DNA Double-Strand Breaks Measured in Individual Cells Subjected to Gel Electrophoresis

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ABSTRACT

Microscopic examination of individual mammalian cells embedded in agarose, subjected to electrophoresis, and stained with a fluorescent DNA-binding dye provides a novel way of measuring DNA damage and more importantly, of assessing heterogeneity in DNA damage within a mixed population of cells. With this method, DNA double-strand breaks can be detected in populations of cells exposed to X-ray doses as low as 5 Gy. The radiation dose-response relationship for initial formation of double-strand breaks was identical for cell lines irradiated in G1, regardless of their sensitivity to killing by ionizing radiation. However, for cells irradiated in S phase, DNA migration was significantly reduced. For Chinese hamster V79 cells, Chinese hamster ovary cells, WiDr human colon carcinoma cells, and L5178Y-R mouse lymphoblastoid cells, S-phase DNA appeared to be about 3 times less sensitive to X-ray damage than DNA from other phases of the cell cycle. However, for the very radiosensitive L5178Y-S cells, the migration of replicating DNA was reduced only slightly. For Chinese hamster V79 and Chinese hamster ovary cells, damage was repaired at a similar rate in all cells of the population, and 85% of the breaks were rejoined within 2 h after irradiation. The radiosensitive L5178Y-S cells repaired damage more slowly than V79 or Chinese hamster ovary cells; 2 h after exposure to 50 Gy, approximately 50% of the damage was still present.

INTRODUCTION

In 1984 Ostling and Johanson (1) described a method to measure DNA damage in individual cells based on migration of DNA in an electric field. Cells embedded in agarose were lysed, subjected briefly to about 4 V/cm, stained with a fluorescent DNA-binding dye, and viewed using a fluorescence microscope. DNA which was broken and therefore smaller or less condensed migrated towards the anode, and the displacement of DNA from the nucleus could be used as an indication of DNA damage. The ability to quantify heterogeneity in DNA damage is an important attribute of this method and allows detection of treatment-resistant cells within a mixed population (2, 3).

We have previously modified the method of Ostling and Johanson to improve reproducibility and sensitivity for video image analysis and to specifically quantify DNA single-strand breaks (3, 4). Our initial modifications included alkaline lysis once cells were embedded in agarose. Previous studies described the effects of ionizing radiation on murine tumor cells and tumor infiltrating macrophages. We argued based on several observations that the alkaline "comet assay," named because of the appearance of the cells following electrophoresis, was able to detect DNA single-strand breaks. First, we could resolve significant amounts of DNA damage by the single-strand breaking agent, hydrogen peroxide. Secondly, DNA repair kinetics was consistent with repair of single-strand breaks. Lastly, as has been observed using other methods which measure single-strand breaks, DNA damage was independent of inherent radiation sensitivity and was not influenced by position of the cell in the cell cycle (4).

However, DSBs are generally considered to be of greater biological consequence than single-strand DNA breaks since they can lead directly to chromosome aberrations and loss of genetic material (5–7). For detection of DSBs, cells embedded in agarose must be lysed in a neutral (non-denaturing) solution able to remove proteins. Ostling and Johanson's original method (1, 2) and the "halo" method of Roti Roti and Wright (8) also used a neutral lysing solution to identify damage in individual cells, but these methods retain the intact "loop" structure of DNA which makes them sensitive to the release of DNA supercoiling caused by single-strand breaks.

The goal of this study was to develop and optimize a method to measure DSBs in individual cells which we could then use to examine cell sensitivity to ionizing radiation and other DNA-damaging agents. In view of several reports indicating a relation between radiation sensitivity and initial DSBs (9, 10) or their rate of repair (10–12), we measured radiation-induced DSB induction and repair in cell lines varying in radiation sensitivity. Since the unique feature of this method is its ability to measure DNA damage in individual cells, we wished to determine whether all cells within a population showed similar amounts of initial damage by ionizing radiation and whether all cells repaired DSBs with similar kinetics.

MATERIALS AND METHODS

Chinese hamster V79–171b lung fibroblasts were maintained in exponential growth by subcultivating twice a week in MEM containing 10% FBS from Gibco. Cells were removed from tissue culture dishes using 0.1% trypsin in PBS. The action of trypsin was stopped by adding MEM plus 10% FBS and cells were recovered by centrifugation. Cells were suspended in PBS at a density of 4 × 10^4 cells/ml and irradiated on ice at a dose rate of 7.2 Gy/min.

