Excision Repair of cis-Diaminedichloroplatinum(II)-induced Damage to DNA of Chinese Hamster Cells

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ABSTRACT

Platinum was lost from the DNA of cis-diaminedichloroplatinum(II) [cis-Pt(II)]-treated exponentially growing Chinese hamster V79-379A cells with a half-life of 28 hr. By contrast, platinum was lost from the DNA of cells treated in stationary-phase culture with a half-life of 4 days. Cells treated in and allowed to remain as a stationary-phase culture with a half-life of 4 days. Cells treated in and allowed to remain as a stationary-phase culture maintained an intact and apparently viable appearance. When the stationary-phase culture was diluted with fresh medium, cell division occurred, and cell survival, as measured by colony-forming ability, could be determined. Dilution of cells immediately after treatment with 40 μM cis-Pt(II) resulted in 0.19% control survival. There was an increase in the ability of the stationary-phase cells to survive cis-Pt(II) damage with time after treatment. Thus, after 3 days, the survival had increased from 0.19 to 15.9%. We demonstrate that this increased ability of stationary-phase cells to survive with time after treatment is due to DNA excision repair and hence that survival is inversely related to the extent of reaction of cis-Pt(II) with the DNA.

INTRODUCTION

Neutral platinum-coordinated complexes are currently being used in the treatment of several neoplastic diseases (3). The cytotoxic effects of these compounds are apparently associated with their ability to bind with cellular DNA (12, 17, 20). Replication of the DNA in the presence of platinum-induced damage to DNA results in alterations in the rate and product of DNA synthesis (9, 10, 22). We wished to determine whether cells were capable of reducing these effects by removing platinum-induced lesions from their DNA.

A number of reports have provided circumstantial evidence for the existence of a mechanism(s) for removing DNA-bound platinum adducts in both prokaryotic and eukaryotic cells. Drobnik et al. (7) and Beck and Brubaker (2) have suggested, on the basis of experiments with various DNA repair-deficient mutants of Escherichia coli, that cis-Pt(II)2-induced damage was reversible and that this might be related to an excision or recombination repair facility. In Chinese hamster V79 cells, alkaline sucrose gradient centrifugation studies of cis-Pt(II)-treated cells demonstrated time-dependent changes in the sedimentation characteristics of DNA, which could be due to the loss of interstrand cross-links during 20 hr by a DNA repair process (21). A comparison of the relative sensitivities of normal and excision repair-deficient human cells in culture to cis-Pt(II) suggested that DNA excision repair contributed greatly to the ability of the cell to survive (8). In addition, the formation and disappearance of interstrand DNA cross-links, measured by the relative rate of alkaline elution of DNA through a polystyrene chloride filter, is consistent with the presence of an excision repair mechanism (23).

We present here data demonstrating that platinum is indeed removed from the DNA of both exponentially growing and stationary-phase Chinese hamster V79 cells. In addition, we show that the survival of stationary-phase cells, plated out at various times after cis-Pt(II) treatment, is directly related to the amount of platinum remaining in the DNA following the action of a DNA excision repair mechanism.

MATERIALS AND METHODS

Cell Culture

Chinese hamster V79-379A (V79) cells, an established line in our laboratory, were grown as a suspension culture at 37°C in Eagle’s minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.; No. F14) supplemented with 7.5% fetal calf serum (Grand Island Biological Co.) as previously described (18).

Estimation of Loss of Platinum from Cellular DNA

In Vivo under Exponential Growth Conditions. The DNA of Chinese hamster V79 cells growing at a cell density of 1.5×106 cells/ml was prelabeled with [14C]thymidine (10 nCi/ml medium) (Radiochemical Centre, Amersham, Buckinghamshire, England; 61.5 mCi/mmol) during 16 hr. The culture was subsequently treated with 80 μM cis-Pt(II) (Johnson Matthey Research Centre, Reading, Oxon, England). After a 1-hr treatment, the cells were pelleted by gentle centrifugation at 37°C (MSE bench centrifuge at 600 rpm) and resuspended in fresh medium at 4×106 cells/ml. Aliquots were removed at various times, the DNA was extracted (8, 14), and the platinum content of the DNA was estimated by atomic absorption spectroscopy (Perkin Elmer, Beaconsfield, Buckinghamshire, England; Model 306) as previously described (8). The extent of dilution of the original DNA content was calculated from the ratio of the specific activity of the DNA (dpm [14C]thymidine per mg DNA) at various times after treatment compared to time zero.

In Vivo under Stationary-Phase Conditions. DNA template-labeled cells (8×106/ml) (see above) were gently centrifuged and resuspended in fresh medium at 106 cells/ml. Following 2 days of stirred incubation at 37°C, a station-
The platinum content of the DNA was determined as previously described (8). Aliquots were removed from the culture medium which has been used to grow cells from 1 x 10⁶ to 1 x 10⁷ cells/ml and then followed by filtration through a 0.22-μm pore size. Aliquots were removed from the culture at various times. The platinum bound to DNA and extent of dilution of the original DNA content were estimated (see above) together with the colony-forming ability and number of cells in the culture (18).

