Inherent Sensitivity and Induced Resistance to Chemotherapeutic Drugs and Irradiation in Human Cancer Cell Lines: Relationship to Mutation Frequencies

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ABSTRACT

Metastatic nonseminomatous testicular germ cell tumors are curable using combination chemotherapy in approximately 80% of patients. In contrast, most other patients with other types of cancer either present with or acquire drug-resistant disease following chemotherapy. Cell lines derived from testis tumors retain hypersensitivity to both drugs and radiation in vitro, thus providing a model system with which to investigate the genetic basis of hypersensitivity to these agents. This study compared the spontaneous and both ethyl methanesulfonate- and cisplatin-induced frequencies of mutation to 6-thioguanine resistance in 3 human bladder and 3 testis tumor cell lines and a bladder and a testis cell line with cisplatin resistance induced in vitro. The two tumor types showed similar frequencies of both spontaneous and induced mutation frequencies at this locus. Therefore, we failed to provide evidence for the hypothesis that the curability of testis tumors is associated with a low frequency of mutation to drug resistance.

INTRODUCTION

Testis tumors rarely develop drug resistance, and approximately 80% of patients with metastatic disease are cured using combination chemotherapy (1). In contrast, in most other types of metastatic cancer, treatment fails due to inherent or acquired drug resistance. Resistance can arise by spontaneous random mutation in bacteria (2) and in mammalian cancer cells (3). One hypothesis to explain the difference in cure rates among tumor types is that curable cancers, such as those arising in the testis, have a low frequency of mutation to drug resistance.

To investigate this hypothesis, we compared the frequencies of mutation at the HGPRT2 locus in three human bladder and three testis tumor cell lines and one bladder and one testis cell line with cisplatin resistance induced in vitro. The HGPRT locus was chosen because mutation to 6-thioguanine resistance is well characterized and measurable, even in cell lines such as those derived from testis tumors, which are difficult to grow and have low plating efficiencies. There is no known relationship between 6-thioguanine and cisplatin resistance, but we assume that mutations at the HGPRT locus will reflect genomic stability and thus the mutation frequency at other loci, such as those responsible for cisplatin resistance. Testis cancer cell lines retain sensitivity in vitro to a broad range of chemotherapeutic drugs (4–6), as well as γ-radiation (7) and UV radiation (8), reflecting the curability of these tumors.

In addition to spontaneous mutation frequencies, those induced by EMS and cisplatin at the HGPRT locus were measured. EMS was chosen because its effect at the HGPRT and other loci has been characterized extensively (9). Cisplatin is the most effective single agent in the therapy of both metastatic testis and bladder cancers (1, 10). In combination with other drugs, cisplatin cures approximately 80% of patients with metastatic testis cancer; yet metastatic bladder cancer is rarely cured.

MATERIALS AND METHODS

Cell Lines. The sources of the cell lines used in this study are shown in Table 1.

Cell Culture. Stock cultures of all cell lines were maintained under identical conditions as monolayers in plastic tissue culture flasks (Nunc, Gibco, Paisley, Scotland) in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal bovine serum (Tissue Culture Services Ltd., Slough, Berkshire, England) and 2 mM L-glutamine (Flow, Irvine, Scotland), in a humidified atmosphere of 5% CO2 in air. The cell lines were used over a restricted range of 10 passages to minimize any changes that might occur during long-term culture.

Cisplatin-resistant Sublines. Cisplatin-resistant cell lines were derived in vitro from the bladder line RT112 and the testicular line SuSa by continuous exposure to increasing concentrations of cisplatin for periods of 14 and 11 months, respectively. The concentrations of cisplatin required to reduce colony formation by 70% were compared, and RT112-CF was 4-fold more resistant than RT112 and SuSa-CF was 4-fold more resistant than SuSa.

Sensitivity to Ethyl Methanesulfonate and Cisplatin. The sensitivities of the cell lines to EMS (Sigma Chemical Co., Poole, Dorset, England) and cisplatin (Neoplatin; Bristol Myers Pharmaceuticals, Langley, England) were determined by an in situ clonogenic assay. After the cells were plated in 9-cm plastic Petri dishes, they were incubated for 48 h prior to a 1- or 2-h exposure to a range of concentrations of cisplatin or EMS, respectively, diluted in medium. Five dishes were set up for untreated controls and each concentration of drug. Following exposure the cells were washed three times with medium and incubated for 14–21 days. Colonies were fixed in methanol (BDH Chemicals, Poole, England) and stained with 10% Giemsa (BDH) and those consisting of 50 or more cells counted. Inhibition of colony formation in treated dishes was expressed as a percentage of colony formation in untreated controls. The data are derived from three separate experiments for each cell line.

