Chromosomal Instability in Unirradiated Cells Induced in Vivo by a Bystander Effect of Ionizing Radiation

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Abstract

Using a bone marrow transplantation protocol in which we transplanted a mixture of irradiated and nonirradiated bone marrow cells that were distinguishable by a cytogenetic marker, we have demonstrated chromosomal instability in the progeny of nonirradiated hemopoietic stem cells. This first demonstration of a link between a bystander effect of ionizing radiation and the induction of genomic instability in vivo clearly poses a major challenge to current views of the mechanisms of radiation-induced DNA damage with mechanistic implications for the health consequences of radiation exposure particularly in the context of the induction of malignancy.

Introduction

Chromosome aberrations and gene mutations induced by ionizing radiation are conventionally attributed to the DNA being irreversibly changed immediately after exposure, either during the processing and enzymatic repair of the damage or during DNA replication. However, this paradigm of genetic alterations being restricted to direct DNA damage has been challenged by a number of recent developments in which effects are observed after cytoplasmic irradiation or, more dramatically, are demonstrated in cells that are not themselves irradiated but are in the neighborhood of irradiated cells. These effects include p53 protein expression, sister chromatid exchanges, micronucleus formation, cytotoxicity, and gene mutation and are defined as bystander effects (1). An additional challenge to the conventional view of genetic alterations requiring the nucleus to be irradiated is the substantial evidence for an enhanced death rate (“delayed reproductive death”) in the progeny of irradiated cells that persists for many generations and the rapidly growing body of evidence for de novo gene mutations and chromosome aberrations arising at very high frequency in cells that were not themselves irradiated but were the progeny of cells exposed to ionizing radiation many cell divisions previously. These delayed responses are collectively termed radiation-induced genomic instability (2). The majority of investigations of both inducible instability and bystander effects have used in vitro systems, and the in vivo relevance of the findings is not known. Previously, the chromosomal instability demonstrated in vitro in the progeny of mouse and human hemopoietic stem cells after low fluences of α-particles (3, 4) was shown to persist for up to a year in vivo by transplantation of α-irradiated mouse bone marrow into syngeneic recipients (5). 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, and this was due to interactions between irradiated and nonirradiated cells, i.e., a bystander mechanism (6). Other examples of α-particle-induced bystander effects (reviewed in Ref. 1) have been demonstrated by exposing cells to low fluences where, on statistical grounds, not all cells are traversed or by irradiating specific cells in a population using single particle microbeams. An important aspect of using low fluences of α-particles is that it models the environmentally relevant dose where most cells in a tissue are not actually traversed by an α-particle. However, transplantation studies are complicated by nonirradiated cells inevitably and unavoidably being transferred with irradiated survivors (because of the Poisson distribution of α-particles), and it is unclear whether any or all chromosomal instability demonstrated in the previous transplantation experiments (5) might be attributed to a bystander mechanism. Accordingly, to investigate the potential for a bystander mechanism in vivo, we have transplanted mixtures of nonirradiated cells with cells exposed to neutrons (a densely ionizing radiation like α—particles) to model the mixture of irradiated and nonirradiated cells in the previous α-irradiation experiments. Mixing irradiated and nonirradiated cells has been used successfully to demonstrate bystander effects (7). A sex mismatch transplantation protocol using CBA/H mice and congenic CBA/H mice that have a stable reciprocal chromosomal translocation (8) provided a three-way marker system allowing us to distinguish not only host-derived cells from donor-derived cells but also irradiated donor stem cell-derived cells from nonirradiated donor stem cell-derived cells. Using this system, chromosomal instability has been demonstrated in the progeny of nonirradiated stem cells. The results provide the first evidence for a role of an in vivo bystander mechanism in the induction of chromosomal instability by ionizing radiation.

Materials and Methods

Irradiation of Cells. CBA/H mice were bred in the Medical Research Council Radiation and Genome Stability Unit and used in this study, which was carried out in compliance with the guidance issued by the Medical Research Council and Home Office Project License number PPL 30/1272. Femoral bone marrow suspensions were obtained from 12-week-old male mice and irradiated with 0.5 Gy neutrons at 0.04 Gy/min using a californium-252 source with a 25% γ component. The mean energy of the neutrons was 2.2 MeV, with a spectrum of energies up to 12 MeV.

Clonal Cytogenetic Analysis. Immediately after irradiation, the cells were washed and resuspended, and an in vitro clonogenic assay operationally defined as the Type A colony-forming unit assay was used to obtain clones of cells derived from members of the hemopoietic stem cell compartment as described previously (3, 5). Cytogenetic preparations were obtained from individual colonies 7–10 days (10–13 cell divisions) after initiation of culture, and chromosomal aberrations were classified from coded slides. Differences between the proportions of aberrant cells in the colonies were analyzed by Fisher’s exact test, and significant differences between the various classes of aberration types were confirmed by a conditional binomial test.

