Cell Cycle Dependence of Sister Chromatid Exchange Induction by DNA Topoisomerase II Inhibitors in Chinese Hamster V79 Cells

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ABSTRACT

The cell cycle dependence of sister chromatid exchange (SCE) induced by topoisomerase II inhibitors was studied in Chinese hamster V79 cells. 4'-[9-Acridinylamino]methanesulfon-m-anisidine, which increases the concentration of covalently linked DNA-topoisomerase II complexes (cleavable complexes), induces SCE strongly in only a short period of the cell cycle. The sensitive period was identified as occurring in early to mid-S phase through the use of labeled thymidine incorporation and flow cytometry. Novobiocin, an inhibitor which prevents formation of the cleavable complex, did not induce SCEs in any part of the cell cycle. However, novobiocin did decrease the level of 4'-[9-acridinylamino]methanesulfon-m-anisidine-induced SCEs. These results indicate that the cleavable complex may be important in 4'-[9-acridinylamino]methanesulfon-m-anisidine-induced SCE.

INTRODUCTION

Although SCE3 is widely used as a measure of genetic toxicity, the mechanism(s) by which DNA lesions result in SCE is not known. Several models of SCE formation (1-3) have postulated the involvement of DNA topoisomerases, enzymes which alter the topology of DNA. Lim et al.4 measured SCEs produced by two inhibitors of DNA topoisomerase II, VM26 and mAMSA, and an inhibitor of DNA topoisomerase I, camptothecin (1-h treatments at 10^{-6} M followed by 2 cell cycles in BrdUrd-containing medium). With all 3 of these agents, there was a wide distribution in the number of SCEs among the cells scored, compared to cells treated with a concentration of mitomycin C that produced approximately the same mean SCE/cell. Because of a distribution of cell cycling times within the population, the cells scored in these experiments may have been at different positions in the cell cycle at the time of treatment. Thus, the heterogeneity in the response to the topoisomerase inhibitors could be due to cell cycle variations in the sensitivities to these agents. These observations prompted us to investigate the cell cycle dependence for SCE induction by mAMSA.

MATERIALS AND METHODS

Cells. Chinese hamster V79 cells were grown in minimal essential medium (Gibco) with 10% fetal bovine serum (Dutchland-Hazelton) and penicillin-streptomycin (Gibco). The medium pH was maintained by incubation at 37°C in a 5% atmosphere except during treatments, when pH was maintained with 0.02 M HEPES added to the medium.

SCE Analysis. In the initial experiments, cells were treated in the second of 2 cell cycles in which BrdUrd was incorporated into DNA (Protocol A). Cells (4 x 10^6) were inoculated into 100-mm Petri dishes. The next day 10 μg/ml of BrdUrd were added. The dishes were wrapped in foil for protection from light. Treatments were performed at various times after BrdUrd addition for the periods indicated in the figure legends. The medium was replaced with HEPES-buffered medium, and the dishes were placed in a 37°C incubator for the treatment period for chemical treatments or irradiated immediately for γ-ray treatments. The same changes except for the chemical or irradiation were performed for mock treatments. The treatment medium was then removed, the dishes were washed once with HEPES-buffered medium, and warmed normal medium was added. Colcemid (0.2 μg/ml; Gibco) was added 22 h after BrdUrd addition. One hour later, cells were detached with trypsin-EDTA (Gibco), and mitotic spreads were prepared as described by Dearfield et al. (4) using the fluorescent DNA stain DAPI. SCE in 20 or 25 spreads were counted for each dish.

In later experiments treatments were performed on synchronous populations of cells which had incorporated BrdUrd in the previous cell cycle (Protocol B). Cells (3 x 10^5) were inoculated into 75-cm^2 flasks, and 10 μg/ml of BrdUrd were added the next day. Colcemid (0.2 μg/ml) was added 9 h after BrdUrd addition, and mitotic cells were detached by shaking 2 h after Colcemid addition. These cells were pelleted by centrifugation and resuspended in fresh medium, and aliquots were distributed to flasks with warm medium. Chemical treatments were carried out as previously described. Colcemid was added 9.5 h after replating of the cells, and mitotic cells were collected by shaking. Preparation of metaphase spreads was the same as in the previous procedure. Poor sister chromatid differentiation was obtained with DAPI staining in these cells which had incorporated BrdUrd in the first but not the second cell cycle. Therefore, a fluorescence plus Giemsa staining technique (5) was used to obtain sister chromatid differentiation. The method followed that of Schneider et al. (6) except that the slides were heated for 30 min at 60°C following the exposure to black light.

