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Decreased Accumulation as a Mechanism of Resistance to *cis*-Diamminedichloroplatinum(II) in Cervix Carcinoma HeLa Cells: Relation to DNA Repair

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SUMMARY

We previously described a cisplatin-resistant HeLa variant cell line that also exhibited cross-resistance to UV radiation and an enhancement in repair of UV-DNA adducts. In this study, excision repair of cisplatin-DNA adducts in the resistant cell line was investigated by different methods. Using a monoclonal antibody specific for cisplatin-DNA adducts, we found a 2–3-fold decrease in the accumulation of cisplatin-DNA adducts in the resistant cells. This was supported by the direct measurement of the number of cisplatin molecules in cells by atomic absorption spectrophotometry. The repair kinetic curves were composed of two phases, i.e., a rapid phase within the first 4 hr of repair incubation, followed by a slow phase for the cisplatin-DNA adducts. There was a 2.6-fold enhancement in the rapid repair rate in the resistant cells. Dose-response curves from these direct measurements indicated a 2.3-fold reduction in adduct accumulation in the resistant cells. In addition, repair-associated DNA strand breaks, measured using alkaline elution, showed a 1.6-fold increase in the resistant cells. Indirect detection of DNA excision repair, using host cell reactivation of transfected plasmid DNA carrying cisplatin damage, also showed 2.4-fold enhancement in the resistant cells. A phenotypic revertant of the cisplatin-resistant cells displayed reduced DNA repair, compared with the resistant cells. Furthermore, immediately after cisplatin treatment the resistant cells accumulated only 50–60% of the cisplatin-DNA adducts of the parental cells. The results suggest multifactorial mechanisms in cisplatin resistance, including reduced adduct formation and improved excision repair. The findings are also consistent with the notion that the early stage of DNA excision repair is a rate-limiting step in drug resistance.

Cisplatin [*cis*-diamminedichloroplatinum(II)] is a widely used chemotherapeutic agent in the treatment of testicular and ovarian tumors (1, 2). Occasionally, resistant cells arise, causing failure in cancer therapy. The mechanism for the acquired resistance of cells to the drug is not clear. It has been experimentally demonstrated that cisplatin is an effective cross-linking agent that generates various forms of cisplatin-DNA adducts, including intra- and interstrand DNA cross-links, DNA-protein cross-links, cisplatin-DNA-glutathione cross-links, and monoadducts (3). The interaction of cisplatin with DNA has been implicated as the major cytotoxic action of the drug (4). Most cisplatin-resistant cells are also resistant to alkylating agents or even cadmium, suggesting that cisplatin resistance may be related to DNA repair or an alteration in the level of free radical scavengers such as glutathione or metallothionein (3, 5–19). The link between the metabolism of cisplatin-DNA adducts and the sensitivity of cells to the drug is supported by studies using cells with genetic mutations in DNA metabolism. Patients with the human autosomal, recessively inherited, syndrome XP are subject to a high incidence of sunlight-induced skin disorders, including cancers (20). XP is characterized genotypically by mutations in at least seven genes (XP groups A-G). Cells from patients with XP exhibit reduced levels of removal of pyrimidine dimers and DNA adducts caused by UV-mimetic agents (20). XP group A is one of the most severe forms of the disease, and XP group A cells are defective in introducing incisions into damaged DNA (21–26). Recently, a doublet of 40- and 42-kDa proteins that can complement DNA repair of XP group A cell extracts have been purified from calf thymus (27), whereas cell extracts from XP group A or E cells display a reduction in recognizing UV-damaged DNA (28–30). These results revealed that the ability of cells to effectively eliminate damaged DNA at the early stage of excision repair plays an important role in determining the sensitivity or resistance of cells to genotoxic agents.

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ABBREVIATIONS: XP, xeroderma pigmentosum; CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; PI, propidium iodide; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); FITC, fluorescein isothiocyanate; MAb, monoclonal antibody; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Using a cell-free repair system, independent investigations have demonstrated that cell extracts can carry out repair synthesis in DNA damaged by UV, psoralens, and platinating agents (31–35) and that this repair synthesis is localized to sites of DNA damage (36), whereas extracts from some XP cells are incapable of repairing damaged plasmid DNA (25, 31, 37). We previously established a cisplatin-resistant HeLa cell line (38), which is phenotypically cross-resistant to UV irradiation and overproduces damaged-DNA recognition proteins (39, 40). Using a cell-free system (31), we have recently demonstrated improved recognition and incision of UV-DNA adducts, but not repair synthesis, in the resistant cells (41). These results suggest that the resistant cells have acquired efficient excision repair determined in the early stage. However, it is not clear whether the characteristics detected *in vitro* are reproduced in cells. In this study, a MAb specific for cisplatin-DNA adducts was used to investigate the accumulation of cisplatin-DNA adducts *in situ* in the resistant as well as the parental HeLa cells. The data are consistent with the measurements by atomic absorption spectrophotometry, alkaline elution, and plasmid reactivation, pointing to the conclusion that reduced adduct formation and enhanced DNA excision repair play important roles in cisplatin resistance of human cells.

