Increased Expression of DNA Topoisomerase I Gene and Collateral Sensitivity to Camptothecin in Human Cisplatin-resistant Bladder Cancer Cells

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ABSTRACT

We established three cis-diaminedichloroplatinum(II) (cisplatin)-resistant cell lines, T24/DDP5, T24/DDP7, and T24/DDP10, by the stepwise exposure of T24 human bladder cancer cells to increasing concentrations of cisplatin. The resistance to cisplatin of T24/DDP5, T24/DDP7, and T24/DDP10 cells was 2.2-, 5.2-, and 8.4-fold that of the parental T24 cells, respectively. The cisplatin-resistant cell lines also showed an increased resistance to vincristine, although their sensitivities to Adriamycin and etoposide resembled that of T24. In contrast, the cisplatin-resistant cells developed a collateral sensitivity to (4S)-4,11-dieethyl-4-hydroxy-9-[(4-piperidino)carbonyloxy]carbonylethylcarboxyloxy]dione hydrochloride trihydrate, a camptothecin derivative, and its active metabolite, 7-ethyl-10-hydroxycamptothecin, that targets DNA topoisomerase I. Both a Northern blot analysis and an immunoblot analysis demonstrated increased cellular levels of DNA topoisomerase I mRNA in the resistant cell lines. However, the expression of DNA topoisomerase I mRNA in the three resistant cell lines did not significantly differ from that in the T24 cells. No significant differences in the glutathione S-transferase θ levels were observed, although the intracellular content of glutathione in the T24/DDP7 cells was slightly but significantly increased. In addition, the intracellular platinum concentration correlated negatively with the degree of cisplatin resistance and was found to be significantly decreased in T24/DDP10 at an external cisplatin concentration of 20 μg/ml. These results suggest that the increased levels of DNA topoisomerase I mRNA in the resistant cell lines. However, only a few cisplatin-resistant bladder cancer cell lines have been previously described. Walker et al. (11) established a cisplatin-resistant human bladder cancer cell line, but they did not clarify the mechanisms of its resistance. Thus, the mechanisms of cisplatin resistance in human bladder cancer remain to be elucidated. Therefore, we established three cisplatin-resistant cell lines by the stepwise exposure of a human bladder cancer cell line to increasing concentrations of cisplatin and then characterized the biochemical properties of these cells.

INTRODUCTION

Treatment with cis-diaminedichloroplatinum(II) (cisplatin) has improved the therapeutic efficacy against a wide variety of solid tumors, including bladder cancer (1). In particular, combination chemotherapy regimens that include cisplatin, such as methotrexate, vinblastine, doxorubicin, and cisplatin, have proven beneficial in patients with advanced bladder cancer (2). However, the response is often limited due to the appearance of drug-resistant tumor cells. A clarification of the mechanisms of drug resistance is therefore needed in order to improve the therapeutic benefit. The establishment and characterization of cisplatin-resistant tumor cell lines are considered to be helpful in elucidating the underlying mechanisms of acquired resistance to cisplatin in human tumors.

Numerous cisplatin-resistant cell lines have been established, thus revealing various mechanisms of cisplatin resistance, including a decrease in drug accumulation (3, 4), an increase in drug detoxification by thiol-containing molecules such as GSH (5, 6) and metallothionein (7, 8), and an increased repair of DNA damage (9, 10). However, only a few cisplatin-resistant bladder cancer cell lines have been previously described. Walker et al. (11) established a cisplatin-resistant human bladder cancer cell line, but they did not clarify the mechanisms of its resistance. Thus, the mechanisms of cisplatin resistance in human bladder cancer remain to be elucidated. Therefore, we established three cisplatin-resistant cell lines by the stepwise exposure of a human bladder cancer cell line to increasing concentrations of cisplatin and then characterized the biochemical properties of these cells.

MATERIALS AND METHODS

Drugs and Chemicals. Eagle's minimum essential medium and all other tissue culture medium components were from Grand Island Biological (Grand Island, NY). Cisplatin and etoposide were obtained from the Nihon Kayaku Co. (Tokyo, Japan); Adriamycin was from the Kyowa Hakko Kogyo Co., Ltd. (Osaka, Japan); vincristine was from the Shionogi Chemical Co., Ltd. (Osaka, Japan). CPT-11 was provided by the Daidichi Sekiyaku Co. (Tokyo, Japan), and its active metabolite, SN-38, was provided by the Yakult Co. (Tokyo, Japan). All other drugs and chemicals not specifically mentioned were purchased from Sigma (St. Louis, MO).

