Metallothionein Content Correlates with the Sensitivity of Human Small Lung Cancer Cell Lines to Cisplatin

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ABSTRACT

We have established cis-diaminedichloroplatinum(II) (cisplatin)-resistant human small cell lung cancer cell lines, H69/CDDP4 and H69/CDDP5, and cisplatin-resistant human small cell lung cancer cell line, H69, and its resistant cell lines with different degrees of cisplatin resistance. Increased levels of GST isozymes have been correlated with the resistance to alkylating agents (10–12). However, there was no difference in GST activities and GST-α, -μ, and -γ contents between in vitro acquired cisplatin-resistant cells and parental cells in our previous studies. Transfection of a GST-γ expression vector into NIH3T3 cells could not induce cisplatin resistance (13). A potential role for GST-γ as a marker of cisplatin resistance remains to be demonstrated (11), although GST-γ by itself may not induce cisplatin resistance. MT is the most prominent of the protein sulfhydrox. Andrews et al. (14) reported elevation of the MT level in cadmium chloride-resistant human ovarian carcinoma cells that were cross-resistant to cisplatin. Furthermore, mouse C127 cells transfected with the human MT II gene became resistant to cisplatin and alkylating agents (15). Thus MT may be one possible mechanism of resistance.

In the present study, we have examined the mechanisms of cisplatin resistance in the human small cell lung cancer cell line, H69, and its resistant cell lines with different degrees of resistance to cisplatin.

INTRODUCTION

Cisplatin is a key anticancer agent for the treatment of human small cell lung cancer (1), although development of resistance to cisplatin limits its efficacy and results in failure of cancer treatment. Thus it is essential to determine the mechanism(s) of cisplatin resistance, not only to improve the present chemotherapy but also to develop new anticancer agents, especially new platinum derivatives. We have already reported characteristics of cisplatin-resistant human non-small cell lung cancer cell lines (2). A variety of mechanisms of cisplatin resistance have been described (3), including decreased drug accumulation (4–6), increased detoxification by thiol containing scavenger molecules such as GSH4 and MT, and increased repair of DNA damage (7–9). Fujiwara et al. (2) reported that the non-small cell lung cancer cell line, PC-9/CDDP5, which showed 28-fold resistance to cisplatin, had higher GSH content than that of parental PC-9. Although dl-buthionine-R,S-sulfoximine depleted the GSH of the resistant line to a level approximately equal to that of the parental line, the I50 of the resistant line was still 21-fold higher than that of the parental line. This indicates that factors other than increased GSH confer cisplatin resistance. Increased levels of GST isozymes have been correlated with the resistance to alkylating agents (10–12). However, there was no difference in GST activities and GST-α, -μ, and -γ contents between in vitro acquired cisplatin-resistant cells and parental cells in our previous studies (2). Transfection of a GST-γ expression vector into NIH3T3 cells could not induce cisplatin resistance (13). A potential role for GST-γ as a marker of cisplatin resistance remains to be demonstrated (11), although GST-γ by itself may not induce cisplatin resistance. MT is the most prominent of the protein sulfhydrox. Andrews et al. (14) reported elevation of the MT level in cadmium chloride-resistant human ovarian carcinoma cells that were cross-resistant to cisplatin. Furthermore, mouse C127 cells transfected with the human MT II gene became resistant to cisplatin and alkylating agents (15). Thus MT may be one possible mechanism of resistance.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: GSH, glutathione; PBS, calcium-free and magnesium-free Dulbecco’s phosphate-buffered saline; ACNU, 3-[4-(4-aminomethyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea; MT, metallothionein; GST, glutathione S-transferase; ICL, interstrand cross-links; ADM, Adriamycin; I50, drug concentration that inhibits cell growth by 50%.

MATERIALS AND METHODS

Drugs and Chemicals. RPMI 1640 and PBS were purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Cisplatin and etoposide were obtained from Bristol Myers Co., Tokyo, Japan. ACNU was purchased from Sankyo Co., Ltd., Tokyo, Japan. ADM was purchased from Nissui Pharmaceutical Co., Tokyo, Japan. CDDP, to investigate the mechanism of acquired resistance to cisplatin.

