Prediction of in Vivo Tumor Response to Chemotherapeutic Agents by the in Vitro Sister Chromatid Exchange Assay

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ABSTRACT

The ability of the in vitro sister chromatid exchange (SCE) assay to predict in vivo tumor drug sensitivity was investigated using a spontaneous hepatocarcinoma in C3H/Kam mice and 3 chemotherapeutic agents: melphalan; cis-platinum; and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). For hepatocarcinoma cells grown in monolayer culture, melphalan was the most efficient at inducing SCEs, and BCNU, the least. cis-Platinum induced a range in SCEs that overlapped those of BCNU and melphalan, suggesting that hepatocarcinoma is not a homogeneous population with intermediate sensitivity, but is a mixture of cis-platinum-sensitive and -resistant cells. According to in vitro cell survival curves, hepatocarcina was most sensitive to melphalan, less sensitive to cis-platinum, and essentially resistant to BCNU. The relative antineoplastic effects of melphalan, cis-platinum, and BCNU in vivo were compared by the response of artificial and spontaneous pulmonary metastases and solid tumors to these agents. For artificial metastases, there was a dose-dependent decrease in the number of lung nodules in mice treated with melphalan or cis-platinum, with melphalan being the more effective. BCNU had no effect. Spontaneous pulmonary metastases generated from hepatocarcinoma leg tumors were reduced in those mice treated with melphalan, unaffected by cis-platinum, and increased by BCNU. In hepatocarcinoma leg tumors (5 to 6 mm in diameter), melphalan induced the longest growth delay, and BCNU the least. Therefore, the relative effects produced by these three drugs in vivo were the same as predicted by SCE induction in vitro. The SCE assay may thus have potential clinical application.

INTRODUCTION

In the laboratory, the in vitro sensitivity of cells to antineoplastic drugs is usually determined from survival curves generated from the CFE assay. As a proposed method for predicting tumor sensitivity in vivo, the CFE assay has been performed recently using a large number of human tumor biopsy specimens. In over 1600 correlations, the in vitro CFE assay was successful in predicting 71% of the sensitive tumors and 94% of resistant tumors (1). However, this assay requires considerable time, 2 to 4 weeks, for the formation of the maximum number of colonies and, in addition, it does not provide information on the heterogeneity in chemosensitivity among tumor cell subpopulations unless extensive cloning studies are performed.

We have begun recently to investigate the ability of the SCE assay to predict the in vitro drug sensitivity of tumor cells. The SCE assay is a sensitive, rapid method for the detection of agents that damage DNA (18). In rat 9L brain tumor cells grown in monolayer, the induction of SCEs by BCNU and other nitrosoureas has been directly correlated with cell kill, with a linear increase in SCEs being detected at doses corresponding to the shoulder region of the cell survival curves (15). 9L cell lines resistant to BCNU-induced cell kill were also resistant to BCNU-induced SCEs (12). Using several other rodent cell lines and human brain tumor cell lines, the SCE assay has also been shown to predict the relative in vitro cell sensitivities to cis-platinum [cis-diaminedichloroplatinum(II)] (14) and BCNU (2). Furthermore, agents (α-difluoromethylornithine, X-rays) that modify drug-induced cell kill were also shown to modify drug-induced SCEs (11, 13).

Thus, for cells grown in vitro as a monolayer and treated with BCNU or cis-platinum, the SCE assay provides the same relative information about drug efficacy as the CFE assay. In addition, because the SCE assay is based on the analysis of individual cells, it can also be used to quantitate the heterogeneity existing in a mixture of drug-sensitive and -resistant cells, a condition present in many in situ tumors. We reported previously the results of experiments in which various proportions of BCNU-sensitive and -resistant 9L cells were mixed in monolayer culture and treated with BCNU (16). When the number of SCEs were counted and data plotted as histograms representing the number of cells versus SCEs per metaphase, 2 regions corresponding to the BCNU-sensitive and -resistant populations were obtained, and the approximate percentages of sensitive and resistant cells in each mixture could be predicted. Similar results were obtained from spheroids grown from mixtures of BCNU-sensitive and -resistant cells (10). These data suggest that the SCE assay may be useful in the analysis of the heterogeneity in drug sensitivity existing among the cell subpopulations of a single tumor.

