Increased DNA Repair as a Mechanism of Acquired Resistance to cis-Diamminedichloroplatinum(II) in Human Ovarian Cancer Cell Lines

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ABSTRACT

A human ovarian cancer cell line, A2780, derived from an untreated ovarian cancer patient and relatively sensitive to cisplatin was treated by stepwise incubation with cisplatin to produce a cisplatin-resistant variant, 2780CP. The relative abilities of these cell lines to repair cisplatin-induced damage to cellular DNA then was examined by measure of [3H]thymidine incorporation into normal density DNA separated from bromodeoxyuridine-substituted DNA on alkaline cesium chloride gradients. These studies revealed that primary cisplatin resistance present in 2780CP was associated with a near twofold-increased ability to repair damage induced by the drug under conditions where 2780CP was approximately 5-fold resistant to cisplatin. Aphidicolin, a specific inhibitor of DNA polymerase α, showed a dose-dependent capacity to inhibit DNA repair in this system with maximum inhibition of 63% at 4 μg/ml. It was also found that inhibition of DNA repair during and shortly after cisplatin exposure resulted in an approximately threefold increase in the cytotoxicity of cisplatin as monitored by clonogenic cell survival in the resistant but not the sensitive parental cell line.

INTRODUCTION

The treatment of many human tumors requires the use of systemic drugs to eliminate metastatic disease not amenable to cure by primary surgery or irradiation. Thus, combination chemotherapy is often indispensable in the effective treatment of cancer. All too often, however, after an initial response to combination chemotherapy, patients are left with residual disease which is unresponsive to further treatment with the original drugs and is also resistant to a wide spectrum of structurally unrelated agents (1). This phenomenon of pleiotropic drug resistance, or multidrug resistance, is particularly evident in ovarian cancer where the response rate to induction chemotherapy is high (70%) but only 20–25% of patients are cured due to the development of resistance to the initial therapy as well as cross-resistance to salvage therapy (2). Insights into the mechanisms of acquired resistance may permit the design of therapeutic strategies which prevent or delay its onset or reverse the resistance once it develops.

To study the problem of acquired resistance to chemotherapeutic agents and cross-resistance to irradiation we have developed ovarian carcinoma cell lines with induced primary resistance to Adriamycin, melphalan,2 and cisplatin from the parental cell line, A2780, derived from an untreated patient (3). These matched pairs of drug-sensitive/resistant cell lines have proven useful to study the potential mechanisms of drug resistance including alterations in drug transport (4), metabolism to less active compounds (5), gene amplification (6), and induction of cellular protective agents such as glutathione (5, 7). In the 2780CP cell line, induced resistance to cisplatin was associated with cross-resistance to the classical bifunctional alkylating agent melphalan, to irradiation, and to Adriamycin. The development of the resistance and cross-resistance phenotype of this cell line is probably by multiple mechanisms but overexpression of the P-170 glycoprotein with associated reduced drug accumulation is not among the mechanisms.

Little is known in regard to the mechanisms operative in the development of resistance to cisplatin, currently one of the most effective drugs for the treatment of human solid tumors including ovarian cancer. The cytotoxicity of cisplatin is accepted to be produced through binding to DNA and attendant interference with transcription and replication. Thus, a cell's only option for continued survival once these covalent interactions have occurred is the recognition and efficient repair of the lesions. Details of the mechanisms by which mammalian cells recognize and deal with such potentially lethal damage remain to be established with the term "excision repair" being generally applied to the processes which doubtless require many enzyme systems (8). A first and critical step in this series of events involves the recognition and incision of the DNA strand near a lesion. This step has special implication with regard to cisplatin as the major DNA lesion produced is a bidentate intrastand cross-link between the N7 position of adjacent guanines (9, 10). This lesion should be quite effective at blocking the transcription so critical to survival but likely would present problems to recognition processes. Thus, the marked activity of this drug in clinical cancer therapy, in part at least, could be explained. These concepts have led us to examine the possibility that the increased ability of 2780CP to survive potentially lethal damage by cisplatin relates to an increase in ability to recognize and repair such damage. This possibility has been studied by exposing the A2780 and cisplatin-resistant 2780CP cell lines to cisplatin and measuring [3H]dThd into normal density DNA as a monitor of repair to the entire genome. In addition, the ability of aphidicolin to inhibit the DNA repair process and influence the cytotoxicity of cisplatin is reported.

