Isolation of Two Chinese Hamster Ovary Cell Mutants Hypersensitive to Topoisomerase II Inhibitors and Cross-Resistant to Peroxides

Stella M. Davies, Sally L. Davies, Adrian L. Harris, and Ian D. Hickson

Department of Clinical Oncology, University of Newcastle upon Tyne, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, United Kingdom

ABSTRACT

We have isolated two Chinese hamster ovary cell lines, designated ADR-4 and ADR-5, which exhibit hypersensitivity to intercalating agents and epipodophyllotoxins. These drugs are thought to exert their cytotoxicity via an interaction with the enzyme topoisomerase II. However, there is no apparent change in the level or catalytic activity of topoisomerase II in the mutant cells. Drug sensitivity does not appear to be due to increased drug transport because accumulation of radiolabeled actinomycin D is similar in mutant and wild-type cells. Both mutant cell lines show enhanced resistance to hydrogen peroxide and to organic peroxides. ADR-4 cells show a degree of temperature sensitivity. ADR-5 cells show mild sensitivity to UV irradiation. Neither cell line shows significant sensitivity to mono- or bifunctional alkylating agents, ionizing radiation, or bleomycin.

Cell fusion studies indicate that the phenotype of each mutant cell line is recessive and that the mutants represent two different genetic complementation groups. These studies also indicate that ADR-4 and ADR-5 are in different complementation groups from the previously described Adriamycin-sensitive mutant, ADR-1. These results indicate that sensitivity to topoisomerase II inhibitors can result from abnormalities in several genes. These drug-sensitive mutants may be useful for studying the mechanisms of cell killing by topoisomerase II inhibitors, free radicals, and heat.

INTRODUCTION

DNA topoisomerases are nuclear enzymes which catalyze topological change in DNA (for a review see Ref. 1). Two classes of enzyme (types I and II) have been isolated from both prokaryotes and eukaryotes. Type II topoisomerases appear to be essential for cell proliferation (2) and are a major constituent of the mitotic chromosome scaffold and of the nuclear matrix (3, 4). There is evidence for an involvement of topo II in the catalysis of key steps in DNA replication, transcription, and recombination, in addition to a structural role in chromatin (5-7).

Type II topoisomerases catalyze topological change by transiently introducing a double strand break into a DNA molecule and passing a second DNA duplex through the break. As part of this reaction, one enzyme subunit is covalently bound to the 5' end of the nicked DNA forming a so-called "cleavable complex" (8). Drugs such as the intercalating agents Adriamycin, actinomycin D, m-AMSA, and mitoxantrone and the epipodophyllotoxins VP 16 and VM26 appear to stabilize the "cleavable complex." This leads to inhibition of strand passage and consequently the accumulation of protein-associated DNA double-strand breaks (9-11).

Study of cell lines with abnormal topo II has provided evidence that the production of topo II-mediated double-strand breaks is important in drug-induced cell killing. For example, the VP16-resistant cell line, Vpm-5, has been shown to be cross-resistant to other topo II inhibitors and to have an abnormal, drug-unresponsive topo II (12). We have previously reported the isolation of a CHO cell line, ADR-1, which is hypersensitive to all classes of topo II inhibitors and which overproduces topo II (13). There is a correlation between DNA strand break frequencies and cytotoxicity in ADR-1 and CHO-K1 cells (14). This suggests that the intracellular level of topo II enzyme is an important determinant of cellular sensitivity to topo II inhibitors.

Despite the evidence that intercalating agents and epipodophyllotoxins share a common intracellular target, their spectrum of clinical activity is somewhat different, as are the side effects caused by these drugs. These differences may be due to drug interactions with intracellular targets other than topo II. For example, intercalating agents are known to be potent generators of free radicals and to cause lipid peroxidation (15, 16).

As part of a study of cellular factors which determine sensitivity to intercalating agents, we have isolated 2 CHO mutant cell lines on the basis of hypersensitivity to Adriamycin and actinomycin D. Unlike the previously described CHO cell line, ADR-1, neither of these cell lines appears to have an abnormality in the level or catalytic activity of topo II.