2 Recipient of a research resident fellowship from the Foundation for Promotion of Cancer Research.

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The abbreviations used are: GSH, glutathione; PBS, calcium-free and magnesium-free Dulbecco’s phosphate-buffered saline; ACNU, 3-[4-(4-aminomethyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea; MT, metallothionein; GST, glutathione S-transferase; ICL, interstrand cross-links; ADM, Adriamycin; I50, drug concentration that inhibits cell growth by 50%.
tured in cisplatin-free medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum [Immu-no-Biochemical Laborato-
ries, Fujioka, Japan], penicillin (100 units/ml), and streptomycin (100 
µg/ml) for at least 1 month. The degree of cisplatin resistance was
stable in cisplatin-free medium for at least 1 year in each cell line (data
not shown). Each cell line was cultured in a humidified atmosphere of
5% CO2 at 37°C. K562/ADM, a human leukemia cell line resistant to
ADM, was kindly provided by Dr. T. Tsuruo at the University of
Tokyo.

Drug Sensitivity Test. The drug sensitivity of each cell line to various
antitumor agents was determined by growth inhibition as previously
reported (17). In brief, a number of 60-mm dishes were set up each
containing 2 × 104 cells. Drugs were added to each dish at appropriate
concentrations. After incubation for 7 days, the cells were counted in a
Microcellcounter CC-108. All assays were conducted using continuous
drug exposure. Each experiment was performed in triplicate and each
experiment was done at least 3 independent times. The degree of drug
sensitivity of cells was determined from the graph of cell number versus
drug concentration and was expressed as IC50 value, defined as the drug
concentration inhibiting cell growth by 50% compared to the control

Cisplatin Accumulation Study. For cisplatin accumulation studies,
exponentially growing cells of the three cell lines were harvested and
seeded into 60-mm culture dishes at a density of 1 × 106 cells/ml. The cells
were incubated with 5 µg/ml [195Pt]cispalatin in RPMI 1640 plus fetal
bovine serum for 1, 2, or 3 h. In efflux studies, cells treated with
5 µg/ml [195Pt]cispalatin for 3 h were washed with cold PBS and
resuspended in fresh medium and incubated for 1, 2, and 3 h. At the
end of each time period, cells were collected by centrifugation and
washed twice with cold PBS. The radioactivity of each sample was
counted with a Packard Auto-Gamma scintillation counter. All results
were normalized to cellular protein as measured by the BCA protein
assay kit (Pierce, Rockford, IL).

Filter Elution Assays. ICL induced by cisplatin were determined by the
filter elution method. In brief, 4 × 104 cells were radiola-
beled with 1.0 µCi/ml of [methyl-3H] thymidine for 60 h, followed by
a 3-h chase period. Labeled cells were incubated with cisplatin at various
concentrations for 3 h. For γ-irradiation, labeled cells were irradiated
on ice with 60Co γ-irradiation at a dose rate of 0.22 Gy/min. In the
experiments of repair of ICL, cells treated with cisplatin were incubated in
cisplatin-free medium for various periods before irradiation. Cells (1
× 106) were layered on a polycarbonate membrane, washed with cold
PBS and lysed with a lysis solution containing 2% sodium dodecyl
sulfate, 50 mM glycine, 25 mM disodium EDTA, pH 10.0, and
0.5 mg/ml proteinase K. The lysis solution was drawn through the
filters and the retained DNA was washed with 0.02 M disodium EDTA
and 0.5 mg/ml proteinase K. The lysis solution was drawn through the

The DNA on the filter was eluted at 0.04 ml/min for 15 h
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and 0.5 mg/ml proteinase K. The lysis solution was drawn through the

