Pergamon

Biochemical Pharmacology, Vol. 47, No. 4, pp. 689–697, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0030-B

ROLE OF PLATINUM–DNA ADDUCT FORMATION AND REMOVAL IN CISPLATIN RESISTANCE IN HUMAN OVARIAN CANCER CELL LINES

STEVEN W. JOHNSON,* RAYMOND P. PEREZ,*† ANDREW K. GODWIN,* ANTHONY T. YEUNG,‡ LAURA M. HANDEL,* ROBERT F. OZOLS* and THOMAS C. HAMILTON*§

*Department of Medical Oncology and ‡Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, U.S.A.

(*Received* 28 *June* 1993; *accepted* 17 *September* 1993)

Abstract—A series of cisplatin-resistant cell lines were used to examine the formation and removal of platinum–DNA adducts from the overall genome and the formation and removal of cisplatin-interstrand cross-links from specific genomic regions. Cisplatin accumulation and DNA platination levels, which correlated linearly, were similar in three of the resistant cell lines despite differences in their primary cisplatin resistance. Increased platinum removal from total genomic DNA was found to be associated with increased resistance. Interstrand cross-link levels were found to be 2- to 4-fold lower in the 28S ribosomal RNA gene and a non-coding genomic region of the resistant cell lines as compared with the parental A2780 cell line. In addition, 1.2- to 2.7-fold more cross-links were formed in the non-coding region than in the ribosomal RNA gene in all of the cell lines. Interstrand cross-links were removed more rapidly from both regions of the highly cisplatin-resistant C80 and C200 cells and from the ribosomal RNA gene only in the cell lines of lower resistance. The results support a role for DNA repair and alterations in interstrand cross-link formation in cisplatin resistance and provide evidence for heterogeneous interstrand cross-link formation in the genome.

Key words: DNA repair; drug resistance; cisplatin; ovarian cancer

Currently the most effective treatment for advanced stage ovarian cancer following cytoreductive surgery is combination chemotherapy with a platinum-containing drug (e.g. cisplatin, carboplatin) [1]. Despite the encouraging clinical complete response rates (40–60%) achieved with platinum-based chemotherapy, the majority of patients eventually relapse and become refractory to further treatment [2]. One approach to alleviate this problem is to elucidate the mechanisms responsible for drug resistance and then develop strategies to effectively circumvent resistance or prevent its occurrence.

The available data indicate that the antitumor effect of *cis*-diamminedichloroplatinum (II) (cisplatin) occurs through its ability to covalently bind to DNA and prevent DNA replication and transcription. Cisplatin reacts primarily at the N7 position of guanine residues to form several types of lesions including (dG)Pt monoadducts, d(GpG)Pt, d(ApG)Pt and d(GpNpG)Pt intrastrand cross-links, and $(dG)_2Pt$ interstrand cross-links [3]. The specific lesion(s) responsible for cisplatin cytotoxicity remains to be established.

Cellular resistance to cisplatin appears to be multifactorial [4–8]. The potential mechanisms responsible for cisplatin resistance, which have been identified, may be classified into two categories: those that influence the formation of cytotoxic DNA lesions and those that minimize their impact. The first category includes decreased drug accumulation [9–13] and enhanced drug inactivation either by thiol-containing proteins (e.g. metallothionein) [14–17] or by non-protein sulfhydryl molecules (e.g. GSH‖) [18–24]. For example, we and others have shown GSH levels to be strongly correlated with increased cisplatin resistance [18–24].

If such mechanisms do not prevent a drug from interacting with DNA, the cell must either tolerate or repair the resulting DNA damage in order to survive. This constitutes a second major category of resistance mechanisms. Evidence for increased DNA repair (2- to 4-fold) in the total genomic DNA of cisplatin-resistant cells has been reported by several laboratories [25–29]. In some model systems, it appears that DNA repair occurs preferentially in transcribed gene regions relative to the overall genome [reviewed in Ref. 30]. It is conceivable that the survival of cisplatin-resistant cells may depend more on the speed or efficiency of repair in essential regions of their genomes than in bulk DNA. If this is the case, differences in gene-specific repair may be more dramatic and correlate more closely to relative cisplatin resistance than does repair in the

† Present address: Department of Medicine, Roswell Park Cancer Institute, Buffalo, NY 14263.

§ Corresponding author: Thomas C. Hamilton, Ph.D., Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Tel. (215) 728-2856; FAX (215) 728-4333.

‖ Abbreviations: AAS, atomic absorption spectrometry; DHFR, dihydrofolate reductase; GSH, glutathione; ICL, interstrand cross-link; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; UDS, unscheduled DNA synthesis; and VNTR, variable number tandem repeat.

overall genome. In fact, it has been reported recently that the gene-specific repair of cisplatin interstrand cross-links may be associated with cellular resistance to cisplatin in human ovarian cancer cell lines [31]. We have measured the formation and removal of total platinum–DNA adducts and one specific platinum–DNA adduct, the interstrand cross-link, in specific DNA sequences including the 28S ribosomal RNA gene and a non-coding DNA region of relatively cisplatin-sensitive A2780 cells and a series of cisplatin-resistant cell lines that were derived from the parental A2780 cell line. These measurements were made in the context of the complex multifactorial resistance mechanisms that are present. In addition to a previously reported role for glutathione in cisplatin resistance, our results indicated that reduced cisplatin accumulation, increased DNA repair, and decreased gene-specific interstrand cross-link formation contribute to cisplatin resistance in these cell lines.

