Characterization of a Novel Epigenetic Effect of Ionizing Radiation: The Death-Inducing Effect

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ABSTRACT

The detrimental effects associated with exposure to ionizing radiation have long been thought to result from the direct targeting of the nucleus leading to DNA damage; however, the emergence of concepts such as radiation-induced genomic instability and bystander effects have challenged this dogma. After cellular exposure to ionizing radiation, we have isolated a number of clones of Chinese hamster-human hybrid GM10115 cells that demonstrate genomic instability as measured by chromosomal destabilization. These clones show dynamic and persistent generation of chromosomal rearrangements multiple generations after the original insult. We hypothesize that these unstable clones maintain this delayed instability phenotype by secreting factors into the culture medium. To test this hypothesis we transferred filtered medium from unstable cells to unirradiated GM10115 cells. No GM10115 cells were able to survive this medium. This phenomenon by which GM10115 cells die when cultured in medium from chromosomally unstable GM10115 clones is the death-inducing effect. Medium transfer experiments indicate that a factor or factors is/are secreted by unstable cells within 8 h of growth in fresh medium and result in cell killing within 24 h. These factors are stable at ambient temperature but do not survive heating or freezing, and are biologically active when diluted with fresh medium. We present the initial description and characterization of the death-inducing effect. This novel epigenetic effect of radiation has implications for radiation risk assessment and for health risks associated with radiation exposure.

INTRODUCTION

The biological effects of radiation were thought to be exclusively because of deposition of energy in the cell nucleus. However, over the years, a number of epigenetic or nontargeted effects of radiation exposure have been described including genomic instability and BSEs. Radiation-induced genomic instability manifests as reduced clonogenic survival, chromosomal aberrations, and/or gene mutations in the progeny of irradiated cells multiple generations after irradiation (reviewed in Refs. 1–4). Although the mechanism for induction of genomic instability is unclear, it appears to involve interactions among oxidative stress-induced free radicals, cell signal transduction pathways, and various epigenetic factors. The bystander family of effects occur in cells that were themselves not irradiated but were either in the vicinity of irradiated cells or were cultured in medium from irradiated cells (5–9). BSEs include reduced plating efficiency (8, 10), increased sister chromosome exchanges (11), micronuclei formation (12, 13) and altered gene expression (14, 15). A number of studies (15, 16) suggest that cell-cell communication plays a role in mediating this phenomenon; however, there is also evidence for a cell contact-independent BSE mediated by secreted diffusible factors (12). Furthermore, medium transfer experiments whereby growth medium from irradiated cells was transferred to nonirradiated cells reduced clonogenic survival significantly in the unirradiated cells (6, 17, 18). It is hypothesized that irradiated cells release factor(s) into their growth medium that can induce the mobilization of intracellular calcium (19), the loss of mitochondrial membrane potential, and an increase in reactive oxygen species that ultimately leads to reduction in cell survival by apoptosis (17, 20).

We described previously radiation-induced genomic instability in human-hamster cell line GM10115 exposed to iron ions or X-rays (21–23). This instability manifests as the dynamic production of subpopulations of cells with unique cytogenetic rearrangements. Our initial rationale for this study was to test the hypothesis that unstable cells perpetuate instability by secreting factors into the culture medium, thus driving the delayed production of chromosomal rearrangements. We report here instead, the identification of a novel epigenetic effect of radiation. Medium from two unstable GM10115 clones, Fe-10-3 and LS12 was cytotoxic to parental GM10115. These clones were expanded from an individual cell surviving exposure to ionizing radiation, but the clones had not themselves been irradiated. This cell-death-inducing phenomenon was termed the DIE and appears to be mediated by the extracellular secretion of DIE factor(s) into the culture medium by these chromosomally unstable clones.

MATERIALS AND METHODS

Cell Culture. GM10115 cells (Human Genetic Mutant Cell Repository, Camden, NJ) contain one copy of human chromosome 4 in a background of 20–24 Chinese hamster ovary chromosomes. Cells were grown as monolayers in DMEM containing 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.2 mM l-proline. All of the cultures were grown at 34°C in humidified incubators containing 5% CO2. Cells were routinely monitored for Mycoplasma (Mycoplasma Detection kit; Molecular Probes, Eugene, OR) and show no evidence of infection.

