Investigation of Resistance to DNA Cross-Linking Agents in 9L Cell Lines with Different Sensitivities to Chloroethylnitrosoureas

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ABSTRACT

The 9L-2, 9L-7, and 9L-8 cell lines, derived from the 9L in vivo rat brain tumor, were treated with nitrosoureas that can alkylate and cross-link DNA and carbamoylate intracellular molecules to various extents. Compared to 9L cells, 9L-2 cells were very resistant to the cytotoxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea, and to 2-[(3-(2-chloroethyl)-3-nitrosoureido)]o-deoxyglucopyranose. The sensitivity of 9L-7 and 9L-8 cell lines to these drugs was intermediate between 9L and 9L-2. Treatment of 9L, 9L-2, 9L-7, and 9L-8 cell lines with 1,3-bis(trans-4-hydroxycyclohexyl)-1-nitrosourea produced approximately the same level of cell kill. Compared to 9L cells, 9L-2 cells are 10-fold more resistant to the cytotoxic effects, 34-fold more resistant to the induction of sister chromatid exchanges, and have 40% fewer DNA interstrand cross-links caused by treatment with 3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-(2-chloroethyl)-1-nitrosourea. In contrast, treatment of 9L and 9L-2 cells with 1-ethylnitrosourea produced approximately the same level of cell kill and induction of sister chromatid exchanges. Our results suggest that the resistance of 9L-2, 9L-7, and 9L-8 cells is related to DNA cross-linking and not to alkylation or carbamoylation.

We studied the effects of other agents that form DNA cross-links with structures different from those formed by treatment with chloroethylnitrosoureas (CENUs) in 9L and 9L-2 cells. In contrast to results obtained with CENUs, 9L-2 cells were 2-fold more sensitive to the cytotoxic effects, 2-fold more sensitive to the induction of sister chromatid exchanges, and had 3-fold more cross-links formed than 9L cells treated with nitrogen mustard. However, the amount of cell kill, number of sister chromatid exchanges induced, and the DNA cross-linking were the same for 9L and 9L-2 cells treated with cis-diaminedichloroplatinum(II).

Our results indicate that cellular resistance to CENUs is highly specific and that the mechanism of resistance does not allow cross-resistance with other DNA cross-linking agents. These and other results suggest that when DNA repair processes mediate cellular resistance to CENUs, other cross-linking agents will not be cross-resistant unless they form alkylation products that are affected by repair processes that mediate resistance to CENUs.

INTRODUCTION

Certain CENUs are hydrolyzed intracellularly to reactive intermediates that alkylate DNA, form DNA interstrand cross-links, and carbamoylate cellular proteins. Because all of these reactions may cause cellular cytotoxicity, but with different efficiencies, it is necessary to establish which of these events is of primary importance to cell killing in sensitive cells and which processes are modified by cells resistant to the cytotoxic effects of these agents. Therefore, in this study we treated 9L cells and lines derived from the in vivo 9L rat brain tumor by BCNU treatment (1) with CENUs and other nitrosoureas with different capacities to alkylate DNA, cross-link DNA, and carbamoylate intracellular molecules (see Table 1). We measured cell kill, SCE induction, and DNA cross-link formation in order to obtain a better understanding of the molecular mechanisms of cell killing and cellular resistance to CENUs.

Preliminary investigations have shown that 9L-2 cells are resistant to the cytotoxic effects of BCNU and to the induction of SCEs compared to 9L-cells treated with the same concentration of BCNU (2). However, from these studies we could not determine if the observed cellular resistance was specific for CENUs, or whether 9L-2 cells were also resistant to other DNA-cross-linking agents. Schabel et al. (3, 4) have performed extensive studies on resistance and patterns of cross-resistance in L1210 and P388 cell lines. Schabel’s results showed that L1210 and P388 cells resistant to BCNU were also resistant to other CENUs but retained the sensitivity of the parent line to phenylalanine mustard, cyclophosphamide, and cis-Pt (3, 4). Erickson et al. (5) have shown that human tumor cells resistant to BCNU are not cross-resistant to cis-Pt. Tew and Wang (6) have reported that Walker 256 rat carcinoma cells highly resistant to HN2 and related derivatives are not cross-resistant with CENUs. These results suggest that cellular resistance to DNA cross-linking agents is highly specific. The experiments reported here were performed to determine whether 9L-2 cells resistant to BCNU are cross-resistant to other DNA cross-linking agents.