MATERIALS AND METHODS

Materials. Cisplatin was obtained from Bristol-Myers Squibb (Syracuse, NY). Chemicals and reagents were obtained from the Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were obtained from GIBCO (Grand Island, NY).

Cell lines. The human ovarian cancer cell line A2780 was derived from a patient prior to treatment [32]. The A2780/C series of cisplatin-resistant cell lines was developed by near continuous, incremental exposure of the A2780 cells to cisplatin (8–200 μM). Cisplatin sensitivities and relative resistances were determined as described previously [24] using the MTT assay (see Table 1). Cell lines were maintained at 37° in 5% CO_2 in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 0.3 mg/mL glutamine and 0.3 U/mL insulin (porcine). For repair experiments, duplicate flasks of cells (grown to approximately 80% confluence) were treated at 37° with various concentrations of cisplatin as follows: A2780 (50 μM for 2 hr), CP70 (200 μM for 2 hr), C30, C80 and C200 (300 μM for 4 hr). Following cisplatin exposure, the medium was replaced with fresh medium and incubated for 4, 8 or 12 hr at 37°. The data for each cell line are reported as the mean values taken from independent measurements on DNA isolated from flasks on two separate occasions.

Cisplatin accumulation. Cell-associated platinum was measured in total cellular extracts by AAS. Triplicate flasks containing subconfluent, exponentially growing cells were treated with cisplatin (20–200 μM) for 2 hr at 37°. Cells were washed twice with phosphate-buffered saline, harvested by trypsinization and counted; 2.5 million cells from each flask were pelleted. Cells were "wet ashed" essentially as described [33] by incubating overnight in 0.5 mL of concentrated nitric acid at room temperature followed by incubating for 5 min at 100°, adding 0.5 mL of 30% (v/v) H_2O_2, and reincubating for 5 min at 100°. Duplicate aliquots (20 μL) of the resulting clear, pale yellow solution were analyzed directly by AAS using a Perkin–Elmer model 3100 atomic absorption spectrometer equipped with a graphite furnace, and platinum was quantitated relative to a standard curve for elemental platinum. The optimized furnace program included an 80-sec drying step at 90°, a 50-sec charring step at 1300°, followed by atomization for 5 sec at 2350°.

DNA isolation. Cells (10–30 × 10^6) were harvested by washing three times with 10 mM Tris–HCl, pH 7.6, containing 0.15 M NaCl and incubated in 8 mL of lysis buffer (10 mM Tris–HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA, 0.1% (w/v) SDS and 200 μg/mL proteinase K) for 3–12 hr at 37°. The lysates were collected into 50-mL polypropylene tubes and mixed with 8 mL of Tris-saturated phenol [0.1 M Tris–HCl, pH 8.0, 0.2% (w/v) hydroxyquinoline and 0.01% (v/v) β-mercaptoethanol]. Samples were centrifuged at 3000 *g* for 8 min, and the resulting supernatants were transferred to fresh polypropylene tubes and mixed with 8 mL of phenol:$CHCl_3$:isoamyl alcohol (25:24:1). Following centrifugation at 3000 *g*, the supernatants were mixed with 0.1 vol. of 3 M sodium acetate, pH 5.2, and 2 vol. of absolute ethanol. DNA was spooled onto glass rods, rinsed with 70% ethanol, and resuspended in 2.5 mL of 10 mM Tris–HCl, pH 7.6, containing 1 mM EDTA (TE buffer). Samples were incubated with 100 μg/mL RNase A for 1 hr at 37°, and the DNA was spooled, as described above, and resuspended in 1 mL of TE buffer overnight at 4°. DNA samples (200–300 μg) were restriction-digested with 400 U of *Hin*dIII (BRL, Gaithersburg, MD) for 8–12 hr at 37°. The DNA was precipitated with ethanol, resuspended in 150–200 μL of TE buffer, and quantitated by the Hoechst fluorescent dye-binding assay [34].

Total DNA platination. Approximately 125 μg of each purified DNA sample was incubated with 100 μg/mL RNase A and 1000 U of RNase T_1 in 200 μL of TE buffer for 1 hr at 37° to ensure the removal of contaminating RNA. SDS (0.05%) and proteinase K (100 μg/mL) were added, and the samples were incubated for 1 hr at 37°. Following an extraction with phenol:$CHCl_3$:isoamyl alcohol (25:24:1), the DNA was reprecipitated with ethanol, resuspended in 130 μL of 5% HCl, and incubated for 20 min at 90–95°. The absorbance at 260 nm was measured in the resulting hydrolysate, and the platinum content was determined in duplicate by AAS. The furnace program included a 110-sec drying step at 100°, a 50-sec charring step at 1400°, followed by atomization for 5 sec at 2350°.