Bone Marrow Transplantation. A simple modification of a previously described protocol (5) was used in which nonirradiated, irradiated, or a mixture of irradiated and nonirradiated cells were washed and resuspended, and an in vitro clonogenic assay operationally defined as the Type A colony-forming unit assay was used to obtain clones of cells derived from members of the hemopoietic stem cell compartment as described previously (3, 5). Cytogenetic preparations were obtained from individual colonies 7–10 days (10–13 cell divisions) after initiation of culture, and chromosomal aberrations were classified from coded slides. Differences between the proportions of aberrant cells in the colonies were analyzed by Fisher’s exact test, and significant differences between the various classes of aberration types were confirmed by a conditional binomial test.
of irradiated and nonirradiated male bone marrow cells were transplanted into female recipients. Irradiated and nonirradiated cells were distinguished by using marrow from CBA/H mice (40XY cells) and the congenic CBA/H strain (40XYT6T6 cells) homozygous for the stable T6 reciprocal translocation between chromosome 14 and 15 (8). Previous studies in our laboratory had demonstrated that transplantation of cell suspensions containing 200 short-term repopulating stem cells assayed in vivo as day-12 CFU-S correlated with long-term survival and donor repopulation in this mouse strain (5). 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 approximately 200/10^5 cells and 50/10^6 cells in O.5 Gy neutron-irradiated bone marrow (25% surviving fraction). Irradiated and sham-irradiated cells were placed on ice immediately after irradiation, mixed in vitro within 10 min, and diluted appropriately, and 0.2-ml aliquots were injected i.v. within 1 h of completion of irradiation into 100 female recipients per treatment that had received 10 Gy X-irradiation less than 2 h before transplantation. All cell suspensions were diluted 20-fold and assayed directly for CFU-S to confirm that a standardized donor population had, in fact, been used for the long-term repopulation studies. The cell mixture was chosen to model the mixture of irradiated and nonirradiated cells in previous studies of 0.5 Gy a-irradiated marrow. As described previously (6), using Poisson statistics, the probability of cells of a particular size being traversed by 0, 1, 2, 3, or more a-particles can be calculated from the particle fluence. From these values and the coefficient of the exponential survival curve, the proportions of cells in the surviving population that are irradiated and not irradiated can be calculated. For convenience, we have used a cell mixture reflecting a cell size of 8 μm because the clonogenic cells detected by the type A colony-forming unit and CFU-S assays have diameters in the range of 7–9 μm (3, 9). This is equivalent to 39% of clonogenic cells exposed to a-irradiation having been hit and survived and 61% of clonogenic cells exposed to a-irradiation not having been hit.

Cytogenetic Analysis of Repopulated Bone Marrow. At intervals of up to 1 year posttransplantation, femoral bone marrow was obtained from three recipient mice per sample time, and direct chromosome preparations were obtained from each animal. The donor origin of cells for analysis was confirmed by the presence of the Y chromosome, and chromosomal aberrations were scored from coded slides. The progeny of irradiated (40XY) and nonirradiated (40XYT6T6) cells were readily distinguished by the stable T6 reciprocal translocation (Fig. 1). The data from the three samples were pooled. Differences between the proportions of aberrant cells were analyzed by Fisher’s exact test, and significant differences between the various classes of aberration types were confirmed by a conditional binomial test.

Results and Discussion

Initial experiments carried out to determine whether densely ionizing neutron irradiation induces chromosomal instability in hematopoietic cells revealed that nonclonal chromosome aberrations were observed in clonal populations derived in vitro from neutron-irradiated bone marrow cells (Table 1). The frequency of cells with such aberrations (14.6%) was significantly greater than that seen in controls (3.2%; P = 0.00021). The aberrations were characterized by chromatid and chromosome breaks typical of induced chromosomal instability and qualitatively and quantitatively similar to the previously reported induction of instability by densely ionizing a-particles (3, 6). Clonal aberrations were not seen in any of the colonies, but this is not unexpected, given the number of colonies studied, and it highlights the general finding that the frequency of induction of instability is considerably greater than the frequency of stable chromosome translocations and deletions and mutations at specific loci induced by direct effects of ionizing radiation (2).