Thus, the SCE assay might serve as a complementary or alternative method to the CFE assay for predicting human tumor chemosensitivity. As indicated above, SCE induction correlates well with in vitro cell survival for several established cell lines treated with BCNU or cis-platinum. However, to our knowledge, the SCE assay in vivo has not been investigated for its ability to predict in vivo tumor response to chemotherapeutic agents. Here, we report that in vitro SCE induction correlates highly with the in vivo response of a highly metastatic murine hepatocarcinoma, grown either as a solitary tumor in the leg or as micrometastases in the lung, to 3 chemotherapeutic agents.
MATERIALS AND METHODS

Mice. Inbred male C3Hf/Kam mice bred and maintained in our own specific-pathogen-free mouse colony were used. Mice were 11 to 15 weeks old at the beginning of each experiment.

Tumors. These studies used a sixth isotransplant generation of a spontaneously developed hepatic carcinoma syngeneic to C3Hf/Kam mice. The tumor, generously provided by Robert Sedlacek, Massachusetts General Hospital, Boston, MA, is highly metastatic and weakly immunogenic. Single-cell suspensions were prepared by trypsin digestion of nonnecrotic tumor tissue (5). Viability of cells was greater than 95%, as determined by phase-contrast microscopy and trypan blue exclusion.

Experiments on the antitumor activity of chemotherapeutic agents were performed with solitary tumors in the leg or with micrometastases in the lung generated by tumor cells injected i.v. To generate tumors in the leg, mice received injections of $5 \times 10^5$ viable tumor cells into the right hind thigh. When tumors grew to 6 or 8 mm in diameter, mice were treated i.p. with graded doses of BCNU, melphalan, or cis-platinum. To obtain tumor growth curves, 3 mutually orthogonal diameters of tumors were measured 3 times a week with a vernier caliper, and the mean values were calculated. Mice treated with the drugs when their tumors were 8 mm in diameter were killed 31 days after tumor injection, the lungs were removed, and the number of lung metastases was determined. Each group contained 7 to 10 mice.

To produce tumor micrometastases in the lung, $1 \times 10^5$ viable hepatocarcinoma cells were suspended in 0.5 ml of Hsu's medium (Grand Island Biological Co., Grand Island, NY) and injected i.v. into mice. Four days later, mice were given i.p. injections with graded doses of each drug. Ten days after treatment, mice were killed, and the number of lung nodules was determined using the method described earlier (4). Each group contained 7 mice.

Cell Culture. From the same single-cell suspension used to grow leg tumors and lung micrometastases, $10^7$ hepatocarcinoma cells were suspended in 0.5 ml of Hsu’s medium (Grand Island Biological Co., Grand Island, NY) and injected i.v. into mice. Four days later, mice were given i.p. injections with graded doses of each drug. Ten days after treatment, mice were killed, and the number of lung nodules was determined using the method described earlier (4). Each group contained 7 mice.

Drug Treatment. Stock solutions of all drugs used in this study were made immediately before use. BCNU was dissolved in ethanol; melphalan was dissolved in Solution A containing 3% IN HCI (v/v); and cis-platinum was dissolved in warm Solution A.

SCE Assay. After 1-h treatments with graded drug concentrations, cells were rinsed, and 15 ml of fresh medium containing 10 μM BrdUrd was added. Cells were allowed to replicate in the presence of BrdUrd for approximately 30 h. Points, mean of 20 well-spread metaphase cells; bars, SE. △, melphalan; ●, cis-platinum; ■, BCNU.

CFE Assay. After treatment for 1 h with graded drug concentrations, cells were trypsinized, counted, diluted, and plated into dishes containing 106 cells. Ten days after treatment, cells were trypsinized, and the number of colonies was determined using the method described earlier (4). Each group contained 7 mice.
RESULTS

SCE Formation. The SCE assay was performed on hepatocarcinoma cells grown in monolayer culture (passages 1 and 2) after treatment with graded doses of melphalan, cis-platinum, and BCNU (Chart 1). The dose-response relationships show that melphalan was the most efficient at inducing SCEs, with cis-platinum inducing fewer, and BCNU inducing essentially none in hepatocarcinoma cells.