**In Vitro DNA Solutions.** DNA from Chinese hamster V79 cells previously treated with either 80 or 160 μM cis-Pt(II) was extracted (8, 14) and dissolved (0.3 mg DNA per ml) in phosphate-buffered saline [136 mM NaCl solution-2.7 mM KCl-8.1 mM Na₂HPO₄-1.47 mM KH₂PO₄ (pH 7.3)]. The solutions were gently agitated by orbital shaking at 37°C. Samples were removed at various times, and the DNA was reextracted by precipitation with 2 volumes of 2-ethoxyethanol. The platinum content of the DNA was determined as previously described (8).

**RESULTS**

**Excision of DNA-bound Platinum in Exponential Growth.** Treatment of exponentially growing Chinese hamster cells with 80 μM cis-Pt(II) resulted in a binding of 45.4 nmol platinum per g DNA and a survival of 0.1%. The platinum content of the cellular DNA isolated at various times after treatment was found to decrease at 38 hr. To calculate what proportion of this decrease represented "true" loss from the DNA, account was taken of the increase in the amount of DNA present in the culture as a result of DNA replication. Thus, 38 hr after cis-Pt(II) treatment, the DNA content was 2.71 times greater than at the time of treatment (Table 1). It was calculated that platinum was removed at a constant rate and had a half-life of 28 hr (Chart 1).

**In Vitro Stability of DNA-bound Platinum.** To establish that DNA-bound platinum is stable in vitro in the absence of DNA excision repair enzymes, DNA was isolated from cells previously treated with cis-Pt(II) (see "Materials and Methods") and was dissolved in phosphate-buffered saline at 37°C. The extent of platinum bound to the DNA, expressed as a percentage of the initial platinum content, was plotted against time after making a DNA solution.

**Excision of DNA-bound Platinum in Stationary-Phase Cells in Relation to Increased Survival.** Stationary-phase Chinese hamster V79 cells treated with 40 μM cis-Pt(II) for 1 hr bound 25.4 nmol platinum per g DNA. During 3 days in

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**Table 1**

A summary of the method of calculating the rate of removal of DNA-bound platinum from both exponential and stationary-phase cells using particular experiments as examples rather than averages from several experiments.

The percentage of survival and cell number data are included for the sake of comparison.

<table>
<thead>
<tr>
<th>Cell growth condition at time of treatment</th>
<th>Dose (μM) for 1 hr</th>
<th>Extent after treatment (hr)</th>
<th>Extent of platinum bound to DNA (nmol platinum/g DNA)</th>
<th>Specific activity of [14C]thymidine-labeled DNA (dpm/mg DNA)</th>
<th>Increase in DNA content (fraction of time zero value)</th>
<th>Extent of retention of platinum bound to DNA (% original)</th>
<th>% cell survival</th>
<th>No. of cells (10^6 x cells/ml)</th>
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<tr>
<td>Exponential</td>
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<td>0.1</td>
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<td>10</td>
<td>21.99</td>
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<td>1.356</td>
<td>66</td>
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<td>13.26</td>
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<td>60.1</td>
<td>1.0</td>
<td>5.67</td>
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<td>29.5</td>
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<td>14.9</td>
<td>8.1</td>
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</table>

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- a) The survival of the stationary-phase cells was calculated as a fraction of the time zero cell number rather than of the cell number at the time of plating out.
- b) The DNA was labeled with [14C]thymidine before reaching stationary phase.
- c) Extent of increase in the DNA content obtained from a culture using preconditioned medium.
- d) Extent of retention of DNA-bound platinum in a culture using preconditioned medium.

**Chart 1.** The rate of excision of DNA-bound platinum from exponentially growing Chinese hamster V79 cells (O) and isolated DNA in vitro (•, □). In exponentially growing cells, the platinum bound to the DNA is expressed as nmol platinum per g DNA or as a percentage of the initial platinum content of the DNA versus time after treatment. The data were corrected for the extent of dilution of the DNA due to DNA synthesis. --- --- --- final-half-life of the DNA-bound platinum lesions. For in vitro studies, solutions of DNA in phosphate-buffered saline extracted from cells previously treated with cis-Pt(II) (80 μM, □; 160 μM, △) were allowed to agitation gently at 37°C. The extent of platinum bound to the DNA, expressed as a percentage of the initial platinum content, was plotted against time after making a DNA solution.
stationary phase, platinum was lost from the cellular DNA with a half-life of 4 days (Chart 2). Account was again taken of the extent of dilution of the platinum content of total DNA by the synthesis of new DNA during the period of the experiment (Table 1). When stationary-phase cells were resuspended in fresh medium, a doubling of the DNA content occurred during 24 hr. However, the cell number did not increase. By contrast, resuspension of the cells in preconditioned medium did not result in any DNA synthesis (Table 1). The addition of preconditioned rather than fresh medium results in a deterioration in the condition of the cells after 2 days but did not appear to alter the rate of excision of platinum from the DNA (Table 1, Footnotes c and d). Since the rate of platinum excision was comparatively slow and the maintenance of viability was important, the use of fresh medium was preferred. Under these conditions, the loss of platinum could be determined over 3 days rather than 2.

