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RAPID COMMUNICATIONS

ENHANCED DNA REPAIR AND RESISTANCE TO CISPLATIN IN HUMAN OVARIAN CANCER

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While cisplatin is an important drug in the treatment of a variety of cancers, the rapid development of resistance in tumor cells is a major problem which frequently limits clinical effectiveness, particularly in ovarian cancer (1). Resistance to cisplatin is incompletely characterized but, based on laboratory studies and patterns of clinical cross-resistance, multiple mechanisms are likely involved. Cell lines with induced resistance to cisplatin have multiple biochemical differences compared to more drug-sensitive cell lines including altered drug accumulation (2-4), increased intracellular levels of glutathione (5-7), increased thymidylate synthetase activity (8), DNA binding (intra- and interstrand crosslink formation) and adduct removal (9-11). In addition, circumstantial evidence in the form of cisplatin hypersensitivity of both microbial and mammalian UV-repair deficient mutants has also suggested the potential for altered DNA repair as a mechanism of cisplatin resistance (12-14).

Experimental attempts to demonstrate a role for DNA repair in recovery from exposure to cisplatin have been reported elsewhere. These include time-dependent recovery of clonogenic growth, measurement of platinum loss by atomic absorption spectroscopy (15) or adduct removal quantitated by alkaline elusion (9,10,14), alkaline sucrose gradient (13), high-pressure liquid chromatography (11) as well as antibody directed immunochemical detection (16,17). In the present study, we measured DNA excision repair using bromodeoxyuridine to separate replicative DNA synthesis from repair synthesis on alkaline cesium chloride gradients (18). DNA repair and its relationship to cisplatin cytotoxicity were examined in a cell line established from a previously untreated patient with ovarian cancer and in the same cell line after cisplatin resistance was induced in vitro. Furthermore, the clinical relevance of DNA repair as a mechanism of resistance to cisplatin-containing chemotherapy was determined by examining two additional cell lines derived from the same ovarian cancer patient before and after the onset of resistance to a cisplatin-containing drug regimen (7). Lastly, to further clarify the relationship between DNA repair and resistance to cisplatin, we examined the ability of aphidicolin to inhibit DNA repair through inhibition of DNA polymerase α (19) and increase the cytotoxicity of cisplatin.

MATERIALS AND METHODS

Cell lines and cultures. A2780, an ovarian cancer cell line derived from an untreated patient, was provided by Dr. S. Aaronson of the National Cancer Institute. 2780^{cp} is a cisplatin-resistant cell line made by intermittent drug exposure to A2780 through

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stepwise increase of concentration up to 70 μM. PEO1 and PEO4 are two cell lines from one patient, one isolated prior to, and the other after, the onset of resistance to cisplatin-containing chemotherapy (7). All the cell lines were maintained in monolayer culture in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS), 0.25 units/ml insulin, 100 μg/ml streptomycin, 100 units/ml penicillin and 0.3 mg/ml glutamine, and incubated at 37° in a humidified atmosphere of 5% (v/v) CO_2 in air.

The magnitude of resistance is defined as the ratio of 50% inhibitory concentration (IC_{50}) of resistant cells to that of sensitive cells, which was assessed by clonogenic cell survival in a two-layer soft agarose system as previously described (20). By this approach 2780^{cp} was 20-fold more resistant than A2780, and PEO4 was 2 to 3-fold more resistant than PEO1.

DNA repair activity determined on alkaline CsCl equilibrium density gradients. Briefly, exponentially growing cells were prelabeled with [^{14}C]dThd (0.004 μCi/ml) for 24 hr, harvested and divided equally into 25 cm^2 petri dishes, and grown to confluence in Minimum Essential Medium (MEM) with 10% FBS. At confluence, cultures were switched to arginine-deficient MEM (ADM) containing 2.5% dialyzed FBS (DFBS) and maintained for 3 days to decrease replicative DNA synthesis to a minimum (21). Cultures were then treated with 10 μM bromodeoxyuridine (BrdUrd) and 2 mM hydroxyurea (HU) (22), and aphidicolin if indicated, 1 hr prior to drug damage. After a 1-hr exposure to cisplatin, the dishes were washed three times with phosphate-buffered saline (PBS) followed by the addition of fresh 2.5% DFBS ADM containing BrdUrd, HU, and aphidicolin (if indicated), and 5 μCi/ml [^{3}H]dThd either immediately or at the indicated time for pulse labeling. After labeling for 4 hr or longer as indicated, all dishes were washed three times with cold PBS and then solubilized with 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing sodium dodecyl sulfate (0.5%, w/v), to which 5 ml of NaCl/sodium citrate (0.15/0.015 M) and 200 μl of 5 N NaOH were added. The lysate was made to a density of approximately 1.72 g/ml with CsCl, and centrifuged to equilibrium in a Beckman type 50 Ti rotor at 100,000 g for 36 hr. Fractionation was accomplished bottom to top. Fractions (0.25 ml) were precipitated with 10% (w/v) trichloroacetic acid (TCA), and precipitated DNA was collected on cellulose filters. Filters were washed with cold TCA, and radioactivity was determined in 10 ml Aquassure in a Beckman LS 2800 scintillation counter with correction for ^{14}C and ^{3}H channel overlap.