Measurement of cisplatin interstrand cross-links in specific DNA sequences. To measure the formation and removal of cisplatin interstrand cross-links in specific genomic regions, we used the renaturing agarose gel electrophoresis assay [35]. DNA samples (0.5 to 5.0 μg) were incubated at 55–60° in 0.1 N NaOH for 5 min and placed on ice for 2 min. Loading buffer was added, and the samples were electrophoresed in 0.5% agarose gels (90 mM Tris-borate, 2 mM EDTA) for 1 hr at 100 V followed by 12–16 hr at 35–40 V. The gels were incubated in 0.25 N HCl for 20 min, equilibrated in 0.4 N NaOH-1.5 M NaCl for 15 min, and transferred to Gene Screen Plus transfer membranes (NEN Research Products, Boston, MA) overnight in 0.4 N NaOH-1.5 M NaCl. The membranes were subsequently

Table 1. Relationship between cisplatin cytotoxicity, cisplatin accumulation and DNA platination in cisplatin-sensitive and -resistant ovarian cancer cell lines

Cell line	Cisplatin IC_{50}* (μM)	Fold resistant	Accumulation† (ng Pt/10^6 cells)	DNA platination† (pg Pt/μg DNA)
A2780	0.35	1	79	106
CP70	5.5	16	39	46
C30	98	280	11	18
C80	144	411	12	14
C200	147	418	12	16

* Cytotoxicity was determined by cisplatin treatment for 72 hr using the MTT assay. The IC_{50} is the concentration that inhibited growth by 50%. The IC_{50} values were measured in triplicate in two independent experiments.

† Cells were incubated with 200 μM cisplatin for 2 hr at 37°.

hybridized with either the ABE_{II} probe (provided by Dr. J. E. Sylvester, University of Pennsylvania), which recognizes a 17 kb *Hin*dIII restriction fragment of the 28S ribosomal RNA gene [36], or the 144D6 probe (D17S34, obtained from the American Type Culture Collection, Rockville, MD), which hybridizes to an approximately 23 kb *Hin*dIII fragment of a non-transcribed VNTR region on chromosome 17. These two probes were selected since they recognize an actively transcribed gene and a non-transcribed, non-gene region, respectively. In addition, these DNA fragments are of similar size and are present in multicopies, which facilitated analysis. Histograms were generated for each lane using an AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, CA), and the fraction of cross-linked strands was determined by weight analysis of the peaks. The average number of ICL per fragment was calculated using the Poisson distribution equation: $\ln (1 - F_c)$, where F_c is the fraction of DNA strands containing cross-links [35].

RESULTS

We have developed a series of cisplatin-resistant ovarian cancer cell lines that exhibit a wide range of cisplatin sensitivity (Ref. 24, Table 1). Cisplatin accumulation in these cell lines is nonsaturable and approximately linear with increasing cisplatin concentrations, up to 200 μM (data not shown). The relatively cisplatin-sensitive A2780 cell line, from which the resistant variants were derived, accumulated 2-fold more platinum than the moderately resistant CP70 cell line and approximately 7-fold more platinum than the highly resistant C30, C80 and C200 cell lines when treated with 200 μM cisplatin for 2 hr (Table 1). Total DNA platination was also linear with increasing cisplatin concentrations, up to 300 μM (data not shown). When treated with equivalent cisplatin concentrations (200 μM), genomic DNA from A2780 cells contained approximately 2-fold more platinum than DNA from CP70 cells and 5- to 7-fold more platinum than DNA from C30, C80 and C200 cells (Table 1). Although cellular platinum accumulation correlated with total DNA platinum ($r = 0.99$), cisplatin cytotoxicity did not correlate as well with accumulation ($r = 0.85$) or DNA platination ($r = 0.83$), suggesting that an additional resistance mechanism(s) may be present.

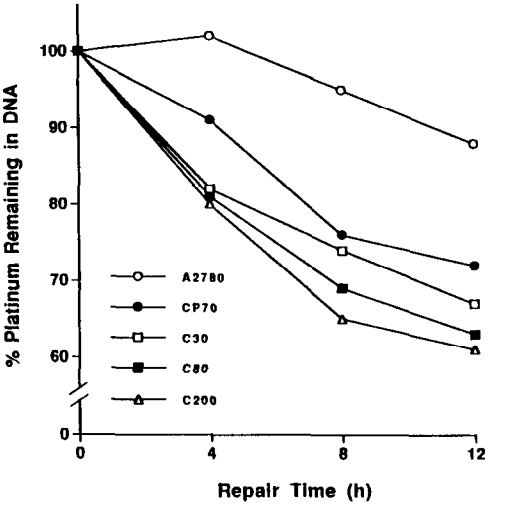

Fig. 1. Removal of platinum from genomic DNA in cisplatin-sensitive and -resistant ovarian cancer cell lines. Cells were treated as described in Materials and Methods to obtain the following initial DNA platination levels: 58 pg Pt/μg DNA (A2780), 46 pg Pt/μg DNA (CP70), 61 pg Pt/μg DNA (C30), 48 pg Pt/μg DNA (C80) and 46 pg Pt/μg DNA (C200). Points indicate the mean of two independent experiments, and errors ranged from 0–20%.

For DNA repair experiments, cisplatin concentrations and treatment times were optimized to obtain similar amounts of platinated DNA (46–61 pg Pt/μg DNA or 0.77 to 1.02 platinum adducts/10 kb). Following cisplatin treatment, cells were either harvested or incubated in fresh medium for 4, 8 or 12 hr. Under these conditions, very little, if any, new replicative DNA synthesis occurred as measured by [^{3}H]thymidine incorporation (data not shown). Figure 1 shows the removal of platinum from genomic DNA in the cisplatin-sensitive and -resistant

cell lines as determined by AAS. The initial rate of platinum removal from DNA was increased in the cisplatin-resistant cell lines relative to the A2780 parental cell line. After 12 hr, the resistant cell lines removed 24–40% of platinum from their DNA, whereas the A2780 cell line removed only 14%.

