Radiation Sensitivity, H2AX Phosphorylation, and Kinetics of Repair of DNA Strand Breaks in Irradiated Cervical Cancer Cell Lines

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ABSTRACT

Six human cervical cancer cell lines [five human papillomavirus (HPV) positive, one HPV negative] for induction and rejoining of DNA strand breaks and for kinetics of formation and loss of serine 139 phosphorylated histone H2AX (γH2AX). X-rays induced the same level of DNA breakage for all cell lines. By 8 hours after 20 Gy, <2% of the initial single-strand breaks remained and no double-strand breaks could be detected. In contrast, 24 hours after irradiation, γH2AX representing up to 30% of the initial signal still present. SW756 cells showed almost four times higher background levels of γH2AX and no residual γH2AX compared with the most radiosensitive HPV-negative C33A cells that showed the lowest background and retained 30% of the maximum level of γH2AX. Radiation sensitivity, measured as clonogenic-surviving fraction after 2 Gy, was correlated with the fraction of γH2AX remaining 24 hours after irradiation. A substantial correlation with γH2AX loss half-time measured over the first 4 hours was seen only when cervical cell lines were included in a larger series of p53-deficient cell lines. Interestingly, p53 wild-type cell lines consistently showed faster γH2AX loss half-times than p53-deficient cell lines. We conclude that cell line-dependent differences in loss of γH2AX after irradiation are related in part to intrinsic radiosensitivity. The possibility that the presence of γH2AX foci may not always signify the presence of a physical break, notably in some tumor cell lines, is also supported by these results.

INTRODUCTION

Phosphorylation of histone H2AX occurs in response to DNA double-strand breaks produced by ionizing radiation and a variety of genotoxic drugs (1, 2). Histone phosphorylation is rapid and extensive, covering large regions of the chromosome adjacent to each break and producing foci that can be visualized microscopically after antibody labeling (3, 4). Although direct double-strand breaks are most efficient in inducing histone phosphorylation, intermediates in repair of damage and double-strand breaks produced during replication may also cause H2AX phosphorylation (5–7). Foci continue to grow for about 1 hour after exposure to X-rays and then disappear slowly over time, consistent with rejoining of DNA breaks but with slower kinetics. Phosphorylation of H2AX is proposed to concentrate repair factors at sites of DNA damage (8). Absence of H2AX is associated with genomic instability and radiation sensitivity (9), and mice deficient for both p53 and H2AX rapidly develop tumors (10). The ability to use serine-139 phosphorylated histone H2AX (γH2AX) foci to locate one double-strand break per nucleus has introduced new possibilities for low-level DNA damage detection, analysis of repair enzyme recruitment, and development of predictive assays for tumor response.

The rate of loss of foci and the presence of residual foci has been correlated with cellular radiosensitivity (11, 12). Using 10 unrelated tumor and normal cultured cell lines, we previously observed a correlation between γH2AX loss in the first few hours following irradiation and relative sensitivity to killing by ionizing radiation (11). To determine whether the correlation might be improved, a series of cell lines of the same type was used, namely six human cervical carcinoma cell lines. Two of these cell lines, Caski and SW756, showed very high background levels of γH2AX foci, and substantial amounts of residual γH2AX were present in most of the cell lines 24 hours after irradiation. To determine whether foci were indicative of unrepaired strand breaks, the comet assay was used to examine these cell lines for induction and rejoining of DNA strand breaks.

MATERIALS AND METHODS

Cell Lines. Cervical cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained as exponentially growing monolayers in MEM containing 10% fetal bovine serum. A standard clonogenic assay was used to measure plating efficiency and cell survival after irradiation by plating approximately 600 surviving cells in 10-cm dishes containing MEM plus 10% fetal bovine serum and allowing colonies to form for approximately 2 weeks before staining and counting. Results from three independent experiments are shown.