For determination of the amount of normal density DNA, fractions of normal density DNA were pooled and dialyzed against water for 24 hr, and DNA content was determined spectrophotometrically. The ^{14}C specific activity of the DNA was then calculated and repair reported as ^{3}H dpm/μg DNA which represented the value of difference between drug treatment and control.

RESULTS AND DISCUSSION

Table 1 represents the results of at least three separate experiments where DNA repair synthesis was calculated from the [^{3}H]dThd incorporated per microgram of DNA in the 4 hr immediately after cisplatin exposure. There was a 2 to 3-fold increase in the repair synthesis of the 2780^{cp} cell line as compared to its relatively cisplatin-sensitive parent when repair synthesis was measured after exposure to cisplatin (10-160 μM). When such repair was measured at equitoxic cisplatin doses, the difference between the resistant 2780^{cp} and sensitive A2780 cell lines was even more prominent. Differences in DNA repair synthesis between a cisplatin-resistant and -sensitive cell line were also found in the PEO1 and PEO4 cell lines. These latter lines with resistance developing in the patient during treatment may be more clinically relevant than cell lines with resistance induced in vitro. Consistent with the level of resistance to cisplatin of PEO4, it was

Table 1. Cisplatin dose dependent repair synthesis

Cell lines	Repair synthesis (^{3}H dpm/μg DNA)			Equitoxic dose*	
	Cisplatin (μM)				
	10	40	160	at IC_{50}	at IC_{90}
A2780	9.9 ± 0.9	25.6 ± 5.7	53.0 ± 8.1	2.4	7.3
2780^{cp}	23.1 ± 3.9	71.9 ± 12.4	129.7 ± 23.3	27	58
PEO1	2.7 ± 1.1	7.4 ± 1.7	19.9 ± 3.9	ND+	ND
PEO4	5.9 ± 2.5	18.0 ± 6.9	47.0 ± 14.2	ND	ND

DNA repair synthesis was calculated from the [^{3}H]dThd incorporated/μg of DNA in the 4 hr immediately after cisplatin exposure. Values which indicate the difference after subtraction of control are means ± SD from at least three separate studies. The control values in A2780, 2780^{cp}, PEO1, and PEO4 were 9.6 ± 3.6, 10.8 ± 3.2, 10.8 ± 1.8 and 7.4 ± 3.1 ^{3}H dpm/μg DNA respectively.

+Not determined.

*Determined by a double-layer agarose clonogenic assay after drug treatment in conditions as described in DNA repair assay.

found as noted in Table 1, that PEO4 had nearly 3-fold more repair synthesis than its more sensitive predecessor PEO1. We also examined the time course of repair by pulse labeling and attempted to gain an insight into total repair capacity by prolonged [^{3}H]dThd labeling after cisplatin damage in A2780 and 2780^{cp} (Fig. 1A). DNA repair was maximal during the initial 4-hr period after exposure to 40 μM cisplatin and slowly decreased to basal levels by 12-24 hr in the sensitive cell line. In contrast, repair synthesis did not return to basal levels in the resistant variant, 2780^{cp}, until 48 hr post-treatment (Fig. 1A). Thus, in addition to the initially greater repair synthesis seen in 2780^{cp} (Table 1), repair was also sustained for a longer period in the resistant variant as indicated by both pulse (Fig. 1A) and prolonged (Fig. 1A inset) labeling experiments.

