Differential Sensitivity of a Mouse Myeloid Leukemia Cell Line and Normal Mouse Bone Marrow Cells to X-Ray-induced Chromosome Aberrations

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ABSTRACT

Cell line ML-1 was established from a myelogenous leukemia of an RFM mouse. The ML-1 cells and in vitro normal mouse bone marrow cells were analyzed to determine if there was a differential sensitivity to X-ray-induced chromosome aberrations in G1 cells and/or differences in postirradiation cell cycle progression. Cells identified as being in G1 at the time of irradiation by their staining pattern after replication in 5-bromodeoxyuridine were analyzed for all types of chromosomal aberrations following X-ray doses of 0.5, 1.0, 1.5, and 2.0 Gy. ML-1 cells showed a greater sensitivity to the induction of both chromosome-type aberrations (dicentrics and terminal deletions) and chromatid-type aberrations (exchanges and deletions) compared to normal mouse bone marrow cells, which only contained chromosome-type aberrations. The presence of chromatid-type aberrations in the ML-1 cells and not normal bone marrow cells suggested a differential progression through the cell cycle for the two cell types after irradiation. Mitotic index and flow cytometric analyses were performed and showed that both cell types have a delay in progression from G2 into mitosis, but only the normal mouse bone marrow cells have a delay in progression from G1 into S, as well as delayed progression through the S phase following X-irradiation. These results indicate that the ML-1 leukemia cells have an increased radiosensitivity. This may be due to a defect in their ability to respond to DNA damage as evidenced by their lack of a G1- and S-phase delay which allows normal cells an increased time to repair DNA damage before replication. These same characteristics have been observed in ataxia telangiectasia cells and may well represent a general feature of cells with increased radiosensitivity.

INTRODUCTION

The relationship between cellular sensitivity to cytotoxic agents, cell cycle delay, and DNA repair has been investigated using mammalian cell lines which show a differential sensitivity to the induction of chromosome damage, cell killing, and/or mutation compared to normal cells. Cells derived from inherited human disorders which show an increased sensitivity to various agents have been examined for possible repair and/or replication defects. These include AT3 cells which are sensitive to X-rays (1-4), bleomycin (5), 4-nitroquinoline 1-oxide (6), and neocarzinostatin (7); FA cells which are sensitive to cross-linking agents (8, 9), bleomycin (9), and X-rays (1); Bloom syndrome cells which are sensitive to X-rays (1); and XP cells which are sensitive to UV (10, 11). There are conflicting reports concerning repair or replication defects in these syndromes, but no specific defect which could completely account for the differential sensitivity has been found except for XP cells, which clearly show a deficient repair of UV-induced pyrimidine dimers (10, 12).

Rodent cell lines have also been used to study mechanisms of differential sensitivity. The V79/79 Chinese hamster cell line has been shown to be more sensitive than the parental V79 line to X-rays (13), UV, methyl nitrosourea, ethyl methanesulfonate (14), methyl methanesulfonate, and HN2 (15). Mouse lymphoma L5178Y sublines show an increased sensitivity to X-rays compared to the parent L5178Y line (16-20). The rat Yoshida lymphosarcoma cell line has an increased sensitivity to methylene dimethane sulfonate, UV (21), X rays, HN2, and sulfur mustard (16). As with the human cell lines, no specific defect has been found in these rodent cell lines which can account for the increased cross-sensitivity to these agents.

The increased radiosensitivity of AT cells compared to normal cells has recently been associated with differences in cell cycle progression for both cell types following X-irradiation (22). AT cells are reported to have less radiation- or bleomycin-induced delay in the initiation of DNA synthesis compared to normal cells (22-24). Therefore, AT cells do not show the normal radiation-induced delay in progression from G1 to S or S to G2 (25-27). FA fibroblasts, following treatment with HN2, showed no delay in S-phase progression (28), and HN2-treated V79/79 cells showed reduced S-phase delay (15). The above reports support the idea of an association between increased cellular sensitivity and reduced G1 and/or S-phase delay. However, results with the human lymphoblastoma cell line TK6, which is sensitive to HN2, showed more S-phase delay than the less sensitive Raji cell line (29), and mouse lymphoma L5178Y cells showed an X-ray-induced delay in DNA synthesis similar to that for resistant cells (20).

The present study was carried out to compare the sensitivities of a mouse myeloid leukemia cell line (ML-1) and normal mouse bone marrow cells to X-ray-induced chromosome aberrations and to examine the effects of X-rays on the cell cycle progression of both cell types. Flow cytometric and MI analyses were used to study the effects of radiation on the cell cycle.

