Genotype-Dependent Induction of Transmissible Chromosomal Instability by γ-Radiation and the Benzene Metabolite Hydroquinone

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Abstract

Although it is well established that ionizing radiation and benzene are epidemiologically linked to acute myeloid leukemia (AML), the underlying mechanisms are not understood. We have shown that γ-radiation can induce a persisting genomic instability in the clonal descendants of hemopoietic stem cells manifested as a high frequency of nonclonal chromosome and chromatid aberrations. A strikingly similar instability is shown after exposure to the benzene metabolite hydroquinone. The CBA/Ca but not the C57BL/6 genotype is susceptible to the induction of instability by both ionizing radiation and hydroquinone and exposure of CBA/Ca, but not C57BL/6, mice to either agent is known to be associated with the development of AML. The results are consistent with the proposal that chromosomal instability induced by either agent may contribute to AML development by increasing the number of genetic lesions in hemopoietic cells. Genotype-dependent chromosomal instability can be induced by hydroquinone doses that are not acutely stem cell toxic and this may have important implications for current assessment of safe levels of exposure to benzene as well as for mechanistic understanding of the hemotoxic and leukemogenic effects.

Introduction

Although benzene has long been known to be hemotoxic and leukemogenic from both epidemiologic studies and laboratory models, the underlying mechanisms remain obscure (1). Benzene is not a classic chemical carcinogen as there is little evidence for the production of highly electrophilic DNA-binding metabolites in vivo (2, 3). The hemopoietic effects are mediated through the generation of reactive oxygen species by its principle metabolite hydroquinone (4, 5), but neither benzene nor the majority of its metabolites are mutagenic in the Ames test (6). However, in addition to ionizing radiation, benzene is one of the few agents known to be associated with acute myeloid leukemia (AML). Exposure of CBA strains of mice to either radiation or benzene leads to the development of AML (7); although, whereas the CBA model of radiation-induced AML has found wide acceptance as a robust model, benzene-induced AML is less well established. In contrast, C57BL/6 mice exposed to radiation or benzene, like most mouse strains, develop various lymphoid malignancies (8, 9) and the majority of benzene toxicity studies have not been conducted with CBA strains.

Cytotoxic and genetic changes are readily shown in irradiated cells but there is now a substantial body of evidence that high frequencies of chromosome aberrations and gene mutations may be shown in unirradiated cells that are the progeny of cells exposed to ionizing radiation many cell divisions previously. These delayed effects are manifestations of radiation-induced genomic instability (10–12). The induction of radiation-induced chromosomal instability in hemopoietic cells from CBA strains of mice can be shown as nonclonal chromosome and chromatid-type aberrations in the clonal progeny of hemopoietic stem cells (13), but the relationship of inducible instability and AML induction is not known. We now report that both γ-irradiation and hydroquinone induce chromosomal instability in a genotype-dependent manner and that induction by hydroquinone can be by doses that are not acutely toxic to stem cells. The induction of this genotype-dependent phenotype by such disparate agents, both of which are implicated in the induction of AML together with the significant susceptibility of the CBA strain both to chromosomal instability and to AML supports the view that such instability may contribute to disease development.

Materials and Methods

Cells and treatments. Single cell suspensions of femoral bone marrow were obtained from 8- to 16-week-old CBA/Ca or C57BL/6 mice. All procedures were carried with local Ethical Committee approval and in accordance with Home Office project license PPL 60/2841. Cells (5 × 10^6/mL) were incubated with 0 to 600 μmol/L hydroquinone (Sigma-Aldrich, St. Louis, MO) hydroquinone at 37°C, washed twice, resuspended, and counted. Alternatively, the same number and concentration of cells were 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 leukemogenic dose for CBA strains of mice (7). Sham-treated controls were also obtained.