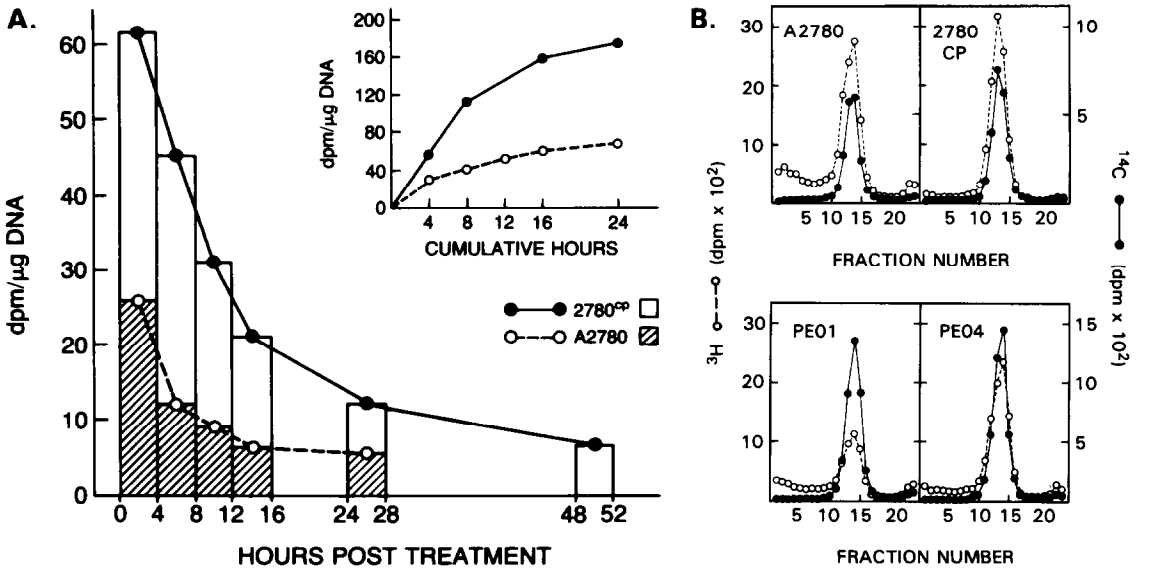

Fig. 1. A) Time course repair studies of 2780^{cp}. After a 1-hr exposure to 40 μM cisplatin, medium was replaced with fresh ADM containing BrdUrd and HU, and [^{3}H]dThd at indicated time for 4 hr in pulse labeling, or for increasing times in prolonged chasing (inset). Cells were washed, solubilized and analyzed for DNA repair synthesis. B) Inducible repair synthesis after 160 μM cisplatin damage in the four cell lines measured as the methods described. No intermediate density DNA was observed.

Lastly, we wished to determine if inhibition of DNA repair could lead to a reversal of resistance to cisplatin. Aphidicolin, a specific inhibitor of DNA polymerase α, inhibited, in a dose-dependent manner, cisplatin-associated DNA repair activity of 2780^{cp} with maximal inhibition of 86% at 10 μg/ml after damage with 40 μM cisplatin. Furthermore, it was found that inhibition of DNA repair by aphidicolin after cisplatin exposure resulted in total inhibition of recovery from cisplatin damage such as occurs when resistant cells are maintained in stationary phase for 4-96 hr prior to placing cells under conditions which permit cell division. Clonogenic cell survival changed from 16%, 4 hr after exposure to 40 μM cisplatin, to 52% after 96 hr in stationary phase prior to subjection to conditions allowing for cell growth. The increase in clonogenic growth was totally inhibited if aphidicolin was placed in the stationary phase cultures continuously for the 96-hr period (data not shown).

The studies reported here used a direct assay of DNA repair to demonstrate that in vitro induced resistance to cisplatin is associated with an increase in DNA repair. Inhibition of DNA repair under the stationary phase culture conditions used for the DNA repair assay prior to a clonogenic growth assay resulted in failure to recover clonogenic growth potential after cisplatin damage. This is direct evidence for the importance of DNA repair to the expression of cisplatin resistance activity and capacity. Furthermore, the potential relevance of DNA repair to clinical drug resistance is supported by the finding of increased repair to cisplatin damage in PEO4 compared to PEO1. Future studies will attempt to clarify which of the repair enzymes, in addition to DNA polymerase α, are involved in repair of cisplatin damage and whether inhibition of repair of cisplatin damage in vivo can increase the activity of cisplatin. The availability of aphidicolin glycinate, a water-soluble formulation of aphidicolin, should allow this latter possibility to be tested.

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