Chromosomal Instability in Unirradiated Hemaopoietic Cells Induced by Macrophages Exposed *In vivo* to Ionizing Radiation

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Abstract

The tumorigenic potential of ionizing radiation has conventionally been attributed to DNA damage in irradiated cells induced at the time of exposure. Recently, there have been an increasing number of reports of damage in unirradiated cells that are either neighbors or descendants of irradiated cells, respectively, regarded as bystander effects and genomic instability and collectively termed nontargeted effects. In this study, we show that descendants of normal murine hemaopoietic clonogenic stem cells exposed to bone marrow–conditioned medium derived from γ-irradiated mice exhibit chromosomal instability unlike the descendants of directly γ-irradiated cells. The instability is expressed in bone marrow cells of the radiation-induced acute myeloid leukemia (r-AML) susceptible strain (CBA/Ca) but not in mice resistant to r-AML (C57BL/6). Furthermore, crossgenetic experiments show the induction of the instability phenotype requires both the producer and responder cells to be of the susceptible CBA/Ca genotype. Macrophages are the source of the bystander signals, and the signaling mechanism involves tumor necrosis factor-α, nitric oxide, and superoxide. The findings show a genotype-dependent chromosomal instability phenotype induced by radiation-induced macrophage-mediated bystander signaling. As the majority of accidental, occupational, and therapeutic exposures to ionizing radiation are partial body exposures, the findings have implications for understanding the consequences of such exposure. [Cancer Res 2008;68(19):8122–6]

Introduction

The major adverse consequences of radiation exposures are attributed to DNA damage in irradiated cells induced at the time of exposure that has not been correctly restored by metabolic repair processes. Recently, however, a number of studies have shown that not all genetic damage is attributable to lesions induced at the time of exposure in the cell nucleus or in the irradiated cell itself. These studies have shown effects, characteristic of radiation responses in directly irradiated cells, in nonirradiated cells as a consequence of communication between irradiated and nonirradiated cells. The findings are broadly grouped into two categories, effects in the unirradiated descendants of irradiated cells, collectively regarded as radiation-induced genomic instability, and effects in unirradiated cells that have received signals produced by neighboring irradiated cells, collectively regarded as radiation-induced bystander effects (1–8). It is not understood whether nontargeted effects have a common underlying mechanism, and as the majority of published studies of nontargeted effects have used *in vitro* model systems, the relevance to *in vivo* responses is not understood. However, delayed chromosome aberrations, characteristic of a chromosomal instability phenotype, have been shown in the bone marrow of irradiated CBA/Ca mice (9), and clastogenic factors, able to induce chromosome damage in unirradiated cells, have been shown in the blood of radiotherapy patients, Japanese atomic bomb survivors, and Chernobyl liquidators (4, 5, 10, 11). The relationship of the production of clastogenic factors to the demonstration of delayed unstable chromosome aberrations *in vivo* has not been established, but clastogenic factors, delayed cellular damage responses, and delayed unstable chromosomal aberrations have all been associated with genotype-dependent inflammatory processes (10, 12, 13). Late-appearing adverse chronic side effects in unavoidably irradiated normal tissues of radiotherapy patients are also associated with such processes (14). The majority of accidental, occupational, and therapeutic human exposures to ionizing radiation are partial body exposures, and a better understanding of the consequences of such exposures requires a better understanding of the interactions between irradiated and nonirradiated cells.

Because the responses of the hemaopoietic system are major determinants of outcome after exposure to ionizing radiation, we are conducting studies to compare responses in CBA/Ca mice and C57BL/6 mice that are, respectively, susceptible or resistant to the development of radiation-induced acute myeloid leukemia (15). In previous studies of the *in vivo* response to a potentially leukemogenic dose of ionizing radiation, we showed a marked genotype-dependent macrophage activation in the hemaopoietic system (12) in which CBA/Ca macrophages have an enhanced classically activated M1-like (proinflammatory) phenotype, whereas C57BL/6 macrophages expressed an enhanced M2-like (anti-inflammatory/wound healing) phenotype (16). In irradiated CBA/Ca mice, there is also a persisting chromosomal instability phenotype (9). In the present study, we have shown that macrophages obtained from the bone marrow of irradiated CBA/Ca, but not C57BL/6 mice, are able to induce chromosomal instability assayed as nonclonal cytogenetic aberrations in the clonal descendants of nonirradiated stem cells as a consequence of proinflammatory cytokine signaling. The findings may also be regarded as a radiation-induced genotype-dependent bystander effect triggering an instability phenotype.

