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Decreased Drug Accumulation and Increased Tolerance to DNA Damage in Tumor Cells with a Low Level of Cisplatin Resistance

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ABSTRACT. In an attempt to examine the cellular changes associated with cisplatin resistance, we selected a cisplatin-resistant (A431/Pt) human cervix squamous cell carcinoma cell line following continuous *in vitro* drug exposure. The resistant subline was characterized by a 2.5-fold degree of resistance. In particular, we investigated the expression of cellular defence systems and other cellular factors probably involved in dealing with cisplatin-induced DNA damage. Resistant cells exhibited decreased platinum accumulation and reduced levels of DNA-bound platinum and interstrand cross-link frequency after short-term drug exposure. Analysis of the effect of cisplatin on cell cycle progression revealed a cisplatin-induced G_2M arrest in sensitive and resistant cells. Interestingly, a slowdown in S–phase transit was found in A431/Pt cells. A comparison of the ability of sensitive and resistant cells to repair drug-induced DNA damage suggested that resistant cells were able to tolerate higher levels of cisplatin-induced DNA damage than their parental counterparts. Analysis of the expression of proteins involved in DNA mismatch repair showed a decreased level of MSH2 in resistant cells. Since MSH2 seems to be involved in recognition of drug-induced DNA damage, this change may account for the increased tolerance to DNA damage observed in the resistant subline. In conclusion, the involvement of accumulation defects and the increased tolerance to cisplatin-induced DNA damage in these cisplatin-resistant cells support the notion that multiple changes contribute to confer a low level of cisplatin resistance. BIOCHEM PHARMACOL **55**;8:1247–1254, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cisplatin; drug resistance; cervix squamous cell carcinoma; apoptosis; DNA damage

The chemotherapeutic agent cisplatin is successfully used for the treatment of ovarian and testicular carcinoma as well as a variety of solid tumors including squamous cell carcinomas [1]. Despite its effectiveness, resistance to the drug often develops [2]. It has been reported that low levels of cisplatin resistance readily emerge in treated patients, which may be sufficient for treatment failure [2]. A rapid emergence of low levels of cisplatin resistance has been documented in human tumor xenografts [3]. Thus, the study of cellular alterations associated with low levels of resistance represents a critical step in understanding the clinically relevant mechanisms of resistance of tumor cells.

Investigations on cellular mechanisms of resistance to cisplatin using pairs of sensitive cell lines and *in vitro* selected variants have shown that resistance is multifactorial. The major alterations underlying resistance to cisplatin include: reduced drug accumulation [4, 5], increased detoxification through cellular thiols [6, 7], and reduced DNA platination and enhanced DNA-platinum adduct removal [8]. Recently, a role for apoptosis-related proteins such as p53, bcl-2 and bcl-x as determinants of cellular response to cisplatin has been proposed [9, 10]. Therefore, factors contributing to cisplatin resistance include cellular defence systems and mechanisms that modulate the capability of cells to deal with drug-induced damage. In this regard, a role for cell cycle regulation in the chemoresistance of cells can be envisaged. Accumulation in G_1 and/or G_2 following exposure to DNA-damaging agents could allow repair of drug-induced DNA lesions before DNA synthesis and mitosis [11, 12].

p53 is involved in regulation of cell cycle and in apoptosis activation following DNA damage [13]. However, the precise role of p53 gene status in acquired resistance to cisplatin is still a matter of debate. In an attempt to study the changes related to the development of a low level of resistance, a tumor cell line with mutant p53 was chosen. Although the relevance of p53-dependent response may be

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cell-specific, this model should allow determination of p53-independent mechanisms of resistance. Our results indicate that reduced drug accumulation resulting in a decreased DNA platination contributes to cisplatin resistance in such cells and suggest an increased tolerance to DNA damage by resistant cells associated with a decreased expression of MSH2. The present results support the notion that multiple alterations can be expressed even in cells characterized by low levels of resistance, thus confirming the multifactorial and complex nature of the cisplatin-resistant phenotype.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human cervix squamous cell carcinoma A431 and a cisplatin-resistant variant were used in the study. The resistant subline, A431/Pt, was selected by exposure of A431 cells to increasing cisplatin concentrations up to 1 μg/mL starting from 0.1 μg/mL up to 1 μg/mL over a period of 9 months; when A431/Pt cells were grown in the absence of cisplatin, no modification of response to the drug was observed for at least 2 months. After selection with 1 μg/mL of cisplatin, resistance was not increased further when cells were grown in the presence of 2 μg/mL of cisplatin for 2 months. Both cell lines were grown as monolayers in RPMI-1640 medium plus 10% fetal calf serum.