Materials and Methods

Chemicals, media, and antibodies. Dulbecco's modified Eagle's medium, fetal bovine serum, and penicillin/streptomycin were obtained from GIBCO (Gaithersburg, MD). Platiamine (cisplatin) was purchased from Farmitalia Carlo Erba Ltd. [^{32}P]dCTP (3000 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol), and [2-^{14}C]thymidine (51.4 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. FITC- and peroxidase-conjugated goat anti-mouse immunoglobulins were purchased from Dako A/S (Copenhagen, Denmark). MAb 62–5 (kindly provided by Dr. H. Huang, National Tsin Hua University, Taiwan, ROC) was raised against cisplatin-treated calf thymus DNA by the method of Sundquist *et al.* (42).

Cell cultures and cytotoxicity. Human cervix carcinoma HeLa and cisplatin-resistant cells (38) and revertant variant cells (43) were maintained in monolayer culture in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin and were incubated at 37° in a humidified atmosphere of 5% (v/v) CO_2 in air. Cisplatin (1 μM) was added in the medium to maintain the resistant phenotype. Before the start of the experiments, the resistant cells were grown continuously for 3 weeks in medium without cisplatin. The sensitivity of cells to cisplatin was determined from the clonogenicity of cells 2 weeks after treatment. The fold resistance of cells was calculated from the ratio of the IC_{50} (cisplatin concentration inhibiting cell proliferation by 50%) values for resistant or revertant cells and parental HeLa cells.

FCM. FCM analysis was performed by a modification of the dual-staining method described by Pollack (44). Briefly, cells were rinsed twice in PBS, resuspended in 1 ml of nuclear isolation buffer (0.5% Nonidet P-40, 0.05 M NaCl, 1.0 mM EDTA, 0.05 M Tris·HCl, pH 7.4), and stained with MAb 62–5 (1/50 dilution in PBS) for 2 hr. One milliliter of FITC-conjugated goat anti-mouse immunoglobulins and 1 ml of PI stock solution (70 μg/ml PI in PBS) were then added. After a 40-min incubation at 4°, the cells were filtered through 0.45-μm nylon mesh and were analyzed on a FACScan flow cytometer (Becton-Dickinson) equipped with a 15-mW, air-cooled, argon ion laser. With the FACScan (488-nm excitation), fluorescence emission was collected after passing through band pass filters (530 nm for green fluorescence of FITC-conjugated antibody and 620 nm for red fluorescence of PI). Data were collected and analyzed on a Hewlett-Packard model 340 computer interfaced with the FACScan.

ELISA. The procedure entailed the transfer of aliquots of antibody solution to DNA-coated polystyrene, flat-bottomed, 96-well, microtiter plates (3.4-mm diameter; Corning). In each well, 5–8 × 10^4 cells were evaluated. The nonspecific binding was blocked by addition of 1% normal goat serum in PBS at 37° for 60 min. After removal of normal goat serum with PBS containing 0.05% Tween, the plate was incubated with 100 μl of diluted MAb 62–5 (1/20) for 30 min at 37°. Secondary antibody (peroxidase-conjugated goat anti-mouse immunoglobulins) (50 μl) was added and incubated at 37° for 30 min. Freshly prepared 1 mM ABTS in ABTS buffer (0.1 M citrate, 0.2 M disodium phosphate buffer, pH 4.2–4.8, 0.01% H_2O_2) was then added for 30 min and the absorbance at wavelength 405 nm was measured with a Biotek microtiter plate reader, as described previously (42, 45, 46). In competition ELISA, the 96-well plates were coated with 3 μg of cisplatin-DNA in 100 μl of PBS and air-dried overnight at room temperature. The cisplatin to nucleotide ratio of the cisplatin-DNA (calf thymus DNA) was about 0.2, as measured by atomic absorption spectrophotometry (47).

Alkaline elution and measurement of single-stranded DNA breaks. Single-stranded DNA breaks were measured using alkaline elution, as described previously (48). Cellular DNA was labeled with 0.01 μCi/ml [^{14}C]thymidine or [^{3}H]thymidine. After treatment of cells with cisplatin, the ^{14}C-labeled cells were incubated for 0–20 min in medium containing 10 mM hydroxyurea and 0.1 mM 1-β-D-arabinofuranosylcytosine. ^{3}H-labeled control cells were also incubated with the inhibitors. Cells were harvested in ice-cold PBS containing 0.2 μg/ml Na_2EDTA. ^{14}C-labeled tested cells were mixed with ^{3}H-labeled control cells and subjected to alkaline elution. The elution conditions and calculation of DNA strand breaks were as described by Rosenstein and Ducore (49).