MATERIALS AND METHODS

Cell Culture Conditions. Cells were routinely maintained in Ham's F-10 medium (Northumbria Biologicals) supplemented with glutamine (3 mM), 5% fetal calf serum, 5% newborn calf serum, and antibiotics (streptomycin, 100 µg/ml; penicillin, 100 units/ml; and nystatin, 50 units/ml). Cells were grown at 37°C under 5% CO₂ and were routinely tested for Mycoplasma contamination.

Mutagenesis. Cells were mutagenized in growth medium for 24 h with ethyl methanesulfonate as described previously (17).

Mutant Isolation. Mutants were isolated using the replica plating technique described previously (17). Briefly, mutagenized cells were plated to yield single colonies which were overlaid with growth medium containing 0.4% Noble agar. After 48 h, when the cells had started to grow into the agar, sterile toothpicks were used to transfer individual colonies onto the surface of gridded agar plates (0.5% Noble agar in normal growth medium) which contained either the selective drug (Adriamycin at 5 ng/ml or actinomycin D at 5 ng/ml) in the agar or no drug. Cells growing on the control but not on the drug-containing plates were restaged on agar and then transferred to liquid culture for accurate survival testing. All mutants were cloned twice before survival determinations were carried out.

Survival Curves. Exponentially growing cells were trypsinized and seeded in 90-mm Petri dishes to yield 500 to 100,000 cells. These were allowed to adhere for 4 h before treatment with a DNA-damaging agent, as outlined below.

Radiation. Treatment with UV or X-rays was as described previously (17).

Drug Treatments. Drug treatments were all for 1 h. Drugs were prepared and stored as described previously (13, 17), with the following additions. VM26 was prepared freshly in dimethyl sulfoxide at 10 mM. Hydrogen peroxide, tetrabutyl hydroperoxide and cumene hydroper-
oxide were diluted with water immediately before use.

The $D_{10}$ value is the drug dose required to reduce survival to 37% of control and represents the average dose required to kill a cell.

Construction of Cell Hybrids. Cells hybrids were constructed as described previously (18). Briefly, one or other of the dominant selectable markers gpt or neo was introduced into each cell line by transfection (19), using pSV5gpt and pSV5neo, respectively. The resultant mycophenolic acid and G418-resistant transfecants were then mixed and fused using polyethylene glycol 6000, essentially as described by Davidson and Gerald (20). Hybrids were selected in medium containing both mycophenolic acid and G418. Flow cytometry was used to confirm that hybrid populations were tetraploid.

Drug Accumulation. Accumulation of $[^{3}H]$actinomycin D was measured essentially by the method of Bates et al. (21).

Western Blotting. Blotting was performed as described previously (14). Briefly, nuclear extracts were prepared from CHO-K1 and mutant cells by the method of Gilsson et al. (12) and their protein content was determined by the method of Bradford (22). Nuclear extracts, equalized for protein content, were loaded on an 8% polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked with bovine serum albumin, exposed to rabbit serum against calf thymus topo II (kindly provided by Dr. L. F. Liu, Johns Hopkins University, Baltimore, MD), washed, reacted with $^{32}P$-labeled protein A, and autoradiographed.

Filter Binding Assay. Measurement of the efficiency of covalent binding of topo II to DNA was carried out essentially as described by Minford et al. (23). Nuclear extracts were incubated with linearized plasmid DNA labeled at the 3’ end, together with various concentrations of m-AMSA, at 37°C for 20 min. The reactions were stopped by the addition of 20 mM EDTA, pH 10, and the mixture was applied to a polyvinyl chloride filter (Millipore; 2-μm pore size). Filters were processed as described by Minford et al. (23).

Site Specificity of DNA Cleavage. Reactions were performed as described previously (14). Following incubation of end-labeled linear DNA with nuclear extract and various concentrations of m-AMSA, the samples were phenol extracted and electrophoresed on a nondenaturing 9% polyacrylamide gel.

Temperature Sensitivity. Growth curves were performed at the temperatures indicated by direct cell counting at intervals over 5 days using a Coulter Counter. Plating efficiencies were measured by seeding between 50 and 1,000,000 cells in 90-mm Petri dishes and incubating at the temperatures indicated for 10 days.