Unstable and stable clones were isolated from parental GM10115 cells surviving exposure to ionizing radiation as described previously (21–23). Chromosomally unstable clones were defined as those having at least three distinct aberrant metaphase subpopulations involving rearrangements of human chromosome 4, of which the total number constitutes ≥5% of the 200 metaphases scored. Clones having <3 rearrangements were considered chromosomally stable. Three unstable (Fe-10-3, LS12, and BNL) clones and one clone that survived irradiation but was cytogenetically stable (RT210B) were investigated. Fe-10-3 and BNL unstable clones were isolated after exposure to 10 Gy and 7.5 Gy, respectively, of iron ions from the alternating gradient synchrotron facility at Brookhaven National Laboratory, Upton, NY. Fe-10-3 and BNL clones demonstrated 18 and 9 unique cytogenetic subpopulations of cells, respectively, having chromosomal rearrangements involving the human chromosome 4. Clones LS12 and RT210B were isolated after exposure to 10 Gy of X-rays. LS12 demonstrated 13 subpopulations having unique rearrangements of chromosome 4, whereas RT210B is stable, having only 2 subpopulations each with a single reciprocal translocation. Maintenance of the stable or unstable phenotype of all of the clones was monitored regularly using fluorescence in situ hybridization (23).

Medium Transfer Experiments and the BSE. Potential BSEs in GM10115 cells were investigated by medium transfer experiments as de-
GM10115 cells were irradiated with 5 Gy X-rays (Coronado Westinghouse; 0.5 Cu +1 Al source, dose rate of 3.1 Gy/min), and 2 h later the medium was removed, filtered (0.2 μm cellulose acetate filter; Corning, Corning, NY) and transferred to 60-mm 2 dishes containing 50 parental GM10115 cells. Irradiated medium and medium from unirradiated cells were used as a control. To investigate whether the number of cells influenced the effect, experiments were carried out irradiating 1 × 10^4, 1 × 10^5, 1 × 10^6, 5 × 10^5, or 1 × 10^6 GM10115 cells, and 2 h later transferring filtered medium to parental cells. In addition, cells were exposed to 1, 2, 4, 6, 8, 10, or 12 Gy of X-rays, and 2 h later, filtered medium transferred to parental cells to determine whether increasing the radiation dose increased the BSE.

Medium Transfer Experiments and the DIE. Control GM10115 cells, stable RT210B cells, and unstable clones Fe-10-3, LS12, and BNL were grown to 90% confluence. Fresh medium was added and cells cultured for 48 h (“48-h medium”). After 48 h, medium was removed, filtered, and transferred to dishes of GM10115 cells plated 2–24 h previously. Cells were grown for 10–14 days for colony formation and stained with 2% crystal violet in 40% methanol. Plating efficiency was calculated as the ratio of the number of cells plated to number of colonies obtained. Controls included filtered medium from stable GM10115 cells, medium from irradiated but chromosomally stable RT210B cells, and fresh medium irradiated in the absence of cells. All of the experiments were carried out in triplicate, and results are expressed as the average of three independent experiments with SE.

Kinetics of secretion and cell killing by the DIE factor were analyzed by time course medium transfer experiments. To determine kinetics of secretion of the factor causing cell killing, Fe-10-3 and LS12 cells were cultured in fresh medium for 2, 4, 8, 24, or 48 h after which time the medium was filtered and transferred to stable GM10115 cells. Cells were cultured in this medium for 10–14 days for colony formation. To determine the length of exposure to DIE factor(s) required for cytotoxicity, 48-h medium from Fe-10-3 and LS12 cells was filtered and transferred to GM10115 cells for 2, 4, 8, 24, or 48 h. After incubation in 48-h medium for the appropriate time, fresh medium was added, and cells were incubated for 10–14 days for colony formation.

Titation of the DIE factor was performed by diluting 48-h medium from Fe-10-3 or LS12 with either fresh medium or medium from unirradiated GM10115 cells (conditioned medium).

Potential temperature sensitivity of the DIE factor was determined by heating 48-h medium from Fe-10-3 or LS12 to 56°C in a water bath for 1 h. After heating, medium was cooled to 37°C and supplemented with 10% fetal bovine serum. Fe-10-3 or LS12 48-h medium was also cooled to 4°C in the refrigerator or frozen at −20°C overnight. These media were transferred to stable cells, and cells were incubated for 10–14 days for colony formation.

RESULTS

Chromosomal Instability

Radiation-induced genomic instability in GM10115 cells is determined cytogenetically and regularly monitored in all of the cell clones. Because of the induced instability the number and type of rearrangements observed are consistently different over time in culture. No rearrangement is common between the two unstable clones. Representative metaphase spreads demonstrating chromosomal instability in an unstable clone are presented in Fig. 1.

Medium Transfer Experiments and the BSE

X irradiation and subsequent medium transfer failed to reveal a BSE in GM10115 cells using reduced plating efficiency as the end point as reported by others using a different cell system (6, 9, 10, 17, 18, 20). The absence of a BSE was not changed by increasing the number of cells irradiated (Fig. 2A), increasing the dose of X-rays used (Fig. 2B) or altering the time between irradiation and medium transfer (Fig. 2C). Likewise, no decrease in plating efficiency was observed when medium irradiated in the absence of cells was transferred to GM10115 cells (data not shown).