MATERIALS AND METHODS

Cell Culture. These studies used the ML-1 cell line derived from X-ray-induced RFM mouse myeloid leukemia (30) and normal mouse bone marrow cells grown in vitro. As previously described by Au et al. (31, 32), the ML-1 cell line, analyzed by the trypsin:Giemsa banding technique,
was found to have a stable, diploid chromosome number of 40 and the following consistent chromosomal alterations: deletion of chromosome 2, Bands C-1 and F1-3; isochromosome 8; trisomy 19; and no Y chromosome. ML-1 cultures were maintained in RPMI 1640 (Gibco) containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Flow Laboratories) at 37°C in an incubator humidified 95–100% in an atmosphere of 5% CO₂ in air. Cultures were initiated at 4 × 10⁵ cells/ml for each experiment.

Normal mouse bone marrow cell cultures were established prior to each experiment using a modified version of the technique described by Nakamura (33). Male RFM mice 9–11 wk old were sacrificed by cervical dislocation, and the femurs were removed. Bone marrow cells were flushed out of the femurs with Hanks’ balanced salt solution (pH 7.0) at 37°C containing 5 units of heparin per ml using a 22-gauge needle. Pooled bone marrow cells were centrifuged at about 350 × g for 4 min and resuspended in 37°C RPMI 1640 plus 25% heat-inactivated fetal bovine serum. An aliquot of cells was treated with ZapogloWnIIR (Coulter Diagnostics) to lyse the RBC, and WBC counts were determined using a Coulter Counter (Model ZM; Coulter Electronics). The cell concentration was adjusted to approximately 3.5 × 10⁶ nucleated cells per ml. Cell cultures were established with 7 × 10⁶ nucleated cells per 2 ml of medium in 35-mm plastic culture dishes and incubated as described for the ML-1 cells. It is appreciated that a small proportion of the cells established in culture from the bone marrow will be represented by the metaphases examined; however, since the population analyzed is dividing, with a cycle time of about 20 h (Chart 1), it can be considered to be somewhat homogeneous.

Slide Preparation. For the cell cycle kinetics and chromosome aberration experiments, Comedol (Sigma) at a final concentration of 1 × 10⁻⁷ M was added 1 h prior to fixation. For the cell cycle kinetics, chromosome aberration, and MI experiments, cells were centrifuged, resuspended in 10 ml of 0.075 M potassium chloride, and incubated at 37°C for 20 min. Cells were centrifuged, and approximately 1 ml of fixative (methanol:glacial acetic acid, 3:1) was slowly added to the pellet. Cells were washed a total of 3 times with fixative, and single drops of cell suspension were air dried onto wet microscope slides. Differential staining of the chromatids was performed using the method of Goto et al. (34).

Determination of Cell Cycle Kinetics. An analysis of the cell cycle kinetics for each cell type was made to determine the time at which each culture should be fixed in order to analyze metaphases that were in G1 at the time of irradiation. To obtain the cell cycle kinetic data shown in Chart 1, 10 μM BrdUrd was added at the time the cultures were established, and the ML-1 cells were fixed 4, 6, 8, 10, 12, 18, 24, or 30 h later. The normal bone marrow cells were fixed 6, 12, 18, 24, 30, 36, 42, or 54 h later. The cell cycle kinetic data indicate that, at the following fixation times, 90% or more of the metaphases were in G1 when the BrdUrd was added: 10–14 h after initiation for the ML-1 cells and 12–25 h after initiation for the bone marrow cells.

To determine the time of irradiation which would allow both cell types an equivalent length of time for repair of DNA damage before entering the S phase, the position of the G1–S border in the cell cycle was determined as shown in Chart 2. The ML-1 cells are a continuous culture, and the data from the experiment described above are replotted in Chart 2 to show the percentages of M1 and M1S cells. Because the normal bone marrow cells are not a continuous culture, the following protocol was used to determine the time after irradiation that cells reach the G1–S border, for those G1 cells which reach mitosis at 24 h. Normal bone marrow cells were fixed 24 h after being established in culture, and BrdUrd was added 4, 6, 8, 10, 12, 18, or 24 h prior to fixation. That is, cells were in culture 20, 18, 16, 14, 12, 6, or 0 h before BrdUrd was added.

Slides were prepared and differentially stained as previously described. At each time point, a minimum of 100 metaphases was analyzed to determine their cell cycle stage at the time of BrdUrd addition. Cells were classified in a similar manner as described by Tice et al. (35) into MO, M1S, M1, M2, M3, or greater, to indicate whether they were in G1, S, or G2, respectively, at the time BrdUrd was added.