Sensitivity to 6-Thioguanine. To determine the optimum concentration of 6TG for selection of 6TG-resistant mutants, the sensitivities of the cell lines to increasing concentrations of 6TG (Sigma) were determined. A single cell suspension was produced by trypsinization and viable cell numbers were counted using a hemocytometer. Viability was determined by trypan blue exclusion. Depending on the plating efficiency of the cell line, between 500 and 4000 cells were added to control dishes, and up to 105 cells were added to dishes containing the highest concentration of 6TG in 10 ml of medium. Ten dishes were used for the controls and 40 dishes for the highest concentrations of 6TG. After 14–21 days the colonies were fixed in methanol (BDH) and stained with 10% Giemsa (BDH). Colonies consisting of 50 or more cells were counted and the surviving fraction (SF) was calculated as follows:

\[
SF = \frac{\text{No. 6TG-resistant colonies}}{\text{No. cells plated} \times \text{colony forming efficiency of untreated cells}}
\]

The data are derived from at least 3 separate experiments for each cell line.

Measurements of Mutation Frequency. The method used for measuring the frequency of mutations was described by Arlett and Harcourt.
MUTATION FREQUENCIES IN HUMAN TUMOR CELL LINES

Table I Cell line sources

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Previous treatment</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT4</td>
<td>TCC*</td>
<td>None</td>
<td>11</td>
</tr>
<tr>
<td>TCCSUP</td>
<td>TCC</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>RT112</td>
<td>TCC</td>
<td>None</td>
<td>13</td>
</tr>
<tr>
<td>RT112-CP</td>
<td>TCC</td>
<td>Cisplatin-resistant sub-line of RT112</td>
<td>14</td>
</tr>
<tr>
<td>833K</td>
<td>NSTGCT</td>
<td>Chemotherapy</td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>NSTGCT</td>
<td>None</td>
<td>15</td>
</tr>
<tr>
<td>SuSa</td>
<td>NSTGCT</td>
<td>None</td>
<td>16</td>
</tr>
<tr>
<td>SuSa-CP</td>
<td>NSTGCT</td>
<td>Cisplatin-resistant sub-line of SuSa</td>
<td></td>
</tr>
</tbody>
</table>

* TCC, transitional cell carcinoma of the bladder; NSTGCT, nonseminomatous testicular germ cell tumor.

(17). Mutation frequencies were compared using both equimolar and equitoxic concentrations of each agent. All cell lines were exposed to 600 µg/ml EMS and 4 µg/ml cisplatin, and all were exposed to concentrations of each agent reducing survival by approximately 10, 50, and 90%.

At the start of the experiment cells growing exponentially in 175-cm² flasks (Nunc) were exposed for 2 h to EMS or for 1 h to cisplatin. The starting number of viable cells was 6 × 10⁷ for controls and the lower concentrations of EMS and cisplatin and 10⁶ for the highest concentrations. Following exposure the cells were washed 3 times with fresh medium.

EMS and cisplatin toxicities were measured at the start of the experiment using an in situ clonogenic assay as described. To allow for recovery from EMS treatment and full expression of mutants, assays were repeated on days 6, 10, 13, 17, and 21 after exposure and, for cisplatin, on days 7, 14, and 21 following exposure. These periods were selected on the basis of earlier experience (18). Cells were detached by trypsinization, a single cell suspension was produced, and the cells were then bulked for each treatment level and counted. The cell suspension was then split into three parts: (a) 500–4000 cells (depending on the cell line) were plated into eight 9-cm dishes (Nunc) to assess the colony-forming efficiency compared with that of the untreated controls; (b) 10⁵ cells were plated into each of 30–40 dishes with 10 ml of medium containing 10 µg/ml 6TG for the selection of 6TG-resistant mutants. This low cell number was selected to avoid metabolic cooperation; (c) each flask for the controls and each treatment level was reinoculated with 3–4 × 10⁶ cells in complete medium for the next time point.

The plates for the selection of resistant mutants and for measuring colony-forming efficiency were fixed, stained, and counted as described. Data are derived from at least two experiments for each cell line and for each drug.