Materials and Methods

Irradiation. CBA/Ca and C57BL/6 mice were bred in-house under conventional conditions. Suspensions of femoral bone marrow were obtained from 8- to 16-wk-old male mice that had been γ irradiated (or sham irrradiated) at a dose rate of 0.4 Gy/min using a Bio International

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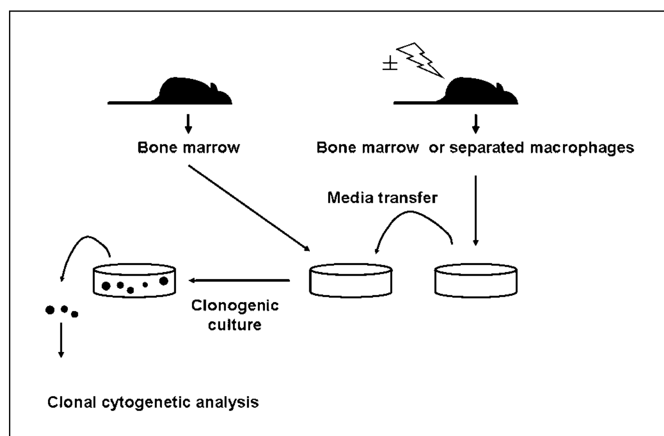

Figure 1. A schematic view of the experimental protocol in which normal unirradiated primary bone marrow cells exposed to conditioned medium generated by bone marrow irradiated *in situ* are cultured in standard semisolid clonogenic cultures. The cultures are incubated for 9 d before harvesting individual colonies for clonal cytogenetic analysis.

637 Cesium irradiator to a total dose of 4 Gy; a potentially leukaemogenic dose for CBA strains of mice (17). Experiments were approved by local ethical review and carried out in compliance with Home Office Project Licences PPL 60/2841 and 60/3564.

Assay of conditioned medium and clonal cytogenetic analysis. Immediately after irradiation, bone marrow cells were suspended at 5.10^6 cells/mL and the supernatant medium obtained by centrifugation at 1,000 rpm for 5 min and filtration through a 0.45-μm membrane to obtain irradiated cell–conditioned medium (ICCM). In parallel with control cell–conditioned medium derived from unirradiated mice (CCCM), ICCM was assayed at cell equivalent concentrations at a ratio of 1:1 producer/assay cells as used previously to study macrophage-derived stem cell proliferation regulators (18). Immediately after mixing, an *in vitro* clonogenic assay, operationally defined as the CFU-A assay, was used to obtain clones of myelo-monocytic cells derived from members of the hemaopoietic stem cell compartment (19). The time from the initiation of irradiation to the initiation of the clonogenic cultures is ~1 h. Cytogenetic preparations were obtained from individual colonies 7 to 9 d (10–13 cell divisions) after initiation of cultures using a previously reported method for karyotyping hemaopoietic colonies (20). Chromosomal aberrations in coded samples were analyzed, data from 3 to 6 replicate experiments were pooled after decoding, and differences between the proportions of aberrant cells were analyzed by the Fisher's exact test (Fig. 1).