The formation and removal of cisplatin interstrand cross-links were measured in the ribosomal RNA gene and a non-coding genomic region by renaturing agarose gel electrophoresis (Fig. 2, Table 2). Although each cell line contained similar levels of total platinated DNA, the level of interstrand cross-links was reduced approximately 2- to 4-fold in the non-coding and ribosomal RNA gene regions of the resistant cell lines relative to that of the A2780 cell line. In addition, the non-coding region initially contained 1.2 to 2.7 times the number of cross-links as the ribosomal RNA gene in these cell lines. The rate of removal of the interstrand cross-links from the 28S ribosomal RNA gene was higher in the cisplatin-resistant cell lines than in the parental A2780 cell line at 4 hr post-treatment (18–33 vs 6%) (Table 2). Repair was also more complete in this gene region of the resistant cells after 12 hr (71–95 vs 56%). The initial (4 hr) removal rates of cisplatin-interstrand cross-links from the non-coding region of the A2780, CP70 and C30 cell lines were similar (21–23%), while the more resistant C80 and C200 cell lines removed 53 and 62% of the interstrand cross-links from this region, respectively.

DISCUSSION

The present investigations were designed to expand on our initial observation [26, 27] and those of others [25, 28, 29] indicating a role for DNA repair in cisplatin resistance. We have used a related series of cell lines with a wide range of *in vitro* selected primary cisplatin resistance and determined the potential for total platinum–DNA adduct repair and DNA sequence-specific interstrand cross-link repair to contribute to cisplatin resistance. This has been done in the context of other factors that may contribute to the formation of platinum-induced DNA damage. Since the measurement of repair generally requires drug or radiation exposure conditions that kill a high percentage of cells, we have individualized platinum exposure conditions in order to achieve equivalent initial total platinum–DNA damage. Therefore, equivalent damage may be anticipated to produce similar cell kill in the absence of differences in the types of lesions formed, damage tolerance and/or DNA repair. In support of the existence of such alterations, we have observed that cell survival under the treatment conditions employed, which yield similar levels of platinum–DNA damage, is much higher in the resistant cell lines relative to the sensitive cell line.

Consistent with many reports [9–13], we show that the *in vitro* selection for cisplatin resistance was associated with decreased cellular cisplatin accumulation. Our studies demonstrated that cell lines (C30, C80, C200) which vary in degree of primary cisplatin sensitivity (IC_{50} values from 98 to 147 μM) exhibit similar cellular accumulation after exposure to equivalent amounts of the drug (Table 1). Therefore, decreases in cellular accumulation are not likely to be the sole determinant of primary cisplatin resistance in this model system. Indeed, the only parameter previously tested by us and others which was found to correlate well with resistance to DNA damaging agents has been cellular GSH [19, 24, 37, 38]. The mechanisms through which GSH contributes to cisplatin resistance are incompletely defined; however, GSH has been postulated to inactivate cisplatin in the cell and/or facilitate DNA repair perhaps by quenching platinum–DNA monoadducts [39–41].

We have also examined the relationship between cisplatin cytotoxicity and total DNA platination. Similar levels of DNA platination were observed in the C30, C80 and C200 cell lines that vary significantly in cisplatin resistance (Table 1). The most straightforward explanation for this finding may be that the more resistant cells have an increased ability to repair cisplatin-damaged DNA. It is also possible that there are differences in the ability of the cells to tolerate DNA damage or differences in the types of platinum–DNA adducts that are formed, assuming the various lesions differ in their cytotoxicity. Our data regarding the repair of total platinum–DNA adducts and the formation and removal of cisplatin interstrand cross-links in specific DNA sequences support these possibilities.

Many factors have the potential to impact on the ability of cisplatin to form potentially cytotoxic DNA lesions. Therefore, when studying the role of DNA repair in cisplatin resistance, it is important to consider the choice of cisplatin exposure conditions. The approach we have taken is to compare the DNA repair abilities of cisplatin-sensitive and -resistant cells under cisplatin exposure conditions that yield similar amounts of total platinum–DNA adducts. DNA repair activity was shown to be dependent on the amount of platinum-induced DNA damage as measured by UDS [26], and our approach should enable the detection of differences in repair rate and/or capacity. Furthermore, we have found that very aggressive platinum exposure conditions, which create substantial amounts of DNA damage, prevent the relative quantitative recovery of high molecular weight DNA. Hence, when cells are treated equivalently with high concentrations of cisplatin, substantially more high molecular weight DNA is recovered from resistant versus sensitive cells. Such differences have the potential to yield artifactual results. In contrast, exposure of cells to different cisplatin concentrations in order to obtain equivalent levels of DNA damage (i.e. total platinum–DNA adducts) results in similar recovery of high molecular weight DNA between resistant and sensitive cells.

Under conditions in which individual cell lines were treated with cisplatin concentrations that yielded similar total platinum–DNA adduct levels (0.77 to 1.02 Pt adducts/10 kb), there was a clear relationship between the ability to remove platinum from DNA and the degree of resistance (Fig. 1). This finding in several related cell lines expands our initial observation of increased DNA repair as monitored by UDS in CP70 cells [26]. Such results in CP70 cells have been confirmed by others using several approaches [27–29]. It is also noteworthy