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RESULTS

Characteristics and Drug Sensitivity of H69 Parental and
Cisplatin Resistant Cell Lines. Two cisplatin-resistant cell lines,
H69/CDDP02 and H69/CDDP, were derived from H69 paren-
tal cells. The doubling times of each cell line were 60, 67 and
67 h, respectively, and the cell sizes were 6.92, 6.59, and 6.96
µm, respectively. There was no significant difference in doubling
times and cell sizes in the three cell lines. Table 1 shows the
sensitivities for various agents of three cell lines. H69/CDDP02
and H69/CDDP were more resistant to cisplatin than H69
with relative resistance (IC50 value of resistant cell line/IC50 of paren-
tal cell line) of 6.2 and 10.9, respectively. H69/CDDP was
cross-resistant to 254-S, a new platinum compound. H69/
CDDP02 and H69/CDDP were more resistant to ACNU, h-
CPA, the active form of cyclophosphamide, and cadmium chlo-
ride. Sensitivities to ADM, vincristine, 7-ethyl-10-[4(1-piperi-
dino)-1-piperidinolcarbonyloxycamptothecin, and etoposide
were not different between H69 and H69/CDDP.

Accumulation of [195Pt]cispalatin. Fig. 1A shows the kinetics of
[195Pt]cispalatin accumulation. Cisplatin concentration, 5 µg/
ml, was chosen in order to obtain measurable platinum levels
in this experimental setting. Accumulation of cisplatin increased linearly
up to 3 h. There was little difference in cisplatin accumulation between
H69 and its cisplatin-resistant cell lines. Similarly, no significant
difference was observed in cisplatin efflux among the 3 cell lines (Fig. 1B).

DNA ICL and Its Repair. We determined the formation of
DNA ICL by the filter elution method. Fig. 2A shows the

| Table 1 Sensitivities for various agents of H69 and its cisplatin-resistant cell lines |
|-----------------|-----------------|-----------------|-----------------|
| ICL (µg/ml)     | H69             | H69/CDDP02      | H69/CDDP        |
| Agents          | IC50            | IC50            | IC50            |
| Asparaginase    | 0.007 ± 0.0034  | 0.07 ± 0.0072   | 1.47 ± 0.0072   |
| ADM             | 0.21 ± 0.006    | ND              | 0.86 ± 0.024    |
| IPC             | 0.26 ± 0.017    | 0.56 ± 0.0062   | 0.73 ± 0.0112   |
| ACNU            | 0.52 ± 0.012    | 1.8 ± 0.2035    | 2.2 ± 0.014    |
| ACC             | 0.058 ± 0.007   | ND              | 0.063 ± 0.005   |
| CRV             | 0.017 ± 0.008   | ND              | 0.017 ± 0.008   |
| CPT-11          | 0.85 ± 0.016    | ND              | 0.79 ± 0.012   |
| VP-16           | 0.82 ± 0.011    | ND              | 0.55 ± 0.019   |
| GdCl3           | 9.6 ± 0.7      | 14.7 ± 1.5      | 19.8 ± 1.0      |

* Each value is the mean ± SD of the three independent experiments.
* Relative resistance value: IC50 value of resistant cells/IC50 value of parental cells.

ND, not determined.
CISPLATIN RESISTANCE MECHANISMS

Fig. 1. Accumulation and efflux of [\textsuperscript{195m}Pt]cisplatin in H69 and its cisplatin-resistant cell lines. A, incorporation of radiolabeled cisplatin into H69 parental and drug-resistant cells. All cells were treated with 5 \(\mu\)g/ml of cisplatin for 1, 2, or 3 h. B, efflux of [\textsuperscript{195m}Pt]cisplatin from H69 cells. Cells treated with 5 \(\mu\)g of cisplatin per ml were washed with cold PBS and incubated for 1, 2, or 3 h in drug-free medium. All data were normalized to that of H69 cells with no drug-free incubation period (control). O, H69; □, H69/CDDP\textsubscript{0.2}; ●, H69/CDDP. Bars, SD.