MATERIALS AND METHODS

Cell Lines. The 9L rat gliosarcoma is a well-established cell line (7). Sublines that are resistant to BCNU were produced by the following procedure. Fisher 344 rats bearing the intracerebral 9L tumor were treated with a single dose of BCNU, either 13.3 or 26.7 mg/kg. Twenty-four h after the dose was administered, rats were sacrificed, and tumors...
Chromosomes were spread on glass microscope slides. The method of fixation involved twice with glacial acetic acid and methanol (1:3), and metaphase cells were collected by centrifugation (1000 rpm for 5 min). The following day, cells were treated with various concentrations of the drug, followed by medium replacement or without a medium change (“continuous treatment”). Cultures were then incubated in a 5% CO2:95% air atmosphere for 1 h followed by drug-free medium at 37°C for an additional 6 h to allow for the formation of DNA interstrand cross-links and then collected for alkaline elution. The CFE- and cis-Pt-treated cells were incubated in drug-free medium at 37°C for an additional 6 h to allow for the formation of DNA interstrand cross-links and then collected for alkaline elution.

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>CFE assay</th>
<th>SCE assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>9L and 9L-2</td>
<td>BCNU</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
<tr>
<td>9L-8</td>
<td>CHLZ</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
<tr>
<td>9L-7</td>
<td>BHCNU</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
<tr>
<td>9L-2</td>
<td>ACNU</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
<tr>
<td>9L-7</td>
<td>ENU</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
<tr>
<td>9L-8</td>
<td>HN2</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
<tr>
<td>9L-2</td>
<td>cis-Pt</td>
<td>60-80%</td>
<td>50-100%</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, references for each agent.

RESULTS

Survival curves for 9L, 9L-2, 9L-7, and 9L-8 cells treated with BCNU and CHLZ are shown in Chart 1A and B. At a concentration of 30 μM BCNU and CHLZ produced a 2-log cell kill in 9L cells. In contrast, treatment of 9L-2 cells with 70 μM BCNU produced only a 1-log cell kill, and treatment with 70 μM CHLZ produced very little cell kill. Treatment with BCNU produced the same intermediate levels of cell kill in 9L-7 and 9L-8 cell lines (Chart 1A). For CHLZ, the levels of cell kill for 9L-7 and 9L-8 cells were intermediate between that for 9L and 9L-2; 9L-7 cells were somewhat more resistant than were 9L-8 cells (Chart 1B). All cell lines were equally susceptible to the cytotoxic effects of BHCNU, however (Chart 1C).

Survival curves for 9L and 9L-2 cells treated with ACNU are shown in Chart 2. 9L-2 cells were very resistant to the cytotoxic effects of ACNU; at a 1 log of cell kill, 9L-2 cells were 10-fold more resistant than 9L cells. ACNU induced SCEs in 9L cells; the dose-response curve is linear with a slope of 15.8 SCEs/metaphase/μM ACNU (all slopes were determined by linear regression analysis) (Chart 2). Treatment of 9L-2 cells with ACNU over the same dose range induced very few SCEs; the slope of the SCE dose-response curve is 0.46 SCEs/metaphase/μM ACNU. Therefore, calculated as the ratio of the slopes for induction of SCEs, 9L-2 cells were 34-fold more resistant to the induction of SCEs caused by treatment with ACNU than were 9L cells. Comparison of the cross-linking index for 9L and 9L-2 cells treated with ACNU shows that there were on the average 40% fewer proteinase K-resistant DNA interstrand cross-links formed in 9L-2 than in 9L cells treated with ACNU (Table 2).

9L and 9L-2 cells were equally sensitive to the cytotoxic effects of [14C]-thymidine, 0.01 μCi/ml (60 mCi/mmol) or [3H]thymidine, 0.1 μCi/ml (82.7 Ci/mmol). One h before drug treatment, medium containing labeled compounds was removed and replaced with fresh medium. 14C-labeled cells were treated with various concentrations of ACNU, cis-Pt, or HN2 for 1 h at 37°C. After treatment, the drug-containing medium was removed. The 1-h treatment period is sufficient for formation of the HN2 DNA cross-links; therefore, after the treatment period, HN2-treated cells were placed in cold Hanks’ balanced salt solution and collected for alkaline elution. The ACNU- and cis-Pt-treated cells were incubated in drug-free medium at 37°C for an additional 6 h to allow for the formation of DNA interstrand cross-links and then collected for alkaline elution. HN2-labeled cells were irradiated with 300 rads, and the 14C-labeled cells were irradiated with 600 rads of X-rays (General Electric Maxitron 300). Comparison of the elution pattern of 14C-labeled cells with control 14C-labeled cells provides an internal control to assure that DNA is eluting off the filter as a function of molecular weight. The elution procedure used as a modification of that described by Kohn et al. (10), and a detailed procedure has been described (2). The general procedure is as follows. Cells were collected on the filters, lysed, and treated with proteinase K for 1 h. DNA is eluted from the filters overnight. Results were calculated as the fraction of [14C]DNA remaining on the filter when 50% of the [3H]DNA remained on the filter.

