Induction of Chromosome Damage by Ultraviolet Light and Caffeine: Correlation of Cytogenetic Evaluation and Flow Karyotype

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Received for publication September 9, 1981; accepted October 23, 1981

Asynchronously growing cells of a M3-1 Chinese hamster line were ultraviolet (UV) irradiated (λ = 254 nm) with UV fluences up to 7.5 J/m². After irradiation, cells were incubated with or without 2 mM caffeine for 20 hr, then mitotic cells were selected by mechanical shaking. Their chromosomes were isolated, stained with Hoechst 33258 and chromomycin A3, and measured flow cytometrically. While the fluorescence distributions of chromosomes (flow karyotypes) from cells treated with UV alone or with caffeine alone were very similar to those of untreated controls, the flow karyotypes of UV + caffeine-treated cells showed a debris continuum that increased with increasing UV fluence suggesting an increased number of chromosome fragments. Visual evaluation of metaphase plates revealed that the percentage of cells with chromosome damage also increased steadily with increasing UV fluence. A high degree of correlation was observed between the relative magnitude of the debris level from flow karyotypes and the percentage of cells with chromosome damage and with generalized chromosome shattering, respectively, as determined from metaphase spreads.

Key terms: Flow cytometry, chromosomes, ultraviolet light, caffeine, chromosome damage

Recently, flow cytometry (12, 17) has been shown to be useful in chromosome classification (1, 2, 10, 11, 15, 22). In this approach, mitotic cells are collected and mechanically sheared to release the chromosomes. These chromosomes are then stained with a DNA specific fluorescent dye and analyzed flow cytometrically to produce a fluorescence distribution pattern showing peaks caused by chromosomes with the same stain fluorescence being superimposed on a continuum that is caused by chromosomes or chromosome fragments with abnormal DNA content. This debris continuum declines rapidly with increasing DNA content and its magnitude has been shown (3) to correlate with chromosomal damage caused by clastogenic agents.

In the present paper, we apply this method to investigate the flow karyotype of Chinese hamster cells after treatment with ultraviolet (UV) light and caffeine. In rodent cells and in a number of other cell types, the clastogenic action of ultraviolet light is known to be synergistically enhanced by caffeine (for review see [14]). An interesting characteristic of Chinese hamster cells is the frequent occurrence of cells with generalized chromosome shattering (GCS) in which all chromosomes are fragmented and/or pulverized (4–8, 13, 14, 16, 19). The incidence of GCS after UV plus caffeine has been studied in detail by microscopic analysis of metaphase spreads (5–7).

In this paper, we demonstrate that the magnitude of the debris in flow karyotypes correlates well with the frequency of cells with GCS and with chromosome damage. These data support the use of flow karyotyping as a quantitative tool for the study of chromosome damage by other clastogenic agents.

Materials and Methods

Cell culture: The present studies were conducted using a derivative (650 A) of the M3-1 Chinese hamster cell line (10). These cells have a well defined flow karyotype as established cytologically and by univariate (10) and bivariate flow cytometry (11, 15). The cell cultures were maintained under standard conditions in minimum essential medium (MEM) (Gibco Grand Island, NY) plus 10% fetal calf serum. The cell cycle transit time of asynchronously growing cells was determined to be approximately 11 hr (9).

UV + caffeine treatment: Asynchronously growing cells were inoculated into large (10 cm diameter) or small (5 cm diameter) plastic Petri dishes at 2 x 10⁶ cells/cm². Two days later, the cells were...
washed once with phosphate buffered saline solution (PBS) and then covered with 1 ml of PBS. After this, the cells were irradiated from above with a germicidal lamp emitting predominantly at 254 nm (fluence rate 0.12 W/m²). Other cultures were treated identically except that they received no irradiation. Immediately after the UV treatment, the cells were incubated at 37°C in MEM with 10% fetal calf serum with or without caffeine (2 mM). Fifteen hours later, Colcemid (Sigma Chem. Co., St. Louis, MO) (0.033 µg/ml) was added.