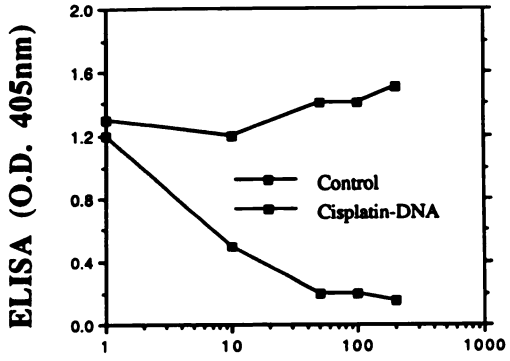
DNA transfection and CAT assays. Twenty micrograms each of plasmid DNA pRSVcat and pSVβ (Clontech Laboratories, Inc.) were co-transfected into cells using the electroporation technique (50). The pRSVcat plasmid was treated with cisplatin *in vitro* to generate various amounts of cisplatin-DNA adducts before being introduced into cells as described previously (39). Cells were seeded at 1 × 10^6 cells/100-mm plate 1 day before electroporation. Cell suspensions (1 ml), in HEPES buffer, were added to a sterile cuvette containing pRSVcat plasmid, gently mixed, and subjected to electroporation with a GenePulser (1000-μF capacity, 200 V; Bio-Rad). The cells were fed with fresh medium the following day and were incubated for another 40 hr to allow transient expression. Cells were then harvested into 1 ml of PBS and centrifuged for CAT and β-galactosidase activity assays (51), using 200 μg and 400 μg of cell extracts, respectively. The CAT assay reaction was incubated at 37° for 1 hr, followed by development on a silica thin layer chromatography plate (Macherey-Nagel, Düren, Germany). After autoradiography, density on the X-ray film corresponding to modified chloramphenicol or unmodified chloramphenicol was quantitated with a scanning densitometer (Hoefer GS300). The average of three scans of each chloramphenicol spot was taken. CAT activity was calculated as the percentage of chloramphenicol converted into acetylated derivatives. After being normalized to β-galactosidase activity, relative CAT activity was determined by setting untreated cells as 100%.

Isolation of cellular DNA and atomic absorption spectrophotometry. Cellular DNA was isolated by a standard method (51), in the presence of a potassium phosphate concentration of 80 mM (52). By this method, recovery of RNA from an hydroxyapatite column was undetectable (i.e., <5% of a similar applied amount of DNA). In brief, cells were lysed, sonicated using a ultrasonic processor (Sonics and Materials VC-600) fitted with a cup-horn (maximum power for 2 min), and then incubated with 10 μg of RNase at 37° for 15 min. After extraction with phenol, the aqueous phase was mixed with 25 ml of 6 M urea/80 mM potassium phosphate, pH 6.8, and 0.5 g of hydroxyapatite (DNA-grade Biogel-HPT; Bio-Rad) for 15 min at room temperature. The gel suspension was transferred to and eluted from a spun

column device. Specific binding of platinum to DNA was determined by atomic absorption spectrophotometry as described previously (53). Briefly, the cells were washed twice with cold PBS, and the cell pellets were either stored at −70° until analysis or immediately dissolved in nitric acid at 80° for 5 hr. The platinum was chelated with sodium diethyldithiocarbamate, followed by extraction with chloroform, and was analyzed on an atomic absorption spectrophotometer. The amount of platinum was normalized to the cellular protein concentration, which was determined by the method of Bradford (54), using the Bio-Rad dye reagent.

Results

Specificity of the MAb. The specificity of MAb 62–5 was characterized by ELISA and FCM (Fig. 1). A 96-well plate was coated with platinated DNA (drug/nucleotide ratio = 0.2) (see Materials and Methods). Exactly 0, 10, 50, 100, or 200 ng of untreated control or platinated DNA were used as competitor. The ELISA absorbance (at 405 nm) was read and competition curves were determined (Fig. 1, *upper*). Control DNA did not affect MAb binding. In contrast, >50% of the binding was inhibited by 10 ng of cisplatin-DNA. Further inhibition was observed with 50 ng or larger amounts of cisplatin. In addition, much larger amounts of single-stranded DNA or double-stranded DNA without cisplatin damage were required to inhibit the binding of MAb 62–5 (Table 1), suggesting that the MAb is preferential for cisplatin-DNA adducts. The dose-response curve for MAb 62–5 binding was also studied (Fig. 1, *lower*). The ELISA absorbance at 405 nm roughly increased with the level of cisplatin-DNA, from 1 to 200 ng of cisplatin-DNA. A dose-response pattern with similar slope was also detected by FCM. The regression lines with similar slopes (0.68047 and 0.56217 for ELISA and FCM, respectively) are indicated in Fig. 1. The relative fluorescence, which reflects MAb binding, increased with the amount of cisplatin-DNA. The results indicate that MAb 62–5 is preferential for cisplatin-DNA adducts, as measured by ELISA and FCM. The antibody also preferentially interacts with cisplatin-DNA adducts formed in the chromosomes of treated cells (data not shown).



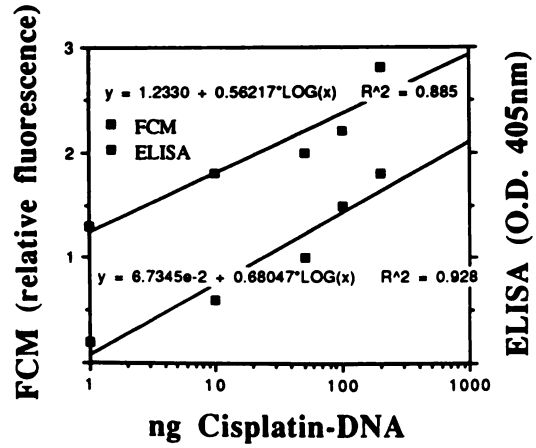


Fig. 1. Specificity of MAb binding to cisplatin-DNA adducts. *Upper*, competition with MAb 62–5 binding by cisplatin-DNA adducts. Exactly 0, 10, 50, 100, or 200 ng of competitors were added to the binding reaction (drug/nucleotide ratio = 0.2), as assayed by ELISA. *Lower*, dose-response curves for MAb 62–5 binding to cisplatin-DNA adducts, as assayed by FCM and ELISA. Exactly 0, 10, 50, 100, or 200 ng of cisplatin-DNA (drug/nucleotide ratio = 0.2) were used. The *lines* were calculated by linear regression. *R*, correlation coefficient.