X-irradiation. For the chromosome aberration, MI, and flow cytometry studies, cell cultures were incubated 12 h before being irradiated with 0.50, 1.0, 1.5, or 2.0 Gy of X-rays at 1 Gy/min. X-irradiations were performed with a 320-kVp Gemini industrial X-ray unit (TFI Corp., New Haven, CT) operated at 250 kV and 12 mA with 2.3 mm of aluminum filtration.

Chromosome Aberration Study. Cultures were fixed 12 and 14 h after X-irradiation for the analysis of chromosome aberrations. Slides were prepared and differentially stained as described previously. One hundred metaphases with the M1 staining pattern were analyzed for each X-ray dose, and all classes of aberrations were recorded. The classes of aberrations were reported as: chromosome-type aberrations which include dicentrics; rings; translocations; and deletions (both terminal and interstitial deletions). Chromatid-type aberrations include all types of deletions and exchanges. Acentric fragments associated with NUPD isochromatid deletions were included in the terminal deletion category.

MI Determination. Giemsa-stained slides were prepared for analysis of the MI every 2 h from 0–14 h following irradiation with 0.5, 1.0, 1.5, or 2.0 Gy. For each sample, 2000 cells were counted, and the MI was expressed as a percentage of the control.

Flow Cytometry Study. A minimum of 1 × 10⁶ cells was fixed every 2 h from 0–14 h postirradiation with 0.5, 1.0, 1.5, or 2.0 Gy for the ML-1 cells and 2.0 Gy for the normal cells, for analysis by flow cytometry. The samples were prepared for analysis by the technique described by Crissman et al. (36). Basically, each sample was resuspended in 2 ml of
Cells were stained with propidium iodide (50 μg/ml) for 10 min. Samples cell sample, and solutions were left at room temperature for 30 min. Cells were centrifuged for 10 min at 1500 rpm and then resuspended in saline:EDTA at room temperature, and this solution was then added to treatment and replication is equivalent (39). To this end, an differences in sensitivity to the induction of chromosome aber

Charts 1 and 2. The dose-response curves for combined chromosome-type and chromatid-type aberrations are shown in

Table 1 shows that the population of dividing normal bone marrow cells analyzed in these experiments has a cell cycle time of 20 h, and the ML-1 cells have a shorter cell cycle time of 15 h. In order to irradiate both cell types in G1 and allow each the same length of time for repair of damage before entering the S phase, a determination was made of the average time that it took cells to pass from the G1-S border to metaphase as described in “Materials and Methods.” Chart 2 shows that the difference in the cell cycle times can be attributed primarily to the longer G1 of the normal bone marrow cells. This finding is in agreement with other reports of cells with different cell cycle times (40). From Chart 2 it can be seen that treatment of both cell types 12 or 14 h before fixation would yield cells in mitosis that were from the same region in G1, approximately 6 or 8 h, respectively, before the midpoint of S.

The frequencies of the different types of aberrations are given in Tables 1 and 2. The dose-response curves for combined chromosome-type and chromatid-type aberrations are shown in Chart 3. Both cell types showed a dose-dependent increase in chromosome-type aberrations, with the ML-1 cells showing a significantly greater sensitivity to the induction of chromosome-type aberrations at all X-ray doses and at both 12- and 14-h times points (Chart 3). There was a 2-fold or greater increase in terminal deletions in the ML-1 cells at all doses and time points compared to the normal cells (Tables 1 and 2). Many of these terminal deletions could be isochromatid NUPD deletions which are indistinguishable from chromosome-type terminal deletions. The terminal deletions contribute the most to the observed increase in the chromosome-type aberrations for the ML-1 cells, but there was a significant increase in the frequency of dicentrics at the 14-h fixation following 2.0 Gy of irradiation (0.44 for the ML-1 cells versus 0.23 for the normal cells). The ML-1 cells, in contrast to the normal cells, also had a significant number of chromatid-type aberrations (deletions and exchanges). The true frequency of chromatid-type aberrations is probably higher, because isochromatid NUPD deletions were classified as chromosome-type terminal deletions as described in “Materials and Methods.” The presence of many chromatid-type aberrations in ML-1 cells irradiated in G1 indicates that the cells are progressing into the S phase with unrepaired DNA damage that is then being converted into chromatid-type aberrations. Since both cell types were treated at the same time prior to entering S, the presence of only chromosome-type aberrations in the normal cells indicates: (a) that they are delayed in G1 where misrepair of the X-ray-induced damage produces chromosome-type aberrations and/or (b) that the normal cells have different DNA repair kinetics compared to the ML-1 cells. To investigate the first possibility, an analysis was made of the X-ray-induced perturbations of the cell cycle.