Macrophage separation. Macrophages were isolated from the femora of CBA/Ca or C57BL/6 mice with or without whole body 4 Gy γ-irradiation 24 h previously. Bone marrow was gently expelled from the diaphysis with air pushed through 23G needles by syringe, and cell clumps were digested with medium containing liberase (1.67 Wunsch units/mL) and 0.2 mg/mL DNase (Roche) for 30 min at 37°C. Cells were collected in lipopolysaccharide-free PBS containing 2 mmol/L EDTA and 0.5% bovine serum albumin (Invitrogen). Macrophages were positively selected using F4/80 antibody followed by anti-rat immunoglobulin microbeads (Miltenyi Biotec; ref. 16). The whole separation procedure took 3 h. Postseparation, cytocentrifuge preparations revealed cells to be >90% monocytes and macrophages, and these were incubated overnight at 37°C, 5%CO2 in air in α-MEM medium containing 20% pretested horse serum. Macrophages were collected in their culture medium, spun at 1,000 rpm for 5 min, and filtered as before to produce irradiated or control macrophage–conditioned medium (IMCM or CMCM). The conditioned medium were assayed as described above using a ratio of producer macrophages to responder bone marrow cells of 1:2.

Signaling studies. A neutralizing antibody directed against tumor necrosis factor (TNF)-α (R &D Systems) and a scavenger of reactive oxygen species and DMSO and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), respectively, (Sigma) were used. To investigate the response to potential signaling molecules, inhibitors/blocking agents were added to the clonogenic culture medium before the addition of unirradiated assay cells, and the conditioned medium was prepared from the separated F4/80-positive cells. Toxicity studies for each inhibitor/blocking agent were performed using the CFU-A assay, and the highest concentration that was nontoxic was selected for these studies: 1 μg/mL anti–TNF-α, 50 μm c-PTIO, and 0.05% DMSO.

Results

The clonal progeny of CBA/Ca short-term repopulating stem cells were studied using a clonogenic assay (operationally defined as CFU-A), and the chromosomal instability phenotype were assessed by the presence of nonclonal aberrations, i.e., in any individual clone, not all cells are karyotypically abnormal and any aberrant cells are not a subclone as each cell exhibits a different aberration. The aberrations that characterize this instability phenotype are unstable aberrations, that is, chromatid breaks, chromosome fragments, and minutes indicative of ongoing

Table 1. Nonclonal aberrations in clonal progeny derived *in vitro* from short-term repopulating stem cells in irradiated or unirradiated CBA/Ca or C57BL/6 mouse bone marrow and in progeny derived from nonirradiated mouse bone marrow exposed to medium conditioned by bone marrow obtained from irradiated (ICCM) or nonirradiated (CCCM) mice

Assay cells	Source of CM	Cells with cytogenetic aberrations	Cells with 0, 1, 2, and ≥3 aberrations (%)				Mean aberrations/cell
			0	1	2	≥3	
CBA/Ca	—	32/312 (10.3)	280	22	6	4	0.147
Irradiated CBA/Ca	—	13/170 (7.6)	157	8	4	1	0.112
CBA/Ca	CBA/Ca	43/479 (9.0)	436	35	6	2	0.117
CBA/Ca	Irradiated CBA/Ca	84/482 (17.4)	398	65	13	6	0.232
C57BL/6	—	23/205 (11.2)	182	22	1	0	0.117
Irradiated C57BL/6	—	15/110 (13.6)	95	12	3	0	0.164
C57BL/6	C57BL/6	20/175 (11.4)	155	16	4	0	0.137
C57BL/6	Irradiated C57BL/6	33/232 (14.2)	199	30	2	1	0.160

Abbreviation: CM, conditioned medium.

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Table 2. Nonclonal aberrations in clonal progeny derived *in vitro* from unirradiated CBA/Ca or C57BL/6 short-term repopulating stem cells exposed to medium conditioned by bone marrow obtained from irradiated (ICCM) or nonirradiated (CCCM) mice of the opposite genotype; i.e., a crossgenetics investigation