Fig. 2. ICL formation by cisplatin in H69 and its cisplatin-resistant cell lines. A, ICL formation after 3 h exposure to cisplatin as a function of the concentration of cisplatin. B, ICL formation as a function of time after treatment with cisplatin for 3 h. H69, H69/CDDP\textsubscript{0.2}, and H69/CDDP were treated with 2, 6, and 10 \(\mu\)g of cisplatin per ml, respectively. O, H69; □, H69/CDDP\textsubscript{0.2}; ●, H69/CDDP. Bars, SD.

Table 2 Detoxification mechanisms of H69 and its cisplatin-resistant cell lines

<table>
<thead>
<tr>
<th>Relative resistance</th>
<th>H69</th>
<th>H69/CDDP\textsubscript{0.2}</th>
<th>H69/CDDP</th>
<th>H69/CDDP\textsubscript{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH content (nmol/mg protein)</td>
<td>16.6 ± 4.7\textsuperscript{a}</td>
<td>14.4 ± 2.9</td>
<td>19.9 ± 3.0</td>
<td>14.4 ± 2.9</td>
</tr>
<tr>
<td>GST activity (nmol/min/mg protein)</td>
<td>11.5 ± 8.7</td>
<td>12.3 ± 4.5</td>
<td>74.3 ± 16.1\textsuperscript{b}</td>
<td>74.3 ± 16.1\textsuperscript{b}</td>
</tr>
<tr>
<td>MT content (pmol/mg protein)</td>
<td>14.5 ± 1.5</td>
<td>22.3 ± 2.0\textsuperscript{c}</td>
<td>32.6 ± 1.6\textsuperscript{d}</td>
<td>32.6 ± 1.6\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± SD.
\textsuperscript{b} \(P < 0.001\) compared to value for H69 and H69/CDDP\textsubscript{0.2} by unpaired Student's \(t\) test.
\textsuperscript{c} \(P < 0.001\) compared to value for H69 by unpaired Student's \(t\) test.
\textsuperscript{d} \(P < 0.001\) compared to value for H69 and H69/CDDP\textsubscript{0.2} by unpaired Student's \(t\) test.

Detoxification Mechanisms. In order to elucidate the role of the potential drug detoxification systems in the acquisition of cisplatin resistance, we measured GSH content, MT content, and GST activity. The results are summarized in Table 2. There was no difference in GSH content among the 3 cell lines. The MT levels in H69/CDDP\textsubscript{0.2} and H69/CDDP were 1.6- and 2.3-fold higher, respectively, than that of H69. The MT levels were positively correlated with IC\textsubscript{50} values to cisplatin and cadmium chloride in these cell lines (Fig. 3). GST activity was not different between H69 and H69/CDDP\textsubscript{0.2}, but H69/CDDP showed an approximately 6-fold increase in GST activity. To investigate the role of GST in cisplatin resistance, we used ethacrynic acid, an inhibitor of GST-\(\alpha\) and GST-\(\pi\) (27), in a growth inhibition assay (Table 3). An ethacrynic acid concentration of 0.1 \(\mu\)g/ml was chosen because this was not cytotoxic in all the cell lines. Ethacrynic acid sensitized each cell line, but the relative resistance of the H69 cell lines was not changed.

