Glutathione Dependence of Neocarzinostatin Cytotoxicity and Mutagenicity in Chinese Hamster V-79 Cells

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ABSTRACT

Neocarzinostatin (NCS) is mutagenic in bacteria, yeast, fungi, and mammalian cells. In cell-free systems, DNA strand breakage induced by NCS requires a reducing agent like 2-mercaptoethanol, unless very high (>100 μg/ml) concentrations of NCS are used. In this study, we have investigated the role of the sulfhydryl compound glutathione (GSH), which is usually the most common intracellular thiol, in the bioactivation of NCS to a toxic and mutagenic species. Chinese hamster V79 cells were pretreated with one of two GSH-depleting agents, buthionine sulfoximine or diethyl maleate. These agents deplete GSH via different mechanisms, but both will lower GSH levels within the cell to <5% of control (untreated) values. GSH-depleted cells and control cells were then exposed to NCS concentrations of 0.5–2.5 μg/ml for 1 h, assayed for survival, and plated for expression of hypoxanthine-guanine phosphoribosyltransferase-negative (HGPRT⁻) mutants. After an expression period of 7 days, during which the cultures were subcultured twice, HGPRT⁻ mutants were selected by plating in hypoxanthine-free medium containing 5 μg of 6-thioguanine per ml, at a density of 2 × 10⁶ cells per 100 mm dish. NCS alone decreased the surviving fraction to about 1% at 2.5 μg/ml and produced dose-related increases in HGPRT⁻ mutants that reached >10 times the spontaneous mutation frequency at 2.5 μg NCS per ml. In GSH-depleted cells, however, NCS was only mildly cytotoxic (60–80% surviving fraction) and did not produce dose-related increases in HGPRT⁻ mutants over cells treated only with diethyl maleate or buthionine sulfoximine. Thus, GSH appears to be the main reducing agent for the bioactivation of NCS to a toxic and mutagenic species in Chinese hamster V79 cells.

INTRODUCTION

Neocarzinostatin is a polypeptide antibiotic that has shown some antitumor activity in clinical trials (1, 2) and which continues to be the subject of clinical investigation (3–5). The biologically active portion of the molecule is a nonprotein chromophore (6), the complete structure of which has recently been proposed (7). The chromophore induces single strand breaks in DNA by abstraction of a hydrogen from the C-5' carbon of deoxyribose, which leads to the production of C-5'-aldehyde and a strand break (8). Chromophore-DNA adducts (9) and alkali-labile breaks (10, 11) are also induced by the NCS chromophore. In cell-free systems, DNA damage by NCS requires the presence of a reducing agent, such as a sulfhydryl group donor like 2-mercaptoethanol (12). NCS is mutagenic in bacteria, yeast, fungi, and mammalian cells (13–17); addition of exogenous sulfhydryls is not necessary, presumably due to the abundance of sulfhydryl group donors within the cell. The tripeptide glutathione is usually the most abundant nonprotein thiol within the mammalian cell (18), and it is therefore a potential source of sulfhydryl groups for the bioactivation of NCS. On the other hand, GSH is important in protecting cells from free radical damage (18), and there is evidence that free radicals are important in the activity of NCS (19). By depleting cells of GSH by treatment with buthionine-sulfoximine, which inhibits γ-glutamylcysteine synthetase (20), or diethyl maleate, which binds thiol groups (21, 22), the role of GSH in the toxicity and mutagenicity of NCS was investigated.

MATERIALS AND METHODS

Exponentially growing cultures of Chinese hamster V79 cells were maintained in F12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. 10⁶ cells were plated in 100-mm dishes and allowed to incubate overnight prior to each experiment. To deplete GSH, cells were treated with 1 mM BSO (Chemical Dynamics Corp.) for 8 h or 0.5 mM DEM (Aldrich Chemical Co.) for 2 h prior to addition of NCS. NCS (National Cancer Institute Drug Synthesis and Chemistry Branch) exposure was done in complete medium for 1 h. Determinations of GSH levels for control and GSH-depleted cells were done for each experiment. Cells were plated and treated with BSO or DEM as described above. After treatment, the cell monolayer was rinsed with Dulbecco’s phosphate-buffered saline (Quality Biological, Inc.) and extracted with cold 0.5% sulfosalicylic acid. GSH levels were then assayed spectrophotometrically by the GSH-reductase procedure (23), which can detect as little as 1–5 ng of GSH. Following NCS exposure, the cells were rinsed, trypsinized, and plated for survival determination, and cultures were started to allow for the necessary expression period for HGPRT⁻ mutants. After a 7-day expression period, during which the cultures were subcultured twice, HGPRT⁻ mutants were selected by plating 2 × 10⁶ cells per 100-mm dish in hypoxanthine-free F12 medium with 5% dialyzed serum and 6-thioguanine (5 μg/ml). A total of 10⁶ cells was plated for each condition. Plating efficiency at the time of selection was determined by plating 100–200 cells in the above medium minus 6-thioguanine. The mutagenicity of BSO and DEM was assayed in the same manner. NCS-treated cells were also assayed for the induction of ouabain-resistant mutants. Cells were treated as above and plated for selection of ouabain-resistant mutants after a 48-h expression period, using 3 mM ouabain (Sigma Chemical Co.). All experiments were done twice.