γH2AX loss half-time and clonogenicity were also measured for a series of human tumor and normal cell lines, some of which have been reported previously (11). These include WiDr human colon carcinoma, A549 lung carcinoma, Du145 prostate carcinoma, HT144 melanoma, WIL2NS lymphoblastoid, U87 glioma, and HCC1937 breast cancer cell lines. In addition to these cell lines, M059J and M059K glioma cells were obtained from American Type Culture Collection. Cell lines with wild-type p53 included A549 and U87 cells from American Type Culture Collection, TK6 cells from Dr. Helen Evans, and two normal primary skin fibroblast cell lines established in our lab. A p53-positive fibroblast line deficient in Ataxia Telangiectasia Mutated (ATM) (AT3BI) and BR3 normal fibroblasts were obtained from Dr. Colin Arlett. Primary human umbilical endothelial cells were obtained from Dr. Aly Karsan.

Irradiation. Cells were irradiated in suspension in glass spinner culture vessels containing MEM plus 10% fetal bovine serum. Irradiation was performed with a 250 kV X-ray unit at a dose rate of 4.7 Gy/minute. Samples were obtained at the indicated recovery times after irradiation (on ice), fixed in 70% ethanol and stored at −20°C until analysis for γH2AX. Alternatively, comet assay was used to examine samples immediately for strand breaks.

Flow Cytometry and Immunohistochemistry for γH2AX. Staining for γH2AX was conducted as described previously (7). Briefly, fixed cells were rehydrated for 10 minutes, centrifuged, and resuspended in 200 μL mouse monoclonal anti-phospho-histone H2AX antibody (1:500 dilution, Upstate Biotechnology Inc., Waltham, MA). Cells were incubated for 2 hours at room temperature, rinsed, and resuspended in 200 μL secondary antibody, Alexa 488 goat antirabbit IgG (H + L)F(ab’)2 fragment conjugate (1:200 dilution, Molecular Probes) for 1 hour at room temperature. A Coulter Elite cell sorter (Coulter, Fullerton, CA) was used to measure γH2AX and DNA content after rinsing and resuspending cells in 1 μg/ml 4’,6-diamidino-2-phenylindole dihydrochloride hydrate (Sigma-Aldrich, St. Louis, MO). WinList software (Verity Software, Topsham, ME) was used to conduct analysis of flow cytometry data. Samples were gated on 4’,6-diamidino-2-phenylindole dihydrochloride hydrate for DNA content and time of flight to eliminate debris and cell doublets before analysis of γH2AX antibody staining intensity. In some cases, only late S-G2 phase cells were analyzed. Apoptotic cells, based on a typical 10-fold increase in H2AX phosphorylation (13), were also eliminated from analysis of γH2AX loss kinetics. Intensity, in arbitrary units, was expressed relative to the control (untreated) cell population.

Received 4/29/04; revised 6/22/04; accepted 7/22/04.

Grant support: National Cancer Institute of Canada with funds provided by the Canadian Cancer Society.

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Alkaline and Neutral Comet Assays. An overnight alkaline lysis method at 5°C was used as described previously (14) to perform the alkaline comet assay and to maximize resolution of radiation-induced strand breaks and alkali-labile sites. Damage remaining 24 hours after irradiation was taken to represent unrepairable single-strand and double-strand breaks. An overnight lysis (22 hours) at 50°C followed by 3 rinses for 30 minutes at room temperature and staining for 30 minutes with propidium iodide was used as described previously (15) to perform the neutral comet assay. Approximately 150 comet images were obtained and analyzed for each dose and time, and experiments were repeated 2 to 3 times. Mean comet tail moment and SD are presented.