Detoxification mechanisms in the 3 cell lines after exposure to various concentrations of cisplatin for 3 h. ICL in each cell line was formed in a concentration-dependent manner. In H69/CDDP\textsubscript{0.2} and H69/CDDP cells, ICL was decreased to 30.0 and 21.1\%, respectively, compared with H69. To elucidate any difference in repair of ICL, we washed platinated cells with fresh medium and incubated them for various times. The concentrations of cisplatin to which H69, H69/CDDP\textsubscript{0.2}, and H69/CDDP were exposed were 2, 6, and 10 \(\mu\)g/ml, respectively, to induce an equal level of ICL. As shown in Fig. 2B, ICL increased during the first 6 h of the repair time and after 6 h ICL decreased in a time-dependent manner. No significant difference was observed in peak levels and rates of decrease of ICL among the 3 cell lines. No single or double strand breaks induced by cisplatin were found in the filter elution assays (data not shown).
A Northern blot analysis was undertaken to investigate the overexpression of genes in the resistant cell lines. Fig. 4 shows the mRNA levels for GST-π, MT, and β-actin. The β-actin probe demonstrated that an equivalent amount of total RNA from each cell line was loaded in each lane. There was no difference in the expression of GST-π mRNA between H69 and H69/CDDP. But it was overexpressed in H69/CDDP. The expressions of MT mRNA were 2.6- and 4.3-fold higher in H69/CDDP and H69/CDDP, respectively. The expression of MT mRNA was inversely correlated with the sensitivity to cisplatin. No evidence for MT gene amplification could be seen in H69/CDDP and no evidence of GST-π gene amplification could be seen in H69/CDDP cells (data not shown). No cell lines expressed mdr-1 mRNA, although all cell lines had mdr-1 gene (data not shown).

**DISCUSSION**

The resistant cells used in this study had equal doubling times and cell sizes, making them suitable to analyze mechanisms of acquired cisplatin resistance. We evaluated five factors that are thought to play a role in cisplatin resistance. The analysis using [195Pt]cisplatin revealed equal amounts of cisplatin accumulation and no difference in efflux. Thus we concluded the difference in sensitivity to cisplatin among the 3
CISPLATIN RESISTANCE MECHANISMS

MTs comprise a class of isoproteins with molecular weights of 6000–7000. They are involved in zinc homeostasis and the detoxification of heavy metal such as cadmium, copper, and mercury (30). Bakka et al. (31) showed that human epithelial cells and mouse fibroblasts, which showed resistance to cadmium chloride and contained a higher MT content, showed cross-resistance to cisplatin. Andrews et al. also reported that the cadmium chloride-resistant cell lines, 2008/MT and COLO/MT, showed cross-resistance to cisplatin and that MT levels were elevated 23-fold in 2008/MT and 9.4-fold in COLO/MT compared to their parental cell lines (14). Although the resistant cells also had increased GSH, depletion of GSH by DETOXIFICATION MECHANISMS

The primary mode of action for cytotoxicity of cisplatin is likely a result of its reaction with DNA. DNA ICL may be one of the important types of cisplatin-DNA adducts. We studied the formation of DNA ICL by the filter elution assay. The formation of DNA ICL significantly decreased in H69/CDDP0.2 and H69/CDDP under the same concentration of cisplatin. However, the equivalent amounts of cross-link formation and DNA repair were observed in all three cell lines at approximately equitoxic concentrations of cisplatin. These data suggest that formation of DNA ICL is important for cisplatin cytotoxicity and that repair of DNA ICL did not contribute to the difference in the resistance in H69 cell lines.

Detoxification mechanisms have been thought to play an important role in cisplatin resistance. GSH may be one of the important factors. In our studies, no significant difference was observed in GSH content. GSH-related enzymes, especially GST, have been considered to be important factors for cisplatin resistance. However, Nakagawa et al. (13) could not demonstrate the induction of cisplatin resistance in GST-ir-transfected NIH3T3 cells. In our resistant cell lines, H69/CDDP showed approximately 6-fold higher GST activity and overexpression of GST-ir mRNA, but in a less cisplatin-resistant cell line, H69/CDDP0.2, from which H69/CDDP was derived, there was no significant difference in GST activity and the expression of GST-ir mRNA was similar to the parental H69. As shown in Table 3, the IC₅₀ values to cisplatin were decreased by treatment with ethacrynic acid, an inhibitor of GST-α and -η, in all cell lines, but the relative resistant values were unchanged. Although the GST activity of H69/CDDP was inhibited by ethacrynic acid, H69/CDDP0.2 and H69/CDDP showed 4.2- and 11.0-fold resistance compared with H69 cells. These data suggest that GST may contribute to cisplatin resistance but other factors must be responsible for the difference between these lines.

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Unpublished data.

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