Increased Expression of Thioredoxin/Adult T-Cell Leukemia-derived Factor in Cisplatin-resistant Human Cancer Cell Lines

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ABSTRACT
Thioredoxin (TRX) is a widely distributed Mr 13,000 protein with a redox-active diethiol/disulfide in the active site. The TRX system, consisting of TRX, TRX reductase, and NADPH, has an intracellular reducing capacity. Another reducing capacity, glutathione (GSH), can be associated with cis-diaminedichloroplatinum (cDDP) resistance. Therefore, we examined the involvement of TRX in cDDP resistance using two cell lines designated St/DDP and HT/DDP, which were established from the human gastric cancer cell line St-4 and the colon cancer cell line HT-29. St/DDP and HT/DDP were seven and five times as resistant to cDDP as their parental lines, and the expression of TRX in these variants was increased by 2.5- and 2-fold, respectively. The expression of TRX in the complete revertant cells of St/DDP was reduced as low as that in St-4 cells. TRX reductase activity was also increased in St/DDP and HT/DDP, suggesting that activation of the TRX system was associated with in vitro-acquired cDDP resistance. Because cDDP is the first-line drug against ovarian cancer, we examined the expression of TRX in 11 human ovarian cancer cell lines not treated with cDDP in vitro. Positive correlation between TRX expression and cDDP resistance was observed in these cell lines (r = 0.76, P = 0.007). This correlation was comparable to that between GSH content and cDDP resistance (r = 0.69, P = 0.019). These results suggest a possible involvement of TRX, as well as GSH, in cDDP resistance.

INTRODUCTION
cDDP is one of the most widely used antineoplastic agents for treatment of malignant solid tumors. It is particularly effective against head and neck (1), testicular (2), bladder (3), and ovarian (4) cancers. However, drug resistance to cDDP occurs frequently and limits its clinical use.

Resistance to cDDP is incompletely characterized, but multiple mechanisms are likely involved. cDDP-resistant cell lines have multiple biochemical differences from the parental lines, including reduced drug accumulation (5, 6), increased levels of intracellular thiols, such as GSH (7) and metallothionein (8), and enhanced DNA repair (5, 9).

Among these mechanisms, the involvement of GSH in cDDP resistance has been well studied. GSH reacts with cDDP in a 2:1 molar ratio and forms a GS-platinum complex (10). Then this complex is eliminated from the cell by an ATP-dependent glutathione-S-conjugate export pump (10). GSH also protects cells by supporting DNA repair, possibly by stabilizing repair enzymes or by promoting the formation of deoxyribonucleotides (11).

TRX/adult T-cell leukemia-derived factor is a Mr 13,000 protein with a redox-active diethiol/disulfide in the active site. TRX is widely distributed in tissues and organs and highly expressed in epithelial, neuronal, and secretory cells (12). Moreover, the expression of TRX is elevated in a large number of tumor cells such as cervical (13), hepatic (14), and lung (15) cancers. TRX, along with TRX reductase and NADPH, composes the TRX system (12). The TRX system, as well as GSH-mediated systems, operates as a major intracellular reducing system. The expression of TRX is induced by various stresses, including hydrogen peroxide (16, 17), ischemia-reperfusion injury (18, 19), and viral infection (13, 20). TRX has reactive oxygen-scavenging and protein-refolding activities in vitro (21). These properties suggest that TRX, as well as GSH, protects against oxidative stresses.

In this report, we show the increased expression of TRX in cDDP-resistant human cancer cell lines and discuss the role of TRX in cDDP resistance.

MATERIALS AND METHODS

Chemicals. cDDP was obtained from Bristol-Myers Squibb Co., Ltd. (Tokyo, Japan). Recombinant human TRX was a gift from Basic Research Laboratory, Ajinomoto Co., Inc. (Kawasaki, Japan). Mouse monoclonal antibody against human TRX was originally established by FUJIREBIO (Tokyo, Japan) and was provided by Basic Research Laboratory, Ajinomoto. All other reagents were of analytical grade.