RESULTS

Six cervical carcinoma cell lines were initially chosen for analysis based on reported differences in their radiation sensitivity (16). They were examined for plating efficiency and for the background level of expression of γH2AX foci. SW756 and Caski showed high endogenous levels of expression of γH2AX relative to the other cell lines (Table 1). This occurred in the absence of significant differences in background DNA strand breaks. More than 60% of the SW756 cells showed ≥50 foci per cell. The high plating efficiency of this cell line (67 ± 4%) indicates that most of the cells with large numbers of γH2AX foci are clonogenic. When flow cytometry was used for analysis, comparable differences in γH2AX were also apparent, and untreated SW756 cells expressed almost four times more γH2AX than the HPV-negative C33A cell line. For the majority of cell lines, 20 to 30% of the cells showed foci. These foci, generally quite small, could be a result of γH2AX expression at endogenous breaks produced transiently during replication and are consistent with our previous elutriation and flow cytometry results describing S-G2-phase increases in γH2AX (13).

Clonogenic assays were performed on the six cell lines following irradiation. The HPV-negative cell line, C33A, was the most radiosensitive, whereas the remaining cell lines showed similar responses to X-rays (Fig. 1A; Table 2). Analysis of the effects of ionizing radiation on cell cycle progression is shown in Fig. 1B. There were some differences in the proportion of cells still blocked in G2 phase 24 hours after irradiation, a result in part to difference in cell-doubling time. Measured doubling times varied from 20 hours for HeLa to 32 hours for SW756 cells.

The alkaline and neutral comet assays were applied to Caski cells exposed to 0 to 40 Gy and then examined immediately for strand breaks or allowed 6 hours for strand break rejoining. Residual single-strand breaks were still present 6 hours after exposure to the higher doses (Fig. 2A), but there was no evidence for the presence of residual double-strand breaks 6 hours after exposure to 40 Gy, although γH2AX level was still 10 times background at this time (Fig. 2B).

Results for all six cell lines are shown in Fig. 3B. The comet assay is sensitive to the presence of replication forks in S-phase cells, and the radiation-induced G2 block becomes substantial at longer times after irradiation. Therefore, DNA content information from the comet and flow histograms limited analysis to G2 phase cells (Fig 3A and B). There was no evidence for the presence of residual DNA strand breaks 24 hours after irradiation. However, small but substantial amounts of residual damage were present for all cell lines 8 hours after 20 Gy. These differences are more easily appreciated in Fig. 2A. The fraction of residual damage is given in Table 2 as dose-equivalent damage remaining. Values of 0.19 to 0.31 Gy, representing <2% of the initial dose, were calculated; these values were not significantly different for the six cell lines.

In contrast to results obtained with the comet assay, expression of γH2AX was cell-line dependent. HeLa cells, known to contain low amounts of histone H2AX (4), exhibited the smallest slope when measured at the peak expression time of 1 hour (Fig. 3A). Both SiHa

<table>
<thead>
<tr>
<th>Cervical cancer cell line</th>
<th>Published HPV status</th>
<th>Plating efficiency</th>
<th>Percentage of DNA in comet tail (n = 150)</th>
<th>Percentage of cells with foci (mean ± SD, n = 32–50 cells)</th>
<th>Average no. foci per cell in cells with foci</th>
<th>Relative DNA content per cell</th>
<th>Relative background γH2AX (flow cytometry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>HPV-18 (10–50 copies)</td>
<td>0.53 ± 0.02</td>
<td>11.5 ± 8.4</td>
<td>31</td>
<td>20 ± 11</td>
<td>1.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Caski</td>
<td>HPV-16 (&gt;500 copies)</td>
<td>0.31 ± 0.09</td>
<td>12.9 ± 11.2</td>
<td>37</td>
<td>25 ± 16</td>
<td>1.6</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>MS571</td>
<td>HPV-45</td>
<td>0.44 ± 0.04</td>
<td>17.2 ± 12.1</td>
<td>32</td>
<td>13 ± 10</td>
<td>1.7</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>C33A</td>
<td>HPV negative</td>
<td>0.43 ± 0.05</td>
<td>17.5 ± 6.3</td>
<td>20</td>
<td>5 ± 4</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SW756</td>
<td>HPV-18 (10–50 copies)</td>
<td>0.68 ± 0.04</td>
<td>12.5 ± 7.9</td>
<td>66</td>
<td>46 ± 8</td>
<td>1.4</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td>SiHa</td>
<td>HPV-16 (1–2 copies)</td>
<td>0.72 ± 0.09</td>
<td>15.2 ± 9.9</td>
<td>28</td>
<td>7 ± 6</td>
<td>1.3</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