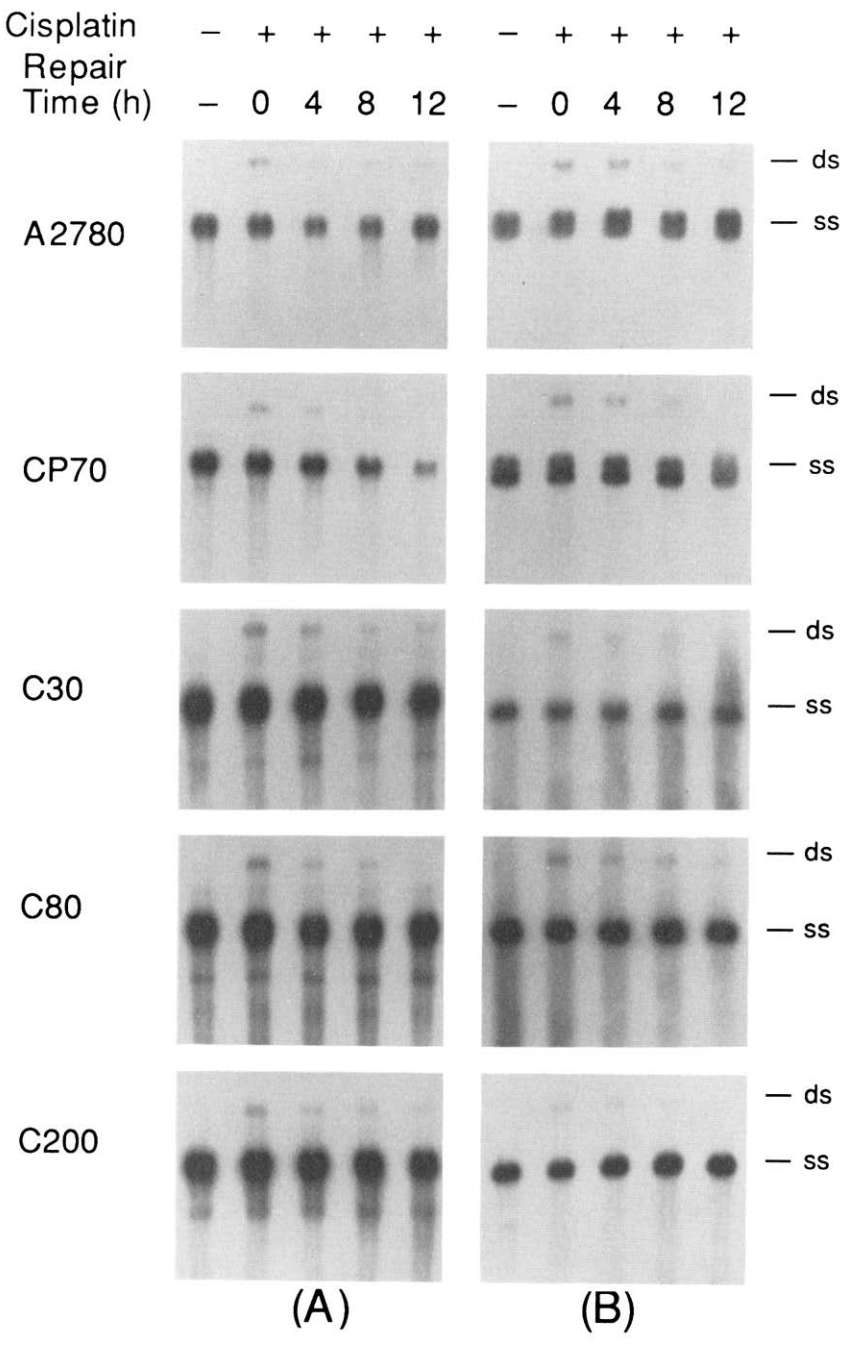

Fig. 2. Removal of cisplatin-interstrand cross-links from (A) the ribosomal RNA gene and (B) a non-coding DNA fragment in cisplatin-sensitive and -resistant ovarian cancer cell lines. DNA was isolated from cells that were treated as described in Materials and Methods and subjected to renaturing agarose gel electrophoresis followed by Southern blotting.

that at the initial repair time point (4 hr) the parental A2780 cells showed little repair of total platinum adducts in contrast to the repair observed in the resistant variants. We have speculated that DNA repair could be a mechanism of cisplatin resistance distinct from a process that would influence the innate sensitivity of cells to cisplatin. This view was based on the observation that the inhibition of cisplatin-induced DNA repair by aphidicolin influenced the cytotoxicity of cisplatin in the moderately cisplatin-resistant CP70 cells but not in the parental A2780 cells from which they were derived [26]. The present data further support this possibility. One would assume that if removal/repair

Table 2. Removal of cisplatin ICL from (a) the ribosomal RNA gene and (b) a non-coding region of cisplatin-sensitive and -resistant ovarian cancer cell lines

(a) Ribosomal RNA gene

Repair time (hr)	ICL/10 kb* (% repair)				
	A2780†	CP70‡	C30§	C80§	C200§
0	0.101	0.060	0.038	0.039	0.024
4	0.095 (6)	0.042 (30)	0.029 (24)	0.032 (18)	0.016 (33)
8	0.049 (51)	0.024 (60)	0.021 (45)	0.009 (77)	0.014 (42)
12	0.044 (56)	0.016 (73)	0.011 (71)	0.002 (95)	0.007 (71)

(b) Non-coding region

Repair time (hr)	ICL/10 kb* (% repair)				
	A2780†	CP70‡	C30§	C80§	C200§
0	0.131	0.073	0.061	0.058	0.065
4	0.103 (21)	0.058 (21)	0.047 (23)	0.027 (53)	0.025 (62)
8	0.075 (43)	0.038 (48)	0.031 (49)	0.024 (59)	0.023 (65)
12	0.027 (79)	0.030 (59)	0.033 (46)	0.008 (86)	0.016 (75)

* Independent replicate determinations consistently yielded values that varied by 0.001 to 0.019 ICL/10 kb.