RESULTS

Cell Survival Studies. The mutant cell lines, ADR-4 and ADR-5, were screened for sensitivity to topo II inhibitors and to a range of other cytotoxic agents. Fig. 1 shows survival curves for the mutants and for parental CHO-K1 cells following exposure to Adriamycin for 1 h. Both mutants are approximately 5-fold more sensitive to this drug, as judged by $D_{10}$ values, than are CHO-K1 cells.

Table 1 shows $D_{10}$ values for CHO-K1, ADR-4, and ADR-5 cells for a range of different cytotoxic agents. ADR-4 and ADR-5 cells show a similar degree of cross-sensitivity to all the drugs which interact with topo II, including actinomycin D, m-AMSA, mitoxantrone, VP16, and VM26. The result (Fig. 2) shows survival of ADR-5 cells after UV irradiation compared with normal cells. ADR-4 is approximately 2-fold more sensitive to UV irradiation than CHO-K1 cells. ADR-4 shows wild-type resistance to UV irradiation (data not shown).

In addition to showing sensitivity to topo II inhibitors, ADR-4 and ADR-5 cells show significantly enhanced resistance to peroxides (Table 2). ADR-4 cells show mild resistance to hydrogen peroxide but more marked resistance to organic hydroperoxides. ADR-5 cells show mild resistance to cumene hydroperoxide but more marked resistance to hydrogen peroxide and tetrabutyl hydroperoxide.

Temperature Sensitivity. ADR-5 cells show wild-type resistance to heat but ADR-4 cells show a degree of temperature sensitivity. Fig. 3 shows growth curves for CHO-K1 and ADR-4 cells at a range of temperatures. At 41°C ADR-4 cells fail to divide while a temperature of 43°C is required to completely inhibit growth of CHO-K1 cells. Table 3 shows the relative plating efficiencies of CHO-K1 and ADR-4 cells at different temperatures. ADR-4 and CHO-K1 cells have similar plating efficiencies at 37°C. There is a marked reduction, relative to wild-type, in the plating efficiency of the mutant cell line at increased temperatures.

Because of the temperature sensitivity of ADR-4 cells, induction of the $M_{r}$ 70,000 heat shock protein was studied. $M_{r}$ 70,000 heat shock protein was induced normally and was not constitutively overproduced in ADR-4 cells (data not shown).

Cell Fusion Studies. Fig. 4 shows survival curves for CHO-K1/CHO-K1, ADR-4/ADR-5 and CHO-K1/ADR-5 hybrids following exposure to Adriamycin. After fusion with CHO-K1 cells, ADR-4 cells display wild-type resistance to drug. Similar levels of resistance were seen with CHO-K1/ADR-4 and ADR-4/ADR-5 cell hybrids. The self-cross mutant hybrid ADR-4/ADR-4 shows mutant levels of Adriamycin sensitivity (data not shown). Fusion studies of ADR-4 and ADR-5 with the Adriamycin-sensitive mutant, ADR-1 (13), show that ADR-4/ADR-1 and ADR-5/ADR-1 hybrids show wild-type Adriamycin resistance (data not shown).

Intracellular Accumulation of Drug. To determine whether the increased sensitivity to topo II inhibitors was due to enhanced intracellular accumulation of drug, we compared the level of actinomycin D accumulation in CHO-K1, ADR-4, and ADR-5 cells. No difference could be detected in either the initial rate of accumulation or the total amount of drug accumulated over 90 min between mutant and wild-type cells (Fig. 5).

Expression of Topo II Protein and Activity. The level of topo II protein in nuclear extracts from ADR-4 and ADR-5 cells was compared with that in CHO-K1 cells using Western blot analysis. The result (Fig. 6) shows that there is no significant difference in the level of topo II protein in mutant and parental cell lines.
Table 1  \( D_s \) values for CHO-K1, ADR-4, and ADR-5 cells after exposure to various cytotoxic agents for 1 h