* Cells with >50 foci per cell were scored as having 50 foci.
and SW756 showed steeper initial dose-response curves that could be related to higher levels of H2AX in these cells. As shown in Fig. 1B, the near-diploid C33A cells showed the smallest DNA content, which explains in part the lower level of \( \gamma H2AX \) measured 1 hour after irradiation. Regardless of these differences, the slopes of the \( \gamma H2AX \) dose-response curves at 1 hour were unrelated to intrinsic radiosensitivity.

Image analysis was used to examine cells for remaining foci 24 hours after 2 Gy. Typical appearances of untreated cells and cells examined 24 hours after irradiation are shown in Fig. 4. Assuming one \( \gamma H2AX \) focus for each double-strand break, 2 Gy should produce \( \geq 50 \) foci per cell, depending on DNA content. For SW756 cells, all cells still contained foci 24 hours after irradiation (Table 2), but the high background level of \( \gamma H2AX \) makes it difficult to determine whether there are actually residual radiation-induced foci in this cell line. In fact, the average number of foci decreased, although average foci size appeared to increase. The number of foci per cell was not significantly

<table>
<thead>
<tr>
<th>Cervical cancer cell line</th>
<th>Surviving fraction after 2 Gy</th>
<th>Slope of dose response curve (comet assay at 0 hr. post-irradiation)</th>
<th>Gy equivalent damage remaining 8 hr. after 20 Gy (comet assay for ( G_2 ) phase)</th>
<th>Slope of ( \gamma H2AX ) dose response curve 1 hr. after irradiation</th>
<th>( \gamma H2AX ) Loss (slope at 24 hr./slope at 1 hr. for ( G_2 ) phase)</th>
<th>Percentage of cells with foci (24 hr. after 2 Gy)</th>
<th>Average no. foci per cell in cells with foci (24 hr. after 2 Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.53 ± 0.06</td>
<td>2.9</td>
<td>0.19</td>
<td>0.31 ± 0.09</td>
<td>5.9 ± 1.8</td>
<td>0.11</td>
<td>33</td>
</tr>
<tr>
<td>Caski</td>
<td>0.59 ± 0.10</td>
<td>2.9</td>
<td>0.27</td>
<td>0.55 ± 0.14</td>
<td>7.1 ± 2.2</td>
<td>0.15</td>
<td>98</td>
</tr>
<tr>
<td>MS751</td>
<td>0.51 ± 0.04</td>
<td>2.6</td>
<td>0.31</td>
<td>0.68 ± 0.09</td>
<td>4.2 ± 1.7</td>
<td>0.12</td>
<td>53</td>
</tr>
<tr>
<td>C33A</td>
<td>0.34 ± 0.05</td>
<td>3.0</td>
<td>0.25</td>
<td>0.57 ± 0.11</td>
<td>6.1 ± 1.2</td>
<td>0.30</td>
<td>54</td>
</tr>
<tr>
<td>SW756</td>
<td>0.53 ± 0.02</td>
<td>3.1</td>
<td>0.29</td>
<td>0.96 ± 0.17</td>
<td>5.7 ± 3.0</td>
<td>0.03</td>
<td>100</td>
</tr>
<tr>
<td>SiHa</td>
<td>0.58 ± 0.06</td>
<td>2.9</td>
<td>0.20</td>
<td>0.83 ± 0.06</td>
<td>4.8 ± 1.2</td>
<td>0.09</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 2: Response of irradiated cervical cancer cell lines

Fig. 3. Measurement of DNA strand break induction and rejoining and \( \gamma H2AX \) formation and loss in six cervical cell lines at various times after exposure to X-rays. The mean and SD for three independent experiments are shown.

and SW756 showed steeper initial dose-response curves that could be related to higher levels of H2AX in these cells. As shown in Fig. 1B, the near-diploid C33A cells showed the smallest DNA content, which explains in part the lower level of \( \gamma H2AX \) measured 1 hour after irradiation. Regardless of these differences, the slopes of the \( \gamma H2AX \) dose-response curves at 1 hour were unrelated to intrinsic radiosensitivity.