Assay cells	Source of CM	Cells with cytogenetic aberrations (%)	Cells with 0, 1, 2, and ≥3 aberrations				Mean aberrations/cell
			0	1	2	≥3	
CBA/Ca	—	32/312 (10.3)	280	22	6	4	0.147
CBA/Ca	C57BL/6	9/109 (8.3)	100	8	0	1	0.110
CBA/Ca	Irradiated C57BL/6	7/111 (6.3)	104	6	1	0	0.072
C57BL/6	—	23/205 (11.2)	182	22	1	0	0.117
C57BL/6	CBA/Ca	11/92 (12.0)	81	9	1	1	0.132
C57BL/6	Irradiated CBA/Ca	8/97 (8.2)	89	6	2	0	0.103

chromosome breakage (1, 2, 3, 24). In colonies derived from bone marrow obtained immediately postirradiation (Table 1), the proportion of cells with nonclonal aberrations was not significantly different from controls (7.6% versus 10.3%; $P = 0.2146$). Using the same *in vitro* assay, the proportion of cells with nonclonal aberrations in colonies initiated from normal CBA/Ca bone marrow exposed to conditioned medium obtained from CBA/Ca mice exposed to 4 Gy γ-rays (ICCM) was significantly increased relative to the control colonies derived from nonirradiated bone marrow (17.4% versus 10.3%; $P = 0.0031$).This increase was not found in colonies derived from CBA/Ca bone marrow exposed to CCCM, i.e., obtained from the bone marrow of nonirradiated CBA/Ca mice (9.0% versus 10.3%; $P = 0.3151$). Thus, exposure to ICCM, but not CCCM, resulted in an increased frequency of cells with nonclonal cytogenetic aberrations in colonies initiated from normal CBA/Ca bone marrow. The mean number of aberrations per cell (0.232) was increased by comparison with the control value (0.147), and the aberrations were characterized by chromatid and chromosome breaks typical of the chromosomal instability phenotype.

The proportion of cells with nonclonal aberrations (11.2%) in colonies initiated from C57BL/6 bone marrow (Table 1) was slightly greater than that of the CBA/Ca cells (10.3%), but the difference was not significant ($P = 0.4172$). In colonies derived from C57BL/6 bone marrow obtained immediately postirradiation, such as the situation with CBA/Ca bone marrow, the proportion of cells with nonclonal aberrations was not significantly different from the controls (13.6% versus 11.2%; $P = 0.3238$). There was no significant effect of treatment with C57BL/6 CCCM (11.4% aberrant cells; $P = 0.5378$), and the frequency of aberrations in colonies initiated from C57BL/6 bone marrow exposed to ICCM was slightly increased (14.2%) but not significantly so ($P = 0.2138$).

Because the frequency of nonclonal aberrations in colonies initiated from CBA/Ca bone marrow exposed to CBA/Ca ICCM was increased relative to controls but not in colonies initiated from C57BL/6 bone marrow exposed to C57BL/6 ICCM, we investigated the potential for ICCM from one genotype to produce the instability phenotype in normal bone marrow obtained from the other genotype. As shown in Table 2, the proportion of cells with nonclonal aberrations in colonies initiated from CBA/Ca bone marrow exposed to conditioned medium obtained from irradiated C57BL/6 mice (6.3%) was not greater than that in colonies initiated from CBA bone marrow exposed to conditioned medium obtained from nonirradiated C57BL/6 mice (8.3%; $P = 0.3833$). In neither case was the aberration frequency greater than the 10.3% in the CBA/Ca control ($P = 0.14794$ and 0.3456, respectively). Similarly, the proportion of cells with nonclonal aberrations in colonies initiated from C57BL/6 bone marrow exposed to conditioned medium obtained from irradiated CBA/Ca mice (8.2%) was not greater than that in colonies initiated from C57BL/6 bone marrow exposed to

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Table 3. Nonclonal aberrations in clonal progeny derived *in vitro* from unirradiated CBA/Ca short-term repopulating stem cells exposed to medium conditioned by bone marrow macrophages isolated from irradiated (IMCM) or nonirradiated (CMCM) CBA/Ca mice in the presence or absence of anti–TNF-α antibody or a scavenger of superoxide (DMSO) or nitric oxide (c-PTIO)