Drug sensitivity of cisplatin-resistant HeLa cells. HeLa cells and a previously established HeLa cell variant resistant to cisplatin were analyzed for drug sensitivity (Fig. 2). Cells were treated with various concentrations of cisplatin for 5 hr, and after 14 days of incubation cells were assayed for clonogenicity. The relative survival, determined by setting untreated cells as 100%, of both cell lines is indicated (Fig. 2, *upper*). There was a "shoulder" in the dose-response curve of the resistant cells (HeLa-CPR), compared with the parental HeLa cells. For better comparison, the survival curve for HeLa cells is replotted in Fig. 2, *lower*. The IC_{50} (cisplatin concentration inhibiting cellular proliferation by 50%) was ~35 μM in HeLa-CPR cells and ~1.5 μM in HeLa cells. The fold resistance was 23 (three experiments).

Reduced cisplatin-DNA adduct formation in the resistant cells. The removal kinetics of cisplatin-DNA adducts were compared between HeLa-CPR cells and the parental HeLa cells using ELISA (Fig. 3) and FCM (Fig. 4). Cells were treated with 50 μM cisplatin for 5 hr, and the amount of cisplatin-DNA adducts was measured at 0, 4, 12, and 24 hr after cisplatin treatment (Figs. 3 and 4, *upper*). Both repair patterns showed a slight increase, with a peak accumulation at 4 hr, followed by a decrease of the relative A_{405} or the relative fluorescence. The patterns were similar, and the relative A_{405} of HeLa-CPR cells was ~35–80% less than that of HeLa cells, whereas the relative fluoresecence of HeLa-CPR cells was ~30–40% less than that of HeLa cells. Cisplatin-treated resistant and parental cells with the same peak accumulation of cisplatin-DNA adducts exhibited nearly identical kinetic patterns (data not shown). The results indicate that the rate of removal of cisplatin-DNA adducts is the same in both cell lines. The dose-response curve was also determined (Figs. 3 and 4, *lower*). Cells were treated with 0, 25, 50, 75, or 150 μM cisplatin. After

TABLE 1

Immunoreactivity of DNA-bound platinum complexes measured by ELISA

Competitor[a]	D/N[b]	IC_{50}[c]
		ng
Cisplatin-dsDNA	0.21	7
Cisplatin-ssDNA	0.14	120
Cisplatin-poly(dG)·poly(dC)	1.0	700
Transplatin-dsDNA	0.42	2500
Transplatin-ssDNA	0.49	2000

[a] ssDNA, heat-denatured single-stranded DNA produced by boiling double-stranded DNA for 10 min, followed by rapid cooling at 4°. dsDNA, double-stranded DNA.
[b] D/N, bound platinum/nucleotide ratio of competitor DNA.
[c] Amounts of platinated DNA inhibiting 50% binding.

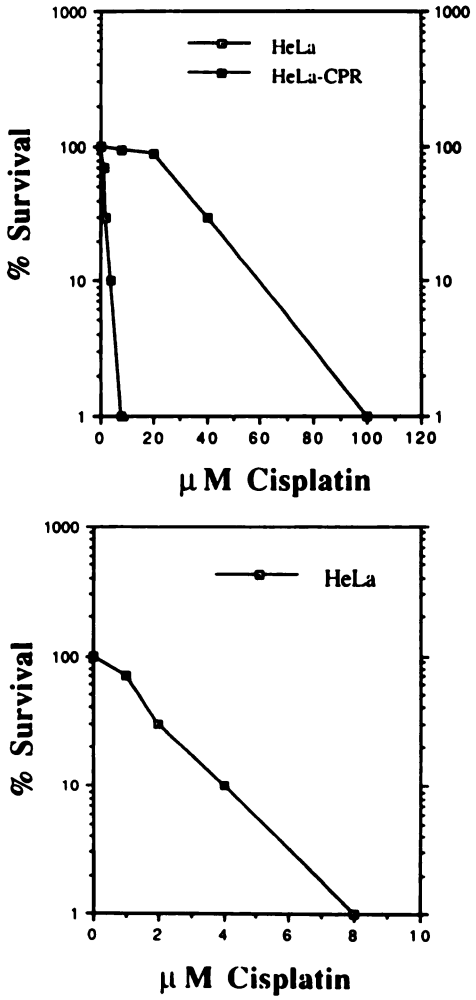


Fig. 2. Toxicity of cisplatin in HeLa cells and resistant cells. Cells were treated with cisplatin for 5 hr, and the clonogenicity was analyzed 14 days after treatment. Relative survival was calculated by setting untreated cells as 100% (three experiments). *Upper*, survival curves for HeLa cells and resistant cells; *lower*, survival curve for HeLa cells.