RESULTS

Treatment of V79 cells with BSO or DEM depleted GSH to levels that were at or below the limit of detection for the GSH-reductase assay in all four experiments. This GSH depletion is similar to that reported previously by this laboratory (24–26). Table 1 shows the results of a mutagenicity assay on BSO and DEM, as well as typical positive and negative controls for the 6-
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Table 1
Results of mutagenicity assay on BSO and DEM, and typical positive and negative controls

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Relative surviving fraction</th>
<th>HGPRT&lt;sup&gt;+&lt;/sup&gt; mutants per 10&lt;sup&gt;5&lt;/sup&gt; clonable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>1.00</td>
<td>0.7</td>
</tr>
<tr>
<td>BSO (1 mw)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94</td>
<td>0.2</td>
</tr>
<tr>
<td>BSO (5 mw)</td>
<td>1.00</td>
<td>0.4</td>
</tr>
<tr>
<td>BSO (10 mw)</td>
<td>1.00</td>
<td>0.7</td>
</tr>
<tr>
<td>DEM (0.5 mw)</td>
<td>0.97</td>
<td>0.5</td>
</tr>
<tr>
<td>DEM (1.0 mw)</td>
<td>0.94</td>
<td>0.6</td>
</tr>
<tr>
<td>MNU (0.5 mw)</td>
<td>0.67</td>
<td>71.5</td>
</tr>
<tr>
<td>MNU (0.5 mw) plus BSO</td>
<td>0.61</td>
<td>90.0</td>
</tr>
<tr>
<td>BSO (1 mw)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Exposure times were 8 h for 1 mw BSO, 4 h for 5 and 10 mw BSO, 2 h for DEM, and 1 h for MNU and MNU plus BSO.

Charts 1 and 2 show the results of NCS treatment on control and GSH-depleted cells. Both charts show the mean of two experiments, with the standard error when it is larger than the symbol. Both toxicity and mutagenicity are considerably reduced in cells that had been depleted of GSH at the time of NCS exposure. Mutation frequencies remained nearly the same when the expression period was extended to 9 days (data not shown). NCS did not induce any ouabain-resistant mutants in either of two experiments. The spontaneous mutation frequency in these experiments was 0.85 per 10<sup>6</sup> clonable cells, and the positive control (10 mw ethyl methane sulfonate) mutation frequency was 53 per 10<sup>6</sup> clonable cells.

DISCUSSION

These results indicate that GSH plays a major role in the activation of NCS to a toxic and mutagenic species in a mammalian cell line. The nearly identical results seen with BSO and DEM, which deplete GSH by different mechanisms, indicate that the reduction in mutagenicity and toxicity seen in GSH-depleted cells is a result of the GSH depletion and not simply inactivation of NCS by the depleting agent. In addition, we have shown that the same reduction in NCS toxicity is seen when BSO is rinsed off prior to NCS exposure (28).

NCS has been reported to be mutagenic in bacteria, yeast,
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fungi, and mammalian cells (13–17). In *Salmonella*, NCS reverses frameshift mutations and base pair substitutions (14) and is a potent mutagen in TA102, which has A-T base pairs at the site of mutation (29). In *Saccharomyces*, NCS induces reverse and possibly forward (suppressor) mutations (16). Recently, Au et al. (17) have reported that NCS induces 6-thioguanine-resistant mutants and chromosome aberrations in Chinese hamster ovary cells. In this study, NCS was assayed for its ability to induce 6-thioguanine-resistant mutants and ouabain-resistant mutants. NCS did not produce detectable numbers of ouabain-resistant mutants. Since the ouabain resistance assay detects primarily base pair substitutions (30), the lack of activity in this assay indicates that NCS does not induce appreciable numbers of base pair substitutions in mammalian cells, while in bacteria it induces both base pair substitutions and frameshifts at frequencies high enough to be easily detected. In mammalian cells, NCS acts much like ionizing radiation, which does not induce ouabain-resistant mutants but does induce 6-thioguanine-resistant mutants (31).

NCS causes a unique type of single strand break in DNA that is the result of the oxidation of the 5'-carbon of deoxyribosyl (8). Thus, it falls into the class of compounds known as "oxidative mutagens." Its mechanism of action appears to involve free radicals (19). In general, GSH protects cells from damage by free radicals, but in the case of NCS, GSH has the opposite role, being essential for the toxic and mutagenic activity of the drug. Another possible nonprotein thiol that could function in the reductive activation of NCS is cysteine. Both glutathione (32) and cysteine (33) have been used to activate NCS in a cell-free assay for DNA strand breakage. We have shown (28), however, that in V79 cells normal intracellular levels of cysteine are extremely low (less than 0.2 μM), making it unlikely that cysteine has any role in the bioactivation of NCS. The nearly complete absence of NCS activity in thiol-depleted cells, and the cysteine data, indicate that GSH is the only reducing agent functioning in the activation of NCS. Using BSO, it may be possible to deplete GSH at a faster rate from a normal human lung fibroblast than from a human lung tumor line, A549, judging from work that is in progress in our laboratory. If this were also true in vivo, non-tumor cells might be protected from the toxic and mutagenic activity of NCS, while tumor cells would still be sensitive to NCS. In addition to making initial treatment with NCS more effective, this could also reduce the risk of secondary tumors being induced by NCS.

REFERENCES

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