† Cells were treated with 50 μM cisplatin for 2 hr at 37°.

‡ Cells were treated with 200 μM cisplatin for 2 hr at 37°.

§ Cells were treated with 300 μM cisplatin for 4 hr at 37°.

of cisplatin from the DNA of the parental A2780 cells had the same impact on survival as in the resistant variants, then the parental cells would have repaired 27% of their cisplatin lesions at 12 hr. This would create a linear relationship between cytotoxicity and DNA repair in the entire panel of cell lines, including A2780. This was not the case, however, since the A2780 cells only repaired 14% of their cisplatin lesions at 12 hr. Therefore, the inclusion of A2780 cytotoxicity and DNA repair data substantially weakens the otherwise linear relationship ($r = 0.98$) between these parameters in the resistant cell lines. Furthermore, the inclusion of A2780 data also weakens a log–linear relationship between these parameters. These data do not exclude a role for DNA repair as a determinant of cisplatin sensitivity. Indeed, the fact that cytotoxicity and DNA repair are both drug concentration dependent infers that a role is present at some level. Our current and previous data do suggest, however, that the relative contribution of DNA repair to cisplatin sensitivity in A2780 is less than that in the cisplatin-resistant cell lines.

Consistent with our finding of a relationship between total platinum–DNA adduct removal and degree of cisplatin resistance, we observed increased DNA sequence-specific removal of cisplatin-interstrand cross-links in resistant versus sensitive cells at 4 hr post-treatment. We report in detail the examination of two categories of DNA sequences, one that contains actively transcribed genes and one that contains non-transcribed, non-gene encoding sequences. The increased repair was more apparent when the transcribed sequences were examined. Repair of interstrand cross-links in the multiple copies of the ribosomal RNA gene occurred more rapidly (18–33 vs 6% at 4 hr) and was more complete (71–95 vs 56% at 12 hr) in the resistant variants as compared with the parental A2780 cell line. The lack of appreciable repair of interstrand cross-links in A2780 cells at the initial 4-hr time was consistent with the lack of removal of platinum from the overall genome in these cells at 4 hr (Fig. 1, Table 2). The data on interstrand cross-link repair in the non-coding DNA sequence are more complex. The increased rate of repair observed for the ribosomal RNA gene in all of the resistant variants was only apparent for the non-coding sequence in the two most cisplatin-resistant cell lines (C80 and C200); however, the extent of repair of this sequence was similar in all cell lines at the 12-hr time point. We did not observe increased removal of interstrand cross-links from the ribosomal RNA genes relative to the non-coding region in any of the cisplatin-sensitive or -resistant cell lines except possibly the C30 cells. The decreased precision with which very low levels of cross-links can be measured, as are present at the later time points in resistant cells, suggests that this apparent difference is not significant. Therefore, our findings are consistent with reports which demonstrate that only genes transcribed by RNA polymerase II are subject to increased time-dependent, gene-specific repair [42, 43].

During the course of the work presented here, an independent report describing overall DNA repair

and repair of specific DNA sequences in A2780 and CP70 was published [31]. Our results complement and expand on these findings. This report described a 2-fold increase in the removal of interstrand cross-links in both actively transcribed (DHFR) and non-transcribed (β-globin and *mdr-1*) genes in CP70 versus A2780 cell lines. Zhen *et al.* [31] treated both cisplatin-sensitive and -resistant cells with the same concentration of cisplatin. Exposure conditions were chosen such that nearly two-thirds of the fragments of interest contained an interstrand cross-link. This was in contrast to our approach on an expanded series of cell lines which used less aggressive cisplatin exposure conditions that yielded similar amounts of total platinum–DNA adducts. Both procedures, however, yielded qualitatively similar data: increased DNA sequence-specific repair of interstrand cross-links in cisplatin-resistant versus -sensitive ovarian cancer cell lines. The small quantitative differences in the amount of repair of DNA sequence-specific interstrand cross-links between this report and that of Zhen *et al.* [31] may be related to the fact that repair in different DNA sequences was measured or they may be due to differences in cisplatin treatment conditions or the choice of time points for repair measurements.

In addition to the results supporting a role for DNA sequence-specific interstrand cross-link removal in cisplatin resistance, these data suggest other unanticipated possibilities as to cisplatin resistance mechanisms. It was of particular interest that, in the presence of near equivalent amounts of total platinum–DNA damage, there was a marked decrease in interstrand cross-link formation within the ribosomal RNA gene as resistance increased. This could be the result of increased gene-specific repair activity occurring in the resistant cell lines, which resulted in an apparent reduction in the initial level of interstrand cross-links. It is also possible that, in addition to the direct role of DNA repair in cisplatin resistance, differences in the types of platinum–DNA lesions formed could contribute to the resistance phenotype. The fact that GSH is elevated markedly in the highly cisplatin-resistant cell lines [24], coupled with this finding of reduced interstrand cross-link formation in these cell lines, indirectly supports the concept that GSH could participate in cisplatin resistance by altering the types of cisplatin–DNA adducts formed. Thus, in the presence of equivalent total DNA platination, there could be a decrease in sequence-specific interstrand cross-links.