Apoptosis

Apoptosis was assessed by fluorescence microscopy 48 hr after a 1-hr drug exposure, as described previously [9]. Briefly, at the end of the experiment cells were fixed in 70% ethanol, stained with propidium iodide solution (30 μg/mL propidium iodide and 66 U/mL of RNase A in PBS), and stored in the dark for 30 min. At least 100 cells in two different smears for each sample were examined. The percentage of apoptotic cells was referred to the total cell number.

Cell Synchronization

Cell cultures were synchronized by double thymidine block [14]. Cells were plated at $7.5 \times 10^4/cm^2$ in 5-cm ∅ dishes and incubated at 37°, in 5% CO_2 in the presence of 3 mM of thymidine for 16 hr (A431 cells) or 18 hr (A431/Pt cells), then for 18 hr in thymidine-free medium. Synchronization of the cells at the G_1/S transition was then achieved by a further 16- or 18-hr incubation in medium containing 3 mM of thymidine. Immediately after release from the second thymidine block, cells were exposed to cisplatin at the indicated concentrations for 1 hr, then washed and allowed to progress through the cell cycle in drug-free medium. At different times after drug exposure, cells were harvested for flow cytometric analysis of DNA content.

Cell Cycle Analysis

Cell cycle distribution was assessed by determining DNA content by propidium iodide staining or DNA content and BrdU incorporation [15]. For determination of propidium iodide-stained cells at different times after treatment, cells were detached, washed with PBS, fixed in 70% ice-cold ethanol and stored at −20°. Cells were then rehydrated in PBS and stained in propidium iodide (30 μg/mL) solution containing RNase A (66 U/mL) for 30 min. Fluorescence intensity was determined by a FACScan flow cytometer equipped with an argon laser (Beckton Dickinson). For BrdU incorporation, cells were incubated with 30 μM of BrdU (Sigma) during the last 2 hr of culture and then fixed. Fluorescence intensity was measured on samples exposed to anti-BrdU monoclonal antibody (Becton Dickinson) and then to FITC-conjugated antimouse antibody by a FACScan flow cytometer (Becton Dickinson).

Determination of GSH Content

Exponentially growing cells were harvested, counted and immediately homogenized in ice-cold 5% trichloroacetic acid. After centrifugation at 5000 *g* for 10 min to remove protein, cellular GSH content was determined according to Ellman [16].

Cisplatin Accumulation

Exponentially growing cells were seeded in Petri dishes in triplicate and after 48 hr were exposed to cisplatin for 1 hr. Cell monolayers were then washed with ice-cold PBS, scraped, harvested and dissolved in 100 μL of 1 N NaOH. Total cellular platinum content was determined by flameless atomic absorption spectroscopy with an instrument characterized by a sensitivity of 5 μg/L (Model 3300, Perkin Elmer). Cellular platinum levels were expressed as nmol platinum/cell number, with cell number determined by counting parallel cultures.

DNA Platination

Cells, grown to near confluence, were exposed to cisplatin for 1 hr. DNA was extracted according to standard procedures involving lysis in the presence of 1 mg/mL of proteinase K overnight at 37°. DNA was then isolated following phenol extraction, ethanol precipitation, RNase treatment and reprecipitation and, finally, was dissolved in 10 mM of Tris-HCl, pH 7.4, and 1 mM of EDTA [17]. DNA content was determined spectrophotometrically, and platinum content was measured by ICP-MS[#] [18].

[#] *Abbreviations:* EGF, epidermal growth factor; IC_{45}, IC_{50}, IC_{75} and IC_{80}, drug concentration inhibiting cell growth by 45, 50, 75 and 80%; ICL, interstrand cross-linking; ICP-MS, inductively coupled plasma mass spectroscopy; UDS, unscheduled DNA synthesis.

Alkaline Elution

[^{14}C]-Labelled cells were exposed to cisplatin for 1 hr; an unexposed control flask was included in all experiments. After drug exposure, cells were incubated for 5 hr in drug-free medium and then processed for DNA ICL measurement by alkaline elution, according to Kohn *et al.* [19] as previously described [9]. DNA ICL was calculated using the following formula:

$$\text{ICL frequency} = [(1 - r_0)/(1 - r)^{1/2}] - 1$$

where r and r_0 are the [^{14}C]-labelled DNA fractions of treated vs control cells remaining on the filter.