To investigate the possibility that these chromosome fragments were due to the mechanical fragmentation of abnormally fragile chromosomes during chromosome isolation, chromosome suspensions obtained from cells homogenized for different times T (20-200 sec) were examined. The time of homogenization was found to have little effect: chromosomes released from UV plus caffeine treated cells appeared fragmented after 20 sec homogenization time (shorter times were not investigated since chromosomes were not released from the cells at shorter times). On the other hand, normal chromosomes were obtained even at T = 200 sec from cells treated with UV alone or caffeine alone. This showed that if chromosomes are mechanically fractured during chromosome isolation, the fragmentation is complete even after the lightest possible shearing. For the flow cytometric experiments described in this paper, a homogenization time of 60 sec was used.

Flow cytometry: The Hoechst fluorescence distributions (flow karyotypes) of the Hoechst-Chromomycin A3 stained chromosome suspensions were measured with the Livermore Flow Cytometer (single beam flow cytometer) (Lawrence Livermore Nat. Lab., Livermore CA) utilizing a Spectra Physics argon-ion-laser (171-05) operating at 351.1 plus 363.8 nm (referred to as UV excitation) with a power of 0.6 W. Fluorescence was collected through a 450-nm-long wavelength pass filter. Typical flow karyotypes showed several peaks superimposed on a debris continuum (Fig. 1). The relative area C of the continuum underlying the peaks in each flow karyotype (C = "debris level") was estimated as indicated in Figure 1. This procedure was chosen because the grossly distorted flow karyotypes obtained after the combined treatment with UV + caffeine (see "Results") could not be fitted in a satisfying way using the normal least squares best-fit techniques (11,18) which approximate the fluorescence distribution as the sum of several normal distributions plus a smooth "continuum" function.

Chromosome preparation for cytogenetic analysis

The cells in the small Petri dishes were exposed for 30 min to hypotonic solution (0.075 M KCl). Then fixative (acetic acid: methanol 1:3) was slowly added with a syringe, the hypotonic solution was gradually replaced by adding fixative. The preparations were air dried and stained with acetic orcein. Microscopic scoring of cells was performed as described (5-7). Briefly, all cells with condensed, chromosome-like chromatin were scored as mitotic. These cells were divided into three classes: normal mitotic cells, cells with some chromosome abnormalities but at least one normal chromosome, and cells with GCS. The percentages of mitotic cells in each class were designated "mitotic", "aberrant" and "GCS", respectively. For each treatment, 200 mitotic cells were scored.

Results

The increase in the frequency of mitotic cells showing GCS and other aberrations with increasing UV exposure is shown in Figure 2. The percentage of mitotic cells with GCS was very low after caffeine treatment alone. However, it increased drastically to almost 90% as the UV fluence increased to 7.5 J/m². At this dose, the percentage of mitotic cells with GCS...
Effects of UV plus caffeine treatment on the debris level of Hoechst 33258 flow karyotypes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Debris Level (%)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV, no caffeine</td>
<td>47.1 ± 4</td>
<td>4</td>
</tr>
<tr>
<td>No UV, 2 mM caffeine</td>
<td>49.1 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>5.0 J/m², no caffeine</td>
<td>49.0 ± 6</td>
<td>3</td>
</tr>
<tr>
<td>7.5 J/m², no caffeine</td>
<td>57.7 ± 3</td>
<td>4</td>
</tr>
<tr>
<td>5.0 J/m², 2 mM caffeine</td>
<td>88.6 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>7.5 J/m², 2 mM caffeine</td>
<td>91.9 ± 7</td>
<td>4</td>
</tr>
</tbody>
</table>

M3-1 cells were UV irradiated and post-treated with/without caffeine for 20 hr as indicated. In flow karyotypes, the “debris level” (given as a percent) as defined in Figure 1 was determined. Given is the mean ± range; n, number of independent experiments.

or other aberrations was almost 100%. UV irradiation alone, however, did not induce GCS (≤1% aberrant ≤5%) in the dose range investigated. These results are similar to those described for Chinese hamster V79 cells (5, 6).

Other experiments (Cremer and Gray, Mutat. Res., in press) showed that the percentages of mitotic cells with GCS in cultures prior to shake-off and in chromosome preparations made from cell suspensions selected by the mitotic shake-off procedure were practically identical. Thus, the mitotic selection technique employed during the chromosome isolation procedure did not potentially select for or against cells with GCS.