Samples removed from the stationary-phase culture at the same time as those used to determine DNA-bound platinum were used to determine the colony-forming ability of cells. Since there was some decrease in the cell number during the 3 days, the surviving fraction was related to the initial cell number of the culture rather than that at the time of plating. This, therefore, gave an accurate reflection of the ability of the cells to survive the cis-Pt(II) treatment. The plating efficiency was 60% throughout the experiment. Plating cells out immediately after treatment with 40 μM cis-Pt(II) resulted in a survival of 0.23% compared to the control. However, assaying samples from the stationary culture 0.5, 1, 2, and 3 days after treatment resulted in a progressive increase in the ability of cells to survive. After 3 days, the survival in this particular stationary-phase culture had risen from 0.23 to 14.9% (Table 1). The average result of 2 experiments is shown in Chart 3 in which the survival increased from 0.19 to 15.9% during 3 days after a 1-hr treatment with 40 μM cis-Pt(II).

Combining the survival and binding data, it is possible to demonstrate that the survival of Chinese hamster cells is directly related to the extent of binding of cis-Pt(II) to the DNA (Chart 4). The results are in good agreement with an experiment in which the extent of platinum binding to DNA and survival were determined following treatment with a number of doses of cis-Pt(II). Hence, with increasing extent of reaction of platinum to DNA, there was a concomitant decrease in the ability of the cells to survive. The removal of platinum from the DNA by an excision repair process correlates directly with an increased ability of the cells to survive. Chart 4 also includes the time zero points from 2 other experiments. However, since no DNA dilution determinations were available in these instances, values for later times have been omitted. Extrapolating the binding data to 100% survival, it can be seen that cells are able to tolerate...
an initial binding of 6 nmol platinum per g DNA without any toxicity. In addition, the $B_i$ dose (where $B_i$ is the extent of binding required to reduce the survival from a fraction, $f$, to 0.37f on the exponential part of the curve) was calculated as 3 nmol platinum per g DNA.

**DISCUSSION**

Chinese hamster V79 cells growing exponentially excise platinum lesions from their DNA 4 times faster than cells maintained in stationary phase. Thus, under our conditions, the rate of loss of platinum was logarithmic in exponential cells with 50% of the induced lesions being removed within 28 hr. Similar studies, using both a number of different cell lines and chemical agents, have been reported previously. In general, they demonstrated that in exponential growth the time required to remove 50% of the DNA adducts introduced by sulfur mustard (1), 4-nitroquinoline-1-oxide (13), 7-BMBA (6, 11), and benzo[a]pyrene (19) was between 24 and 33 hr. In addition, the relatively slow rate of removal of platinum from the DNA in stationary-phase cells is in agreement with reports using 7-BMBA. Thus, nondividing lymphocytes required 12 hr to remove 16% of 7-BMBA-induced adducts from their DNA (16). Similarly, 7-BMBA is excised from stationary-phase Chinese hamster cells with a half-life of 4 days (11). Although these data imply a similar DNA excision repair pathway for all of these agents, the results could merely reflect a common rate-limiting step for a number of different pathways.

Although we have demonstrated that platinum is removed from the DNA, the precise spectrum of products and the relative proportion of these removed are unknown. Thus, only a proportion of the DNA-platinum lesions might contribute to cytotoxicity. In this case, the data suggest that the cytotoxic lesions are excised from the DNA at a rate similar to those of other nontoxic lesions. Hence, there is a linear relationship between the number of platinum lesions removed from the DNA during a given period and the increase in the logarithm of the survival of stationary-phase cells. Assuming a weight of 10 pg of DNA per cell and the $B_i$ value for stationary phase-treated cells shown above, we calculate that a population of cells will have to remove 1.87 x 10^4 platinum molecules from each cell genome to increase the survival from 0.37f to f. However, exponentially growing cells are less sensitive to cis-Pt(II)-induced damage of DNA ($B_i$ dose 8.5 nmol Pt per g DNA (17)) compared to stationary- and $G_1$-phase-treated cells (9, 10). Hence, for cells in exponential growth, 5.12 x 10^4 platinum lesions would have to be removed from each cell in a culture to increase the survival from 0.37f to f. Cells in exponential growth need to remove 2.74 times more platinum from their DNA compared to cells in stationary phase to increase their survival by the same amount. The apparently higher rate of DNA excision repair of platinum in cells in exponential growth compared to that in cells in stationary phase (3.4 times) does not therefore confer a greatly different ability of the former to survive.

Konz-Thomas et al. (15), using confluent human fibroblasts and UV-induced DNA damage, demonstrated an increase in survival from 20 to 100% compared to a control during 16 hr posttreatment. Over the same period, 90 to 100% of the thymine dimer lesions were excised from the DNA. These results and those presented here indicate that cell killing results from the presence of unexcised DNA damage. Thus, our data strongly support the view that DNA is the cytotoxic target of the neutral platinum compounds.

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**REFERENCES**


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