Cell Culture and Establishment of Cisplatin-resistant Cell Lines. The T24 cell line, established from a human transitional cell carcinoma of the urinary bladder (12), was provided by Dr. H. Hisazumi (Kanazawa University, Kanazawa, Japan) and was used as the parent line. The cells were grown as monolayer cultures in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and a 2-fold vitamin solution at 37°C in a humidified atmosphere of 5% CO2 and were usually subcultured once a week with 0.25% trypsin-0.02% EDTA solution. Three sublines, T24/DDP5, T24/DDP7, and T24/DDP10, were established by the continuous exposure of T24 cells to a medium containing progressively increasing concentrations of cisplatin at 0.2–1.0, 1.4, and 2.0 μg/ml, respectively. These sublines were then cloned in the presence of cisplatin after they had been maintained for 1 month at each concentration of cisplatin. Continuous maintenance of the three resistant cell lines in a drug-free medium for at least 2 months did not change their drug-resistant phenotypes. The cells from each resistant line were then grown in cisplatin-free medium and were stored in liquid nitrogen. For the experiments, cells from the T24 and cisplatin-resistant lines were thawed and subcultured at least twice before use. Both the cisplatin-resistant and parent T24 cell lines were usually subcultured once a week.

Colony Formation and Growth Curves. Two hundred T24 cells, 500 T24/DDP5 cells, 700 T24/DDP7 cells, and 1000 T24/DDP10 cells seeded onto 35-mm dishes and cultured in the absence of any drugs gave rise to 100–500 colonies. Therefore, in the colony formation assays the same numbers of cells were seeded, incubated in the absence of drugs for 24 h, and then continuously incubated for an additional 10 days with anticancer drugs at various concentrations. The drugs used in each group of cells were not changed throughout the entire period of colony formation. The colonies were then counted after performing Giemsa staining. Triplicate dishes were used for each drug con

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3 The abbreviations used are: GSH, glutathione; CPT-11, (4S)-4,11-dieethyl-4-hydroxy-9-[(4-piperidino)carbonyloxy]carbonylethylcarboxyloxy]dione hydrochloride trihydrate; SN-38, 7-ethyl-10-hydroxycamptothecin; GST, glutathione S-transferase; PAGIE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; SSC, standard saline citrate.

4 S. Kotoh, unpublished data.
Amersham Japan, Tokyo, Japan). The filter was hybridized with each previously (17). The cytosolic fractious from each cell line were subjected to portions of the extract were stored in 40% glycerol with 1 mg/mI of bovine serum albumin at -80°C. After being washed twice with ice-cold phosphate-buffered saline, the cells were harvested and sonicated. Platinum concentration was determined by flameless atomic absorption spectrophotometry (Atomic Absorption Spectrophotometer 180-70; Hitachi, Tokyo, Japan). All results were normalized to cellular protein as measured with Coomassie Brilliant Blue G-250 (14).

Intracellular Platinum Concentration. The exponentially growing cells of each line were incubated for 2 h with cisplatin at a concentration of 10 or 20 µg/ml. After being washed twice with ice-cold phosphate-buffered saline, the cells were harvested and sonicated. Platinum concentration was determined by flameless atomic absorption spectrophotometry (Atomic Absorption Spectrophotometer 180-70; Hitachi, Tokyo, Japan). All results were normalized to cellular protein as measured with Coomassie Brilliant Blue G-250 (14).

Northern Blot Analysis. The total RNA was extracted from the exponentially growing cells according to standard procedures (15, 16). Human GST-π cDNA, human DNA topoisomerase I cDNA, human DNA topoisomerase II cDNA, and mdr-1 cDNA were used as probes (15, 17—19). RNA (20 µg) was subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and then was transferred to a nylon membrane (Hybond N'; Amersham Japan, Tokyo, Japan). The filter was hybridized with each 32P-labeled cDNA probe in 50% deionized formamide, 10× Denhardt's buffer, 5× SSC (1× SSC = 0.15 M NaCl-0.015 M sodium citrate, pH 7.0), and 0.1% SDS at 37°C for 3 h. The filter was then washed twice in 2× SSC and 0.1% SDS at room temperature. Autoradiography was performed for 12 h with an intensifying screen at -70°C.

Preparation of Crude Nuclear Extracts. Crude nuclear extracts were prepared from 1 × 10⁶ cells in an early log phase culture according to the previously published method (15, 18, 20). The protein concentration in the extracts was immediately determined by the method of Bradford (14). Small portions of the extract were stored in 40% glycerol with 1 mg/ml of bovine serum albumin at -80°C.