SCE frequency histograms (number of cells versus SCEs/metaphase) were generated for hepatocarcinoma cells after treatment with the highest concentration tested of each drug (Chart 2). Treatment of cells with melphalan (3 μM) and BCNU (4 μM) resulted in cells with a range in SCEs/metaphase of 67 to 157 and 10 to 35, respectively. cis-Platinum treatment (4 μM), to which hepatocarcinoma is of intermediate sensitivity according to in vitro cell survival and SCE dose responses, resulted in cells with a range of SCEs/metaphase of from 22 to 161, a distribution overlapping that of BCNU and melphalan.

In Vitro Cell Survival. The in vitro cell survival curves after 1-h treatments with the 3 drugs are shown in Chart 3. According to these survival curves, hepatocarcinoma cells are the most sensitive to melphalan, less sensitive to cis-platinum, and essentially resistant to BCNU. These are the same relative cell sensitivities as predicted by the SCE assay, indicating that the induction of SCEs by melphalan, cis-platinum, and BCNU in hepatocarcinoma cells correlates with cell survival.

In Vivo Response of a Solid Tumor and Micrometastases. To compare the antineoplastic abilities of melphalan, cis-platinum, and BCNU in vivo, the effects of these 3 agents on artificial and spontaneous lung metastases and solid tumor growth were determined. Artificial lung metastases were generated by injecting hepatocarcinoma cells into the tail veins of mice. Four days after injection, when lung colonies were microscopic, graded doses of each drug were administered i.p.; 10 days later, the mice were killed, and the number of tumor nodules in the lung was determined (Chart 4). As compared to controls, there was a dose-dependent decrease in the number of lung nodules for both melphalan and cis-platinum, with melphalan being the most effective. BCNU treatment had no antimetastatic effect. In fact, the number of tumor nodules present in the lungs in some groups was higher than that in controls. These results are similar to the relative effects of chemotherapy as determined by the in vitro
SCE AS A PREDICTOR OF TUMOR CHEMORESPONSE

Chart 5. Growth of hepatocarcinoma leg tumors after treatment with melphalan, cis-platinum, and BCNU. Primary tumors were generated in muscles of the right hind thigh of mice with $5 \times 10^5$ hepatocarcinoma cells. Four days later, when leg tumors were between 5 and 6 mm in diameter, mice received graded doses of each drug i.p. Points, mean of 7 to 10 mice; bars, SE. A, melphalan; B, cis-platinum; C, BCNU.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>No. of lung nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>40.7 ± 6.8^a</td>
</tr>
<tr>
<td>Melphalan</td>
<td>5</td>
<td>14.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>5.1 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.9 ± 2.8</td>
</tr>
<tr>
<td>cis-Platinum</td>
<td>7</td>
<td>38.8 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>55.9 ± 15.8</td>
</tr>
<tr>
<td>BCNU</td>
<td>11</td>
<td>93.1 ± 19.6</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>81.3 ± 17.2</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>85.1 ± 22.1</td>
</tr>
</tbody>
</table>

^a Mean ± SE.

The effects of these agents on spontaneous lung metastases were also examined (Table 1). Hepatocarcinoma cells were injected into the right hind thighs of mice to generate leg tumors. When tumors were 8 mm in average diameter (9 days), mice were treated with graded doses of melphalan, cis-platinum, or BCNU. Only a slight decrease in tumor growth rate was detected after melphalan and cis-platinum treatments; BCNU had no effect on tumor growth. Tumors were allowed to grow for 22 days after treatment, at which time the mice were killed, and the number of lung metastases was determined. The mean number of lung metastases was 40.7. cis-Platinum treatment (7 and 10 mg/kg) had essentially no effect on spontaneous pulmonary metastases. The higher dose of cis-platinum (13 mg/kg), under these conditions, induced cell death in all mice by 8 days after treatment. Melphalan significantly reduced the number of lung metastases, to essentially the same level at all 3 doses tested. Treatment of mice with BCNU resulted in a significant increase in the number of pulmonary metastases.

The lack of a significant effect on tumor growth when the 3 chemotherapeutic agents were administered at a tumor size of 8 mm may have been the result of the large tumor cell burden at the time of treatment. The experiment was repeated with the exception that mice were treated with graded doses of melphalan, cis-platinum, and BCNU when hepatocarcinoma tumors were between 5 and 6 mm in diameter (Chart 5). The administration of melphalan or cis-platinum temporarily reduced tumor size, followed by the resumption of tumor growth. Treatment with cis-platinum (13 mg/kg) did not result in death as found for mice treated when tumors were 8 mm in diameter. The highest dose of BCNU (22 mg/kg) induced only a slight delay in tumor growth.