Medium Transfer Experiments and the DIE

Transfer experiments using medium from the unstable clone BNL resulted in a reduction in plating efficiency of GM10115 cells (PE = 0.63; Fig. 3A). However, 48-h medium from Fe-10-3 or LS12 cells was completely cytotoxic to parental GM10115 cells (Fig. 3A). We termed this cell-killing phenomenon the DIE. Medium from irradiated but chromosomally stable RT210B cells or parental GM10115 did not cause a reduction in plating efficiency. In fact, colonies cultured in medium from parental GM10115 cells were generally larger and more robust, reflecting a “conditioned medium" response (Fig. 3B). Conditioned medium from GM10115 cells was used as a control in all of the subsequent experiments. No pH change was observed in medium from unstable cells relative to conditioned medium from stable cells. Increasing the number of GM10115 cells exposed to medium from unstable clones up to 1 × 10^6 did eventually

Fig. 1. Fluorescence in situ hybridization using a biotinylated probe against human chromosome 4. A, parental GM10115 metaphase chromosomes; B–F, metaphase chromosomes from an unstable clone showing the multiple subpopulations of cells with unique rearrangements involving human chromosome 4 that characterize radiation-induced chromosomal instability in GM10115 cells.
yield surviving colonies, but plating efficiency was never restored (Table 1).

Characterization of DIE

Kinetics of Secretion of the DIE Factor. To determine the kinetics of secretion of factors that led to DIE, Fe-10-3 and LS12 cells were cultured in fresh medium for different time periods after which the medium was filtered and transferred to GM10115 cells. GM10115 cells exposed to 8-h medium from Fe-10-3 have a reduced plating efficiency (PE = 0.5), and by 48 h no GM10115 cells survived (Fig. 4). GM10115 cells to LS12 medium for 8 h yields a plating efficiency of 15%, and 24-h exposure is completely cytotoxic (Fig. 4). These data indicate that exposure of GM10115 cells to LS12 or Fe-10-3 medium caused cell death by 24 or 48 h, respectively, and medium from Fe-10-3 cells requires a longer period of time to effect total cell killing.

Titration of the DIE. To determine the cytotoxicity of DIE factor when diluted, medium from Fe-10-3 and LS12 cells was titrated with fresh or conditioned medium. When diluted with fresh medium as little as 1% of medium from either unstable cell clone resulted in 100% cytotoxicity (Fig. 6). However, when medium from either

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Table 1  Effect of increased cell numbers on DIE

<table>
<thead>
<tr>
<th>Number of cells plated</th>
<th>Fresh medium</th>
<th>Fe-10-3 medium</th>
<th>LS12 medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^2</td>
<td>0.96</td>
<td>0.004</td>
<td>0</td>
</tr>
<tr>
<td>1 x 10^3</td>
<td>Innumerable^a</td>
<td>0.001</td>
<td>0.0003</td>
</tr>
<tr>
<td>2 x 10^3</td>
<td>Innumerable</td>
<td>0</td>
<td>0.0003</td>
</tr>
<tr>
<td>5 x 10^3</td>
<td>Innumerable^a</td>
<td>0.0015</td>
<td>0.000006</td>
</tr>
<tr>
<td>1 x 10^4</td>
<td>Innumerable^b</td>
<td>0.0011</td>
<td>0.0003</td>
</tr>
<tr>
<td>2.5 x 10^4</td>
<td>Monolayer^b</td>
<td>0.0019</td>
<td>0.0007</td>
</tr>
<tr>
<td>5 x 10^4</td>
<td>Monolayer</td>
<td>0.0023</td>
<td>0.0008</td>
</tr>
<tr>
<td>7.5 x 10^4</td>
<td>Monolayer</td>
<td>0.0016</td>
<td>0.00018</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>Monolayer</td>
<td>0.00038</td>
<td>0.0009</td>
</tr>
<tr>
<td>1 x 10^6</td>
<td>Monolayer</td>
<td>0.00055</td>
<td>0.00032</td>
</tr>
</tbody>
</table>

^a Too many colonies to count.
^b After 10–14 days in culture GM10115 cells formed a monolayer in the culture vessel.
conditioned medium; bars, LS12; factor(s) from stable cells that inactivate the DIE. cell rescue is because of secretion of cell survival signals or anti-DIE medium is able to rescue cells. It remains to be determined whether the DIE factor is able to induce cell death, whereas conditioned these data suggest that in fresh medium, even at low concentrations, unstable clone was diluted with conditioned medium from GM10115 cells, 25% of conditioned medium was able to inhibit DIE (Fig. 6). medium from Fe-10-3 and LS12, bars, ±SE. Temperature Sensitivity of the DIE Factor. Heating or cooling the medium inhibits DIE. However, DIE factors maintain activity at 34°C or ambient temperature up to 96 h (Table 2).