Source of macrophages (conditioned medium)	Inhibitory factor	Cells with cytogenetic aberrations (%)	Cells with 0, 1, 2, and ≥3 aberrations				Mean aberrations/cell
			0	1	2	≥3	
CBA/Ca	—	10/137 (7.3)	127	9	1	0	0.080
Irradiated CBA/Ca	—	25/181 (13.8)	156	23	2	0	0.149
CBA/Ca	Anti-TNFα	4/63 (6.4)	44	2	1	0	0.085
Irradiated CBA/Ca	Anti-TNFα	5/80 (6.3)	75	4	1	0	0.075
CBA/Ca	DMSO	4/58 (6.9)	54	4	0	0	0.069
Irradiated CBA/Ca	DMSO	6/74 (8.1)	68	5	1	0	0.095
CBA/Ca	c-PTIO	4/83 (4.8)	79	4	0	0	0.048
Irradiated CBA/Ca	c-PTIO	4/77 (5.2)	73	2	2	0	0.078

conditioned medium obtained from nonirradiated CBA/Ca mice (12%; P = 0.2725) and not significantly different from the C57BL/6 control (11.2%; P = 0.2725 and 0.4972, respectively). Thus, both signal production and signal response exhibit the same genotype dependency with CBA/Ca, but not C57BL/6, mice able to produce and respond to signals that lead to the expression of delayed chromosomal aberrations.

To investigate the potential for macrophages to be the cellular source of the activity in the CBA/Ca ICCM, macrophages were isolated from the bone marrow of irradiated or nonirradiated CBA/Ca mice using the F4/80 pan-macrophage antibody and used to produce macrophage conditioned medium (IMCM and CMCM, respectively). Exposure to IMCM (Table 3) increased the proportion of cells with nonclonal cytogenetic aberrations in colonies initiated from normal bone marrow relative to the proportion in colonies derived from bone marrow exposed to CMCM (13.8% versus 7.3%; P = 0.04701). The effect induced by IMCM (Table 3) was not significantly different from that shown in Table 1 as induced by ICCM (13.8% versus 17.4%; P = 0.1583), indicating that products of macrophages obtained from irradiated CBA/Ca bone marrow were capable of producing the same effect as products of the total irradiated bone marrow from the same strain. TNF-α was implicated in the mechanism underlying the macrophage-mediated chromosomal instability phenotype by the addition of a neutralizing antibody. The treatment reduced the proportion of CBA/Ca cells with nonclonal aberrations in cultures treated with CBA/Ca IMCM to control levels (13.8–6.3%; P = 0.0436), a value indistinguishable from that resulting from exposure to CMCM in the presence of the antibody (6.4%; P = 0.6395). To investigate the potential involvement of reactive oxygen/nitrogen species, DMSO or c-PTIO (respectively, a scavenger of reactive oxygen species and nitric oxide) was added simultaneously with the conditioned medium. Either treatment (Table 3) was shown to reduce the proportion of cells with nonclonal aberrations in cultures treated with IMCM to control levels, 13.8% to 8.1% and 5.2% for DMSO and c-PTIO, respectively. Thus, there was no significant difference between the proportion of aberrant cells in colonies treated with IMCM and DMSO, or CMCM and DMSO (8.1% versus 6.9%; P = 0.1794), and similar findings were recorded for IMCM and c-PTIO, or CMCM and c-PTIO (5.2% and 4.8%; P = 0.7516).