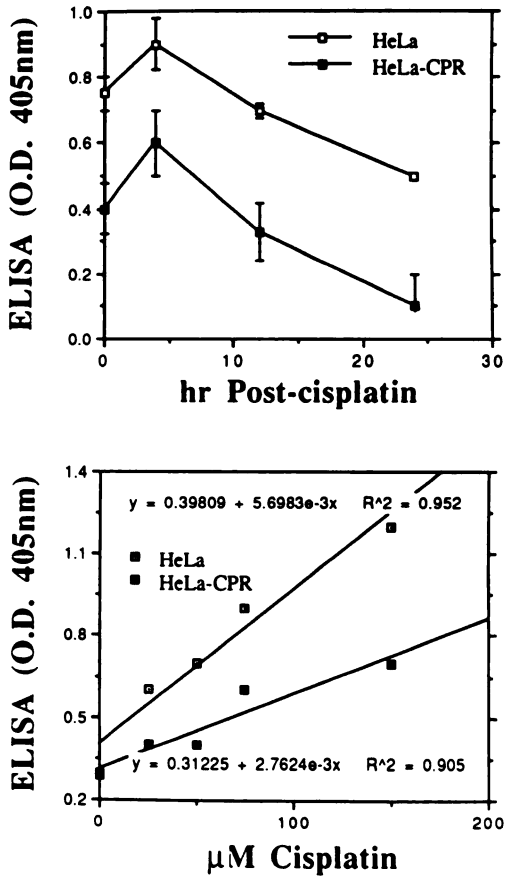


Fig. 3. Repair of cisplatin-DNA adducts in HeLa cells and resistant cells, as measured by ELISA. *Upper*, kinetics of repair after cisplatin treatment. The A_{405} (mean ± standard deviation, three experiments) (tested versus untreated cells) for each time point is shown. Cells were treated with 50 μM cisplatin for 5 hr and incubated in drug-free medium for 0, 4, 12, or 24 hr. *Lower*, dose-response curves for MAb binding to cisplatin-treated cells. Cells were treated with various concentrations of cisplatin for 5 hr and incubated for 24 hr, and the relative A_{405} was determined. The *lines* were calculated by linear regression. *R*, correlation coefficient.

5 hr of incubation, the level of cisplatin-DNA adducts in cells was analyzed by ELISA or FCM. The regression lines of the dose-response curves are indicated, showing 2–3-fold enhancement of DNA repair in HeLa-CPR cells.

The kinetics of removal of cisplatin-DNA adducts and the dose-response curve were also investigated using atomic absorption spectrophotometry (Fig. 5). After the same treatments of cells as for the ELISA and FCM, the DNA was extracted from cells and the amount of platinum was measured. The percentage of platinum remaining was calculated (Fig. 5, *upper*). The repair rate is composed of two phases, i.e., a rapid phase within the first 4 hr, followed by a slow phase. The difference in repair rates in the two cell types was detected only in the rapid phase. There was a ~2.6-fold reduction of adduct frequency in the resistant cells within the first 4 hr of repair incubation, with 15% and 40% of adducts being removed in HeLa and HeLa-CPR cells, respectively. It should be noted that cisplatin treatment (50 μM, 5 hr) produced 250 and 150 ng of cisplatin/mg of protein in HeLa and HeLa-CPR cells, respectively. The dose-response curve for intracellular platinum was also determined (Fig. 5, *lower*). The regression lines of the dose-response curves estimated a ~2.3-fold reduction of platinum in the resistant cells. Taken together, the results based on ELISA, FCM, and atomic absorption spectrophotometry demonstrated that the amount of cisplatin-DNA adducts in chromosomes is reduced 2–3-fold in HeLa-CPR cells.

Demonstration of enhanced repair in the resistant cells by indirect methods. To further evaluate results obtained by immunoassays, DNA repair in the resistant and parental cells was measured by alkaline elution. Cells were treated with 50 μM cisplatin for 5 hr, and repair-associated DNA strand breaks were measured. The excess DNA strand breaks (breaks due to 1-β-D-arabinofuranosylcytosine and hydroxyurea had been substracted) of HeLa and HeLa-CPR cells versus incubation time were determined (Fig. 6). There were ~1.6 excess strand breaks/10^{10} Da of HeLa DNA after a 1-min incubation and ~3.1 breaks under the same assay conditions in HeLa-CPR cells. There was a ~2-fold increase in total repair-associated DNA breaks in the resistant cells. However, the slopes of the regression lines were 0.94 and 0.58 for the resistant and parental cells, respectively, indicating a 1.6-fold enhancement of the repair rate in HeLa-CPR cells. The data also suggest that the initial increase in the accumulation of DNA breaks is 2-fold higher in the resistant cells.