RESULTS

EMS Sensitivity

The dose responses for EMS are shown in Fig. 1. The bladder cells are more resistant to EMS than the testicular cells. The parent and CP-resistant RT112 cell lines have similar sensitivities to EMS but the SuSa-CP cell line is more resistant than its parental line. Due to the differences in sensitivity among the cell lines, induced mutations were compared at both equitoxic and equimolar EMS concentrations.

Cisplatin Sensitivity

The cisplatin sensitivities of two bladder (RT112, RT4) and two testicular (SuSa, 833K) tumor cell lines are shown in Fig. 2. Both the SuSa and 833K cell lines were similar in sensitivity to cisplatin and both were more sensitive than the bladder cancer cell lines.

6-Thioguanine Sensitivity

The sensitivities of the cell lines to 6TG are shown in Fig. 3. The dose-response curves are biphasic and the exponential part of the curves represent killing of nonmutant sensitive cells. The plateau region shows the survival of mutant 6TG-resistant cells. For mutation frequency measurements a concentration of 10 µg/ml 6TG was used to select resistant cells because the dose-response curves of all cell lines had plateaued at this concentration.

Characterization of 6TG-resistant Colonies

A single colony from each cell line resistant to 10 µg/ml 6TG was isolated from the 6TG sensitivity experiments. Depending on the cell line between 500 and 4000 cells were plated into medium containing hypoxanthine, aminopterin, and thymidine.
Mutation Frequencies in Human Tumor Cell Lines

10

10

10

10

10

10

10

10

10

10

10

The HGPRT enzyme is necessary for growth in this medium (19), and none of the 6TG-resistant colonies survived.

Spontaneous Mutation Frequencies

The numbers of mutations to 6TG resistance occurring naturally, the spontaneous mutation frequencies, are shown in Table 2. These are derived from the EMS mutation experiments. For the bladder cells the frequencies range from 2.3 to 18.7 mutants/10⁶ cells. For the testicular cells the spontaneous frequencies were intermediate and in the range of 6.2 to 8.2/10⁶ cells. The bladder and testicular tumor cell lines were compared using Student's unpaired t test; there were no significant differences in the mean spontaneous mutation frequencies (P = 0.846).

Mutation Induction

Effect of Recovery on Mutation Frequency. An example of the relationship between recovery (measured by increasing colony-forming efficiency in EMS-treated cells) and mutation frequency is shown in Fig. 4 for RT112. Colony-forming efficiency of the EMS-treated cells recovers to the level of untreated controls by days 17–21. Recovery in all cell lines occurred at this time and estimations of mutation frequencies are given at day 17 or 21.

EMS-induced Mutation Frequencies. The induced mutation frequencies were compared using equitoxic and equimolar EMS concentrations (Fig. 5).

By taking concentrations of EMS that reduced survival in all cell lines by 50%, we found that the mutation frequencies for the bladder cell lines ranged from 3.7 to 57 mutants/10⁶ cells. The testicular cell lines were intermediate and mutation frequencies ranged from 10 to 15.5 mutants/10⁶ cells (Table 2A).

Mutation frequencies were compared using 600 ng/ml EMS, the highest concentration at which all cell lines were tested. There were no significant differences between the bladder and the testicular cell lines (P = 0.490). The data are shown in Table 2A.

Table 2. Spontaneous and induced mutation frequencies in bladder and testis tumor cell lines at equitoxic and equimolar concentrations of EMS (A) and cisplatin (B)