MATERIALS AND METHODS

Cell Lines and Routine Culture Conditions. A2780 is an ovarian carcinoma cell line derived from an untreated patient (11). This cell line was made resistant to cisplatin (from the Pharmaceutical Resources Branch, NCI) by stepwise incubation with the drug (3, 12, 13) up to final concentration of 70 μM. Once resistance was induced, the resistant variants 2780CP and A2780 were maintained in Roswell Park Memorial Institute 1640 medium augmented to contain 10% (v/v) FBS, 0.25 units/ml insulin (Eli Lilly, Indianapolis, IN), 100 μg/ml streptomycin, 100 units/ml penicillin, and 0.3 mg/ml glucose. Basal medium and all additives except insulin were from Gibco, Grand Island, NY. Growth was at 37°C in a humidified atmosphere of 5% (v/v) CO2 in air.
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Determination of Resistance to Cisplatin and Effect of Aphidicolin on Cisplatin Cytotoxicity. Clonogenic cell survival was assessed by determination on a Bausch and Lomb Omnicon FAS II Image Analysis System of colonies which formed after 7 to 10 days from single cell suspensions plated in RPMI growth medium modified to contain 0.3% (w/v) agarose and a range of concentrations of the drugs to be tested (14). In the case of cytotoxicity studies which included aphidicolin, cells were grown as described below for assessment of DNA repair activity, i.e., grown to heavy confluence in 25-cm² dishes and switched to MEM (GIBCO) without arginine and with 2.5% dialysed FBS. After three days in arginine deficient medium with 2.5% dialysed FBS, cisplatin alone was added or in combination with aphidicolin which was added 1 h prior to cisplatin. One h after exposure to cisplatin cultures were washed with PBS and fresh arginine deficient medium with 2.5% dialysed FBS and with or without aphidicolin was added to cultures. At times indicated in text and figure legends, cells were harvested with trypsin and assessed for clonogenic cell survival as described above.

Assessment of DNA Repair Activity by Equilibrium Sedimentation Analysis. Cell lines were plated in T-75 culture flasks in MEM with 10% FBS, and after 24 h of culture prelabeled for 24 h with 0.004 μCi/ml [14C]dThd (50 mCi/mmol, NEN). Cultures were harvested 48 h later; the cells from a given cell line then were combined, placed in culture dishes at high density (10⁶ cells/25 cm² dish) in the presence of MEM with 10% FBS and grown to heavy confluence (72 h). At this point the cells were treated medium with 2.5% dialysed FBS and treated for 4 h with 0.15 M NaCl/sodium citrate (0.15 M/0.015 M) and pH adjustment by addition of 200 μl of 5 N NaOH (15-17). Digests then were made to a density of 1.72 g/ml with cesium chloride and centrifuged to equilibrium in a Beckman type 50 Ti rotor at 100,000 × g. Fractionation of gradients was bottom to top with 24 fractions of 0.25 ml collected. DNA was precipitated by the addition of 2 ml of cold trichloroacetic acid (10% w/v) and collected on cellulose filters. Filters were washed with cold trichloroacetic acid (5% w/v) followed by water and ethanol. Radioactivity was determined in a Beckman LS 2800 liquid scintillation counter using Aquassure (NEN) as scintillant and with correction for 14C and 3H channel overlap. DNA repair activity was determined as the ratio of [3H]dThd incorporation into normal density DNA in the presence of drug to incorporation in its absence after normalization of 3H dpm by 14C dpm.

RESULTS

The concentration of cisplatin that inhibited clonogenic cell survival by 50% was found to be 1.02 μM for A2780 and 38.5 μM for 2780CP under continuous exposure conditions. If exposure to cisplatin, however, was for 1 h under the conditions used for assessment of DNA repair capacity with subsequent cloning in agarose, the degree of resistance to cisplatin in 2780CP was found to be approximately 5-fold (20 μM IC₅₀ 2780CP/4 μM IC₅₀ A2780).

The ability of cisplatin to induce dose-dependent increases in DNA repair activity, as monitored by [3H]thymidine incorporation into normal density DNA in near stationary phase cultures in the presence of hydroxyurea to help block residual semiconservative DNA synthesis and bromodeoxyuridine to shift the density of any DNA synthesized as part of the replicative process away from the normal density region of the gradient, is shown in Fig. 1A. As indicated in the figure, both the parental and resistant cell lines show cisplatin inducible capacity for DNA repair. The resistant cells, however, on a dose for dose basis show a nearly twofold greater capacity to repair cisplatin damage. This difference was confirmed by analysis of the data as dpm/μg DNA where it was found for example that after damage with 160 μM cisplatin the parental A2780 cell line incorporated 53 ± 8 dpm/μg of DNA and the resistant cell line 130 ± 23 dpm/μg of DNA. The relationship between the relative cytotoxicity of cisplatin in A2780 and 2780CP to their respective repair activities at equitoxic cisplatin dose is shown in Fig. 1B. Cisplatin doses which allow for 10% clonogenic cell survival in the respective cell lines result in a near threefold greater capacity to repair drug-induced damage in the resistant versus the sensitive cells.