DNA Synthesis Rate. In some experiments using Protocol B, [3H]-thymidine (0.001 μCi/ml) was added for the first day after plating the cells. [3H]Thymidine (0.4 μCi/ml) was added for 30 min just prior to treatment (or mock treatment) with mAMSA. After collection of mitotic cells for SCE analysis and centrifugation, a sample of the resuspended pellet was counted by liquid scintillation. The relative rate of DNA synthesis in the samples was determined from the ratio of 3H to 14C cpm.

Flow Cytometry. Position of cell populations in the cell cycle was determined by flow cytometry. At various times cells were collected by trypsinization, the DNA was stained by the ethidium bromide-RNase method (7), and the cells were analyzed on a Becton, Dickinson fluorescence-activated cell sorter Model II.

RESULTS

In the first protocol (A) used to study the cell cycle dependence of mAMSA-induced SCE, cells were treated for 1-h periods at various times during the second cycle after BrdUrd addition. When cells were treated with 1000 nM mAMSA many mitotic spreads contained such extensive chromosomal damage that scoring for SCE was impossible. Fig. 1 shows that the percentage of severely damaged cells was highest for the 14- to 15- and 20- to 21-h treatment periods. At the concentrations used in subsequent experiments (100 or 150 nM), some chromosome aberrations were observed, but there were no severely damaged cells.

At 150 nM, mAMSA strongly induced SCEs only for the 14- to 15-h treatment period (Fig. 2). The results with mAMSA...
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contrast sharply with those for γ-rays (Fig. 3). Similar levels of SCE were observed for irradiations between 10 and 16 h, after which sensitivity decreased. Fig. 3 also shows the results for 1-h treatments with 0.33 mM novobiocin. Novobiocin inhibits DNA topoisomerase II (8), but by a different mechanism from that of mAMSA (see “Discussion”). At this concentration, novobiocin inhibited DNA synthesis by 56% in a 1-h treatment of asynchronous cells. The novobiocin treatments did not significantly increase (P > 0.10 by t test) SCE above background for any time period.

To better define the mAMSA-sensitive region of the cell cycle, a new protocol (B) was used in which [3H]thymidine incorporation was used to indicate the relationship of the treatment times to the S phase of the cell cycle. Cells were labeled with [14C]thymidine and then grown in BrdUrd-containing medium for approximately one generation time. Mitotic cells were obtained by shaking and plated in BrdUrd-free medium. At various times [3H]thymidine was added to the medium for 30 min. After the [3H]thymidine-containing medium was removed, cultures were treated with 100 nM mAMSA (or mock treated) for 1 h. Cells reaching the next mitosis were harvested by shaking. Half of the sample was used to determine SCE/cell, and the other half to determine 3H and 14C cpm. The [3H]-thymidine labeling was done prior to the mAMSA treatment, since flow cytometry measurements (see below) indicated that DNA synthesis is partially inhibited during the treatment. The amount of 3H incorporated will depend on both the fraction of cells in S phase during the labeling and the rate of DNA synthesis in these cells. The ratio of 3H to 14C in the samples should indicate the average rate at which DNA synthesis had been occurring just prior to the treatment time in the collected cells. Figs. 4 and 5 show the SCE and DNA synthesis results, respectively, of one experiment using this protocol. Similar results were obtained in a duplicate experiment. As with the previous protocol, the cells were most sensitive when treated between 7 and 8 h prior to harvest (4 to 5 h after replating). The DNA synthesis rates and total 14C counts were similar for the control and treated samples at each period. These results indicate that the mAMSA treatments did not greatly perturb the cycling of the cells. Comparison of the results in Figs. 4

Fig. 1. Percentages of cells with severely damaged chromosomes as a function of treatment time. Cells were treated with 1000 nM mAMSA at various times after BrdUrd addition. Each point is plotted as the midpoint of the 1-h treatment period.

Fig. 2. mAMSA-induced SCE as a function of treatment time. Cells were treated with 150 nM mAMSA (○) or mock treated (■) at various times after BrdUrd addition. Each point is plotted at the midpoint of the 1-h treatment period.

Fig. 3. γ-Ray- and novobiocin-induced SCE as a function of treatment time. Cells were irradiated (○) with 2 Gy of γ-rays or mock irradiated (■) at various times after BrdUrd. In the same experiment cells were treated with 0.33 mM novobiocin (△) for 1 h starting at the time indicated.

Fig. 4. mAMSA-induced SCE as a function of time after plating of mitotic cells. Cells were treated with 100 nM mAMSA (○) or mock treated (■) for 1 h. Each point is plotted at the midpoint of the 1-h treatment period.

