chromosomal instability was shown in 7.56% of the 40XY cells (i.e., in cells derived from transplanted, irradiated stem cells; Table 1). Thus, the expression of chromosomal instability in 40XY cells was independent of the total number of 40XY stem cells irradiated (7.56 ± 0.29 and 8.46 ± 0.49, respectively, for 40% and 100%; $P = 0.2991$). At all times posttransplantation, chromosomal instability was also shown in cells carrying the T6 marker (i.e., in cells derived from the nonirradiated, transplanted stem cells). The overall frequency of 40XYT6T6 cells expressing instability (3.77 ± 0.63%), although lower than that in the 40XY cells, was significantly greater than in controls (1.26%) transplanted with nonirradiated 40XYT6T6 marrow ($P < 10^{-7}$). That there are significantly fewer aberrations in the control transplantation argues against aberrations in the unirradiated 40XYT6T6 hemopoietic cells being attributed to

Table 1. Cytogenetic aberrations in bone marrow cells obtained from mice transplanted with unirradiated, irradiated, or a mixture of 4 Gy γ-irradiated (40XY) and nonirradiated (40XYT6T6) bone marrow cells show chromosomal instability in the progeny of both irradiated and nonirradiated stem cells (mean ± SE)

Time	Donor cells	Total cells	Normal cells	Cells with chromatid breaks, minutes, and chromosome fragments		% Aberrant cells
				40XY	40XYT6T6	
10 d	Unirradiated	1,002	993	—	9	0.89
	Irradiated	609	557	52	—	8.54
	Mixture					
	40XY	267	247	20	—	7.20
	40XYT6T6	584	564	—	20	3.42
30 d	Unirradiated	658	649	—	9	1.36
	Irradiated	378	350	28	—	7.41
	Mixture					
	40XY	60	55	5	—	8.33
	40XYT6T6	364	345	—	19	5.22
100 d	Unirradiated	1,270	1,251	—	19	1.50
	Irradiated	608	553	55	—	9.05
	Mixture					
	40XY	162	150	12	—	7.41
	40XYT6T6	672	650	—	22	3.27
Total	Unirradiated	2,930	2,893	—	37	1.26 ± 0.19
	Irradiated	1,595	1,460	135	—	8.46 ± 0.49
	Mixture					
	40XY	489	452	37	—	7.56 ± 0.29
	40XYT6T6	1,620	1,559	—	61	3.77 ± 0.63
Unirradiated nontransplanted control	40XY	500	491	9	—	1.80 ± 0.49
	40XYT6T6	300	295	—	5	1.67 ± 0.67

interactions between unirradiated hemopoietic cells and the more radioresistant stromal cells that would survive the conditioning irradiation.

In scoring consecutive metaphases in preparations of bone marrow obtained from mice transplanted with the mixture of irradiated cells, it would be expected that the relative proportions of 40XY and 40XYT6T6 metaphases should reflect the 40:60 ratio of stem cells in the donor cell suspension. However, it was apparent that 40XY cells were underrepresented and 40XYT6T6 overrepresented (Table 2). At 10 days posttransplantation, the observed ratio of 31:69 represents a significant deviation from the expected ($P = 0.0001$), and the ratios at 30 and 100 days (14:86 and 19:81, respectively) are major deviations from the expected 40:60 ratio ($P < 10^{-7}$ in both cases). To determine whether this deviation was a reflection of similar processes in the functional clonogenic cells of the stem cell compartment, the *in vitro* CFU-A assay was used to assess directly the ratio of 40XY/40XYT6T6 clonogenic stem cells in the repopulated bone marrow (Table 3). At both 30 and 100 days posttransplantation, there was a significant deviation from the expected ratio (24:76 and 31:69, respectively; $P = 0.0246$ and 0.0078). In a parallel study in which a 40:60 ratio of unirradiated 40XY/unirradiated 40XYT6T6 stem cells was transplanted, the observed ratio of 45:55 was not significantly different from that expected ($P = 0.2419$). Therefore, the data are consistent with a deficit of the 40XY stem cells derived from irradiated ancestors.