Immunoblot Analysis of GSTs and Topoisomerases I and II. An immunoblot analysis of GST-α, GST-π, and GST-τ was performed as described previously (17). The cytosolic fractions from each cell line were subjected to 12% SDS-PAGE, and the proteins were transferred to a nitrocellulose filter. The filters were incubated with rabbit antibodies to human GST-α, GST-π, and GST-τ, as well as with biotinylated goat antibodies to rabbit IgG; and then were developed according to the instructions of the manufacturer (Vecstain ABC-Go kit; Vector Laboratories, Inc., Burlingame, CA). Immunoblot analysis of DNA topoisomerases I and II was performed as described previously (15, 18). Briefly, nuclear protein fractions extracted from 10⁶ cells were run in 8% SDS-PAGE. The protein fractions from the gel were electroblottedically transferred to nitrocellulose filters in 25 mM Tris-HCl (pH 8.3)-92 mM glycine-20% methanol for 2 h at 20 V. The nitrocellulose membranes were then further incubated with antibody against human DNA topoisomerases I and II (1:2000) for 1 h at room temperature. The membranes were rinsed with phosphate-buffered saline, treated with biotinylated secondary antibody, and developed according to the manufacturer’s specifications (Vecstain ABC-Go kit). Antibodies to the human DNA topoisomerases I and II were gifts of Dr. L. F. Liu (Johns Hopkins School of Medicine, Baltimore, MD).

Statistical Analysis. All means were compared with Student’s t test.

RESULTS

The doubling times of the T24/DDP5, T24/DDP7, and T24/DDP10 cells were 27.3, 52.3, and 36.5 h, respectively. These cisplatin-resistant cells grew significantly slower than the T24 cells, whose doubling time was 24.9 h. Table 1 shows the sensitivities of these four cell lines to various anticancer drugs. The resistance of T24/DDP5, T24/DDP7, and T24/DDP10 cells to cisplatin was 2.2-, 5.2-, and 8.4-fold that of T24 cells, respectively (Fig. 1). The cisplatin-resistant lines showed cross-resistance to vincristine, while their sensitivities to Adriamycin and etoposide were similar to that of the T24 cells. In contrast, the cisplatin-resistant cell lines showed collateral sensitivity to CPT-11, a camptothecin derivative that targets DNA topoisomerase I, and also SN-38, an active metabolite of CPT-11 (Fig. 1, Table 1).

The intracellular total GSH content of T24/DDP5 and T24/DDP10 cells was not significantly different from that of T24 cells, whereas that of T24/DDP7 cells was increased significantly (Table 2). The intracellular platinum concentration in the three cisplatin-resistant cell lines was correlated negatively with the degree of cisplatin resistance. The intracellular platinum concentration of T24/DDP10 cells was significantly lower (P < 0.05) than that of T24 cells at an external cisplatin concentration of 20 μg/ml (Fig. 2).

A Northern blot analysis revealed no significant difference in the expression of GST-π mRNA among the resistant lines and the parent line (Fig. 3). No transcripts of the multidrug resistance-1 gene were detected in any of the cell lines (data not shown). Regarding T24, all the resistant lines overexpressed the mRNA of DNA topoisomerase I, which is considered to be the target of CPT-11 and SN-38. In contrast, the expression of DNA topoisomerase II mRNA was observed at similar levels in T24, T24/DDP5, T24/DDP7, and T24/DDP10 cells (Fig. 3).

<table>
<thead>
<tr>
<th>Drug</th>
<th>T24 cell</th>
<th>T24/DDP5</th>
<th>T24/DDP7</th>
<th>T24/DDP10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>0.25 (± 0.02)</td>
<td>2.2°</td>
<td>5.2°</td>
<td>8.4°</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.33 (± 0.04)</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Etoposide</td>
<td>420 (± 32)</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>CPT-11</td>
<td>4.0 (± 0.2)</td>
<td>0.4°</td>
<td>0.3°</td>
<td>0.2°</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.0016 (± 0.000075)</td>
<td>0.6°</td>
<td>0.5°</td>
<td>0.5°</td>
</tr>
<tr>
<td>Vincristine</td>
<td>1.6 (± 0.2)</td>
<td>2.0</td>
<td>7.5°</td>
<td>8.1°</td>
</tr>
</tbody>
</table>

Table 1 The sensitivity of cisplatin-resistant T24-derived cell lines to various anticancer agents

The degree of resistance differs significantly (P < 0.01) from that of the T24 cells (unpaired Student’s t test).