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Act. D. (( \mu g/ml ))</th>
<th>m-AMSA (ng/ml)</th>
<th>VM26 (( \mu g/ml ))</th>
<th>VP16 (( \mu g/ml ))</th>
<th>Mitox. (ng/ml)</th>
<th>Vincristine (( \mu g/ml ))</th>
<th>MMC (( \mu g/ml ))</th>
<th>MMS (( \mu g/ml ))</th>
<th>Bleomycin (( \mu g/ml ))</th>
<th>Chlorambucil (( \mu g/ml ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>0.80</td>
<td>210</td>
<td>0.8</td>
<td>17.2</td>
<td>6.7</td>
<td>11.5</td>
<td>2.5</td>
<td>125</td>
<td>75</td>
<td>148</td>
</tr>
<tr>
<td>ADR-4</td>
<td>0.15</td>
<td>45</td>
<td>0.16</td>
<td>3.2</td>
<td>2.8</td>
<td>5.9</td>
<td>1.5</td>
<td>80</td>
<td>95</td>
<td>112</td>
</tr>
<tr>
<td>ADR-5</td>
<td>0.14</td>
<td>50</td>
<td>0.25</td>
<td>3.2</td>
<td>2.7</td>
<td>11.0</td>
<td>2.0</td>
<td>170</td>
<td>40</td>
<td>96</td>
</tr>
</tbody>
</table>

* Act. D, actinomycin D; Mitox, mitoxantrone; MMC, mitomycin C; MMS, methyl methanesulfonate.

Table 2 Fold resistance of ADR-4 and ADR-5 cells relative to CHO-K1 cells following exposure to peroxide for 1 h

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Hydrogen peroxide</th>
<th>Tetrabutyl hydroperoxide</th>
<th>Cumene hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ADR-4</td>
<td>1.6</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>ADR-5</td>
<td>2.5</td>
<td>3.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Topo II activity was measured using a specific filter-binding assay which uses \( ^{32}P \) end-labeled DNA as substrate. m-AMSA is used to stimulate binding of topo II to DNA. Fig. 7 shows that ADR-4 and ADR-5 cells express a level of topo II activity similar to that of CHO-K1 cells.

Sequence-specific DNA Cleavage. Topo II demonstrates sequence preference for cleavage sites. It is possible that a relaxation of this sequence specificity could lead to an increased level of DNA damage and hence drug sensitivity. To examine this possibility, sequence specific DNA cleavage was examined using \( ^{32}P \)-end-labeled linearized plasmid DNA as a substrate. DNA was cleaved by topo II in nuclear extracts from mutant and parental cell lines in the absence and presence of drug, and the specific cleavage products were examined by polyacrylamide gel electrophoresis. Fig. 8 shows that only a small amount of...
TOPO II INHIBITOR-SENSITIVE MUTANTS

Fig. 8. Site specificity of DNA cleavage by topo II from CHO-K1, ADR-4, and ADR-5 cells. DNAs in Lanes 1, 4, 7, and 10 have been treated with topo II from CHO-K1 cells; DNAs in Lanes 2, 5, 8, and 11 with topo II from ADR-4 cells; and those in Lanes 3, 6, 9, and 12 with topo II from ADR-5 cells. Lanes 1, 2, and 3, no drug; Lanes 4, 5, and 6, 8 μg/ml m-AMSA; Lanes 7, 8, and 9, 20 μg/ml m-AMSA; Lanes 10, 11, and 12, 40 μg/ml m-AMSA. Lane 13, end-labeled pSV2gpt substrate.

DNA cleavage takes place in the absence of drug. DNA cleavage generating specific products is seen in the presence of increasing doses of m-AMSA. The molecular weights and relative amounts of the various products are clearly the same with topo II from all three cell lines. Taken together, these results indicate that there is no difference in the level of topo II protein and no apparent change in catalytic activity of topo II in mutants ADR-4 and ADR-5 to explain drug sensitivity.

DISCUSSION

We have described the isolation of 2 CHO cell lines which show hypersensitivity to drugs which interact with topo II. Both cell lines also show cross-resistance to peroxides. Despite the apparent similarity in their phenotypes, cell fusion studies show that they represent two different genetic complementation groups. Similar studies performed with the previously described mutant, ADR-1, show that this cell line represents a third complementation group of Adriamycin-sensitive mutants. Consistent with this, the mutants show phenotypic differences in that ADR-4 cells show moderate temperature sensitivity while ADR-5 cells show mild UV sensitivity.