<table>
<thead>
<tr>
<th>Line</th>
<th>Spontaneous MF*</th>
<th>MF at 600 µg/ml EMS</th>
<th>MF at IC₅₀ EMS concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT4</td>
<td>4.6 ± 0.2</td>
<td>4.55 ± 0.94</td>
<td>3.72 ± 1.17</td>
</tr>
<tr>
<td>TCCSUP</td>
<td>2.26 ± 0.16</td>
<td>3.45 ± 1.41</td>
<td>4.9 ± 1.57</td>
</tr>
<tr>
<td>RT112</td>
<td>18.74 ± 9.9</td>
<td>26.43 ± 6.0</td>
<td>56.9 ± 2.6</td>
</tr>
<tr>
<td>RT112-CP</td>
<td>18.1</td>
<td>22.0</td>
<td>45.2</td>
</tr>
<tr>
<td>833K</td>
<td>6.24 ± 0.70</td>
<td>13.7 ± 2.26</td>
<td>13.7 ± 2.46</td>
</tr>
<tr>
<td>GH</td>
<td>8.0 ± 0.5</td>
<td>26.0 ± 9.02</td>
<td>15.5 ± 4.85</td>
</tr>
<tr>
<td>SuSa</td>
<td>8.2 ± 0.5</td>
<td>14.1 ± 3.2</td>
<td>9.9 ± 2.0</td>
</tr>
<tr>
<td>SuSa-CP</td>
<td>5.5</td>
<td>9.0</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>P = 0.846</td>
<td>P = 0.490</td>
<td>P = 0.644</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line</th>
<th>Spontaneous MF</th>
<th>MF at 4 µg/ml cisplatin</th>
<th>MF at IC₅₀ cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT112</td>
<td>11.5 ± 5.5</td>
<td>55 ± 35</td>
<td>55 ± 35</td>
</tr>
<tr>
<td>RT4</td>
<td>4.3 ± 0.3</td>
<td>4.3 ± 0.2</td>
<td>5.2 ± 1.4</td>
</tr>
<tr>
<td>833K</td>
<td>3.8 ± 0.5</td>
<td>13.2 ± 3.2</td>
<td>6.0 ± 1.2</td>
</tr>
<tr>
<td>SuSa</td>
<td>4.6 ± 0.3</td>
<td>9.5 ± 1.5</td>
<td>4.8 ± 0.3</td>
</tr>
</tbody>
</table>

A | B

Fig. 3. Dose-response curves to 6TG. A, parental lines; B, parental and cisplatin-resistant lines.

Fig. 4. Survival (A) and mutation frequencies (B) of RT112 cells after exposure to a range of concentrations of EMS. Right ordinate, recovery periods of 0, 6, 10, 13, 17, and 21 days; points, means; error bars, 1 SE either side of mean.
Mutation to 6TG Resistance in the Cisplatin-resistant Sublines. Using the same experimental design, we measured spontaneous and EMS-induced mutation frequencies in the RT112-CR and SuSa-CP sublines. The spontaneous frequencies (Table 2A) in these cell lines were not significantly different from their parental lines. Mutations at equimolar and equitoxic EMS concentrations were also compared (Table 2A), but these were also similar to the parental cell lines.

DISCUSSION

Spontaneous and induced mutation frequencies at the HGPRT locus were measured in three human bladder and three testis tumor cell lines. The mutation frequencies of the testis tumor cells were within the range of those of the bladder cancer cells, and thus we failed to demonstrate any difference between the two cell types.

The clinical implication of these results is that curability of testis tumors is not due to a low mutation frequency to drug resistance. This conclusion is based on limited data and two assumptions: (a) that mutation frequencies at the HGPRT locus reflect the degree of genetic stability throughout the genome, particularly at those loci controlling sensitivity to cisplatin and (b) that relative mutation frequencies between different cell types in vitro reflect those in vivo. While these are reasonable assumptions, there is no conclusive evidence that they are valid.

When it is possible to measure mutation frequencies in vivo at the loci controlling cisplatin sensitivity in tumors, then the implications of these data can be tested directly.

The hypothesis that tumors which are curable have a low rate of mutation to drug resistance is a corollary of the Goldie-Coldman theory (20). It was proposed that cure rate is inversely proportional to mutation rate, i.e., tumors with a high mutation rate will develop resistance earlier than those with a low mutation rate. While the data from this study imply that the corollary does not hold, this is not a test of the Goldie-Coldman theory, which is concerned with the evolution of drug resistance rather than the genetic basis of drug sensitivity.

The Goldie-Coldman theory was extended to explain the relationship between drug resistance, tumor growth rate, and PSR (21). In essence, the higher the PSR, the fewer cell divisions will be required to reach a stated size. Assuming that the mutation frequency is constant for each cell division, then relatively small differences in PSR will have a major influence on the rate at which drug resistance develops (21). This provides a further possible explanation for the curability of testis tumors; if they have a high PSR this would explain not only their rapid doubling time but also why drug resistance rarely develops. However, time lapse studies of testis and bladder tumor cell lines showed no differences in intermitotic times or cell loss rates.3

Another theoretical possibility is that testis tumors have a smaller stem cell fraction than, for example, bladder cancers. Circumstantial evidence from two sources indicates that this explanation is unlikely. First, testis and bladder cancers have similar clonogenic capacity in the human tumor colony-forming assay. Second, in patients, testis tumors have a 2- to 3-fold faster doubling time than bladder cancers.