DNA repair in the resistant and parental cells was also

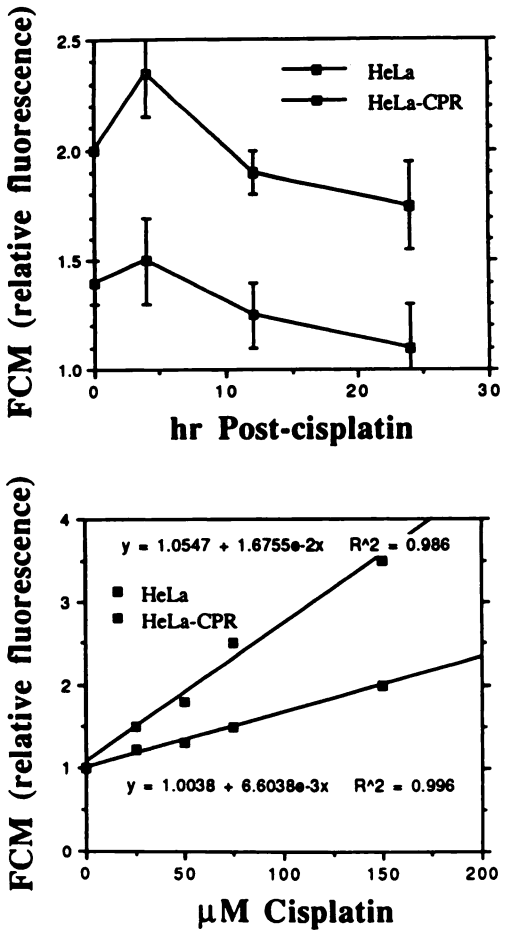


Fig. 4. Repair of cisplatin-DNA adducts in HeLa cells and resistant cells, as measured by FCM. *Upper*, kinetics of repair after cisplatin treatment. The relative fluorescence (mean ± standard deviation, three experiments) (tested versus untreated cells) is shown. Cells were treated with 50 μM cisplatin for 5 hr and incubated in drug-free medium for 0, 4, 12, or 24 hr. *Lower*, dose-response curves for MAb binding to cisplatin-treated cells. Cells were treated with various concentrations of cisplatin for 5 hr and incubated for 24 hr, and the relative fluorescence was determined. The *lines* were calculated by linear regression. *R*, correlation coefficient.

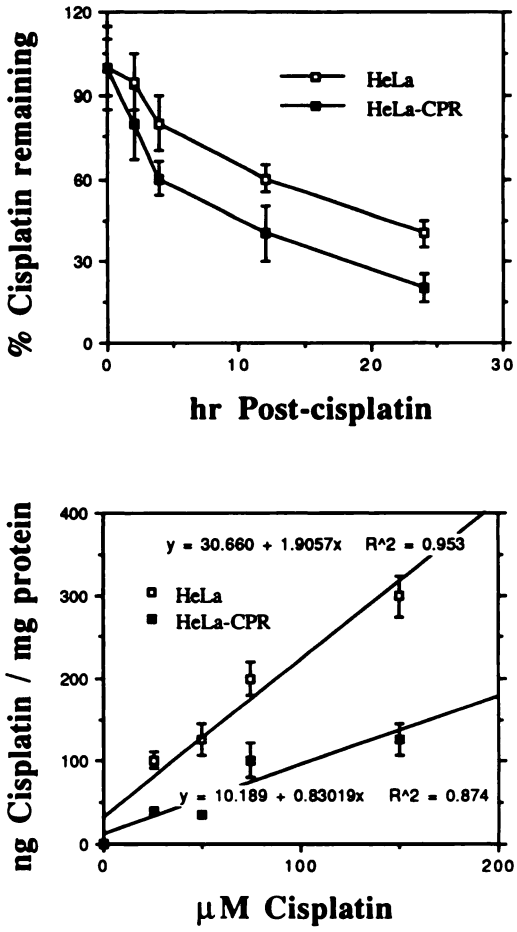


Fig. 5. Repair of cisplatin-DNA adducts in HeLa cells and resistant cells, as measured by atomic absorption spectrophotometry. *Upper*, kinetics of repair after cisplatin treatment. The percentage of cisplatin remaining (mean ± standard deviation, three experiments) (tested versus untreated cells), normalized to the amount of protein, for each time point is shown. Cells were treated with 50 μM cisplatin for 5 hr and incubated in drug-free medium for 0, 4, 12, or 24 hr. There were 250 and 150 ng of cisplatin/mg of protein in HeLa and HeLa-CPR cells, respectively, immediately after drug treatment. *Lower*, dose-response curves for the adduct frequency in cisplatin-treated cells. Cells were treated with various concentrations of cisplatin for 5 hr and incubated for 24 hr, and the relative adduct frequency was determined. The *lines* were calculated by linear regression. *R*, correlation coefficient.

measured by plasmid reactivation, an indirect measurement of DNA excision repair (13). Plasmid DNA pRSVcat with 0, 0.001, 0.002, or 0.004 cisplatin molecules/phosphate was co-transfected with untreated pSVβ for transient expression (Fig. 7). The CAT and β-galactosidase activities were measured 40 hr after transfection. Shown is a typical example of the CAT activity expressed from pRSVcat carrying 0, 0.001, 0.002, or 0.004 cisplatin molecules/phosphate (Fig. 7, A and B, *lanes 2-5*) in HeLa (Fig. 7A) or HeLa-CPR (Fig. 7B) cells. Fig. 7, A and B, *lanes 1*, shows the CAT reaction without cell extracts. After being normalized to the β-galactosidase activity, the relative CAT activity was calculated by setting the untreated pRSVcat as 100% (Fig. 7C). The IC_{50} values (cisplatin concentration inhibiting CAT activity by 50%) for HeLa and HeLa-CPR cells were 0.0007 and 0.017 cisplatin molecules/phosphate, respectively. There was a ~2.4-fold enhancement of the plasmid reactivation in the resistant cells.