Fig. 5. Average rate of DNA synthesis as a function of time after plating of mitotic cells. The DNA synthesis rate was measured by exposing cultures to [3H]thymidine medium for a 30-min period prior to 1-h mAMSA (○) or mock (■) treatments. Cells had been prelabeled with [14C]thymidine. At the time indicated cells were harvested by mitotic shaking, and SCE/cell determined from an aliquot (results in Fig. 5). The ratio of 3H to 14C cpm was determined in another aliquot. This ratio for each sample is plotted at the midpoint of the 30-min [3H]thymidine incubation period.
and 5 shows that the period of maximum sensitivity is prior to mid-S phase.

Flow cytometry was also used to define the sensitive period. Following replating after the first mitotic shaking, cultures were harvested by trypsinization at various times corresponding to the beginning of the treatment periods. DNA per cell was determined from ethidium bromide fluorescence by flow cytometry. This allowed the positions of cells in the cycle and the degree of synchrony at the treatment times to be measured. Fig. 6 shows a sample DNA histogram in which most of the cells are near the midpoint of S phase. A small peak is also seen at the position corresponding to a G₁ DNA content. This could either be due to some nonmitotic cells obtained during the shaking or to cells which stopped cycling after replating. Since a mitotic shaking was again used when the synchronous cells reached mitosis, the unsynchronous cells should not have contributed substantially to either the SCE or [³H]thymidine incorporation measurements. For various harvest times, the channel number corresponding to the maximum of the main peak was divided by the G₁ channel number. The resulting ratios are plotted in Fig. 7 versus harvest time for control cells and for cells which had grown in BrdUrd-containing medium for one cell cycle prior to mitotic shaking (same conditions as Protocol B). It can be seen that BrdUrd substitution causes a small delay in progression through S. Comparing these results with those of Fig. 4, it can be seen that the period of highest sensitivity for SCE induction by mAMSA occurs early in S and that, during the second half of S (beginning about 7 h after replating), the sensitivity is low.

Two different kinds of experiment suggested that novobiocin could partially suppress the mAMSA sensitivity (Table 1). Since cells are sensitive to mAMSA in only a short period of the cycle, it is possible that an apparent suppression by another agent could be due to perturbation of cycling, such that cells collected for the combined treatment were not at the sensitive stage during mAMSA treatment. However, with the conditions used in these experiments, we do not think this is a likely explanation of the novobiocin effect. In the first experiment, the mAMSA treatment time was increased to 2 h to ensure that more of the cells would pass through the sensitive phase during the treatment. That this actually was the case is suggested by the observation that the distribution of SCE per cell was not as wide as for the 1-h treatments. In the second experiment, only 1-h treatments were used, but the flow cytometer was used to monitor cell cycle progression. These measurements indicated that the novobiocin treatment slowed the rate of progression through S about 10% compared to mAMSA alone. This delay is unlikely to result in the observed suppression of mAMSA-induced SCEs.

**DISCUSSION**

The functioning or misfunctioning of DNA topoisomerases has been suggested to play a role in several models of SCE formation. Painter (1) postulated that an SCE could form at the junction of a replicated replicon cluster and an adjacent replicon cluster which had not yet completed replication, and that DNA topoisomerase II was involved. Cleaver (2) postulated that SCE occur within replicons between the diverging replication forks and result from faulty unraveling by DNA topoisomerase II of the intertwined daughter double helical strands. Pommier et al. (3) have refined this later model by postulating that the SCE results from exchange of topoisomerase II subunits while they are covalently bound to adjacent double strands. During the normal functioning of topoisomerase II there is an intermediate state in which the topoisomerase is covalently linked to the DNA at the site of a double-strand break (9). In this so-called cleavable complex each of the two topoisomerase subunits is covalently linked to a 5'-phosphate at the break. Certain DNA intercalators (such as mAMSA) and nonintercalators (VM26 and VP16) inhibit topoisomerase II by trapping the enzyme in the complex (9). In the subunit exchange model, SCEs would result if (a) cleavable complexes formed on each of the newly replicated strands, (b) the topoisomerases dissociated into subunits, and (c) each subunit then reassociated with a subunit from the other double strand. Pommier et al. (3) compared two intercalators and found that the one which produced a larger increase in the cellular concentration of the cleavable complex also induced more SCE. However, they measured SCEs in cells that had been treated with the intercalators for 1 h and then allowed to replicate for two S phases in BrdUrd-containing medium. With this protocol, the cells in which SCE are scored would not be in S phase

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**Table 1** Suppression of mAMSA-induced SCE by novobiocin

<table>
<thead>
<tr>
<th>Experiment</th>
<th>SCE/cell</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>mAMSA</td>
<td>41</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>8</td>
</tr>
<tr>
<td>mAMSA and novobiocin</td>
<td>16</td>
</tr>
</tbody>
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* mAMSA, 100 nM, and 1-h treatment time.  
* mAMSA, 100 nM, and 1-h treatment time.
during the treatment period. Since they postulated that SCE occur in newly replicated DNA (S phase), it is not clear what the significance of the observed correlation is for their model.