While mutation rate and PSR theoretically can influence the curability of testis tumors, neither explains the inherent sensitivity of testis tumors to anticancer agents. Because testis tumor cells are inherently sensitive, a ‘resistant’ testis tumor cell can

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3 J. R. W. Masters and M. C. Walker, unpublished observations.
still be more sensitive than a bladder cancer cell. Experience of developing cisplatin-resistant cell lines in vitro supports this hypothesis. After 11 months of exposure to increasing concentrations of cisplatin a 4-fold increase in the resistance of a testis tumor cell line was obtained, but the line was now only as resistant as an untreated bladder cancer cell line (22). The inherent sensitivity of a tumor may reflect the sensitivity of the normal tissue from which it was derived (23).

The HGPRT locus was chosen for the measurement of mutation frequency because the locus has been extensively characterized (24) and mutants can be selected relatively easily. For example, spontaneous mutation frequencies at the ouabain resistance loci are approximately an order of magnitude lower than those obtained with the HGPRT locus (25). Mutations at the HGPRT locus can be induced by a wide variety of chemotherapeutic drugs (26) and have been used as a marker to compare the mutagenicity of new anticancer agents (27).

EMS was chosen as a mutagen because its mechanism of action is well described (9). However, because EMS is not used to treat tumors, the chemotherapeutic drug cisplatin was also used. Cisplatin, which is the most active single agent for bladder and testis tumors (1, 10), had a similar mutagenic effect to EMS at the HGPRT locus in two bladder and two testicular tumor cell lines.

In this study mutation frequencies were measured rather than mutation rates. Frequencies quantify the number of mutations in a given number of cells at a given time. Rates estimate the number of mutations per cell per locus per cell generation time and are a more sensitive measure of mutability. Rates may be more useful for identifying small differences in mutability between cell types, while frequencies give an overall measure of mutability. In this study, mutation frequencies between the two cell types overlapped. In the cell lines made resistant to cisplatin the mutation frequencies were similar to those of the parental cell lines. There is little published work with tumor cell lines relating drug sensitivity and resistance in vitro to differences in mutability. Drobetsky and Meuth (28) studied spontaneous mutation rates at 3 independent loci in Chinese hamster ovary cells resistant to the cytotoxic drugs 5-fluorodeoxyuridine and methotrexate. Spontaneous mutation rates to 6TG, ouabain, and emetine resistance were examined. Rates of mutation to ouabain resistance were elevated in the 5-fluorodeoxyuridine-resistant cells, but 6TG resistance was unchanged compared to control cells. In the methotrexate-resistant cells, elevated mutation rates were observed at all three loci. Therefore, mutation induction differed according to the locus studied and the chemotherapeutic drug used to induce resistance. The present study was limited to one locus and two drugs and failed to demonstrate differences in mutation frequency.

Some studies indicate that neoplastic transformation results in higher mutation rates, but the evidence is limited and contradictory. Comparison of spontaneous mutation rates in 3 normal and 3 malignant human lymphoblast cell lines showed elevated mutation rates in the cancer cells, indicating that transformation may be associated with increased mutability (29). However, spontaneous mutation rates at the HGPRT and ouabain resistance loci in normal and chemically transformed human fibroblast cells were similar (30).

Similar contradictory evidence exists when trying to relate changes in mutability to changes in metastatic potential of tumor cells. Spontaneous mutation rates at the HGPRT and ouabain resistance loci in clones of high and low metastatic potential derived from the murine fibrosarcoma UV-2737 have been measured. Clones with high metastatic potential had elevated spontaneous mutation rates compared to nonmetastatic cells (31). However, similar spontaneous mutation rates have been observed in variants of NIH 3T3 cells with metastatic capacity acquired following transfection with H-ras or exposure to 5-azacytidine and hydroxyurea, compared to untreated cells (32). In addition, similar spontaneous mutation rates at the HGPRT locus in mouse mammary tumors of different metastatic potential have been observed (33).

It is important that the molecular basis for drug sensitivity is identified. Extrapolating the in vitro data for cisplatin (4), a dose killing 10^12 clonogenic testis tumor cells would kill only between 3 and 4 logs of clonogenic bladder cancer cells. If these results accurately reflect clinical response, they explain the differences in inherent resistance to chemotherapy. It has been suggested by Mackillop (23) that inherent sensitivity or resistance may reflect that of the normal tissue from which the tumor is derived. For example, testicular tumors and some leukemias are curable with chemotherapy (1) and both normal tissues are hypersensitive to many chemotherapeutic drugs (34–37).

REFERENCES


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