![Fig. 1. Effect of cisplatin and CPT-11 on colony formation by T24, T24/DDP5, T24/DDP7, and T24/DDP10 cells. Two hundred T24 cells (C), 500 T24/DDP5 cells (●), 700 T24/DDP7 cells (□), and 1000 T24/DDP10 cells (■) were seeded and incubated in the absence of any drugs for 24 h. The cells were then exposed to various drug concentrations for 10 days. The number of colonies was counted after performing Giemsa staining. Points, means from three independent experiments; bars, SD.](image-url)
CISPLATIN-RESISTANT BLADDER CANCER CELLS

Table 2 Intracellular GSH content

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>GSH content (nmol/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td>15.9 ± 2.7</td>
</tr>
<tr>
<td>T24/DDP5</td>
<td>14.2 ± 1.8</td>
</tr>
<tr>
<td>T24/DDP7</td>
<td>21.1 ± 1.3b</td>
</tr>
<tr>
<td>T24/DDP10</td>
<td>18.7 ± 2.1</td>
</tr>
</tbody>
</table>

* Mean ± SD of six independent experiments.

Fig. 2. Intracellular platinum accumulation in T24 ( ), T24/DDP5 ( ), T24/DDP7 ( ), and T24/DDP10 ( ) cells. The exponentially growing cells were incubated in the presence of cisplatin at a concentration of either 10 or 20 μg/ml at 37°C for 2 h. The intracellular platinum concentration was determined by atomic absorption spectrophotometry. Points (bars), means (±SD) from three independent experiments. *, significantly different (P < 0.05) from T24 (unpaired Student's t test).

Fig. 3. Northern blot analysis of GST-α, DNA topoisomerase I (Topo I), and DNA topoisomerase II (Topo II) mRNAs. Samples of total RNA (20 μg) extracted from T24 (Lane 1), T24/DDP5 (Lane 2), T24/DDP7 (Lane 3), and T24/DDP10 (Lane 4) cells were all subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and hybridized with each 32P-labeled cDNA probe. The equivalent loading of total RNA was shown by ethidium bromide staining of the gels.

Fig. 4. Immunoblot analysis of GST-α, topoisomerase I (Topo I), and topoisomerase II (Topo II) from 124 (Lane 1), T24/DDP5 (Lane 2), T24/DDP7 (Lane 3), and T24/DDP10 (Lane 4) cells. For the analysis of GST-α, cytosolic fractions from each cell line were subjected to 12% SDS-PAGE, and the proteins were transferred to a nitrocellulose filter. The filters were incubated with rabbit antibody against human GST-α and biotinylated goat anti-rabbit IgG. For the analysis of topoisomerases I and II, nuclear protein fractions from each cell line were run in 8% SDS-PAGE and then were transferred to nitrocellulose filters. The filters were incubated with rabbit antibodies against human topoisomerases I and II and a biotinylated goat anti-rabbit IgG. Fifty μg of nuclear proteins were applied to each lane.

An immunoblot analysis revealed similar levels of GST-α expression in three resistant sublines and their parental cells (Fig. 4), while no expression of either GST-α or GST-β was observed in any of the cell lines (data not shown). As shown in Fig. 4, the cellular levels of topoisomerase I were apparently higher in the three resistant sublines than their parental T24. However, the cellular level of topoisomerase II was similar among all the cell lines. The amounts of topoisomerases I and II in T24 and its three resistant lines were also comparable to their mRNA levels (Figs. 3 and 4).

DISCUSSION

The cytotoxicity of cisplatin appears to result from the interaction of the drug with DNA (21—23), and various mechanisms have been shown to contribute to the cellular resistance to cisplatin. These mechanisms include a decrease in drug accumulation (3, 4); the overproduction of detoxification factors such as GSH (5, 6), its related enzymes (7, 8); and the enhancement of DNA repair (9, 10).

Resistance to anticancer agents is often attributed to changes in cytosolic thiol groups (24, 25). Hamilton et al. (5) demonstrated that the intracellular GSH content influences cisplatin resistance in human ovarian cancer cells and that the GSH depletion mediated by buthionine sulfoximine can overcome such resistance. Cisplatin sensitivity apparently decreased in T24 cells that had been incubated with GSH ester.3 However, we previously detected no changes in the GSH content in cisplatin-resistant cell lines that were derived from Chinese hamster ovary cells (17). Thus, the role of GSH in acquired cisplatin resistance remains unclear. In the present study, the intracellular content of GSH was slightly but significantly increased in the cisplatin-resistant cell line, T24/DDP7. However, the GSH level of T24/DDP10 cells was lower than that of T24/DDP7 cells and did not differ significantly from that of the T24 parental line. Thus, no direct correlation between the GSH content and the extent of cisplatin resistance was observed to exist for our bladder cancer cell lines. We determined only the GSH contents in this study and not the total sulphydryl contents. Thus, it remains to be studied whether or not the increases in the total sulphydryl may be involved in the acquisition of cisplatin resistance in our cell lines.