Reduction of DNA repair in a revertant cell variant. Enhanced DNA repair in the resistant cells suggests that the phenotypic alterations in the resistant cells result from the efficacy of removing DNA damage. By assuming that this is the major or the only cause of the resistance, one may speculate the reduction of enhanced DNA repair in revertant cells. To test this hypothesis, a revertant cell line derived from the resistant cells (43) was used. The revertant cells (HeLa-rev), along with the HeLa parental cells and the resistant cells, were analyzed for cytotoxicity and DNA repair (Table 2). The IC_{50} values for cytotoxicity were 1.5, 35, and 3.1 μM in parental, resistant, and revertant cells, respectively. The 23-fold resistance was reduced to 2.1-fold in the revertant cells. A majority of the resistance was lost in the revertant cellls. The cisplatin concentration required to generate a relative value of cisplatin-DNA adducts in the FCM assay was 56, 151, and 100 μM for parental, resistant, and revertant cells, respectively (also see Fig. 4). The fold increase, obtained by setting HeLa cells as 1, was 2.7 in the resistant cells and 1.8 in the revertant cells. Similar results were also obtained by ELISA. Coincidentally, the fold enhancement of plasmid reactivation was 2.43 and 1.43 for the resistant and revertant cells, respectively. In addition,

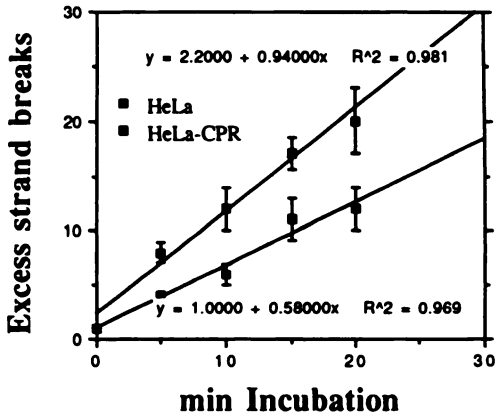


Fig. 6. Repair of cisplatin-DNA adducts in HeLa cells and resistant cells, as measured by alkaline elution. Cells were treated with 50 μM cisplatin for 5 hr, incubated for 0, 5, 10, 15, or 20 min for repair synthesis, and loaded for alkaline elution. The excess strand breaks/10^{10} Da of genomic DNA (mean ± standard deviation, three experiments) were calculated. The *lines* were calculated by linear regression. *R*, correlation coefficient.

alkaline elution studies also demonstrated a partial loss of repair-mediated DNA strand breaks in the revertant cells, compared with the resistant cells (data not shown). The data indicate that disapperance of the enhanced DNA repair is associated with the loss of phenotypic resistance in the revertant cells. The data support the notion that the amount of cisplatin-DNA adducts in cells is important for the sensitivity of cells to the drug.

Discussion

In this study, DNA repair in relation to cisplatin resistance was investigated. Using a cisplatin-DNA-specific MAb in association with ELISA or FCM assay, I have demonstrated a 2–3-fold reduction of the accumulation of cisplatin-DNA adducts in HeLa-CPR cells. However, the kinetic pattern of adduct frequency in the resistant cells is the same as that in the parental cells, except that the initial level of cisplatin-DNA adducts is ~50% lower in the resistant cells. The results are consistent with the direct measurement of the number of platinum atoms by atomic absorption spectrophotometry, which shows 2.3-fold less platinum in HeLa-CPR cells. In addition, treatment of cells with the same cisplatin concentration (50 μM, 5 hr) resulted in 250 and 150 ng/mg of protein in HeLa and HeLa-CPR cells, respectively, further confirming that the resistant cells have developed a characteristic of reduced adduct formation. In contrast to the immunoassays, kinetic studies using atomic absorption spectrophotometry demonstrated two phases of adduct removal, i.e., a rapid phase within the initial 4 hr, followed by a slow phase. The slow repair rates in the two cell types are similar, if not identical. However, there is a 2.6-fold increase in the initial repair rate in HeLa-CPR cells. Nevertheless, it is not clear why immunoassays detected only differences in adduct accumulation and not the difference in repair rate. A likely explanation is that sample preparation somehow causes loss of epitopes for the antibody, because the difference in repair rates is observed only in the intial repair time, as measured by atomic absorption spectrophotometry. Furthermore, there is a 2.5-fold enhancement of the plasmid reactivation in DNA repair in the resistant cells, based on the assumption that the CAT activity is not efficiently expressed in transfected cells when cisplatin-DNA adducts remain on the gene. These findings strongly suggest that earlier steps such as recognition (40) and incision may be altered in the resistant cells, as shown for UV resistance (41). This speculation is supported by the measurement of repair-associated single-stranded DNA breaks using the alkaline elution method. Taken together, these results strongly suggest that the ability of cells to remove cisplatin-DNA adducts plays a critical role in the overall response of cells to the drug. The conclusion is supported by the partial loss of the reduced accumulation of cisplatin-DNA adducts in a revertant cell line derived from the resistant cells (see Table 2). However, one cannot rule out the possibility that, in addition to the direct role of DNA repair, decreased uptake, increased cisplatin conjugation with metallothioneins or glutathione, or differences in the types of cisplatin-DNA lesions formed in cells could contribute to the resistant phenotype.