It has been reported that aphidicolin, a tetracyclic diterpenoid antibiotic isolated from the fungus Cephalosporium aphidicola can inhibit DNA repair by nature of its ability to inhibit DNA polymerase α, an enzyme of importance to the DNA excision repair process (18). Aphidicolin was evaluated for this activity in A2780 and 2780CP. As shown in Fig. 2, the antibiotic inhibited cisplatin-induced DNA repair in 2780CP in a dose-dependent manner with maximum inhibition at 4 μg/ml. DNA repair as assessed with the gradient shift methodology was similarly inhibited by aphidicolin in A2780 cells.

The relationship of DNA repair capacity to the cisplatin resistance phenotype of 2780CP was evaluated by an assay for clonogenic cell survival after aphidicolin-mediated inhibition of repair of cisplatin-induced DNA damage. A2780 and 2780CP treated with equitoxic doses of cisplatin but doses which did not markedly inhibit clonogenic cell survival were either held in stationary phase culture for 24 h in the absence or presence of aphidicolin then subjected to the assay for clonogenic cell survival. As shown in Fig. 3A aphidicolin alone showed no dose-dependent toxicity in either cell line, and this approach to inhibition of repair had no effect on the cytotoxicity of cisplatin in the parental A2780 cell line. Inhibition of repair after cisplatin damage in 2780CP, however, was associated with a marked increase in the cytotoxicity of the drug. To determine the magnitude of the aphidicolin effect on cisplatin cytotoxicity in the resistant cell line, dose-response curves to cisplatin after maintenance in the absence and presence of aphidicolin (2 μg/ml) for 24 h in stationary phase were prepared by subsequent clonogenic assay. These data revealed an aphidicolin mediated shift of the IC₅₀ for cisplatin of 3.5-fold; similar experiments in the parental cell line resulted in a negligible aphidicolin effect on cisplatin cytotoxicity using aphidicolin doses to 10 μg/ml.

More detail of the timeframe during which repair of cisplatin damage occurs and hence ability to grow and form colonies in agarose was established for 2780CP cells through treatment with cisplatin of near stationary phase culture in the absence or presence of aphidicolin. Cell viability was assessed by clonogenic assays at various times after cisplatin treatment. As can be seen in Fig. 3B, treatment of cells with 40 μM cisplatin resulted in a clonogenic cell survival of less than 20% at 4 h after cisplatin treatment at the time when cells were harvested for the DNA repair assay. Furthermore, these data show that maintenance in stationary phase for 96 h prior to culture conditions which require replicative DNA synthesis allows for substantial recovery from cisplatin damage, a process totally inhibited by aphidicolin.

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Fig. 1. DNA repair after cisplatin damage of human ovarian cancer cell lines. A, repair indexes of the sensitive (○) and cisplatin-resistant variant (△) cell lines show with respect to the concentration of cisplatin used to damage the cells. B, repair indexes of the sensitive (○) and cisplatin-resistant (△) cell lines shown with respect to percentage clonogenic cell survival. For B, the data points were generated using actual repair indexes for X-coordinates and determination of Y-coordinates graphically from repair index and clonogenic cell survival data. DNA repair activity was determined as the ratio of [3H]dThd incorporation into normal density DNA in the presence of drug to incorporation in its absence after normalization of [3H] dpm by [14C] dpm.

Fig. 2. Inhibition of DNA repair by aphidicolin in the cisplatin-resistant human ovarian cancer cell line 2780CP. Cells were treated as for the standard DNA repair assay except that aphidicolin was added 1 h prior to cisplatin and continued during the 4-h [3H]TdThd exposure. Cisplatin treatment was at 40 μM. DNA repair activity was determined as the ratio of [3H]TdThd incorporation into normal density DNA in the presence of drug to incorporation in its absence after normalization of [3H] dpm by [14C] dpm.