It is noteworthy that the amount of initial interstrand cross-link formation was lower in the actively transcribed ribosomal RNA gene than in the non-coding DNA sequence. These changes were particularly apparent in the more resistant cell lines (i.e. C30, C80 and C200). We have also examined the formation of cisplatin interstrand cross-links in the non-transcribed β-interferon gene with the interferon-β1 probe (provided by Dr. P. Pitha-Rowe, Johns Hopkins, Ref. 44). Using the same A2780 DNA preparations as above (Table 2), we found fewer cross-links formed in the β-interferon gene (0.051 ICL/10 kb) than in the ribosomal RNA gene (0.101 ICL/10 kb) or the non-coding sequence (0.131 ICL/10 kb). These results suggest that certain regions of the human genome may be more tolerant of, or accessible to, damage than others, an hypothesis that has been proposed [30, 45]. This could be due to the presence of altered chromatin structure in various regions of the genome or the existence of GC-rich sequences in certain genomic regions that may be more conducive to cisplatin interstrand cross-link formation.

In conclusion, the studies reported here expand our understanding of the complex mechanisms responsible for cisplatin resistance. They suggest the likely possibility that changes in the types of DNA lesions formed, such as interstrand cross-links, may prominently contribute to resistance and continue to support a role for increased total and sequence-specific DNA repair in cisplatin resistance.

Acknowledgements—We thank Katharine A. Jackson, Pamela A. Swiggard and Lisa A. Vanderveer for skillful technical assistance and Catherine Thompson for preparation of the manuscript. This work was supported by Grants CA51228, CA06927, CA52181 and CA51175 and a grant from the Mary L. Smith Charitable Trust.

REFERENCES

1. Ozols RF, Ovarian cancer, Part I: Treatment. *Curr Probl Cancer* **16**: 65–126, 1992.
2. Perez RP, Hamilton TC, Ozols RF and Young RC, Mechanisms and modulation of resistance to chemotherapy in ovarian cancer. *Cancer* **71**: 1571–1580, 1993.
3. Eastman A, Separation and characterization of products resulting from the reaction of *cis*-diamminedichloroplatinum(II) with deoxyribonucleosides. *Biochemistry* **21**: 6732–6736, 1982.
4. Hospers GAP, Mulder NH and DeVries EGE, Mechanisms of cellular resistance to cisplatin. *Med Oncol Tumor Pharmacother* **5**: 145–151, 1988.
5. Perez RP, Hamilton TC and Ozols RF, Resistance to alkylating agents and cisplatin: Insights from ovarian carcinoma model systems. *Pharmacol Ther* **48**: 19–27, 1990.
6. Hamilton TC, Ovarian cancer, Part I: Biology. *Curr Probl Cancer* **16**: 1–57, 1992.
7. Vendrick CPJ, Bergers JJ, DeJong WH and Steerenberg PA, Resistance to cytostatic drugs at the cellular level. *Cancer Chemother Pharmacol* **29**: 413–429, 1992.
8. Johnson SW, Hamilton TC and Ozols RF, Mechanisms of drug resistance in ovarian cancer. *Cancer* **71**: 644–649, 1993.
9. Andrews PA, Velury S, Mann SC and Howell SB, *cis*-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res* **48**: 68–73, 1988.
10. Kuppen PJK, Schuitemaker H, van't Veer LJ, de Bruijn EA, van Oosterom AT and Schrier PI, *cis*-Diamminedichloroplatinum(II)-resistant sublines derived from two human ovarian tumor cell lines. *Cancer Res* **48**: 3355–3359, 1988.
11. Andrews PA, Jones JA, Varki NM and Howell SB, Rapid emergence of acquired *cis*-diamminedichloroplatinum(II) resistance in an *in vivo* model of ovarian carcinoma. *Cancer Commun* **2**: 93–100, 1990.
12. Kelland LR, Mistry P, Abel G, Loh SY, O'Neill CF, Murrer BA and Harrap KR, Mechanism-related circumvention of acquired *cis*-diamminedichloroplatinum(II) resistance using two pairs of human