Cell assays. An in vitro clonogenic assay, operationally defined as the colony-forming units in agar (CFU-A) assay, was used to obtain clones of cells derived from members of the hemopoietic stem cell compartment as described previously (14). Cells were plated in 45-mm vented Petri dishes (Sterilin, Stone, United Kingdom) containing 2 mL modified alpha Eagles medium (Life Technologies, Gaithersburg, MD) supplemented with 25% pretested horse serum (Sera-Lab), 50 μg/mL streptomycin, 50 μU penicillin, 2 mmol/L l-glutamine, and 0.3% low melting point agarose (Life Technologies) and conditioned medium from the AFl1.19T and L929 cell lines. A batch test was performed to determine maximum colony-stimulating activity. Cultures were incubated for 11 to 12 days at 37°C in a fully humidified atmosphere of 5% CO2 in air to obtain mixed lineage colonies of ≥2-mm diameter. An in vivo clonogenic assay, operationally defined as the spleen CFU (CFU-S) assay that detects the same cell type as the in vitro CFU-A assay (14), was also used to obtain clones of cells derived from members of the hemopoietic stem cell compartment. Male cells were resuspended at 2.5 × 10^6 cells/mL and 0.2-mL aliquots injected into the dorsal tail vein of each of six to eight female recipients conditioned with 9-Gy γ-radiation. On days 10 to 11, spleens were...
obtained, individual colonies dissected out, and cell suspensions obtained for cytogenetic analyses. In addition to confirming the presence of Y chromosome in scored metaphases, each colony was checked for evidence of donor origin using a PCR-based technique. A small aliquot of each colony (~50,000 cells) was added to two volumes of NSD buffer [10 mmol/L Tris-HCl (pH 8.0), 0.5 mmol/L EDTA (pH 8.0), 1% N-lauryl sarcosylyl], vortexed, and kept on ice for 10 minutes before proteinase K (5 μg/mL overnight at 42°C) and RNAse A treatment (100 μg/mL for 1 hour at 37°C). DNA was extracted using phenol/chloroform and was precipitated overnight at −20°C in 0.3 mol/L sodium acetate, 65% ethanol. Samples were spun at 31,500 rpm (43,000 × g) for 1 hour at 4°C, washed once with 70% ethanol and spun for a further 20 minutes at 4°C before air drying and redissolving the pellet in 10 μL TE 1/0.1 pH 8.0. PCR reactions were done on a Hybaid Omni thermal cycler in 25-μL reaction volumes using the primers YF, CCTATTGCATGGACTG-CAGCCTTA and YB, GACTAGACATGTCTTTACATCTG (Sigma Genosys, The Woodlands, TX) at 0.5 μmol/L using 1.25 units HotStarTaq (Qiagen, Chatsworth, CA), 1.5 mmol/L MgCl2, and 200 μmol/L deoxynucleotide triphosphate’s. Following a 15-minute cycle at 95°C, 30 cycles of 1-minute segments with the following reaction conditions were done: 95°C, 60°C, and 72°C. A product of ~200 bp was visualized by UV light on a 3% agarose gel using ethidium bromide. Positive and negative controls plus blank samples were included in each run.

Cytogenetic analyses. Cells to which Colcemid had been added at final concentration of 0.02 μg/mL were incubated for one hour at 37°C. The cells were resuspended in 5 mL hypotonic potassium chloride (0.5%) for 30 minutes before adding 2 mL sodium citrate/potassium chloride and incubating for a further 9 minutes. Two to 3 mL of fixative (methanol/acetic acid, 3:1) was added and the sample inverted once. After 10 to 15 minutes, the cells were resuspended in fixative. At least two further washes in fixative were done before the cells were dropped onto precleaned glass slides. The slides were air dried and aged for 14 days before stained with Giemsa (diluted 1:4 with distilled water and filtered) for 15 to 20 minutes. Coded samples were scored for karyotypic abnormalities and at the completion of scoring, treatments were assigned to the coded data and differences between the proportions of aberrant cells at each harvesting point were analyzed by the Fisher’s exact test.

Results

Hemotoxicity of hydroquinone. In a preliminary study, femoral bone marrow from CBA/Ca mice was incubated with hydroquinone at concentrations of 0, 200, and 600 μmol/L and samples taken at 60, 120, and 240 minutes. Two hundred cells were examined for trypan blue exclusion in each group at each time point and those cells that took up dye were regarded as dead or dying cells. The number of such cells increased with time and dose with higher doses resulting in a more rapid cell death. Exposure to 200 μmol/L hydroquinone resulted in 70%, 40%, and 23% survival at 60, 120, and 240 minutes, respectively. Increasing the dose to 600 μmol/L decreased these survivals to 40%, 25%, and 28%, respectively. These data provide an indication of overall toxicity for total bone marrow where most of the cells are differentiating/differentiated effector cells. Thus, additional studies using functional assays of primitive multipotential clonogenic stem cells, as more relevant target cells, were undertaken. Using an in vitro clonogenic (CFU-A) assay, the fractions surviving 2-hour exposures to various hydroquinone doses were determined and shown in Fig. 1. There was no significant toxicity at 10 μmol/L but doses >200 μmol/L resulted in <30% survival (Fig. 1). Accordingly, cytogenetic studies were conducted using bone marrow exposed to 10 and 300 μmol/L hydroquinone.