Table 2. The proportions of 40XYT6T6 cells proliferating in the bone marrow of recipients of a 40:60 mixture of 4 Gy γ-irradiated (40XY) and nonirradiated (40XYT6T6) hemopoietic stem cells show a greater than expected frequency of 40XYT6T6 cells

Time	Total cells	40XY/40XYT6T6		
		Observed ratio	Expected ratio	
Day 10	851	267:584 (31:69)	340:511 (40:60)	$P = 10^{-4}$
Day 30	424	60:364 (14:86)	170:254 (40:60)	$P < 10^{-7}$
Day 100	834	162:672 (19:81)	334:500 (40:60)	$P < 10^{-7}$
Total	2,109	489:1,620 (23:77)	844:1,265 (40:60)	$P < 10^{-7}$

The hemopoietic stem cell compartment is a developmentally structured continuum in which the most primitive members have the greatest long-term repopulating ability and are the most resistant to proliferation and differentiation stimuli. When these cells do replicate, in succeeding divisions they give rise to stem cells with decreasing self-renewal capacity and increasing probability of becoming committed to the various hemopoietic lineages and, in the steady state, differentiating to provide functional blood cells. In transplantation recipients there are two phases of bone marrow engraftment: an initial but transient engraftment (essential for survival following the conditioning irradiation) followed by a delayed but long-term reconstitution of the hemopoietic system. These two phases can be attributed respectively to the later and earlier members of the stem cell compartment, and functionally their progeny can be assayed at 30 or 100 days posttransplantation

Table 3. The proportions of 40XYT6T6 clonogenic cells obtained from the bone marrow of recipients of a 40:60 mixture of 4 Gy γ-irradiated (40XY) and nonirradiated (40XYT6T6) bone marrow cells show a greater than expected frequency of 40XYT6T6 cells

Time	Total colonies	40XY/40XYT6T6		
		Observed ratio	Expected ratio	
Day 30	86	21:65 (24:76)	34:52 (40:60)	$P = 0.0246$
Day 100	382	120:262 (31:69)	153:229 (40:60)	$P = 0.0078$
Total	468	141:330 (30:70)	187:281 (40:60)	$P = 0.00031$
Control mixture (no cells irradiated)	88	40:48 (45:55)	34:53 (40:60)	$P = 0.2419$

(17, 18). Thus, the relative deficit of 40XY cells at these two time points (Tables 2 and 3) reflects a generalized stem cell deficit and could be explained by radiation-induced lethal mutations (also known as delayed reproductive death). This delayed death phenomenon, characterized by an elevated incidence of cell death in the progeny of irradiated cells, is well documented for mammalian cells irradiated *in vitro* and is considered a manifestation of the radiation-induced genomic instability phenotype (6, 19). Thus, the present study shows the *in vivo* persistence of the instability phenotype in the descendants of the transplanted irradiated hemopoietic stem cells and, additionally, a delayed bystander-induced chromosomal instability in the progeny of the nonirradiated stem cells.

In this transplantation model, cells derived from the small number of transplanted donor stem cells will have reestablished a stem cell compartment and reconstituted the hemopoietic system (20), and it is improbable that any cells examined cytogenetically were those present in the original irradiated population. Whereas some transmitted delayed effect of irradiation might explain the chromosomal instability and deficit in contribution to repopulation in the progeny of irradiated stem cells, chromosomal instability in the progeny of unirradiated 40XYT6T6 stem cells cannot be explained by such a mechanism. Effects in unirradiated hemopoietic cells *in vitro* that are characteristic of radiation-induced bystander effects are induced by factors produced very rapidly by irradiated cells (13). Comparable bystander interactions in these *in vivo* experiments would have to take place at the time of mixing irradiated and nonirradiated cells before transplantation and/or by the nonirradiated cells interacting with the irradiated radioresistant recipient stromal cells. The levels of cytogenetic damage when control cells alone are transplanted are not significantly different from unirradiated control animals (Table 1). Therefore, the irradiated recipient stroma is not the source of damaging bystander signals. To investigate the possibility that interactions during cell mixing before transplantation are responsible for the observed genomic damage to unirradiated cells, unirradiated bone marrow was preincubated in irradiated cell–conditioned medium as a source of potential bystander signals, then transplanted, and the repopulated bone marrow was examined for cytogenetic abnormalities (Tables 4 and 5). At both 30 and 100 days posttransplantation, ~2% to 3% of cells exhibit cytogenetic abnormalities irrespective of whether the donor cells were incubated with irradiated cell– or control cell–conditioned medium (Table 4), and overall there was no significant difference between the recipients of cells exposed to either of the two conditioned media (2.6% and 2.8% for control cell–conditioned medium and irradiated cell–conditioned medium, respectively; $P = 0.54001$). In addition, although the preincubation of cells resulted in an increased frequency of cells with aberrations posttransplantation, there was no significant difference between the irradiated cell–conditioned medium data (2.8%) and the control data shown in Table 1 (1.26%; $P = 0.9518$). These data are not consistent with any bystander induction of a transmissible chromosomal instability phenotype due to initial mixing, or with the irradiated stromal cells providing significant levels of damaging signals.