ovarian carcinoma cell lines by ammine/amine platinum(IV) dicarboxylates. *Cancer Res* **52**: 3857–3864, 1992.
13. Mistry P, Kelland LR, Loh SY, Abel G, Murrer BA and Harrap KR, Comparison of cellular accumulation and cytotoxicity of cisplatin with that of tetraplatin and amminedibutyratodichloro(cyclohexylamine)-platinum(IV) (JM221) in human ovarian carcinoma cell lines. *Cancer Res* **52**: 6188–6193, 1992.
14. Kelley S, Basu A, Teicher B, Hacker MP, Hamer DH and Lazo JS, Overexpression of metallothionein confers resistance to anticancer drugs. *Science* **241**: 1813–1815, 1988.
15. Kasahara K, Fujiwara Y, Nishio K, Ohmori T, Sugimoto Y, Komiya K, Matsuda T and Saijo N, Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Res* **51**: 3237–3242, 1991.
16. Schilder RJ, Hall L, Monks A, Handel LM, Fornace AJ Jr, Ozols RF, Fojo AT and Hamilton TC, Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. *Int J Cancer* **45**: 416–422, 1990.
17. Andrews PA, Murphy MP and Howell SB, Metallothionein-mediated cisplatin resistance in human ovarian cancer cells. *Cancer Chemother Pharmacol* **19**: 149–154, 1987.
18. Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Louie KG, Knutsen T, McKoy WM, Young RC and Ozols RF, Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* **47**: 414–418, 1987.
19. Mistry P, Kelland LR, Abel G, Sidhar S and Harrap KR, The relationships between glutathione, glutathione-*S*-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. *Br J Cancer* **64**: 215–220, 1991.
20. Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC and Ozols RF, Augmentation of adriamycin, melphalan and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian cancer cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* **34**: 2583–2586, 1985.
21. Louie KG, Behrens BC, Kinsella TJ, Hamilton TC, Grotzinger KR, McKoy WM, Winker MA and Ozols RF, Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. *Cancer Res* **45**: 2110–2115, 1988.
22. Lewis AD, Hayes JD and Wolf CR, Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: Intrinsic differences and cell cycle effects. *Carcinogenesis* **9**: 1283–1287, 1988.
23. Batist G, Behrens BC, Makuch R, Hamilton TC, Katki AG, Louie KG, Myers CE and Ozols RF, Serial determinations of glutathione levels and glutathione-related enzyme activities in human tumor cells *in vitro*. *Biochem Pharmacol* **35**: 2257–2259, 1986.
24. Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC and Anderson ME, High resistance to cisplatin in human ovarian cancer cells is associated with marked increase of glutathione synthesis. *Proc Natl Acad Sci USA* **89**: 3070–3074, 1992.
25. Eastman A and Schulte N, Enhanced DNA repair as a mechanism of resistance to *cis*-diamminedichloroplatinum(II). *Biochemistry* **27**: 4730–4734, 1988.
26. Masuda H, Ozols RF, Lai G-M, Fojo A, Rothenberg M and Hamilton TC, Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. *Cancer Res* **48**: 5713–5716, 1988.
27. Lai G-M, Ozols RF, Smyth JF, Young RF and Hamilton TC, Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. *Biochem Pharmacol* **37**: 4597–4600, 1988.
28. Masuda H, Tanaka T, Matsuda H and Kusaba I, Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res* **50**: 1863–1866, 1990.
29. Parker RJ, Eastman A, Bostick-Burton F and Reed E, Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J Clin Invest* **87**: 772–777, 1991.
30. Bohr VA, Gene specific DNA repair. *Carcinogenesis* **12**: 1983–1992, 1991.
31. Zhen W, Link CJ Jr, O'Connor PM, Reed E, Parker R, Howell SB and Bohr VA, Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. *Mol Cell Biol* **12**: 3689–3698, 1992.
32. Eva A, Robbins KC, Anderson PR, Srinivasan A, Tronick SR, Reddy EP, Ellmore NW, Galen AT, Lautenberger JA, Papas TS, Westin EH, Wong-Staal F, Gallo RC and Aaronson SA, Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. *Nature* **295**: 116–119, 1982.
33. McGahan M and Tyczkowski K, The determination of platinum in biological materials by electrothermal atomic absorption spectroscopy. *Spectrochim Acta* **42b**: 665–668, 1987.
34. Labarca C and Paigen K, A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* **102**: 344–352, 1980.
35. Vos J-M and Hanawalt PC, Processing of psoralen adducts in an active human gene: Repair and replication of DNA containing monoadducts and interstrand cross-links. *Cell* **50**: 789–799, 1987.
36. Sylvester JE, Whiteman DA, Podolsky R, Pozsgay JM, Respess J and Schmickel RD, The human ribosomal RNA genes: Structure and organization of the complete repeating unit. *Hum Genet* **73**: 193–198, 1986.
37. Moore WR, Anderson ME, Meister A, Murata K and Kimura A, Increased capacity for glutathione synthesis enhances resistance to radiation in *Escherichia coli*: A possible model for mammalian cell protection. *Proc Natl Acad Sci USA* **86**: 1461–1464, 1989.
38. Britten RA, Green JA and Warenius HM, Cellular glutathione (GSH) and glutathione *S*-transferase (GST) activity in human ovarian tumor biopsies following exposure to alkylating agents. *Int J Radiat Oncol Biol Phys* **24**: 527–531, 1992.
39. Micetich K, Zwelling LA and Kohn KW, Quenching of DNA:platinum(II) monoadducts as a possible mechanism of resistance to *cis*-diamminedichloroplatinum(II) in L1210 cells. *Cancer Res* **43**: 3609–3613, 1983.
40. Dedon PC and Borch RF, Characterization of the reactions of platinum antitumor agents with biologic and nonbiologic sulfur-containing nucleophiles. *Biochem Pharmacol* **36**: 1955–1964, 1987.
41. Alaoui-Jamali MA, Panasci L, Centurioni GM, Schecter R, Lehnert S and Batist G, Nitrogen mustard–DNA interaction in melphalan-resistant mammary carcinoma cells with elevated intracellular glutathione and glutathione-*S*-transferase activity. *Cancer Chemother Pharmacol* **30**: 341–347, 1992.
42. Christians FC and Hanawalt PC, Inhibition of transcription and strand-specific DNA repair by α-

amanitin in Chinese hamster ovary cells. *Mutat Res* **274**: 93–101, 1992.
43. Carreau M and Hunting D, Transcription-dependent and independent DNA excision repair pathways in human cells. *Mutat Res* **274**: 57–64, 1992.
44. Raj BK and Pitha P, Two levels of regulation of *beta* interferon gene expression in human cells. *Proc Natl Acad Sci USA* **80**: 3923–3927, 1983.
45. Islas AL, Vos J-MH and Hanawalt PC, Differential introduction and repair of psoralen photoadducts to DNA in specific human genes. *Cancer Res* **51**: 2867–2873, 1991.