L5178Y-S murine leukemic lymphoblasts were cultivated in Fisher's medium supplemented with 10% FBS. Cells were maintained in exponential growth by subculturing every 2–3 days by removing a portion of the suspended cells and reseeding cells at a density of 10^4 cells/ml. WiDr human colon carcinoma cells were obtained from the American Type Culture Collection and maintained as exponentially growing monolayers in MEM plus 10% FBS. CHO cells were also grown as monolayers in MEM plus 10% FBS. Standard clonogenic measurements following exposure to ionizing radiation were performed by seeding approximately 500 surviving cells into 100-mm tissue culture dishes containing 10 ml MEM plus 10% FBS and staining colonies 8–14 days later.

Following irradiation, 1.5 ml of low gelling temperature agarose (Sigma type VII, 1% in H2O) at 40°C were added to a tube containing 0.5 ml of cold cell suspension. The contents were pipetted onto a fully frosted microscope slide positioned on an ice-cold surface and allowed to gel for about 1 min. Slides were then carefully immersed in lysing solution consisting of 30 mM EDTA, 0.5% sodium dodecyl sulfate, pH 8.3. The slides were then placed in an incubator at 50°C for 4 h. Initial

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2 To whom requests for reprints should be addressed.
experiments were performed using 0.5 mg/ml proteinase K in the lysis solution. If a lysis period of 4 h or longer at 50°C was used, proteinase K could be omitted from the lysis solution without affecting the sensitivity of the assay. Following lysis, slides were thoroughly rinsed in large volumes (250 ml/10 slides, replaced 3 times) of 90 mM Tris-90 mM boric acid-2 mM EDTA buffer. After a rinse period of 4–16 h, slides were placed in a Bethesda Research Laboratory model H-4 horizontal electrophoresis chamber containing 1.8 liters 90 mM Tris-90 mM boric acid-2 mM EDTA buffer and exposed to 0.66 V/cm for 25 min. Following electrophoresis, DNA was stained by immersing slides in 2.5 μg/ml propidium iodide for 60 min. Slides were then briefly rinsed and placed on wet towels in air- and light-tight boxes.

Individual cells resembling “comets” were viewed using a Zeiss epifluorescence microscope attached to an intensified solid state CCD camera and image analysis system. Slides were illuminated with green light (510–560 nm) from a 100-W mercury source. Individual comets were viewed using a 25× objective and images were analyzed using a fluorescence image processing system previously described (3, 4). Images were selected randomly and only overlapping comets were omitted from analysis. Each digitized image was analyzed using a program written by Dr. Ralph Durand. The program included an algorithm which first applied an edge filter to define the limits of the comet, then subtracted the background, defined as the image intensity at the edge of the comet, and subsequently formed head and tail distributions for analysis as previously described (3, 4).

Several properties or “features” were calculated for each individual comet image. The most important was the “tail moment” which was defined as the product of the percentage of DNA in the tail multiplied by the tail length. The total intensity of propidium iodide fluorescence was used as an indication of the DNA content per comet. Finally, the amount of DNA in the comet tail was used as an indication of extent of migration. Approximately 100 comets could be individually identified, adjusted for optimum focus, digitized, and analyzed within 20 min.

Fluorescence-activated cell sorting in conjunction with Hoechst 33342 staining was used to separate cells from different phases of the cell cycle. Monolayers of asynchronous cultures were incubated for 30 min with 5 μM Hoechst 33342 at 37°C. Although Hoechst 33342 can be toxic to some cells under some treatment conditions (13, 14), the choice of treatment protocol was based on previous results from our laboratory showing no killing or DNA single-strand breakage of V79 cells at these Hoechst 33342 concentrations (14). In addition, we observed no difference in DSB induction in irradiated cells stained with Hoechst either before or after cell sorting, indicating no significant damage caused by laser light. Single cell suspensions were prepared and analyzed using a Becton-Dickinson FACS 440 dual laser cell sorter. Windows were selected from various phases of the cell cycle based on Hoechst fluorescence intensity under UV excitation using the 350–360-nm lines from an argon laser operated at 40 mW power. Cells sorted directly into medium were then centrifuged and resuspended in ice-cold PBS for irradiation on ice using a 270-kV X-ray unit. For DSB-rejoining studies, cells were sorted into ice-cold complete medium, irradiated on ice, and then allowed to repair damage by incubation at 37°C.