Overexpression of topo II has been shown to cause hypersensitivity to the antitumor drugs which interact with topo II (14). Both of the cell lines described here show normal levels of topo II protein as judged by Western blotting. Moreover, no abnormality in catalytic activity could be detected. This raises the possibility that a cellular target other than topo II is altered in these mutants. Enzymes which interact with topo II prior to the induction of DNA damage, or enzymes involved in the processing and repair of topo II-induced DNA strand breaks, are possible candidates. Further studies are in progress to examine these possibilities. Alkaline elution studies indicate that both ADR-4 and ADR-5 cells incur more DNA strand breaks following treatment with Adriamycin than do parental CHO-K1 cells (data not shown). Further work is required to determine whether this increased level of drug-induced DNA damage is mediated via topoisomerase II.

Both cell lines show resistance to hydrogen peroxide and organic peroxides. In the presence of reduced metals, such as ferrous ions, hydrogen peroxide causes hydroxyl radical formation (24). Enzymatic breakdown of hydrogen peroxide is catalyzed by both glutathione peroxidase and catalase. Further work is required to determine whether or not the increased level of drug-induced DNA damage is mediated via topoisomerase II.

Both cell lines show resistance to hydrogen peroxide and organic peroxides. In the presence of reduced metals, such as ferrous ions, hydrogen peroxide causes hydroxyl radical formation (24). Enzymatic breakdown of hydrogen peroxide is catalyzed by both glutathione peroxidase and catalase. Further work is required to determine whether either of the mutant cell lines has an abnormality in free radical formation or detoxification, although it appears that catalase levels are wild-type in both mutants.3 Intercalating agents and epipodophyllotoxins can also generate free radicals or reactive intermediates (15, 25); thus an abnormality in the handling of these reactive species may contribute to sensitivity to these agents. It is also

3 Unpublished observation.
possible that sensitivity to topo II inhibitors and resistance to peroxides are conferred by independent mutations and resistance to more than one gene.

Resistance to a wide range of structurally unrelated drugs (the so-called multidrug-resistant phenotype) is generally found to be due to an abnormality of drug transport mediated via overexpression of a membrane protein, P-glycoprotein, which functions as an energy-dependent drug efflux pump (26). An abnormality in P-glycoprotein is unlikely to be responsible for the drug hypersensitivity of these mutants inasmuch as intracellular accumulation of radiolabeled actinomycin D is similar in mutant and parental cells. However, further studies are required to confirm this.

ADR-4 cells show a moderate degree of temperature sensitivity. Warters and Brizgys (27) have shown that treatment of CHO and HeLa cells with the topo II inhibitors novobiocin and nalidixic acid results in sensitization to hyperthermic cytotoxicity. The authors suggested that heat inactivation of a number of enzymes, perhaps including topo II, may cause the characteristic changes in nuclear protein mass associated with heat-induced cytotoxicity. If ADR-4 cells have an as yet unidentified abnormality in topo II, or enzymes associated with it, this may be related to the temperature-sensitive phenotype. It is also possible that an abnormality in a broader stress response to cytotoxic agents underlies both the drug and the temperature sensitivity of ADR-4.

It is of interest that ADR-5 cells show mild sensitivity to UV irradiation, inasmuch as a role for topo II in excision repair of UV-induced damage has been suggested previously. Mattern et al. (28) have provided evidence that novobiocin inhibits DNA incision at damaged sites, suggesting that topo II may play a role in the preincision step of excision repair. However, Downes et al. (29) suggested that the effects of novobiocin are more likely to be due to a nonspecific effect on ATP metabolism. Dresler and Robinson-Hill (30) have shown that in a permeabilized cell system (where exogenous ATP is supplied) novobiocin still partially inhibits accumulation of UV-induced DNA strand breaks. It is therefore possible that topo II does play some role in excision repair, and study of ADR-5 cells may help to identify this.

In conclusion, we have isolated 2 mutants hypersensitive to topo II inhibitors. The phenotypes of these mutants suggest possible interactions between the pathways controlling cellular resistance to topo II inhibitors, hyperthermia, free radicals, and UV light. Further study of these cell lines may provide useful information concerning these interactions.

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REFERENCES


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