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The correlation between SF2 (surviving fraction after 2 Gy) and significantly different for the six cell lines. However, a significant greater loss rate of type cells have been analyzed separately and show a significantly within a larger series of human tumor cells (Fig. 6).

Six hours after irradiation, all cell lines retained approximately half of the γH2AX measured at the peak time of 1 hour (Fig. 3A). The half-time of loss of γH2AX was determined from data obtained after exposure of cell lines to 5 to 50 Gy with γH2AX levels measured from 1 to 4 hours after irradiation (Fig. 5). This value was not significantly different for the six cell lines. However, a significant correlation between SF2 (surviving fraction after 2 Gy) and γH2AX loss half-time was found when the six cervical cell lines were included within a larger series of human tumor cells (Fig. 6B). The p53-wild-type cells have been analyzed separately and show a significantly greater loss rate of γH2AX after irradiation compared with cell lines deficient in p53 (Fig. 6A). The average loss half-life for all of the p53-wild-type cells, regardless of radiosensitivity, was 3.8 ± 1.4 hours compared with 5.9 ± 1.9 for the p53-deficient cell lines. Loss rate for p53-wild-type and p53-deficient cells correlated independently with SF2 ($r^2 = 0.75$).

By 24 hours after irradiation, relative residual damage was significantly reduced and was essentially negligible for SW756 cells. In contrast, 30% of the original amount of γH2AX remained in C33A cells (Table 2). Therefore the cell line most sensitive to killing by ionizing radiation also showed the most residual γH2AX 24 hours after irradiation. A weak correlation ($r^2 = 0.59$) was found between SF2 and the fraction of residual γH2AX measured as the ratio of slopes at 1 and 24 hours after irradiation for the six cell lines (Fig. 6C). However, as mentioned earlier, the high background of γH2AX in the SW756 cells is likely to interfere with accurate measurement of residual damage. The correlation coefficient without this cell line increased to 0.75.

**DISCUSSION**

A primary aim of this study was to determine whether either of two methods that detect DNA damage and repair after irradiation might be useful in detecting differences in the radiosensitivity of a series of human cancer cell lines of the same type. In previous experiments in which the comet assay was used, we were unable to identify differences in induction or short-term rejoicing of strand breaks that could stratify cell lines according to their radiosensitivity (17). However, the comet assay (18) or other methods (19) have been shown to correlate radiosensitivity with residual damage, measured at longer times after radiation. To achieve the greatest sensitivity for detecting radiation-induced DNA damage, we used the alkaline comet assay that measures the presence of single-strand breaks, double-strand breaks, and alkali-labile lesions. The assumption has been made previously that residual strand breaks are likely to be double-strand breaks (20). Other evidence suggests that both unrepair single-strand and double-strand breaks are probably detected (21), and data shown in Fig. 2 using Caski cells to compare alkaline and neutral methods supports this result. When the neutral method was used, no damage could be detected after 6-hour repair; and even when the more sensitive alkaline method was used, <2% of the initial damage remained. An important point to bear in mind is that both comet assays demonstrate, under optimum conditions, a detection limit on the order of 50 breaks per cell so that persistent DNA damage can go undetected below this threshold. Our results are in agreement with previous results by Dikomey et al. (22) who found that cell line-dependent differences in residual damage could not be detected at 24 hours after 20 Gy, although differences were observed after higher doses. There were no apparent differences between the extent of rejoining for the different...
suggestions that many residual foci may not be associated with a physical break. This is perhaps not surprising considering the high background levels of these foci within some tumor cell lines that cannot be explained by high S-phase content, low-plating efficiency, or a high level of background damage in the comet assays. Moreover, γH2AX foci were shown to persist through cell division producing patterns reminiscent of the “mirror-like similarity of chromosome territories” seen in daughter cell pairs (23). The similar patterns of γH2AX in daughter cells suggest the intriguing possibility that breaks or chromatin structural changes that act as signals for ATM activation and subsequent H2AX phosphorylation might be inherited.