DISCUSSION

The initial rationale for transferring medium from unstable Fe-10-3 or LS12 clones to parental GM10115 cells was to test the hypothesis that radiation-induced chromosomally unstable clones produce factor(s) that stimulate and/or perpetuate instability in parental cells. Instead of stimulating the production of novel chromosome changes, this medium was completely cytotoxic to parental, nonirradiated GM10115 cells. Interestingly, LS2 or Fe-10-3 cells when cultured in their own medium do not exhibit DIE (Fig. 3A). Furthermore, culturing one unstable clone in medium from the other reduced PE but did not completely eliminate cell survival, indicating that unstable clones are refractory to the cell-killing factors produced. Consequently, it remains a reasonable hypothesis that factors produced by unstable cells may drive the dynamic production of chromosomal rearrangements that characterize the instability phenotype. The possibility that the DIE is because of altered gene expression of a gene on human chromosome 4 as a result of chromosomal rearrangements cannot be excluded at this time. In addition not all of the cells are equally efficient at inducing DIE. Medium from BNL cells will reduce PE in parental GM10115 cells but was never completely cytotoxic.

Because GM10115 cells that had never been irradiated were either unable to produce a BSE signal or unable to respond to bystander factors, DIE is a novel and distinct effect of ionizing radiation. DIE is likely because of secretion of a factor, or factors, by unstable Fe-10-3 and LS12 cells, and the release of this factor(s) into the medium is toxic to parental GM10115 cells.

The precedence for epigenetic effects after cellular exposure to ionizing radiation includes extensive literature on BSE and radiation-induced clastogenic factors. Evidence has accumulated suggesting that irradiated cells may secrete BSE factor(s) that can affect unirradiated cells present in the same radiation environment (5, 8, 12, 15, 16), and that transfer of medium from irradiated cells can affect unirradiated cells (6, 17, 18). These BSEs appear to be mediated by both cell-cell gap junction communication (15, 16) and transmissible soluble factors (12, 17, 18). Our study complements these reports and demonstrates that clones exhibiting radiation-induced genomic instability are capable of secreting cytotoxic factors many generations after irradiation that can affect unirradiated cells. Because only medium is transferred from unstable to stable cells, this suggests that DIE is cell contact-independent.

Other nontargeted effects of radiation exposure apparently involving transmissible factors are “clastogenic factors.” Clastogenic factors are found in plasma of some irradiated individuals and cause chromosomal damage in unirradiated cells (24–27). It was hypothesized that a “chromosome breakage factor” was present in plasma of irradiated individuals that could “mimic the direct effect of X-rays on chromosomes” (25). Chromosome aberrations in unirradiated cells were also induced by plasma from rats exposed to whole-body irradiation (24), atomic bomb survivors from Hiroshima (28), and salvage
personnel exposed during cleanup after Chernobyl (29). These data support the concept that extracellular radiation-induced DNA-damaging factor(s) may persist for prolonged periods after exposure to ionizing radiation (30).

To date, no extracellular factor responsible for the induction of genomic instability, BSEs, or clastogenic factors has been identified. Studies implicate transforming growth factor β and interleukin 8 in the production of α-particle-mediated reactive oxygen species production, and support the hypothesis that cytokines may play a key role in the generation of ROS, which in turn may stimulate the production of BSE and genomic instability (31–34). Additionally, ginko biloba extract and antioxidant plant phenols have been reported to have anticlastogenic activity (35, 36). Although it is not certain whether genomic instability, BSEs, or clastogenic effects are manifestations of the same phenomenon, there is speculation that the bystander factor(s), or signal, may be involved in the induction of genomic instability (20, 37).

BSE, clastogenic factors, and DIE are non-targeted effects of radiation seemingly mediated by extracellular release of factor(s) that are able to act on unirradiated cells and elicit a response, including cell death. It remains to be determined what overlap, if any, exists among secreted factors involved in DIE, BSE, clastogenic factors, and genomic instability, and whether they are distinct phenomena or are manifestations of a similar process. Whatever the process, these effects associated with radiation exposure indicate that detrimental effects can manifest outside the irradiated area. Radiation can cause cancer, and is widely used to treat and cure cancer. These epigenetic effects suggest that potential health risks associated with radiation exposure may be greater than originally thought and ultimately impact on human radiation risk assessment.

REFERENCES

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