Cytogenetic analysis. Using an in vivo clonogenic (CFU-S) transplantation assay, stem cells exposed in vitro were stimulated to proliferate and differentiate in vivo for 10 to 11 days and donor-derived clones (confirmed by PCR analysis) of ~10^6 cells were processed for cytogenetic studies; the results are summarized in Table 1. In the in vivo colonies derived from sham-treated control CBA/Ca bone marrow, the frequency of cells with unstable aberrations (chromatid breaks, chromosome fragments and minutes) was ~3%. In colonies initiated from cell exposed to 10 or 300 μmol/L hydroquinone, the incidence of unstable aberrations was significantly increased relative to the control group (8.5%, P = 6 × 10^-5 and 7.0%, P = 1.6 × 10^-5, respectively). However, there was no evidence of dose dependency as there was no significant difference between the level of instability after 10 μmol/L treatment and after 300 μmol/L treatment (8.5% and 7.0%, P = 0.2045).

In colonies derived from sham-treated control C57BL/6 bone marrow, the frequency of cells with unstable aberrations was ~2%; not significantly different from CBA/Ca controls (P = 0.1361). In colonies initiated from cells exposed to 10 or 300 μmol/L hydroquinone, the incidence of unstable aberrations was not significantly increased relative to controls (3.0%, P = 0.8912 and 1.3%, P = 0.2858, respectively).

Colonies derived from γ-irradiated clonogenic cells of both genotypes exhibited levels of instability greater than their respective controls (for CBA/Ca cells, 12.9% versus 3.1%, P < 10^-7; and for C57BL/6 cells, 4.4% versus 1.9%, P = 0.0127). However, the level of instability in colonies derived from irradiated C57BL/6 clonogenic cells was significantly lower (P = 7.0 × 10^-4) than in the corresponding CBA/Ca-derived colonies. Comparing the expression of unstable aberrations in colonies derived from hydroquinone-treated cells of the two genotypes, it is evident that the CBA/Ca-derived colonies exhibited a greater percentage of cells with aberrations than in the corresponding C57BL/6-derived colonies. For the 10 μmol/L treatments, 8.5% of CBA/Ca and 3.0% of C57BL/6 cells (P = 1.16 × 10^-3) and for the 300 mmol/L treatments, 7.6% of CBA/Ca cells and 1.3% of C57BL/6 cells (P = 4.0 × 10^-4).

Discussion

This study has shown a high frequency of de novo chromosome aberrations in cells that were not themselves exposed to hydroquinone but in the progeny of clonogenic stem cells exposed many cell populations previously. This phenotype is directly comparable to
that of radiation-induced chromosomal instability, an untargeted effect of ionizing radiation in which aberrations that are readily and expectedly observed in cells at the first division post-irradiation are unexpectedly shown as nonclonal abnormalities in the clonal descendants of irradiated cells many cell divisions after exposure (12). No clonal aberrations were recorded after hydroquinone exposure, but this is not unexpected given the relatively small number of colonies studied and it is consistent with previous observations that the frequency of induction of instability in hemopoietic cells of a susceptible genotype is considerably greater than the frequency of induction of stable clonal aberrations (10, 11). The cytogenetic results have another significant implication, in that instability induction per treated cell (i.e., per initial cell at risk) is not dose dependent. This can be seen within the context of clonogenic cell survival (Fig. 1). For example, the instability induced by a highly toxic dose of 300 μmol/L hydroquinone (10% survival) is not significantly greater than that induced by a dose of 10 μmol/L that has no significant acute toxicity (Table 1). Consequently, low-dose exposure that has relatively little acute toxicity may, nevertheless, have important long-term biological and potentially clinical consequences. However, an additional implication of this study is that the expression of instability is strongly influenced by genetic factors; the CBA/Ca genotype may be regarded as highly susceptible and the C57BL/6 genotype as relatively resistant. Interestingly, bladder epithelium obtained from these same mouse strains irradiated in vitro, reflected the range of responses shown by similarly treated samples of human urothelium (15). These laboratory studies clearly show the importance of genetic influences on the expression of the phenotype and raise the important issue of genetic predisposition in the human population.