Measurement of UDS

UDS was measured by a modification of a previously reported method [20]. Cells were seeded at near confluent density in 96-well plates and left for 5 days at 37° in 5% CO_2. Cell monolayers were then washed twice with arginine-deficient RPMI and incubated in the same medium supplemented with 5% fetal bovine serum. After 24 hr, the medium was replaced with fresh identical medium for an additional 24-hr incubation. Cells exposed for 1 hr to 10 mM of hydroxyurea were then incubated with cisplatin for 1 hr. After washing, cells were incubated in arginin-deficient RPMI with [^{3}H]thymidine (10 μCi/mL 45 Ci/mmol, Amersham) in the presence of hydroxyurea for 3 hr at 37° in 5% CO_2. Cells were collected on glass fiber filters by a cell harvester. [^{3}H]thymidine incorporation was determined by liquid scintillation counting.

Western Blot Analysis

Cell lysates from exponentially growing cells were prepared as previously described [9]. In brief, samples (80 μg/lane) were fractionated by SDS-PAGE (polyacrilamide gel electrophoresis) and blotted on nitrocellulose sheets. Blots were preblocked for 4 hr at room temperature in PBS containing 5% (w/v) dried nonfat milk. Filters were incubated overnight at 4° with antibody to MSH2, MLH1 (Oncogene Research Products) or EGF-receptor (Upstate Biotechnology Incorporated); a rabbit antiactin antibody (Sigma) was used as a control for loading. Antibody binding to the nitrocellulose blots was detected using chemiluminescence procedures (Amersham). In experiments involving EGF expression, immunoreactive EGF receptor bands were revealed by [^{125}I] Protein A (Amersham) and quantified by a PhosphoImager (Molecular Dynamics).

RESULTS

Features of Cisplatin-Sensitive and Resistant Cells

A human cervix squamous cell carcinoma cell line designated A431/Pt was selected by exposure of the parental A431 cell line to increasing cisplatin concentrations. As shown in Table 1, the resistant cells, which had a resistance index of 2.5, exhibited a slightly increased doubling time compared to parental cells. Expression levels of the EGF receptor, whose downregulation has been reported to be associated with the cisplatin-resistant phenotype of selected models [21], were slightly, but not significantly reduced in A431/Pt cells (Fig. 1). Parental cells had a mutant p53 (codon 273), and the same mutation was found in A431/Pt cells (data not shown).

TABLE 1. Features of A431 and A431/Pt cells

Parameter	A431	A431/Pt
Doubling time* (hr)	17 ± 1.3	24 ± 2
Saturation density† (cells/cm^2)	2 × 10^5	1.5 × 10^5
Diameter‡ (μm)	15.4 ± 0.6	16.0 ± 1.0
Morphology	Epithelial-like	Epithelial-like
Cisplatin sensitivity§ (IC_{50})	10.7 ± 2.5	27 ± 5.5
EGF receptor‖	100	74 ± 16¶
p53 status#	Mutant	Mutant
GSH** (nmoles/10^6 cells)	9.5 ± 0.9	13.7 ± 1.4

*Assessed by cell counting on exponentially growing cells.
†Assessed by cell counting in confluent cultures.
‡Mean value (± SD) measured with a chanalizer-equipped cell counter.
§Measured by cell counting 48 hr after a 1-hr drug exposure; IC_{50} values (drug concentration inhibiting cell growth by 50%, μg/mL) are the means ± SD of 2–3 independent experiments.
‖Evaluated by Western blot analysis ($N = 2$). Similar results were obtained with cytofluorimetric analysis.
¶Percentage in comparison to A431.
#Determined by DNA sequencing of 5–9 p53 exons.
**Determined spectrophotometrically from cellular lysates.

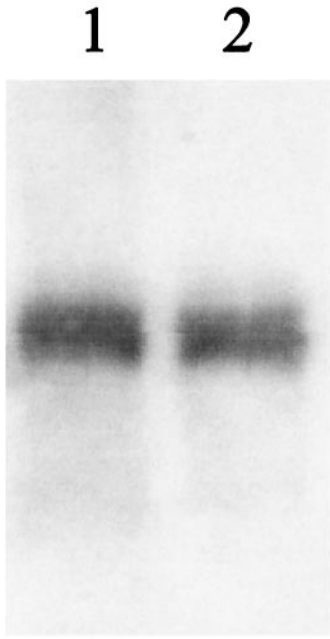

FIG. 1. Western blot analysis of EGF receptor in A431 (1) and A431/Pt (2) cells.