Using two different methods, we found that cells were sensitive to SCE induction by mAMSA in the first half of S phase, but insensitive in the last half. mAMSA produces only a transient increase in the cleavable complex. The concentration of the cleavable complex (as measured by protein-associated DNA breaks) reaches a plateau about 15 min after mAMSA addition and returns to normal by 45 min after mAMSA removal in both V79 (3) and mouse LI210 (10) cells. If the subunit exchange model of SCE is correct, our results indicate that this process occurs much less in late-replicating DNA. This could possibly be due to an absence of topoisomerase II, inaccessibility to the DNA by topoisomerase II, or that the cleavable complexes are not formed, or some factor that prevents subunit exchange. It is unlikely that topoisomerase II is absent from the cell at this phase of the cell cycle. Tricoli et al. (11) observed no variation in the cell cycle for total cellular topoisomerase II in mouse embryo fibroblasts. It is possible that mAMSA may not be able to increase the concentration of the cleavable complex in newly replicated DNA in late S phase. Early replicating and late-replicating chromatin differ in their chromatin structure and function (12), with the former transcribing genes replicating early. Thus, genes early and late-replicating DNAs differ in their accessibilities by the transcriptional apparatus and they could differ in their accessibilities by topoisomerase II during replication. Nelson et al. (13) have found that cleavable complexes are concentrated on newly replicated DNA (compared to non-newly replicated DNA) following treatment with VM26. We are currently studying whether this concentration is uniform throughout S phase.

Some other studies indicate that DNA damage induced in late S phase will not result in SCE. Schwartzman and Guiterrez (14) reported that the SCE induced by visible light in BrdU-containing cells increase through G1, reach a maximum in early S, and are absent in late S. Latt and Loveday (15) observed that 8-methoxyxpsoralen and near-ultraviolet light and found that the SCE induction was at a maximum in early S and fell to near zero with about 20% of S remaining. An exception to the insensitivity of late S cells to SCE induction is the report by Hernandez and Gutierrez (16) that short treatments with caffeine induced the same level of SCE in early, mid-, or late-S-phase onion meristem cells, but not in G1 or G2.

In the topoisomerase II subunit exchange model of SCE, the failure of subunits to complete the exchange could result in a chromatid or chromosome break. Pomnier et al. (3) observed that 55% of the chromatid breaks with mAMSA coincided with an SCE. At 1000 nM mAMSA, we observed the highest percentage of severely damaged cells for the same treatment period which had the highest sensitivity to SCE at lower concentrations. There was also a high frequency of severely damaged cells for the treatment period just prior to mitotic collection. Deaven et al. (17) observed that Chinese hamster cells were most sensitive to chromosome damage by mAMSA at the G1-S border and in late S-G2. Our results suggest that, although there may be a connection between SCE and aberrations in early S, aberrations occurring late in the cell cycle are not associated with SCE.

Novobiocin inhibits topoisomerase II activity by interfering with its ATPase function (18). Marshall et al. (19) observed that novobiocin treatment of mouse mastocytoma cells did not produce double-strand DNA breaks, but did prevent breaks from being produced by mAMSA. Similarly, Yang et al. (8) found that novobiocin, which did not induce an increase in the cleavable complex, blocked the increase in the cleavable complex induced by VM26. Thus, our result showing that novobiocin can partially suppress the mAMSA-induced SCE lends support to the idea that the cleavable complex is important for SCE formation. Ishii and Bender (20) found novobiocin to be a weak inducer of SCE in Chinese hamster ovary cells when present for 2 cell cycles up to a concentration (0.158 mM) which prevented progression of cells to metaphase. We found that 1-h treatments at a higher concentration (0.33 mM) of novobiocin at various times in the cell cycle did not increase SCE above background. This observation further suggests that it is the formation of the cleavable complex, and not simply the inhibition of topoisomerase II activity, which favors SCE formation.

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