After transplantation with neutron-irradiated cells, for an inclusive period of 3–13 months, translocations and deletions were observed; however, in addition, there was a significantly greater frequency of unstable aberrations in the recipient bone marrow (P = 0.036) than in controls (Table 2). These findings clearly demonstrate that chromosomal instability persisted in the hemopoietic system after transplantation of irradiated stem cells. The hemopoietic stem cell compartment is very heterogeneous, and the proportion of stem cells responsible for the long-term reconstitution of hemopoiesis in the recipient mice is of the order of 10 per 10^5 of the viable cells present in the bone marrow (10); therefore, long-term repopulating stem cells were approximately 0.001% of the total cells transplanted. At the sampling times chosen, cells derived from the transplanted donor stem cells have reconstituted the hemopoietic system, and the recipients have steady-state hemopoiesis, in which, at any one time, all hemopoietic cells would be derived from 1–15 long-term repopulating stem cells (10–14). Thus, it is exceedingly unlikely that any cells examined were those present in the original irradiated population. Moreover, it is unlikely that the cells studied were the direct progeny of irradiated stem cells. Rather, they would be the descendants of stem cells that were themselves the progeny of the original transplanted long-term repopulating stem cells; the exact cell types cannot, of course, be distinguished in metaphase preparations. Thus, the transplantation study demonstrates the persistence of the instability phenotype in vivo in the descendants of the transplanted hemopoietic stem cells that had been neutron irradiated before transplantation.

It is important to note that using an in vitro system (Table 1), the frequency of cells expressing chromosomal instability is greater than that found in vivo (Table 2). Moreover, a small but significant number of cells express multiple aberrations in vitro, but such cells are rare in

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4 The abbreviation used is: CFU-S, spleen colony-forming unit.
vivo. These differences are also true for α-particle-induced chromosomal instability (3–5) and may be explained by the more effective recognition and removal of abnormal cells in vivo than in cell culture systems. Such differences highlight the importance of in vivo studies particularly when considering the potential health effects of inducible instability because mechanisms that have evolved to recognize and remove damaged cells in vivo would be expected to reduce the frequency of any pathological consequences relative to the high level of expression of instability.

After transplantation of a mixture of irradiated and nonirradiated bone marrow, chromosomal instability was demonstrated in 3.6% of the 40XY cells; i.e., in cells derived from transplanted, irradiated stem cells (Table 3). Thus, comparing the results with those in Table 2, the induction of chromosomal instability in 40XY cells was independent of the total number of 40XY cells irradiated because the frequency of cells with unstable aberrations was the same after irradiating 39% (3.6 ± 1.1) or 100% (3.2 ± 0.6) of the cells ($P = 0.96$). Furthermore, at all times posttransplantation, chromosomal instability was also demonstrated 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 (2.2%), although lower than that in the 40XY cells, was significantly greater than that in controls (0.38%) transplanted with nonirradiated 40XYT6T6 marrow ($P = 0.0054$).

Using a congenic system enabled us to have a syngeneic transplantation model in which cells derived from irradiated (40XY) or non-irradiated (40XYT6T6) donor CBA/H stem cells could be unequivocally identified; clearly, chromosomal instability in 40XYT6T6 cells (Table 3) cannot be explained by a direct transmission of instability from an irradiated stem cell to its descendants. The findings support a bystander mechanism in which the descendants of 40XY irradiated stem cells are able to induce instability in the 40XYT6T6 descendants of nonirradiated stem cells. Thus the data presented in Table 3 may reflect two distinct mechanisms for the induction of chromosomal instability: (a) a direct transmission of instability from an irradiated stem cell to its progeny; and (b) an indirect bystander mechanism. The demonstration of instability in cells derived from irradiated donor stem cells is, of course, not necessarily inconsistent with an indirect mechanism downstream of the irradiated stem cells, where a cell derived from an irradiated stem cell might induce instability in a bystander cell derived from a different irradiated stem cell.

Currently, the mechanisms underlying the induction and persistence of the various manifestation of radiation-induced genomic instability are not understood, and the induction of chromosomal instability in vivo by a bystander mechanism adds further complexity because the mechanism underlying bystander effects also is not understood. Because some studies of bystander effects implicate gap junction-mediated intercellular communication in transmitting bystander signals immediately after irradiation in vitro (15, 16), and others have implicated extracellular cytokine-like factors that are able to increase intracellular levels of reactive oxygen species in nonirradiated cells (17, 18), it is possible that more than one mechanism is involved and

### Table 1: Nonclonal aberrations characteristic of chromosomal instability in clonal progeny derived in vitro from short-term repopulating stem cells in CBA/H mouse bone marrow cell suspensions exposed to 0.5 Gy neutron irradiation