TABLE 2. Cytotoxicity and apoptosis induced by cisplatin in A431 and A431/Pt1*

	Cytotoxicity		% Apoptosis at	
	IC_{50}†	IC_{80}†	IC_{50}	IC_{80}
A431	28 ± 3.6	64	5 ± 0.5	10
A431/Pt	61 ± 7	140	6 ± 1	13

*Cells were exposed to cisplatin for 1 hr and processed for apoptosis and cytotoxicity evaluation 48 hr later. Values are derived from dose-response curves.
†Expressed as μg/mL.

Susceptibility to Apoptosis

The appearance of apoptotic cells was examined in A431 and A431/Pt cells 48 hr after a 1 hr exposure to the drug (Table 2). Low levels of basal apoptosis were found, and even with highly cytotoxic concentrations of drug, the apoptosis percentage never exceeded 20% in sensitive or resistant cells. Such a finding suggests that apoptosis is not the major mode of cell death in this cell system.

Effect of Cisplatin on A431 and A431/Pt Cell Cycle Progression

Cisplatin treatment is known to induce an arrest in the G_2 phase of the cell cycle independently of p53 status [22]. Since a protective role against DNA damage has been assigned to the G_2 checkpoint [22], we compared cell cycle progression in control and cisplatin-treated cells to investigate whether differences between the two cell lines while in G_2 could account for A431/Pt cell drug resistance. The two synchronized cell lines were exposed to cisplatin for 1 hr. Flow cytometric histograms at different times after exposure are shown in Fig. 2. The sensitive and resistant control cell lines progressed similarly and synchronously through the cell cycle and recovered, 24 hr after release of the thymidine block, a DNA content distribution typical of the asynchronous cell population (Fig. 2A and Fig. 2C). A431 cells exposed to a 30 μg/mL, corresponding to the IC_{75} (drug concentration inhibiting cell growth by 75%) were found accumulated in the G_2M phase (Fig. 2B). The G_2M block was evident at 24 hr from the G_1S block release and persisted for at least 5 days (56% and 30% of total cells for treated and control cells, respectively, Fig. 3). In A431/Pt cells, the same drug concentration, corresponding to the IC_{45} (drug concentration inhibiting cell growth by 45%), did not induce any arrest at the G_2M phase (Fig. 2D), whereas an accumulation similar to that in A431 cells was obtained with the equitoxic concentration, corresponding to the IC_{75} (75 μg/mL, Fig. 2E). Here as well, the G_2M block was persistent for at least 5 days (44% and 21% of total cells for treated and control cells, respectively, Fig. 3). An increase in drug-resistant cells with an S–phase DNA content was found after exposure to cisplatin (Figs. 2 and 3). In fact, starting at 6 hr after the G_1/S block release,