Mitotic Delay. Chart 4 shows the results of the MI analysis. Both cell types had a similar delay in the progression of cells into mitosis following irradiation, and this delay was more pronounced with increasing X-ray dose. For example, 2 h after irradiation of the ML-1 cells, the MI decreased from 54% of the control at 0.50 Gy to 25% at 1.0 Gy, 15% at 1.5 Gy, and finally 0% at 2.0 Gy. With time, the MI of both cell types recovered to control levels, and except for the normal cells at 1.5 Gy, the MI exceeded the 100% level due to the temporarily delayed cells progressing through mitosis in a synchronous wave.

Flow Cytometry. Flow cytometry was used to further investi-
X-RAY SENSITIVITIES OF LEUKEMIC AND NORMAL CELLS

Chart 3. Aberration frequencies per cell following 0.5, 1.0, 1.5, or 2.0 Gy of X-ray. A and B present the results from the 12-h fixation time, and C and D present the results from the 14-h fixation time. In A and C, the chromosome-type aberrations are shown; in B and D, the chromatid-type aberrations are shown for the normal cells (•) and the ML-1 cells (o). Points, mean; bars, SE.

Chart 4. MI as percentage of control. MI in A is after 0.5 Gy, B is after 1.0 Gy, C is after 1.5 Gy, and D is after 2.0 Gy of X-ray for the normal cells (•) and ML-1 cells (o).

Chart 5. Representative DNA histograms showing cell cycle distributions of ML-1 cells 2, 4, 6, 9, and 12 h after exposure to 0.5, 1.0, 1.5, or 2.0 Gy of X-ray. The left peak contains G1 cells, the right peak contains G2-M cells, and the shaded region contains the S-phase cells. For each individual histogram, the ordinate is the frequency of cells, and the abscissa is the fluorescence.

Gate the X-ray-induced perturbations of the cell cycle. Chart 5 shows the DNA histograms for the ML-1 cells following X-irradiation. The cell cycle phase distributions from these histograms were computer analyzed, and for comparative purposes, the control, 0.5-, and 2.0-Gy data are graphed in Chart 6. In Chart 5, very little perturbation of the cell cycle is seen at the lowest dose of 0.5 Gy, but at increasing doses, there was a rise in the G2-M peak which returned to normal at later times (data not shown). The MI at these time points was very low (Chart 4); therefore the increase in the G2-M peak is caused by G2 cells which are being delayed in progressing into mitosis. Thus the flow cytometry data confirm the MI data, showing a G2 delay in the ML-1 cells following irradiation. The continued progression of G1 cells into S, together with the inhibition of the progression of G2-M cells through mitosis and back into G1, resulted in a decrease in the proportion of cells in G1. For example, 6 h after irradiation with 2.0 Gy, the percentage of cells in G1 was decreased to 28% of the total population (Chart 6). As cells continue through the cell cycle, there are fewer G1 cells moving into the S phase, resulting in a decrease in the proportion of S-phase cells with time (Chart 6). Chart 5 shows that, as expected, the decrease in the proportion of S-phase cells begins with the late-G1-early S-phase cells. This is represented in the histogram by the "dip" in the population of cells in the left-hand region of the shaded S-phase area. As this population of cells passes through the S phase, there is a subsequent decrease in the proportion of cells in late S phase of the cell cycle. These results along with
the aberration data indicate that the ML-1 cells are progressing without delay into the S phase with unrepaired DNA damage where misrepair of this damage can lead to chromatid-type aberrations.

Charts 7 and 8 show the DNA histograms and cell cycle phase distributions, respectively, for the normal bone marrow cells following irradiation with 2.0 Gy. The X-ray-induced perturbation of the cell cycle is not as dramatic in the normal bone marrow cells as it was in the ML-1 cells. The G₂ delay indicated by the M₁ data is not evident in these DNA histograms (Chart 7). With the normal bone marrow cell cycle time being 20 h, only 2–3% of the cells occupy the G₂-M region, and the X-ray-induced G₁- and S-phase delays, discussed later, caused fewer cells to progress into the G₂-M phase. Therefore, any X-ray-induced G₂ delay would cause only a very small change in the G₂-M peak, and this will not be observed in the DNA histograms.

In contrast to the ML-1 cells, which show a decrease in the proportion of S-phase cells after irradiation, the proportion of normal cells in the S phase increased with time after irradiation, indicating that the cells are delayed in their progression through this phase. From Chart 7, it appears that the accumulation of late G₁-early S cells is contributing the most to the overall increase in the proportion of S-phase cells seen in Chart 8. These results along with the aberration data indicate that the normal bone marrow cells are delayed in G₁ and early S, where misrepair of the X-ray-induced damage can lead to chromosome-type aberrations.