DISCUSSION

In separate communications, we have reported that 2780CP is cross-resistant to melphalan, Adriamycin, and irradiation (7, 12, 13) thus making it a suitable model to study clinical drug resistance as the resistance mimics the clinical situation where patients treated aggressively with cisplatin are very unlikely to respond to any of the other modalities (1, 2). The cytotoxic lesions produced by cisplatin are generally considered to be at the nuclear level through formation of DNA intra- and interstrand cross-links and perhaps DNA-protein cross-links with the most prominent lesion as discussed earlier being the N7 guanine bidentate intrastrand adduct (9, 10, 19). In the cancer cell there exists multiple potential sites where cisplatin can be inactivated prior to the drug's interaction with DNA, but once the lesion in DNA has been produced it is possible the cell's primary option, if continued survival is to be achieved, would be to repair the damage efficiently. Thus, we have attempted to determine if cisplatin resistance is associated with an increased ability to repair DNA damage produced by the drug.

Our results clearly show that more DNA repair activity is present in resistant cells versus sensitive cells after exposure to equal amounts of cisplatin and the differences become greater if repair activity at equitoxic doses is compared. It is notable that under the stationary phase conditions needed for analysis of DNA repair the magnitude of resistance to cisplatin is only 5-fold in 2780CP. Thus, a twofold difference in repair activity between the sensitive and the resistant cell lines could account for a substantial portion of the resistance observed under these conditions especially when it is considered this assay gives no indication as to the fidelity of the repair process nor any insight into preferential repair of genes undergoing active transcription (19).

An indication that a portion of the resistance to cisplatin in 2780CP is causally related to the increased cisplatin-associated DNA repair activity of the cell line is demonstrated by studies with aphidicolin, a specific inhibitor of DNA polymerase α which acts through competitive inhibition of binding of dCTP to the enzyme. Although aphidicolin showed an equivalent ability to inhibit repair activity in the sensitive and the resistant cell lines, its ability to influence cisplatin cytotoxicity was markedly different between A2780 and 2780CP. Whereas aphidicolin had no effect on cisplatin cytotoxicity in the parental cell line, its effect in the resistant cells was substantial. The combination was greater than three times as effective as cisplatin alone as monitored by change in IC50 concentration of cisplatin. Thus, there appears to be a close relationship between the difference in repair activities of the cell lines at equitoxic doses of cisplatin and the effect of inhibition of DNA repair on the cytotoxicity of cisplatin in the resistant cell line. The failure of aphidicolin to modulate cisplatin cytotoxicity in the sensitive cell line in the presence of its ability to reduce repair activity in that cell line is of interest. A close analysis of the data reveals that the amount of repair activity induced in the parental cell line by a dose of cisplatin relevant to survival is relatively low compared to that required to remove the more abundant potentially lethal lesions anticipated to occur in the resistant cells at the higher drug dose needed to achieve equivalent survival (Fig. 1B). Thus, if damage tolerance is neglected as a possibility, these data support the concept that the sensitive cells cannot recognize or otherwise effectively deal with the finite number of DNA lesions which result in a decrease in cell survival to a
given set point. In contrast, the resistant cell's repair machinery is such that a percentage of the considerably greater number of lesions formed by the higher drug dose required to achieve the survival set point are repaired bringing the number of lesions down to that of the sensitive cell line and yielding a similar survival. Additionally, repair of cisplatin damage might be expected to be a relatively rapid process with completion in 12–24 h. Thus, inhibition of repair in this timeframe should be sufficient to limit the transcription necessary for continued homeostasis even in the absence of a signal for replicative DNA synthesis. Indeed, clonogenic cell survival (Fig. 3 A and "Results") for the resistant cells was markedly inhibited by this approach. It is noteworthy that in the resistant cells recovery from potentially lethal damage continued for 96 h and could be inhibited by aphidicolin (Fig. 3 B). Thus, it might be anticipated that if a more extensive time for recovery from cisplatin damage of the sensitive cells were examined along with an attempt at aphidicolin inhibition, an aphidicolin effect might be uncovered. This, however, appears unlikely based on pulse labeling studies performed in both cell lines (data not shown) to determine the time course of measurable DNA repair. Consistent with Fig. 3 B, we found that DNA repair in the resistant cells did not return to basal level until at least 48 h postcisplatin treatment. In contrast, aside from a lower initial amount of repair activity in the sensitive cell line, activity returned to control values by 12 h posttreatment. These data suggest that 24-h aphidicolin-mediated inhibition of repair should be more than adequate to impact on survival if indeed the repair processes in the sensitive cell lines are relevant to survival. Thus, it may be that this differential aphidicolin effect relates not only to the relative level of repair but to the fidelity of the processes either in the genome as a whole or more probably in the transcriptionally active region of chromatin (20).

Our results demonstrate that DNA repair is a contributor to the resistance phenotype of a human carcinoma cell line made resistant to the clinically important anticancer drug cisplatin. The studies with aphidicolin in this system further indicate the potential to circumvent this mechanism of resistance in a clinical setting.

REFERENCES


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