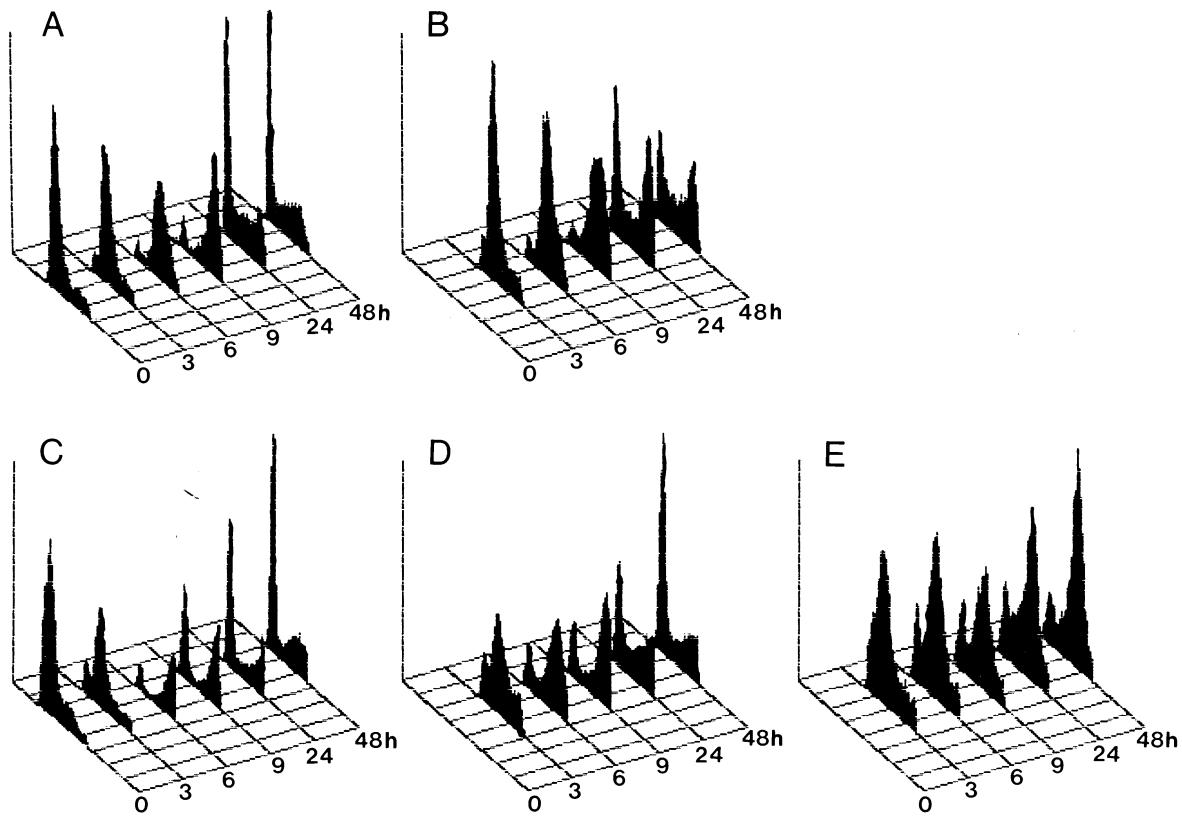

FIG. 2. Cell cycle profile of synchronized A431 and A431/Pt cells exposed to cisplatin. After a 1 hr exposure to the drug, cells were processed at different time intervals for FACScan analysis. A, untreated A431 cells; B, A431 cells + 30 μg/mL of cisplatin; C, untreated A431/Pt cells; D, A431/Pt cells + 30 μg/mL of cisplatin; E, A431/Pt cells + 75 μg/mL of cisplatin.

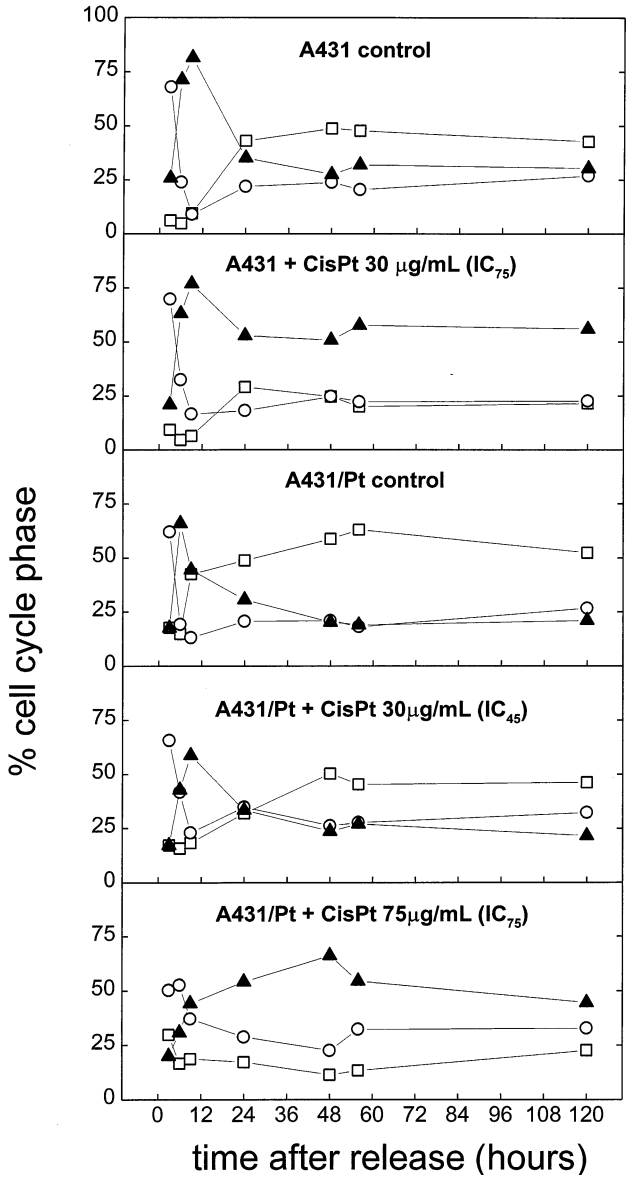

FIG. 3. Cell cycle distribution of synchronized A431 and A431/Pt cells exposed to cisplatin (cisPt). Cells were treated as in Fig. 2. The percentage of cells in the different phases of the cell cycle (square, G1; circle, S; triangle G2M) was obtained by computer analysis of DNA histograms.

the percentage of treated cells in S phase was consistently above that of control cells. The effect, barely appreciable in A431/Pt cells exposed to 30 μg/mL of cisplatin (IC_{45}), was clearly evident in A431/Pt cells exposed to 75 μg/mL of cisplatin (IC_{75}) and could be interpreted as a persistent slowdown in S phase transit, because the cells were actively synthesizing DNA, as observed by BrdU incorporation (not shown).