\[
\left(\frac{1 - R_0}{1 - R_1}\right)^{1/2} - 1
\]

where \( R_0 \) and \( R_1 \) are the relative retention for untreated and treated cells, respectively. Relative retention was defined as the fraction of the [14C]DNA remaining on the filter when 50% of the [3H]DNA remained on the filter.

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Chart 1. The survival of 9L (○), 9L-2 (▲), 9L-7 (■), or 9L-8 (◆) cells after continuous treatment with BCNU (A), CHLZ (B), or BHCNU (C).

Chart 2. The cellular survival and induction of SCEs in 9L (○) and 9L-2 (▲) cells after a 1-h treatment with ACNU. The number of SCEs induced is calculated by subtracting the number of SCEs induced by 10 μM bromodeoxyuridine alone.

Table 2

<table>
<thead>
<tr>
<th>Drug concentration (μM)</th>
<th>Cross-linking index for 9L cells</th>
<th>Cross-linking index for 9L-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACNU</td>
<td>50: 96 ± 10^a, 100: 275 ± 8</td>
<td>9L: 68 ± 3, 9L-2: 128 ± 18</td>
</tr>
<tr>
<td>HN2</td>
<td>0.5: 72 ± 8, 1: 152 ± 19, 2: 333 ± 53</td>
<td>9L: 210 ± 25, 9L-2: 472 ± 79, 810 ± 99</td>
</tr>
<tr>
<td>cis-Pt</td>
<td>30: 228 ± 85, 60: 454 ± 155, 90: 572 ± 147</td>
<td>9L: 285 ± 42, 9L-2: 436 ± 35, 646 ± 54</td>
</tr>
</tbody>
</table>

* Mean ± SD.

of ENU (Chart 3). Treatment of both cell lines with 5 mM ENU produced a 1-log cell kill and induced a similar number of SCEs in both cell lines (Chart 3). Slopes of the SCE dose-response curves are 0.115 SCEs/metaphase/μM ENU for 9L cells and 0.086 SCEs/metaphase/μM ENU for 9L-2 cells.

Survival curves for 9L and 9L-2 cells treated with HN2 are shown in Chart 4. In contrast to results found for treatment with CENUs, 9L cells were approximately 2-fold more resistant to the cytotoxic effects of HN2 than were 9L-2 cells. Slopes of the SCE dose-response curves are 308 SCEs/metaphase/μM HN2 for 9L cells and 660 SCEs/metaphase/μM HN2 for 9L-2 cells. Calculated as the ratio of the slopes, 9L cells are 2-fold more resistant to the induction of SCEs than are 9L-2 cells treated with HN2. Treatment of either 9L or 9L-2 cells with HN2 for 1 h produced high levels of proteinase K-resistant DNA interstrand cross-links (Table 2). The extent of DNA cross-linking in 9L-2 cells was approximately 3-fold higher than for 9L cells treated with equimolar concentrations of HN2. This result is in good agreement with both the differences in the number of SCEs induced and the differences in cell kill in 9L and 9L-2 cells treated with HN2.

The level of cell kill and the number of SCEs induced is the same in 9L and 9L-2 cells treated with equimolar concentrations of cis-Pt (Chart 5). Slopes of the SCE dose-response curves are 11.1 SCEs/metaphase/μM of cis-Pt for 9L and 9.1 SCEs/metaphase/μM for 9L-2 cells. The values of the cross-linking index for 9L and 9L-2 cells treated with equimolar concentrations of cis-Pt are also similar (Table 2).