RESULTS

When cells are embedded in agarose, lysed, and subjected to an electric field, broken DNA is able to migrate towards the anode. Subsequent staining of the DNA using propidium iodide allows visualization of DNA from individual cells using fluorescence microscopy. Each cell has the appearance of a “comet” with a brightly fluorescent head and a tail with an intensity that is related to the amount of damage sustained by the cell. To obtain the photographs shown in Fig. 1, cells were embedded in agarose, lysed for 4 h in 0.5% sodium dodecyl sulfate-30 mM EDTA, pH 8.3, at 50°C, rinsed, and electrophoresed at 0.66 V/cm for 25 min. Unlike previous results using the alkaline lysis method, the electric field strength did not appear to influence the fraction of migrating DNA after neutral lysis but did increase the distance of migration. Similar amounts of DNA in the tail were detected in irradiated cells using 0.66 V/cm for 25 min, 1.2 V/cm for 15 min, and 1.8 V/cm for 8 min. For voltages greater than 1.5 V/cm, the migrating fraction of DNA was separated from the head of the comet and DNA moved as a compression band for radiation doses above 20 Gy, the length of the comet tail was fairly constant for a given voltage of electrophoresis and was independent of the dose of radiation. Instead, there was an increase in the fraction of DNA in the tail (Fig. 1). However, below 20 Gy, tail length was proportional to dose so that “tail moment” [the product of tail length and the amount of DNA in the tail, (3, 4)] was found to be a better overall measure of DNA damage than fraction of DNA in the tail. The slope of the dose-response curve for tail moment versus radiation dose was optimum using 0.66 V/cm for 25 min. For higher voltages/shorter times, the comet tail retracted slightly with time after electrophoresis. Applying a fluorescence image-processing system and a program designed to analyze the tail moment, a linear dose-response curve was obtained over the dose range of 0–100 Gy. The low dose region is shown in Fig. 2 for 100 comets/dose point, and the slope of the line through these points is comparable to the slopes routinely obtained for the 0–100-Gy range. Statistically significant damage to the population by doses as low as 5 Gy could be detected.

Unlike our previous results obtained using alkali lysis after hydrogen peroxide treatment (4), we were unable to detect any damage caused by exposure of V79 cells to 10⁻⁴ M hydrogen peroxide for 20 min on ice followed by neutral lysis and electrophoresis. This result indicates that the neutral lysis method is measuring double- and not single-strand breaks. In addition, treatment of irradiated cells with hydrogen peroxide did not increase the fraction of migrating DNA, nor did we observe any additional damage when we exposed irradiated V79 cells to peroxide after the cells had been allowed a 20-min repair period at 37°C. These results indicate that single-strand breaks cannot be readily detected after this neutral lysis method and do not reduce DNA tangling or influence the migration of DNA containing double-strand damage.

Variability in response of different cells within a population was quite low when near-confluent cultures were examined (Fig. 2B). However, considerable heterogeneity was observed when we used asynchronous cells (Fig. 3C) which could be attributed primarily to the presence of S-phase cells. The slope of the tail moment dose response for Chinese hamster V79 cells in the process of replicating their DNA was about 3 times smaller than the slope for cells irradiated in G1 or G2-M. In addition, the tail moment for unirradiated cells was about twice as large for cells from G1, as for cells from S phase, indicating that the difference in S-phase cells is inherent to the cells and not a result of radiation damage. The decrease in the slope of the dose response for asynchronous cultures versus G1 or G2 cells therefore reflects the distribution of cells in the cell cycle for each culture (about 30% S-phase cells for the V79 culture shown in Fig. 3). DNA content of individual cells, measured as the total intensity of propidium iodide fluorescence in each comet, verifies the ability of cell sorting to provide cells from various phases in the cell cycle (Fig. 3B). In Fig. 3C, which shows heterogeneity in tail moment for cells exposed to 50 Gy, the presence of the 30% resistant S-phase cells is clearly visible in the lower histogram marked “all.” “Early” S phase in both V79 and L5178Y-S cells appeared most resistant (Fig. 4). However,
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Fig. 1. Fluorescence photomicrograph of 4 Chinese hamster V79 cells, exposed to the radiation doses shown, and then examined for DNA double-strand breaks using the neutral comet assay. Cells were irradiated, embedded in agarose, lysed, and exposed to 0.66 V/cm in a horizontal gel electrophoresis apparatus for 25 min prior to staining with propidium iodide and examination using a fluorescence microscope. Negatively charged DNA migrates from the head of the comet positioned at the right toward the anode at the left. Note that as radiation dose increases, the amount of DNA in the tail increases.

for the more radiosensitive L5178Y-S cells, migration was much less affected by position in the cell cycle (Fig. 4).