Table 4. Cytogenetic aberrations in bone marrow cells obtained from mice transplanted with bone marrow incubated *in vitro* with conditioned medium obtained from suspensions of 4 Gy irradiated (irradiated cell–conditioned medium) or unirradiated (control cell–conditioned medium) bone marrow before transplantation

Time	Total cells	Normal cells	Cells with chromatid breaks, minutes, and chromosome fragments	% Aberrant cells	
30 d					
CCCM	150	146	4	2.67	
ICCM	150	145	5	3.30	$P = 0.5000$
100 d					
CCCM	50	49	1	2.00	
ICCM	100	98	2	2.00	$P = 0.74225$
Total CCCM	200	195	5	2.55	
Total ICCM	250	243	7	2.80	$P = 0.54001$

Abbreviations: CCCM, control cell–conditioned medium. ICCM, irradiated cell–conditioned medium.

Table 5. Phosphorylated histone H2AX foci in bone marrow cells obtained from mice transplanted with bone marrow incubated *in vitro* with conditioned medium obtained from suspensions of 4 Gy irradiated (irradiated cell–conditioned medium) or unirradiated (control cell–conditioned medium) bone marrow before transplantation

Time	Total cells	γH2AX foci/cell									Foci per cell (mean ± SE)	
		0	1	2	3	4	5	6	7	8		
30 d												
CCCM	1,611	1,445	97	35	15	11	6	1	0	1	0.18622 ± 0.01692	
ICCM	1,568	1,371	113	49	22	9	1	2	1	0	0.21492 ± 0.01717	$P = 0.2727$
100 d												
CCCM	2,142	1,939	122	45	23	9	3	0	1	0	0.15826 ± 0.01256	
ICCM	2,121	1,895	144	53	19	8	1	0	1	0	0.16549 ± 0.01222	$P = 0.6336$
Total CCCM	3,753	3,384	219	90	38	20	9	1	1	1	0.20038 ± 0.01205	
Total ICCM	3,689	3,266	257	102	41	17	2	2	2	0	0.18650 ± 0.01014	$P = 0.2319$

The ongoing chromosome breakage associated with the chromosomal instability phenotype will be associated with newly arising double-strand breaks and/or stalled replication forks in the progeny of irradiated cells, and these can be detected by the presence of foci of the phosphorylated histone H2AX, commonly designated γH2AX (21, 22). Although there were no detectable cytogenetic aberrations after transplantation of cells exposed to irradiated cell–conditioned medium, it is conceivable that an underlying potential for genomic instability might be detected by the presence of increased numbers of γH2AX foci (Fig. 1). However, when the presence of such foci was investigated (Table 5), there was no significant difference between the recipients of cells exposed to either of the two conditioned media at either time point [0.1862 and 0.2149 ($P = 0.2727$) and 0.1583 and 0.16549 ($P = 0.6336$) for control cell–conditioned medium and irradiated cell–conditioned medium, respectively, at 30 and 100 days]. These data also support the conclusion that an instability phenotype is not induced by a direct bystander mechanism. Thus, although what might be considered a "conventional" bystander effect is highly improbable, a more complex bystander-type model has to be invoked to explain the expression of instability in the descendants of unirradiated stem cells (i.e., it is the descendants of irradiated cells, rather than irradiated cells themselves, that are responsible for the bystander signal(s) in this transplantation model).

Given this interpretation that signals from the descendants of irradiated cells are responsible for the damage in the descendants of unirradiated cells, the explanation of cytogenetic damage in cells derived from irradiated donor stem cells need not be restricted to a transmissible instability model as such instability is not necessarily inconsistent with an indirect mechanism downstream of the irradiated stem cells. It is possible that a cell, such as a macrophage, derived from an irradiated stem cell might induce instability in a bystander cell derived from a different irradiated stem cell. Alternatively, an as yet unrecognized aspect of *in vivo* untargeted effects might be the capacity of a cell derived from an irradiated stem cell to interact with the irradiated stromal microenvironment and induce signals that secondarily induce damage in cells descended from irradiated or unirradiated hemopoietic stem cells. Thus, the radiation-induced genomic instability phenotype *in vivo* need not necessarily reflect intrinsically unstable cells but responses to ongoing production of damaging signals. Further investigations are required to investigate the various possibilities; however, previous studies (23) that identified tissue macrophages expressing increased levels of reactive nitrogen and/or oxygen species as a delayed and persisting consequence of the hemopoietic tissue responses to radiation damage would be consistent with such a model and also with the general view that free radical–mediated processes underlie untargeted effects (23–27). Overall, it is now clear that, in addition to targeted effects of damage directly induced in cells by irradiation, a variety of indirect untargeted effects perpetuated long-term *in vivo* may also make important contributions to determining the consequences of radiation exposures. As the majority of human exposures to ionizing radiation are partial body irradiations, an *in vivo* γ-radiation–induced delayed bystander instability phenotype could have significant implications for mechanisms underlying the health consequences of such exposures.

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