Defects in nucleotide excision repair of damaged DNA are well established for cells of XP groups A through G. Using cell extracts from XP cells, extensive studies have been accumulated showing reduced repair synthesis in most of these hypersensitive cell lines, whereas some cells featuring hypersensitivity to genotoxic agents showed normal repair synthesis (20), suggesting that defects in post-repair synthesis like ligation also play a role in mediating cellular sensitivity to the damage. Furthermore, reduced repair synthesis in XP group A cells has been assigned to the incision step (21–26). We have previously

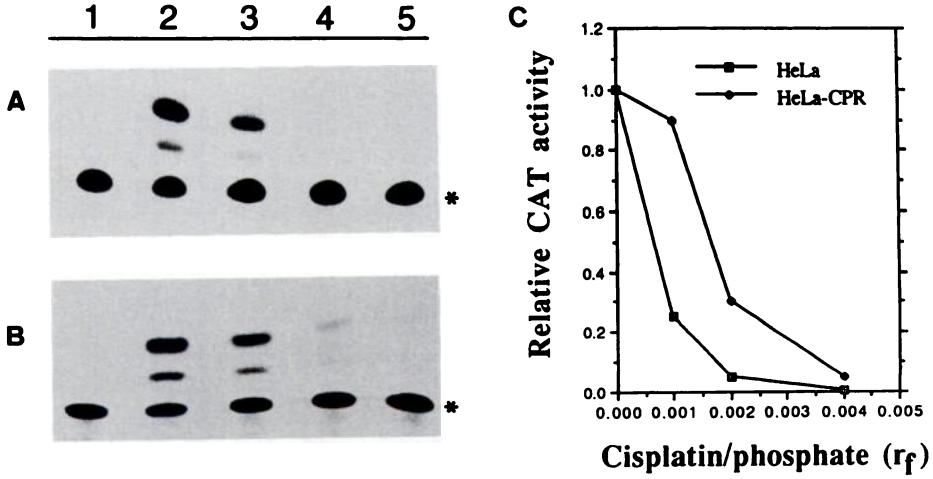


Fig. 7. Repair of cisplatin-DNA adducts in HeLa cells and resistant cells, as measured by plasmid reactivation. Twenty micrograms of pRSVcat were platinated *in vitro* to generate different amounts of cisplatin-DNA adducts (r_f) before being introduced into cells for transient expression. Relative CAT activity, determined by setting untreated pRSVcat as 1, was calculated. The standard deviation was 15–20% (three experiments).

TABLE 2

Comparison of DNA repair measured by different methods, in the parental, resistant (HeLa-CPR), and revertant (HeLa-rev) HeLa cells

Cells	Cytotoxicity IC_{50}[a]	Adduct formation concentration[b]	Plasmid reactivation IC_{50}[c]
	μM	μM	cisplatin/phosphate
HeLa	1.5 ± 0.2 (1)	56 ± 8 (1)	0.0007 ± 0.00012 (1)
HeLa-CPR	35 ± 3 (23)	151 ± 20 (2.7)	0.0017 ± 0.0003 (2.43)
HeLa-rev	3.1 ± 1 (2.1)	100 ± 15 (1.8)	0.001 ± 0.0002 (1.43)

[a] The data are expressed as cisplatin IC_{50} (mean ± standard deviation, three experiments). Numbers in parentheses are the fold resistance, setting HeLa cells as 1.

[b] The data are expressed as the average cisplatin concentration required to generate a relative fluorescence in cells as assayed by FCM (mean ± standard deviation, three experiments) (also see Fig. 4). Numbers in parentheses are the fold increase, setting HeLa cells as 1.

[c] The data are expressed as the IC_{50} (mean ± standard deviation, three experiments), i.e., estimated cisplatin-DNA adducts required to inhibit CAT activity by 50%, as assayed by plasmid reactivation in cells (also see Fig. 7). Numbers in parentheses are the fold enhancement, setting HeLa cells as 1.

demonstrated the cross-resistance of HeLa-CPR cells to UV damage that is associated with enhanced recognition and incision of UV-DNA adducts in a cell-free system (41). The results from this study also suggest that the improved excision repair of cisplatin-DNA adducts in the resistant cells is largely determined in the early stage of the repair process. Thus, HeLa-CPR cells have likely acquired an enhanced ability to circumvent damaged DNA that is deficient in XP cells.

The sensitive detection of cisplatin-DNA adducts *in vivo* using a MAb has also been demonstrated by others (52). MAb 62–5 should be useful for measuring cisplatin-DNA adducts in mammalian cells. The immunoassay represents a direct and quantitative measurement of the cisplatin-DNA adducts in cells. A potential limitation is that the interaction between the cisplatin-DNA epitope and the antibody may be affected by the architecture of chromosomes under different physiological conditions of the cells. Changes due to DNA replication, for example, may shield the target site from recognition by the antibody, leading to underestimation of the intact cisplatin-DNA adducts. However, this may not be a problem, because there was only slight or no significant variation of the adduct frequency in both cell types treated at different cell growth phases (data not shown). In addition, the antibody competitively binds to adducts with damage-recognition proteins in cells.[1] Thus, low adduct frequency in cells may be masked by the intrinsic damage-recognition proteins, causing underestimation of the adducts.

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