HPV viral E6 protein effectively inactivates p53 function through binding, which leads to degradation of p53 via the ubiquitin-proteosome system. Five of the six cell lines express HPV and the one exception, C33A, contains a mutated p53 (24). The presence of mutant p53 has been associated with decreased cell killing and decreased local tumor control following irradiation (25). Interestingly, C33A showed the lowest level of background foci (Table 1). Duesing and Munger (26) also reported that expression of HPV-16 E7 protein in normal human keratinocytes was associated with an increase in the fraction of cells with γH2AX foci that could not be accounted for by apoptosis. Cells containing functional p53 lose foci faster than cells lacking p53 following irradiation. Of some importance, the correlation between SF2 and γH2AX loss half-time was only evident when cells were analyzed according to p53 status. When 10 cell lines, representing rodent and human as well as p53-wild-type and p53-deficient cells, were used previously, they showed a good correlation ($r^2 = 0.7$) between SF2 and rate of loss of γH2AX measured between 1 and 4 hours after irradiation. However, results in Fig. 6 for a larger series of p53-wild-type and p53-deficient human cell lines indicate that p53 status appears to be relevant to γH2AX loss rate after irradiation. A possible explanation for the p53 effect is related to its ability to transactivate genes involved in apoptosis; p53-positive cells with high levels of γH2AX could undergo apoptosis, and loss rate might then appear faster. However, loss half-time was measured over the first 4 hours when little apoptosis was apparent, and many of the cell lines were fibroblasts and resistant to apoptosis. Transcription of dozens of genes is regulated by p53, and these might influence γH2AX formation or loss (27). Alternatively, functions of p53 unrelated to transcriptional activation may be responsible; p53 has been implicated in nucleotide excision repair and homologous recombination through interactions with Rad51 as well as other repair proteins in this pathway (28, 29). Perhaps lack of p53 influences the repair process by reducing accurate rejoining, and mis-rejoined breaks cause longer retention of foci. Alternatively, chromatin conformational changes appear to influence p53 function (30) just as they influence activation of ATM (31), the kinase that phosphorylates both H2AX and p53. Changes in chromatin structure at sites of misrepair may affect p53 function locally (30), and it has been suggested that mutant p53 might modulate nuclear structure and function (32). Finally, it is also possible that p53 may be only indirectly involved, and genomic instability subsequent to loss of p53 could produce additional changes that alter foci retention. Experiments are in progress with isogenic cell lines to try to resolve this question.

In summary, induction and rejoining of radiation-induced strand breaks were the same for six cervical cancer cell lines that varied in radiation sensitivity. Phosphorylation of histone H2AX at sites of radiation-induced strand breaks was cell line dependent. Although background expression of γH2AX and induction of foci in response to radiation showed no obvious relationship with radiosensitivity, the residual level of γH2AX measured 24 hours after irradiation was correlated with SF2. Results also identify a role for the tumor sup-

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Fig. 6. Comparison between γH2AX loss after irradiation and clonogenic surviving fraction after 2 Gy (SF2). A and B show γH2AX loss half-time measured between the peak time of 1 hour and 4 hours for p53-wild-type and p53-deficient cell lines (from Fig. 5). In B, the cervical cell lines are shown (A) along with p53-wild-type regression line and 95% CI (A). C shows the residual γH2AX measured as the ratio of the slope at 24 hours divided by the slope measured 1 hour after irradiation (from Fig. 3B).
pressor p53 in γH2AX loss rate and they question the assumption that background or residual γH2AX foci are necessarily associated with un rejoined double-strand breaks.

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Cancer Res 2004;64:7144-7149.

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