Currently, the mechanism of induction and perpetuation of radiation-induced genomic instability is not understood but is thought to be driven by epigenetic factors that secondarily produce genetic lesions. There is evidence that the expression of the phenotype involves intercellular communication (16, 17) and a number of studies have pointed to an association between radiation-induced chromosomal instability and free radical-mediated processes (18–20). Previous studies in vivo had indicated that macrophages in the murine hematopoietic system become activated after irradiation and produce excess levels of reactive oxygen and nitrogen species and it was proposed that the production of reactive species by macrophages in the longer term after irradiation might be involved in nontargeted and delayed effects of irradiation as part of an ongoing tissue response to radiation injury (21). Previous studies have implicated increased free radical activity in bone marrow as contributing, at least in part, to the toxic and leukemogenic effects of benzene (22, 23). The bone marrow of mice treated with benzene produced 50% more hydrogen peroxide in response to the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate than did cells from control animals (24) suggesting that phagocyte activation may contribute to hydroquinone-induced effects and that ionizing radiation and benzene may share common indirect mechanisms of micro-environmentally mediated secondary cell damage.

This study has shown that chromosomal instability can be a high-frequency delayed consequence of exposure to both ionizing radiation and hydroquinone with the same genotype dependency. Previous studies, using CBA/Ca mice have implicated genetic instability as a factor in benzene- and radiation-induced leukemia (25) and the present study clearly shows that after either exposure, the clonal descendants of stem cells are genetically unstable in susceptible CBA/Ca mice but not in resistant C57BL/6 mice. The induction of this phenotype by such disparate agents, both of which are implicated in the induction of AML, sometimes years after the exposure, and the significant susceptibility of the CBA strain to both chromosomal instability and AML add weight to the possible biological importance of such instability in disease development. It should be noted, however, that many AMLs in humans show normal karyotypes; thus, instability is not a universal finding and this may well reflect different responses to different leukemogenic insults. In addition, low-penetration susceptibility and resistance loci and target stem cells numbers are factors that also impinge on the relative risk for radiation-induced AML in mice (26). In this study, the nonclonal aberrations seen in the clonal progeny of stem cells have the characteristics of spontaneous damage. This implies that induced instability would lead to a greater number of “spontaneous” mutations in the progeny of hemopoietic stem cells put under proliferative stress. Thus, inducible chromosomal instability may

Table 1. Nonclonal aberrations in stem cell-derived colonies derived from hydroquinone-exposed or γ-irradiated bone marrow assayed in triplicate using the in vivo CFU-S assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. clones</th>
<th>No. cells with unstable aberrations</th>
<th>% Cells with unstable aberrations (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/Ca cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>31</td>
<td>21/685</td>
<td>3.1 (1.8-4.4)</td>
<td></td>
</tr>
<tr>
<td>10 μmol/L hydroquinone</td>
<td>32</td>
<td>64/752</td>
<td>8.5 (6.5-10.5)</td>
<td>6 × 10⁻⁶</td>
</tr>
<tr>
<td>300 μmol/L hydroquinone</td>
<td>23</td>
<td>33/470</td>
<td>7.0 (4.7-9.3)</td>
<td>1.6 × 10⁻³</td>
</tr>
<tr>
<td>4 Gy γ-irradiation</td>
<td>17</td>
<td>39/302</td>
<td>12.9 (9.1-16.7)</td>
<td>&lt;10⁻⁷</td>
</tr>
<tr>
<td>C57BL/6 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>12</td>
<td>11/571</td>
<td>1.9 (0.8-3.1)</td>
<td></td>
</tr>
<tr>
<td>10 μmol/L hydroquinone</td>
<td>7</td>
<td>8/264</td>
<td>3.0 (1.0-5.1)</td>
<td>0.8912</td>
</tr>
<tr>
<td>300 μmol/L hydroquinone</td>
<td>10</td>
<td>6/469</td>
<td>1.3 (0.3-2.3)</td>
<td>0.2858</td>
</tr>
<tr>
<td>4 Gy γ-irradiation</td>
<td>14</td>
<td>25/548</td>
<td>4.4 (2.7-6.1)</td>
<td>0.0127</td>
</tr>
</tbody>
</table>

NOTE: Differences between exposed and control groups were analyzed by the Fisher’s exact test.
be a permissive event in the development of AML rather than directly causative. The induction of delayed chromosomal instability by hydroquinone doses that are not acutely stem cell toxic may have important implications for current assessment of safe levels of exposure to benzene as well as for mechanistic understanding the hemotoxic and leukemogenic effects.

References


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