Discussion

This study was designed to investigate the potential of bone marrow exposed to γ-radiation *in vivo* to produce factors able to induce a chromosomal instability phenotype shown as nonclonal cytogenetic aberrations in the clonal progeny of short-term repopulating hemaopoietic stem cells. The bone marrow cells studied were obtained from CBA/Ca and C57BL/6 mice, strains that are, respectively, susceptible and resistant to radiation-induced acute myeloid leukemia, and 4Gy γ-irradiation is the optimal leukaemogenic dose. A chromosomal instability phenotype (i.e., an increased incidence of nonclonal unstable abberations) has been shown after exposure of clonogenic cells to medium conditioned by bone marrow (Table 1) or bone marrow macrophages (Table 3) obtained from irradiated CBA/Ca mice but not from C57BL/6 mice. These findings are consistent with previous studies that showed expression of the chromosomal instability phenotype being strongly influenced by genetic factors (21–23). Previously, we showed radiation-induced chromosomal instability in primary clonogenic hemaopoietic cells after exposure to α-particles when, in the same study, the phenotype was not detected after exposure to 3 Gy X-rays (24). Therefore, the question arises as to why chromosomal instability is detected in the same *in vitro* clonogenic system after exposure to α-particles but not X-rays or γ-rays. A particular feature of the *in vitro* α-irradiation study was that more colonies exhibited instability than the number of clonogenic cells traversed by the Poisson distribution of α-particles, and the data were consistent with an indirect (bystander) mechanism contributing to the α-particle–induced instability phenotype. This was subsequently confirmed by direct experiment in which the absence or presence of a shielding grid between the source of α-particles and the cells was used to manipulate the ratio of irradiated to nonirradiated clonogenic cells, and the instability phenotype was shown in the descendants of nonirradiated cells mediated by a bystander mechanism (25). In the present study, all the cells assayed in the conditioned medium investigations are derived from nonirradiated clonogenic cells, and expression of the instability phenotype in primary bone marrow cells requires some genotype-dependent interaction of nonirradiated and irradiated cells.

The genotype dependency could be due to the ability to produce the relevant signal(s) and/or the ability to respond to the signal(s). In the present study, we have shown that chromosomal instability is not exhibited in colonies initiated from C57BL/6 bone marrow exposed to conditioned medium obtained from irradiated CBA/Ca mice or in CBA/Ca bone marrow exposed to conditioned medium obtained from irradiated C57BL/6 mice (Table 2). Thus, both signal production and signal response exhibit a genotype dependency such that CBA/Ca-type, but not C57BL/6-type, signaling, and response, allows expression of the phenotype. The results of the cell separation studies support macrophages being the source of the signals mediating the effect in CBA/Ca cells (Table 3), and TNF-α signaling is implicated in the mechanism (Table 3).

TNF-α is a major proinflammatory cytokine secreted by macrophages (26) and its wide range of biological activities include the ability to induce DNA damage, including DNA strand breaks, at concentrations that are not acutely toxic (27). The reduction in the proportion of CBA/Ca cells with nonclonal aberrations in cultures treated with IMCM by DMSO treatment additionally implicates oxidative stress as contributing to the development and/or maintenance of the genomic instability phenotype as suggested by previous findings of increased levels of reactive oxygen species associated with instability (28–30). Reactive nitrogen species are also implicated in the chromosomal instability phenotype as the presence of c-PTIO, a scavenger of nitric oxide, similarly reduces the proportion of cells with nonclonal aberrations in cultures treated with IMCM (Table 2). The involvement of other signaling molecules is not excluded by this study as it is known that TNF-α may induce the expression of other cytokines and cytokine receptors (31).

TNF-α and oxidative stress have previously been implicated as a component of the poorly characterized mechanisms underlying the toxicity of clastogenic factors (32) where there is considerable interindividual variation in both production of, and response to, such factors consistent with genotype-dependency (33). The present findings support proposals (11) that inflammatory processes provide a mechanistic link between radiation-induced chromosomal instability, bystander effects, and clastogenic factors. Our recent demonstration of genotype-specific gene and protein expression profiles of CBA/Ca and C57BL/6 macrophages also support an underlying genotype-dependent inflammatory mechanism as C57BL/6 macrophages in haemopoietic tissues have an

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anti-inflammatory M2-like phenotype after irradiation *in vivo*, whereas CBA/Ca macrophages express a proinflammatory M1-such as phenotype (16, 34). Overall, our data are consistent with macrophages having the potential to contribute secondary damage as a consequence of genotype-dependent inflammatory responses to the initial radiation-induced injury and, therefore, of contributing to the differential expression of nontargeted and delayed radiation effects. Furthermore, these processes may be considered as part of the "danger" signaling that mobilizes the innate and acquired immune system to maintain the integrity of the body after exposure to a variety of pathologic, chemical, or physical agents that mediate local tissue recovery or under certain circumstances contribute to a damaging tissue microenvironment (35).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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