GSH Level, Cellular Platinum Accumulation, DNA-Bound Platinum and ICL

Among the mechanisms of resistance to cisplatin, an important role has been attributed to defence factors,

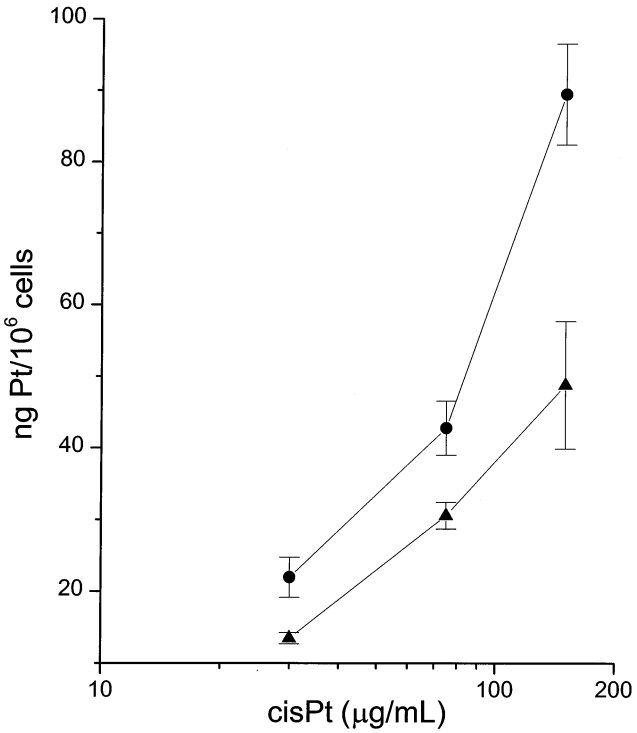

FIG. 4. Cellular accumulation of platinum after a 1-hr cisplatin exposure. Platinum was measured by atomic absorption spectroscopy. A431 (circles); A431/Pt (triangles). Mean values (±SD) of triplicate determinations are shown.

including cellular thiols and drug transport [4, 7]. We therefore measured the levels of intracellular glutathione of A431 and A431/Pt cells. No pharmacologically relevant differences ($P > 0.05$) were found between sensitive and resistant cells, GSH levels being 9.5 ± 0.9 and 13.7 ± 1.4 nmol/10^6 cells, respectively (Table 1). The measurement of cisplatin accumulation after a 1-hr exposure to drug concentrations ranging from 30 to 150 μg/mL revealed an approximately 2-fold lower accumulation in cisplatin-resistant cells than in sensitive cells ($P < 0.05$, Fig. 4). At equitoxic cisplatin concentrations (30 μg/mL for A431 cells, 75 μg/mL for A431/Pt cells), similar amounts of platinum (21.92 ± 2.8 ng/10^6 cells for A431 cells, 30.53 ± 1.8 ng/10^6 cells for A431/Pt cells) were found.

Because DNA is the major cellular target of cisplatin, DNA platination and interstrand cross-link formation were analyzed. ICP-MS analysis of DNA after a 1-hr exposure to cisplatin revealed that DNA-bound platinum increased with drug concentration in the parental cells up to 150 μg/mL. At similar concentrations, the amount of DNA-bound platinum was lower in A431/Pt cells than in A431 cells. Drug concentrations equivalent to the IC_{75} (30 μg/mL for A431 cells and 75 μg/mL for A431/Pt cells) determined similar levels of platination in the two cell lines. No dose-dependent increase was observed in the resistant cells. Similarly, experiments measuring the ICL frequency in the two cell lines showed a reduced level of DNA lesions in the resistant cells, thus confirming the observation of ICP-MS studies (Fig. 5).