To compare directly the effects of melphalan, cis-platinum, and hepatocarcinoma on solid tumor growth, the time for tumors to grow to 12 mm after each drug dose was calculated and plotted in Chart 6. Melphalan was the most effective in delaying tumor growth, followed by cis-platinum, and then BCNU; therefore, in this experimental setting, the relative effects produced by the 3 drugs were the same as those predicted by the SCE assay.

DISCUSSION

Since the administration of effective antineoplastic drugs plays a crucial role in the success of cancer therapy, a great deal of interest has been focused on the development of methods that would enable the selection of appropriate drugs prior to the initiation of therapy. Earlier in vitro studies (2, 10–16) suggested that the SCE assay may be useful in this regard. However, if the validity of the SCE assay as a clinical method is to be established, it must be directly compared to in vivo tumor response. In this paper, we have shown that the relative effectiveness of 3 chemotherapeutic agents as determined by in vitro SCE induction correlated well with not only in vitro survival of hepatocarcinoma cells, but also the in vivo response of these cells grown as solitary tumors or micrometastases.
Hepatocarcinoma cells, according to the data presented herein, are essentially resistant to BCNU. In addition, treatment of mice bearing 8-mm hepatocarcinoma leg tumors with BCNU actually increased the number of spontaneous pulmonary metastases. At the doses of BCNU administered, there were no overt signs of drug toxicity and no effects on the growth rate of solid tumors. Poupon et al. (8) found an increase in spontaneous pulmonary metastases in rats bearing a rhabdomyosarcoma, a mammary adenocarcinoma, or a nickel-induced soft tissue tumor after treatment with chloroethyl nitrosourea of cysteamine and 2 other nitrosoureas, chlorozotocin and hydroxyethyl nitrosourea, even when the growth of the primary tumor was reduced. It has been reported earlier by several groups (6, 9, 17) that prior treatment of mice with different chemotherapeutic agents increased artificial pulmonary metastases formation. Thus, a method that could predict drug sensitivity would greatly reduce the probability of administering a drug with little or no antineoplastic activity and, therefore, reduce or eliminate the potential for the iatrogenic promotion of tumor spread.

Based solely on dose-response curves, SCE data provide the same relative information as the CFE assay with respect to the antitumor activity of melphalan, cis-platinum, and BCNU. However, additional information can be gained from the SCE assay owing to its potential ability to evaluate tumor heterogeneity. Many neoplasms, both human and animal, consist of cell subpopulations that can be heterogeneous with respect to a number of biological characteristics, including drug sensitivity (3). This heterogeneity in drug sensitivity among tumor cell subpopulations is now regarded as a major obstacle to the successful chemotherapeutic management of cancer. Thus, a method that could evaluate the tumor heterogeneity of drug sensitivity would be of obvious therapeutic advantage. Since the SCE assay examines the drug sensitivity of individual cells, apparently, it can be utilized for such purposes. In the SCE frequency histograms generated from the treatment of hepatocarcinoma cells with cis-platinum (Chart 2), there was a range in SCEs per metaphase that overlapped those of hepatocarcinoma cells treated with BCNU, to which hepatocarcinoma is resistant, and melphalan, to which hepatocarcinoma is sensitive. This SCE frequency histogram suggests that hepatocarcinoma is not a homogeneous population with respect to cis-platinum sensitivity, but rather consists of cell types with a range of sensitivities, including cells that are resistant to cis-platinum. Information of this type could potentially be of clinical importance, since the design of treatment protocols for a tumor consisting entirely of cells with intermediate drug sensitivity would not be the same as a treatment protocol for a tumor that contained a subpopulation of resistant cells.

In conclusion, our results show that the in vitro SCE assay can be used for the prediction of in vitro and in vivo response of murine hepatocarcinoma cells to treatment with melphalan, cis-platinum, and BCNU. In addition, the method can provide information on the heterogeneity in the response of tumor cell populations to a single chemotherapeutic agent. As such, the SCE assay appears to have a potential application in the clinical prediction of tumor sensitivity to chemotherapy.

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REFERENCES


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