Three cell types, varying in their radiation sensitivity (Fig. 5A), were examined for DNA double-strand break induction by ionizing radiation. Because the slope of the line describing tail moment as a function of radiation dose is dependent on the fraction of cells in S phase and the degree of retardation of S-phase DNA, we obtained dose-response curves for sorted G1 cells as well as sorted S-phase cells. As shown in Fig. 5C, the responses of G1 cells from these different cell lines to ionizing radiation, as measured by this assay, were identical. However, S-phase sensitivity to DSB induction was much greater in the radiosensitive L5178Y-S cells than in the V79 cells (Fig. 5D). Heterogeneity in damage was similar for V79 and CHO cells but was routinely greater in the sensitive L5178Y-S cells (Fig. 5B). A comparison of the responses of S-phase cells from several different cell types is shown in Table 1. L5178Y-S cells, the most radiosensitive line tested, showed a significantly higher sensitivity in S phase than the other cell types.

DNA repair kinetics following X-irradiation was not first order but demonstrated at least two components. Data from both G1 and G2 cells were pooled since repair rates for these cells were not found to be significantly different. The half-time for repair for V79 and CHO cells was about 12 min and that for L5178Y-S was about 30 min (Fig. 6). Approximately 85% of the DSBs in V79 and CHO cells were repaired rapidly and only 10% of the damage remained after 2 h. Heterogeneity in DNA repair rates was quite small, and the coefficient of variation (i.e., standard deviation divided by the mean) varied from 10 to 15%. All cells appeared to repair damage at a similar rate. However, in the more radiosensitive L5178Y-S cells, heterogeneity was much greater and DSBs were rejoined more slowly; about one-half of the initial number of strand breaks was still present after 2 h. Fifteen min after exposure to 50 Gy, a small fraction of L5178Y-S cells (5–10%) was observed which showed more than double the initial level of damage. Because

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Fig. 3. Cell cycle effects on double-strand break detection using the neutral comet assay. Cells in various phases of the cell cycle were sorted on the basis of Hoechst 33342 fluorescence intensity. Cells were then irradiated and examined for initial DNA damage. A, tail moment determined for 40 comets/dose point: •, S-phase cells; T, unsorted cells; A, G2-M cells; A, G, cells; points, mean; bars, SD. B, heterogeneity in DNA content (arbitrary units) for 160 cells from each sorted population. C, heterogeneity in tail moment for 40 cells/phase exposed to 50 Gy.

Fig. 4. Effect of cell cycle position on DNA damage by ionizing radiation, measured using the neutral comet assay. V79 (bars only) or L5178Y-S (x) cells were sorted on the basis of Hoechst 33342 fluorescence intensity. Sorted cells were irradiated and analyzed for DNA damage using the comet assay. The slope of the tail moment dose-response curve (5 points, 40 comets/dose point) was calculated. Points, mean for 3–6 separate experiments; bars, SD. Data points without error bars were performed only once. × “error” bars, size of the sort windows which can be compared to the typical DNA histogram for V79 cells on the right.

these cells were considered to be in the early stages of lysis or apoptosis, they were not included in the analysis of repair rates.

DISCUSSION

Measurement of DNA double-strand breaks in individual cells is now possible using microscopic analysis of cells subjected to gel electrophoresis. The neutral lysis assay is able to detect double-strand damage by X-ray doses as low as 5 Gy which is equivalent to about 200 double-strand breaks/cell (15). This sensitivity is comparable to that of other gel electrophoresis methods used to detect DSBs in populations of cells (16, 17). Single-strand breaks are not detected by this method and do not influence subsequent measurement of double-strand breaks. At the level of the individual cell, however, heterogeneity limits resolution to damage produced by 10 Gy or more. Within a mixed population of cells, it should be possible to detect a major subset of cells varying in DNA damage by a factor of 3 or more. We base this conclusion on the fact that we could discriminate S-phase cells (constituting about 30% of the population and appearing to be 30% less damaged) from cells within other phases of the cell cycle (Fig. 3C). This sensitivity will provide, for the first time, a method to detect cell subpopulations resistant to radiation-induced double-strand breaks (e.g., hypoxic cells) within a heterogeneous population of cells.