Cell Lines. Human gastric carcinoma cell line St-4 was established in our laboratory as described previously (22). Human colon carcinoma cell line HT-29, human ovarian carcinoma cell lines A2780, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SKOV-3 were supplied by the National Cancer Institute (NIH, Bethesda, MD). Human ovarian carcinoma cell lines RMG-I, RMG-II, RMUG, and RTSG were obtained from Professor S. Nozawa (Keio University, Tokyo, Japan) and HAC2 was provided...
IMMUNOBLOT ANALYSIS. Cells were solubilized with 2% SDS, 1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-HCl (pH 7.5). The protein content was determined using a Bio-Rad Protein Assay kit. Whole cell lysates were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After electroblotting, gels were stained with Coomassie brilliant blue to confirm that equal amounts of the lysates were loaded. Blots were incubated with anti-TRX antibody that was shown to be highly specific to human TRX (26) and visualized with horseradish peroxidase-conjugated sheep antimouse immunoglobulin under chemiluminescence detection (Amersham Japan, Ltd.). The exposed films were analyzed by an Ultrascan XL computerized densitometer (Pharmacia, Uppsala, Sweden).

ASSAY OF TRX REDUCTASE ACTIVITY. The activity of TRX reductase was assayed according to the method described by Luthman and Hormgren (27), with slight modifications. The assay mixture contained 100 mM Tris-HC1 (pH 7.5), 2 mM EDTA, 0.22 mM NADPH, 90 μg/ml recombinant human TRX, and 160 μM bovine insulin. Cells were sonicated in a buffer containing 50 mM Tris-HCl (pH 7.5) and 2 mM EDTA. Ten μl of the sonicated cell lysate were added to a cuvette containing 110 μl of the assay mixture at 25°C. The reaction rate was determined from the oxidation of NADPH at the absorbance 340 nm. The TRX reductase activity in the cell lysates was calculated as μmol NADPH oxidized per min from the relation ΔA340/min × 0.12 × 10²/6.2 (units/ml). The protein content of the lysates was determined using a Bio-Rad Protein Assay kit, and the TRX reductase activity was expressed as unit/mg protein.

MEASUREMENT FOR GSH CONTENT. Cells were sonicated in 5% (w/v) metaphosphoric acid. GSH content of the centrifugation supernatant (3000 × g, 4°C, 10 min) was measured by using a GSH-400 GSH assay kit (BIOXYTECH S A). The precipitate was redissolved in 0.2 M NaOH, and the protein content was measured with a Bio-Rad DC Protein Assay kit.

RESULTS

ESTABLISHMENT OF CDDP-RESISTANT CELL LINES. The CDDP-resistant variants of St-4 and HT-29, St/DDP and HT/DDP, were established by continuous exposure to CDDP. These variants could grow in the complete medium containing 5 μM CDDP. The revertant variants St/R6 and St/R12 were obtained from St/DDP by continuous growth in cDDP-free medium for 6 and 12 months, respectively. HT/DDP was stable after a 4-month culture in CDDP-free medium.

We measured the IC₅₀ values of St-4, HT-29, and their variants for CDDP by using the MTT assay (Table 1). St/DDP cells were seven times and HT/DDP cells were five times more resistant than their parental cell lines. St/DDP cells had gradually lost their CDDP-resistant phenotype by drug-free culture; St/R6 cells were twice as sensitive to CDDP as St/DDP variants for CDDP by using the MTT assay (Table 1). St/DDP, St/R6, and St/R12 were obtained from St/DDP by culturing the resistant variants in medium without CDDP for 6 and 12 months, respectively. All cell lines were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin (complete medium) and were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Table 1 cDDP sensitivity, TRX reductase activity, and GSH content of St-4, HT-29, and their cDDP-resistant variants**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ of cDDP* (μM)</th>
<th>TRX Reductase b (×10⁻⁴ units/mg protein)</th>
<th>GSH content b (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St-4</td>
<td>2.8 ± 0.2</td>
<td>12 ± 0.4</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>St/DDP</td>
<td>20 ± 2.3</td>
<td>16 ± 1.7</td>
<td>125 ± 13</td>
</tr>
<tr>
<td>St/R6</td>
<td>9.3 ± 0.7</td>
<td>15 ± 1.4</td>
<td>112 ± 4</td>
</tr>
<tr>
<td>St/R12</td>
<td>4.2 ± 0.7</td>
<td>10 ± 1.4</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>HT-29</td>
<td>4.2 ± 0.3</td>
<td>9.9 ± 0.5</td>
<td>120 ± 8</td>
</tr>
<tr>
<td>HT/DDP</td>
<td>23 ± 7.0</td>
<td>15 ± 0.1</td>
<td>128 ± 25</td>
</tr>
</tbody>
</table>

* IC₅₀ values of CDDP were determined by using a MTT assay, as described in "Materials and Methods." The values are means ± SEs of more than three independent experiments performed in quadruplicate.

b Mean ± SDs of the triplicate.
Increased Expression of TRX in Cells with cDDP Resistance Acquired in Vitro. The expression of intracellular TRX was examined using the immunoblot analysis with mouse antihuman TRX monoclonal antibody (Fig. 1). The cDDP-resistant variant St-4/DDP expressed a 2.5-fold higher level of TRX than the parental St-4, and HT/DDP expressed a 2-fold higher level of TRX than HT-29. Furthermore, the expression of TRX in the revertant variants St/R6 and St/R12 decreased in proportion to the decreased resistance to cDDP.