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>Colonies with aberrant cells</th>
<th>Aberrant cells/total cells scored (%)</th>
<th>Chromatid breaks</th>
<th>Chromosome fragments and minutes</th>
<th>Translocations and deletions (%)</th>
<th>Cells with more than one aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3/16</td>
<td>5/162 (3.1)</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Neutrons (0.5 Gy)</td>
<td>8/13</td>
<td>23/158 (14.6)</td>
<td>22</td>
<td>10</td>
<td>0</td>
<td>4 (2.5)</td>
</tr>
</tbody>
</table>

### Table 2: Cytogenetic aberrations in bone marrow cells derived from long-term repopulating stem cells in mice transplanted with CBA/H mouse bone marrow cell suspensions exposed to 0.5 Gy neutron irradiation demonstrate the persistence of chromosomal instability in vivo

<table>
<thead>
<tr>
<th>Time posttransplantation (months)</th>
<th>Aberrant cells/total cells scored (%)</th>
<th>Chromatid breaks, minutes, and chromosome fragments (%)</th>
<th>Translocations and deletions (%)</th>
<th>Cells with more than one aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 Gy neutron-irradiated donor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1/895 (0.1)</td>
<td>1 (0.1)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Control unirradiated donor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 3–13 months</td>
<td>1/895 (0.1)</td>
<td>1 (0.1)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3: Cytogenetic aberrations in bone marrow cells derived from long-term repopulating stem cells in mice transplanted with a mixture of 0.5 Gy neutron-irradiated (40XY) and nonirradiated (40XYT6T6) CBA/H bone marrow cells demonstrate the induction of chromosomal instability in the progeny of nonirradiated stem cells

<table>
<thead>
<tr>
<th>Time posttransplantation (months)</th>
<th>Total cells scored</th>
<th>Chromatid breaks, minutes, and chromosome fragments (%)</th>
<th>Translocations and deletions (%)</th>
<th>Cells with more than one aberration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total 61% unirradiated 40XYT6T6 and 39% irradiated 40XY cells in donor population</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>117</td>
<td>0/17 (0)</td>
<td>2/100 (2.0)</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>4.5</td>
<td>158</td>
<td>0/12 (0)</td>
<td>2/294 (2.1)</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>7.5</td>
<td>438</td>
<td>0/134 (3.7)</td>
<td>7/304 (2.3)</td>
<td>8/134 (6.0)</td>
</tr>
<tr>
<td>9</td>
<td>202</td>
<td>3/84 (3.6)</td>
<td>2/118 (1.7)</td>
<td>5/84 (6.0)</td>
</tr>
<tr>
<td>Total 67/894 (7.5)</td>
<td>1021</td>
<td>0/281 (3.6)</td>
<td>16/740 (2.2)</td>
<td>17/281 (6.0)</td>
</tr>
</tbody>
</table>

Cells with translocations demonstrating clonal expansion: a, clone 1-2/8 cells and clone 2-2/8 cells; b, clone 1-2/4 cells; c, clone 1-2/5 cells; d, clone 1-2/6 cells; e, clone 1-5/10 cells; f, clone 2-2/10 cells; g, clone 1-2/5 cells and clone 2-3/5 cells.

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that this mechanism may be a function of the cell type being studied. The demonstration of instability in single cell suspensions of primary hemopoietic cells by a bystander mechanism (6) would argue against a gap junction mechanism for the initiation of the process in these particular cell types, although we do not yet know whether the instability phenotype can be induced by medium obtained from irradiated cells. However, in hemopoietic cultures expressing induced genomic instability, increases in intracellular oxidants and oxidative DNA base damage have also been demonstrated (19). These various findings are consistent with oxidative processes contributing, at least in part, to the expression of instability in hemopoietic cells. Because similar oxidative effects have been demonstrated in cultures of X-ray-irradiated cells (19), and chromosomal instability can be demonstrated in the progeny of X-ray-irradiated hemopoietic cells, (20) there is no reason to assume that bystander-induced chromosomal instability is restricted to densely ionizing radiations. Thus, it is likely that a medium-transmitted mechanism similar to that described by Lehnert and colleagues (17, 18) for bystander-mediated short-term effects may be able to induce long-term chromosomal instability. The study was designed using different recipients at different time points to identify evidence for a bystander effect and not to follow the pathological consequences in the hemopoietic systems of a large cohort of individual animals. However, this first demonstration of a link between a bystander effect of ionizing radiation and inducible genomic instability in vivo clearly poses major challenges to widely held views concerning radiation-induced DNA damage and the mechanisms underlying health consequences of radiation exposures because current models for initiation of radiation-induced malignant transformation consider only lesions in irradiated cells.

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References

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