It is clear that several factors must be responsible for differ-

Table 1 Comparison between cell killing and S-phase DNA damage by ionizing radiation

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Slope* (S phase only)</th>
<th>D0% (asynchronous)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79</td>
<td>0.025 ± 0.006 (6)</td>
<td>8.0 ± 0.5 (8)</td>
</tr>
<tr>
<td>WiDr</td>
<td>0.025</td>
<td>7.4 ± 0.2 (4)</td>
</tr>
<tr>
<td>CHO</td>
<td>0.026 ± 0.003 (4)</td>
<td>5.1 ± 0.2 (5)</td>
</tr>
<tr>
<td>L5178Y-R</td>
<td>0.028 ± 0.009 (3)</td>
<td>3.5 ± 0.2 (3)</td>
</tr>
<tr>
<td>L5178Y-S</td>
<td>0.059 ± 0.012 (4)</td>
<td>1.4 ± 0.2 (3)</td>
</tr>
</tbody>
</table>

* The slope of the curve describing tail movement as a function of radiation dose for cells sorted from S phase [mean ± SD for (n) experiments]. The slope for G1-G2 cells was similar for all cell lines (0.075 ± 0.05).
* D0% dose of radiation in Gy required to reduce clonogenicity of asynchronous cultures to 10%. Mean ± SD for (n) experiments.
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Enances in radiation sensitivity among different cell lines. Even if one makes the simplifying assumption that unrepaired DNA double-strand breaks are ultimately responsible for cell inactivation, these lesions might arise because of inherent physical or chemical differences in the target DNA, or differences in type or activity of repair enzymes. Some radiosensitive cell lines show increases in apparent DSB induction (9, 10), others show decreases in rates of rejoining of DSBs (10–12, 18), and still others show no change in either induction or repair of lesions (19) suggesting that fidelity of repair could be compromised. In the cell lines examined here, only the very radiosensitive L5178Y-S cells showed a significant decrease in repair rate as well as a difference in the initial response of cells in S phase. The response of a closely related subline, L5178Y-R cells, was similar to that of more radiation-resistant cell lines (Table 1).

The extent of retardation of S-phase DNA may be indicative of radiation sensitivity in some cell types although the reason why replicating DNA is less able to migrate during electrophoresis is not yet known. There is little doubt that replicating DNA adopts a different configuration, and extensive tangling could prevent migration of a significant fraction of the DNA. Tightly bound proteins associated with DNA replication may also inhibit migration, although proteinase K treatment (which may not remove these proteins) did not enhance migration of DNA from S-phase cells. DNA in cells at the G1-S border seemed particularly susceptible to this effect. Interestingly, there is evidence to suggest that those regions of the genome the chromatin of which is least condensed during interphase, and therefore most accessible to the replication enzymes, is synthesized in early S phase. Highly condensed heterochromatin, on the other hand, is generally replicated very late in the S phase (20). However, while removing S-phase cells considerably reduced heterogeneity in tail moment for V79 cells (Fig. 3C), this was not true for L5178Y-S cells (Fig. 5B) since about 20% of the cells sorted from G1 and G2 migrated at the rate of S-phase cells. Therefore, the factor which inhibited migration primarily in early S phase in V79 cells might also be present in some L5178Y-S cells in other phases of the cell cycle. If so, one could argue that inherent differences in DNA “packaging” play a role in the sensitivity of some cell types to DNA-damaging agents. A possible role for DNA higher order structure in radiation-induced DNA damage and repair has previously been suggested (21–24), and the concept that DNA organization might be one determinant of radiation sensitivity requires further study.

There are important similarities between the NFE and the neutral comet assay. Both methods detect double-strand breaks, and both require effective lysis of the cells for maximum sensitivity. Both methods show less apparent damage to cells irradiated in S phase (17, 25), and both exhibit non-first order kinetics for DNA repair (11, 18). However, NFE at pH 9.6 often appears able to detect a type of initial DNA damage which is related to cell sensitivity to ionizing radiation, at least for repair-proficient cells (9–11). For the NFE method, cell lysis is followed by expansion of the DNA on the filter, and it is the rate or extent of expansion which appears to be critical for the sensitive detection of DSBs and probably for providing the distinction between cell lines of different radiosensitivities (21). This does not appear to be true for the neutral comet assay which uses more stringent lysis conditions (4 h at 50°C); with the exception of the effects occurring in replicating DNA, DSB induction by ionizing radiation was similar for cells varying considerably in sensitivity to killing by ionizing radiation.

In conclusion, electrophoresis of individual cells after neutral lysis provides a sensitive, albeit indirect, indication of the number of DNA double-strand breaks in individual mammalian cells. The rate of repair of DSBs was slower for radiosensitive L5178Y-S cells than for V79 or CHO cells. Changes in DNA packaging or association with proteins which occur during early S phase appear to inhibit the migration of DNA, and these changes may also be related to differences in radiation sensitivity.

REFERENCES

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