We also demonstrated that there were no significant differences in the expression of GST-α mRNA among the cisplatin-resistant lines and the T24 parental line, while GST-α and GST-β were not expressed either in any of the cell lines. Therefore, GSTs do not appear to contribute to cisplatin resistance in the resistant T24 cell lines.

A reduced accumulation of cisplatin is the most consistent obser-

3 S. Kotoh and A. Yokomizo, unpublished data.
vation in cisplatin-resistant cells (26). Nakagawa et al. (27) recently showed that cisplatin-resistant prostate cancer cell lines have decreased intracellular platinum concentrations. For our cisplatin-resistant bladder cancer cell lines, the intracellular platinum concentration decreased as the degree of cisplatin resistance increased; however, the platinum concentration only decreased significantly (by about 50%) in the T24/DDP10 line, relative to the T24 parent line, when the cells were exposed to cisplatin at a concentration of 20 μg/ml. Although the extent of the decrease in intracellular platinum concentration does not appear to be sufficient to completely explain the resistance of T24/DDP10 cells (8.4-fold more resistant than T24 cells), the decreased cisplatin accumulation may contribute to the acquisition of cisplatin resistance in these cells.

The cellular levels of DNA topoisomerases I and II are known to regulate cellular sensitivity to topoisomerase-targeting agents such as camptothecin, etoposide, and teniposide (28). Because DNA topoisomerases catalyze DNA conformational changes such as unwinding, catenation-decatenation, and condensation-decondensation (29), a change in the cellular levels of topoisomerases I and II would thus also be expected to modulate the cellular sensitivity to the DNA-targeting agent cisplatin. Indeed, several observations have suggested that DNA topoisomerase II plays a role in the production of cisplatin resistance in cancer cell lines. Cellular sensitivity to cisplatin increases in human cancer cell lines in conjunction with increasing resistance to etoposide or teniposide (15, 18). Furthermore, DNA topoisomerase II is increased in some cisplatin-resistant human cancer cell lines (29, 30) and in a cisplatin-resistant murine leukemia cell line (31). In addition, Eder et al. (32) showed that the transfection of teniposide-resistant Chinese hamster ovary cells, which expressed a mutant topoisomerase II, with topoisomerase II cDNA resulted in increased etoposide sensitivity and a small but significant degree of resistance to cisplatin, which thus indicated a direct relationship between topoisomerase II and cellular resistance to cisplatin.

In contrast, no studies to date have yet suggested a possible role for topoisomerase I in cisplatin resistance. However, because DNA topoisomerase I can substitute for topoisomerase II in most functions (29), topoisomerase I might also be expected to contribute somewhat to cisplatin resistance. In our present study, all of the cisplatin-resistant cell lines overexpressed DNA topoisomerase I mRNA and also showed collateral sensitivity to CPT-11. Thus, DNA conformational changes mediated by topoisomerase I may alter the accessibility of DNA to cisplatin and thus result in a modification of the cellular sensitivity to this drug. Topoisomerases I and II are involved in DNA replication, transcription, recombination, and mitosis (33). The repair of cisplatin-damaged DNA is enhanced in cisplatin-resistant cell lines (9, 10), and repair-deficient mutants are hypersensitive to cisplatin (34, 35). The enhanced expression of topoisomerase I might promote the repair of cisplatin-damaged DNA, which would result in the loss of drug sensitivity to cisplatin in cisplatin-resistant T24 cell lines. However, further studies are called for to determine whether or not topoisomerase I plays a role in the removal of cisplatin from the damaged DNA. Although the topoisomerase I mRNA levels did not differ in the three resistant cell lines (Fig. 3), T24/DDP7 and T24/DDP10 were more collaterally sensitive to CPT-11 than T24/DDP5 (Fig. 1). The formation of cleavable complexes might be different between T24/DDP5 and T24/DDP7 or T24/DDP10 cells, but this possibility has not yet been determined.

In conclusion, our cisplatin-resistant sublines derived from T24, a human bladder cancer cell line, showed increased levels of topoisomerase I and a decreased drug accumulation, which suggested that pleiotropic mechanisms may therefore contribute to the acquisition of cisplatin resistance in these cells. The collateral sensitivity to CPT-11 as shown by our cisplatin-resistant lines suggests that CPT-11 may prove to be clinically efficacious for the treatment of individuals with cisplatin-resistant bladder cancer.

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