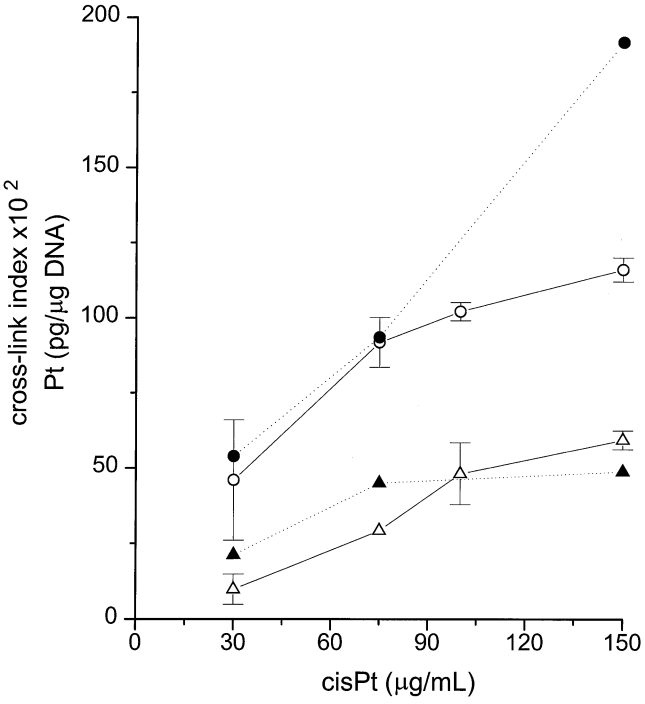

FIG. 5. DNA platination level and interstrand cross-link frequency in cisplatin-exposed A431 and A431/Pt cells. Platination (dotted line and closed symbols) was measured by inductively coupled plasma spectroscopy after a 1-hr drug exposure. A431, circles; A431/Pt, triangles. Interstrand cross-linking frequency (solid line and open symbols) was assessed by alkaline elution. A431, circles; A431/Pt, triangles.

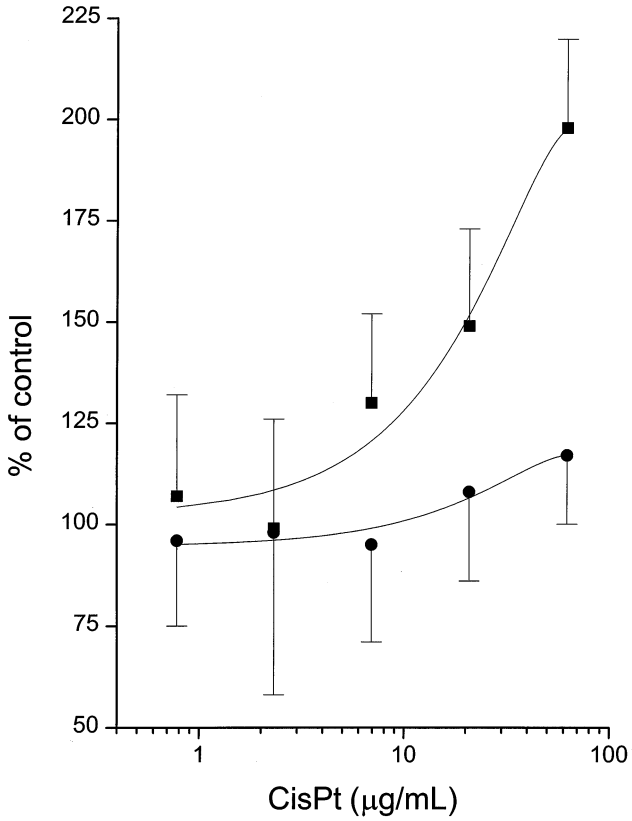

FIG. 6. Unscheduled DNA synthesis. Repair capability was measured as described in Materials and Methods in A431 (circles) and A431/Pt (triangles) cells after a 1-hr exposure to cisplatin. ^{3}H-thymidine incorporation measured in untreated cells was taken as 1 unit.

Effect of Cisplatin on UDS

Cell survival depends on enzymatic mechanisms of DNA repair. The process is also thought to play an important role in recovery from cisplatin cytotoxicity [23]. To establish whether a different ability of cells to repair drug-induced DNA damage could contribute at least in part to the different sensitivity of A431 and A431/Pt cells to cisplatin, we measured the nonsemiconservative UDS induced by the drug in both cell lines. Figure 6 shows that 1 hr exposure to cisplatin caused a dose-dependent increase in UDS in A431 cells at drug concentrations >10 μg/mL, but not in A431/Pt cells even with highly cytotoxic concentrations. It can be inferred from the plots that drug concentrations causing similar DNA platination and ICL (e.g. 30 μg/mL in A431 vs 75 μg/mL in A431/Pt cells) likely induced a lower repair activity in A431/Pt than in A431 cells, thereby suggesting that resistant cells were able to tolerate higher levels of cisplatin-induced DNA damage than their parental sensitive counterparts.