Figure 3 shows the effect of UV + caffeine treatment on the flow karyotype. The flow karyotypes of cells exposed to UV irradiation alone (Fig. 3c) and of cells treated only with caffeine (Fig. 3b) are very similar to those for untreated control cells (Fig. 3a). However, the debris continuum in the flow karyotype of UV irradiated cells (5 J/m²) postincubated with caffeine is increased so drastically (Fig. 3d) that no clear chromosome peaks are obtained. Table 1 gives a quantitative evaluation of the “debris level” of flow karyotypes after treatment with 5.0 and 7.5 J/m² and posttreatment with and without caffeine, respectively. To examine the reproducibility, a number of independent experiments was carried out and isolation and flow cytometric measurements were performed on different days. The results show that the “debris level” is not or is only slightly enhanced compared with controls (no UV, no caffeine) after UV treatment alone or caffeine treatment alone while it is drastically increased after the combined treatment. For a given treatment, the relative variation in the “debris level” was observed to be ≤10%. This variation includes differences in preparation quality or optical alignment of the flow cytometer. Figure 4 shows the relative debris level in the flow karyotypes (defined to be the ratio of the “debris level” (C) for sample flow karyotypes (Fig. 1) to the “debris level” of the flow karyotypes for untreated controls) as a function of UV fluence (J/m²).
function of the UV fluence for cells treated with UV and postincubated with or without caffeine.

While no marked increase of the relative debris level was observed following UV irradiation alone or caffeine treatment alone, the relative debris level was increased strongly with UV fluence after the combined treatment.

In Figure 5A the relative debris level following UV plus caffeine treatment is compared to the percentage of cells with GCS as obtained by microscopic examination of chromosome preparations of cells after an identical treatment. The two measurements are linearly correlated relative debris level: = \(1.055 \times 10^{-2} \times \%\) GCS + 1.081; correlation coefficient 0.98. Very similar results are obtained if the relative debris level is plotted as a function of the percentage of all cells with chromosome damage (% GCS + % aberrant; Figure 5B). In this case, the linear regression curve was found to be: relative debris level: = \(0.97 \times 10^{-2} \times \%\) GCS + % aberrant + 1.035; correlation coefficient 0.98. If only cells with aberrations other than GCS (% aberrant) were considered, the degree of correlation was observed to be considerably lower (correlation coefficient 0.82). This might be ascribed to the fact that under the conditions investigated, GCS was the predominant event (Fig. 2).

Discussion

The results presented here show that UV irradiation (up to 7.5 J/m²) of Chinese hamster M3-1 cells and posttreatment with caffeine (2 mM) for an appropriate time (20 hr) results in a drastic change of the flow karyotype while no marked alterations were observed following UV irradiation alone or caffeine treatment alone. The relative debris level underlying the flow karyotype of caffeine treated cells increased steadily with UV dose suggesting an increasing number of mitotic cells with abnormal chromosomes (3). Microscopic examination of chromosome suspensions revealed a high frequency of chromosome fragments.

These results do not allow determination of whether the effects observed are due to a high number of chromosome breaks existing prior to chromosome isolation or to an increased chromosomal fragility. They do show, however, that if an increased chromosome fragility is the predominant factor, the chromosomes are so fragile that the disruption is complete after the minimum shearing time required to release normal chromosomes. Both possibilities are consistent with models for the induction of chromosome damage or GCS, by ultraviolet light and caffeine (7, 8, 14, 21). The high degree of correlation observed between the flow karyotype debris level and the percentage of cells with chromosome damage and with GCS following UV plus caffeine treatment suggests that flow cytometry might be used as a semiquantitative tool for rapid monitoring of effects of UV plus caffeine treatment. Since a flow cytometric measurement can be performed within a small fraction of the time required for conventional cytogenetic analysis, this might facilitate investigations on the action of ultraviolet light (or other mutagens) and caffeine on chromosomes.

Acknowledgments

The authors thank Dr. Marvin Van Dilla for helpful discussions, Dr. Frank Dolbeare and Karen Glickmann for providing the cell cultures, and John Poco and Mary Look for assistance in flow cytometry.

Literature Cited