**DISCUSSION**

ML-1 cells, established from an X-ray-induced myeloid leukemia in an RFM mouse, showed an increased sensitivity to X-ray-induced chromosome aberrations compared to *in vitro* normal mouse bone marrow cells. Specifically, G₁ irradiated ML-1 cells had twice as many chromosome-type aberrations as did normal cells, many of which were in the terminal deletion category. ML-1 cells also had a significant number of chromatid-type aberrations, and the true frequency is probably higher since isochromatid NUPD deletions are included in the terminal deletion category. This is analogous to X-irradiated G₁ AT cells, which are reported to have chromatid-type aberrations and a high frequency of fragments reported as terminal deletions (3, 4, 25, 41, 42).

Normal cells after treatment with a DNA-damaging agent have been shown to undergo an inhibition of DNA synthesis (22-24, 43) and G₁- and S-phase delays (25-27, 44, 45). RB cells, which do not have a greater sensitivity to X-ray-induced chromosome aberrations and may or may not be sensitive to killing by X-rays (25, 46), also show a radiation-induced delay in the initiation of DNA synthesis and a G₁ block (25). In contrast, AT cells do not show the same radiation-induced inhibition of DNA synthesis or G₁- and S-phase delays (22-27). Painter and Young (22) proposed that cell cycle delays may serve as a protective mechanism, which allows normal cells or cells with normal radiosensitivity like RB cells additional time to repair DNA damage, and that AT cells have increased radiosensitivity because they fail to undergo normal X-ray-induced cell cycle delays. However, this
is not consistent with the increased S-phase delay in the HN2-sensitive TK6 cell line (29) or the similar X-ray-induced delay in DNA synthesis in the mouse L5178Y cells (20), suggesting that there may be different mechanisms for the increased cellular sensitivity of these cells.

The results obtained by flow cytometry with the ML-1 and normal bone marrow cells following irradiation indicate that the normal cells are delayed in G1 as well as S while the ML-1 cells do not have the same G1 and S delays. These results explain the presence of chromatid-type aberrations in the ML-1 cells and not the normal cells by showing that the normal cells undergo a G1- and S-phase delay following irradiation, which allows them extra time in G1 where misrepair of the X-ray-induced damage leads to chromosome-type aberrations. The ML-1 cells, like AT cells, progress into the S phase with unrepaired DNA damage where misrepair of that DNA damage leads to chromatid-type aberrations. There is the possibility that a repair defect exists in the ML-1 cells, which would result in more DNA damage remaining when the cell enters the S phase, thus contributing to the formation of chromatid-type aberrations and the overall increased sensitivity observed for these cells. Additional experiments are in progress to analyze DNA repair kinetics for the ML-1 and normal cells.

There are conflicting reports concerning the relationship between a G2 delay and cellular sensitivity. X-ray-sensitive L5178Y sublines have a greater X-ray-induced G2 delay than the parent line (18, 19). FA cells after mitomycin C treatment have a greater G2 delay than normal cells (47), but HN2-treated FA cells have less G2 delay (28). AT fibroblasts have been shown to have less X-ray-induced mitotic delay (46, 48), while AT lymphoblastoid cells do not show this decreased G2 delay (27, 49). The ML-1 and normal cells have a similar dose-dependent delay in the progression of cells from G2 into mitosis, and the ML-1 recovered in about the same time after irradiation for both cell types.

We describe here a mouse myeloid leukemia cell line, ML-1, which has an increased sensitivity to X-ray-induced chromosome- and chromatid-type aberrations and decreased X-ray-induced cell cycle delays compared to normal mouse bone marrow cells. ML-1 cells, in response to radiation, have characteristics similar to irradiated AT cells, suggesting that these cells may have similar defects contributing to their increased radiosensitivities. In addition, it would be expected that these differences in aberration induction between the ML-1 and normal cells would be paralleled by differences in cell killing, as for AT cells versus normal human fibroblasts, since the majority of aberrations observed are cell lethal. It is also expected that there would be similar differences in sensitivity between the two cell types to the induction and transmission of non-cell-lethal aberrations. It is proposed to determine from banded chromosome preparations if there is a difference in the frequency of transmission of aberrations to subsequent cell generations in the ML-1 and normal bone marrow cells. Experiments are also in progress to establish whether other tumor cells have similar differences in X-ray sensitivity, cell cycle progression, and repair abilities.

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