Expression of Proteins Involved in Mismatch Repair

Loss of mismatch repair has been associated with low levels of cisplatin resistance [24]. To establish whether alterations of the mismatch repair machinery could be related to the observed increased tolerance to cisplatin-induced DNA damage in the studied cell system, we examined the expression of MLH1 and MSH2 (Fig. 7). MSH2 expression was reduced in A431/Pt cells, whereas MLH1 level remained unchanged as compared to parental cells.

DISCUSSION

We examined the cellular changes associated with the development of a low degree of cisplatin resistance (2.5-fold) in a human cervix carcinoma cell line. The development of a low degree of resistance may be clinically relevant for therapy with platinum compounds, because it could

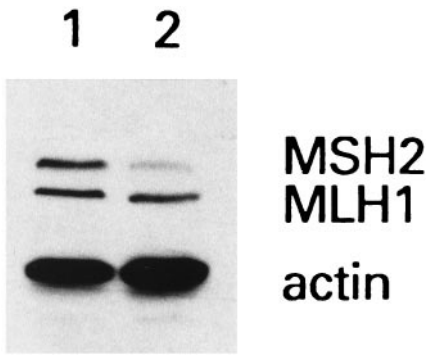

FIG. 7. Western blot analysis of MLH1 and MSH2 in A431 (1) and A431/Pt (2) cells. Control loading is shown by actin.

account for loss of drug efficacy. Previous studies have addressed the same aspect [25, 26].

The cell line A431 was chosen for selecting a cell subline system characterized by a low and stable degree of resistance since it exhibited a relatively low sensitivity to cisplatin. The cell line and, as expected, the resistant subline expressed a mutant p53. The role of p53 as a determinant of cell chemosensitivity to DNA-damaging agents is still a matter of debate [13]. The limited sensitivity of the parental cell line to cisplatin and the low level of drug-induced apoptosis are consistent with the presence of a mutant p53. The A431/Pt cell line is 2.5 times less responsive to cisplatin, and we did not obtain higher levels of resistance with additional exposure to increasing concentrations of the drug (data not shown).

Cisplatin has been described to induce perturbations of cell cycle progression [12]. In our model, equitoxic concentrations induced a similar accumulation of A431 and A431/Pt cells in the G_2M phase of the cell cycle. Such a perturbation appears to be the major drug-induced change described in several tumor cells [27]. Interestingly, a prolonged accumulation in S phase peculiar of the resistant cells was observed. After this slowdown transit in S phase, cells appeared to undergo a more persistent G_2M block.

Our present results support the view that multiple alterations are expressed in A431/Pt cells. Indeed, a reduction of drug accumulation was found in resistant compared to sensitive cells, and the event was directly correlated to the extent of cisplatin-induced DNA damage, as assessed by ICP-MS and alkaline elution. Thus, upregulation of defence factors, as reflected by a reduction in drug accumulation, might be a critical determinant of this cisplatin-resistant phenotype. Several studies have related cisplatin resistance to detoxification mechanisms and to repair efficiency [4, 8, 28, 29]. In contrast, it is unlikely that GSH, another cellular defence factor involved in cellular pharmacology of cisplatin [7, 8], plays a role in this system, since a pharmacologically irrelevant difference in the levels of the thiol was found between sensitive and resistant cells. In addition, A431/Pt cells exhibited a lower cisplatin-induced UDS than A431 cells, thus ruling out a greater efficiency of the repair apparatus in the resistant phenotype. At comparable levels of DNA platination in sensitive and resistant cells, a low cisplatin-induced UDS was observed in A431/Pt cells. Such a finding suggests that resistant cells have an increased tolerance to DNA damage, probably related to a reduced capability of A431/Pt cells to recognize DNA damage, thus allowing cell survival by replicating DNA on a damaged template. This interpretation is consistent with the slow progression of treated A431/Pt cells through the S phase.

It is conceivable that changes in the cellular pathways regulating DNA damage recognition and/or cell death may account for the increased tolerance. Relevant to this point is the finding of a decreased expression of MSH2 in resistant cells. In this regard, a failure of cellular mechanisms involved in repair of DNA mismatches could underlie the increased tolerance. Previous studies have shown that cisplatin-resistant cell lines are characterized by loss of DNA mismatch repair [24]. The precise role of this alteration remains to be defined.

In conclusion, in spite of the low level of cisplatin resistance, multiple resistance factors are expressed in our cell system, including cellular defence factors (such as reduced drug accumulation) as well as factors probably involved in dealing with drug-induced DNA damage such as an increased tolerance to cisplatin-induced DNA damage.

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