DISCUSSION

The cytotoxic response of 9L, 9L-2, 9L-7, and 9L-8 cells treated with BCNU and CHLZ have been measured. In contrast to 9L, 9L-2 cells were very resistant to the cytotoxic effects of these nitrosoureas, and 9L-7 and 9L-8 cells had intermediate responses. The dose-response curve for 9L cells treated with ACNU was very similar to 9L cells treated with BCNU and CHLZ. 9L-2 cells were more sensitive to treatment with BCNU than they were to ACNU or CHLZ, however, suggesting that carbamoylation of proteins by BCNU may either decrease the degree of cellular resistance or lead to additional cytotoxicity by itself. Similar results have been obtained by Erickson et al. (12) with IMR-90 and VA-13 cells treated with these nitrosoureas.

A good correlation between SCE induction and cellular sensitivity to BCNU has been found (2). In agreement with this, 9L-7, and 9L-8 cells have been shown to have an intermediate response to SCE induction caused by BCNU and CHLZ (13). Compared to 9L cells, 9L-2 cells were 34-fold more resistant to SCE induction and 10-fold more resistant to cell killing by ACNU treatment. These results suggest that the biochemical events that lead to SCE induction and cell killing are related.

Treatment of cells with certain CENUs results in DNA alkylation, formation of DNA interstrand cross-links, and carbamoylation of cellular proteins (Table 1). Biochemical modification of any
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**Chart 3.** The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with ENU.

**Chart 4.** The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with HN2.

**Chart 5.** The cellular survival and induction of SCEs in 9L (●) and 9L-2 (▲) cells after a 1-h treatment with cis-Pt.

of these processes could result in cellular resistance. To obtain a better understanding of how the resistance of 9L-2 cells to the cytotoxic effects of these agents is expressed, we investigated the effects of other nitrosoureas that were deficient for either alklylation, cross-linking, or carbamoylation.

9L, 9L-2, 9L-7, 9L-8 cells were treated with BHCNU, which does not alkylate or cross-link but does carbamoylate (14). The cytotoxic response of the 4 cell lines was similar indicating that all were equally susceptible to cytotoxicity caused by carbamoylation. 9L and 9L-2 cells were treated with ENU, a nitrosourea that alkylates (15) and presumably carbamoylates (16) but does not form DNA interstrand cross-links (17). 9L and 9L-2 cells were equally susceptible to both the cytotoxic effects of ENU and to the induction of SCEs. The results obtained with ENU and BHCNU suggest that cellular resistance to CENUs is associated with the DNA cross-linking properties of the CENUs.

Results obtained with CENUs indicate that cellular resistance in 9L-2 cells is related to reduced levels of DNA cross-links. We determined whether this resistance was specific for CENUs or was part of a general pattern of resistance to DNA cross-linking agents. The cytotoxicity, number of SCEs induced, and number of DNA cross-links formed in 9L and 9L-2 cells treated with cis-Pt were very similar. Therefore, 9L-2 cells were not cross-resistant to cis-Pt. The results obtained with HN2, however, were in sharp contrast to results obtained with CENUs. 9L cells were 2- to 3-fold more resistant to the cytotoxic effects, induction of SCEs, and formation of DNA cross-links than were 9L-2 cells. Recent studies have shown that 9L cells contain approximately twice as much reduced glutathione as do 9L-2 cells, which correlates with their increased resistance to NH2 (24). These results show clearly that the cellular resistance to CENUs is highly specific and does not result in cross-resistance to other bifunctional agents. Similar results have been obtained by Schabel et al. (3, 4) using L1210 and P388 cell lines resistant to BCNU.

The reduced formation of DNA interstrand cross-links in 9L-2 cells (2) and resistant human cells treated with CENUs appears to be the result at least in part of increased repair of O\(^2\)-chloroethyl guanine (12, 19); the initial alklylation product leading to formation of the CENU induced DNA interstrand cross-link 1-[N\(^2\)-deoxycytidyl]-2-[N\(^1\)-deoxyguanosiny]ethane (25). The cross-link formed by HN2 is bis(2-guanin-7-ylethyl)methylamine (26), which is different in structure from the cross-link caused by CENUs (1, 23, 25). Work with semipurified mammalian enzymes

has shown that recognition and removal of alkylation products is very specific (27). Therefore, the results of this study suggest that when resistance to a particular chemotherapeutic agent is mediated by a specific repair process(es), other cross-linking agents will not be cross-resistant unless they form DNA alkylation products that are also substrates for that particular repair process(es). When cellular resistance is mediated by drug uptake, glutathione levels, glutathione-S-transferase, or gene amplifications, however, different patterns of sensitivity and resistance probably will be observed.

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