TRX Reductase Activity and GSH Content of cDDP-resistant Cell Lines. TRX reductase activity of the cDDP-resistant cell lines was also increased, but the increase was not as high as that observed in TRX (Table 1). TRX reductase activity of St/DDP and HT/DDP was 1.3 and 1.5 times as high as that of parental cell lines, respectively.

The content of GSH in St/DDP was increased (1.3 times as high as St-4), but the content of GSH in HT/DDP was unchanged compared with its parent line (Table 1).

Correlation between cDDP Resistance and TRX Expression in Ovarian Cancer Cell Lines. cDDP and its analogues are clinically used as the first-line chemotherapeutic drug against ovarian cancer, but the degree of tumor response to cDDP differs. To clarify whether increased expression of TRX in cDDP-resistant cells happened along with cDDP treatment in vitro or cDDP-resistant phenotype in situ, we examined the IC_{50} values of cDDP and TRX expression among 11 ovarian cancer cell lines.

The treatment profiles of the patients at the time when the cell lines were established and the IC_{50} values determined by the 72-h growth inhibition assay (using a Coulter counter) are listed in Table 2. The ovarian cancer cell lines exhibited a wide range of sensitivity to cDDP. The most resistant cell line RTSG was 18-fold as resistant to cDDP as the most sensitive cell line OVCAR-4. The in vitro resistance of these cell lines to cDDP did not necessarily correlate with the chemotherapy the patients had received.

The expression of TRX in the ovarian cancer cell lines was determined by immunoblot analysis (Fig. 2). The expression of TRX in ovarian cancer cell lines was lower than that of St-4 or HT-29. For example, the level of TRX in A2780 was about one third of that of St-4 (data not shown). The level of TRX in each cell line was calculated as absorbance × band area of the cell line absorbance × band area of A2780 in the immunoblot film. The values are means ± SDs of three independent experiments. A significant correlation

Table 2 Treatment profile, cDDP sensitivity, TRX expression, TRX reductase activity, and GSH content of human ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment profile</th>
<th>IC_{50} of cDDP( \mu M )</th>
<th>Fold resistant</th>
<th>TRX expression ( \times 10^{-3} ) units mg protein</th>
<th>Fold increase</th>
<th>TRX reductase ( \text{nmol/mg protein} )</th>
<th>Fold increase</th>
<th>GSH ( \text{nmol/mg protein} )</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>Untreated</td>
<td>0.33 ± 0.10</td>
<td>1</td>
<td>1</td>
<td>9.5 ± 1.0</td>
<td>1</td>
<td>189 ± 6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HAC2</td>
<td>Untreated</td>
<td>0.67 ± 0.03</td>
<td>2.0</td>
<td>0.87 ± 0.07</td>
<td>8.1 ± 0.1</td>
<td>0.85</td>
<td>224 ± 3</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>CPA, dADM, cDDP</td>
<td>0.24 ± 0.07</td>
<td>0.73</td>
<td>0.91 ± 0.13</td>
<td>10.7 ± 0.7</td>
<td>1.1</td>
<td>154 ± 18</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>OVCAR-4</td>
<td>CAP, ADM, cDDP</td>
<td>0.22 ± 0.07</td>
<td>0.67</td>
<td>0.53 ± 0.21</td>
<td>8.7 ± 0.6</td>
<td>0.92</td>
<td>185 ± 10</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>OVCAR-5</td>
<td>Untreated</td>
<td>1.8 ± 0.26</td>
<td>5.5</td>
<td>1.46 ± 0.21</td>
<td>17.1 ± 2.1</td>
<td>1.8</td>
<td>199 ± 6</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>OVCAR-8</td>
<td>Carboplatin</td>
<td>0.77 ± 0.10</td>
<td>2.3</td>
<td>1.89 ± 0.42</td>
<td>10.6 ± 0.8</td>
<td>1.1</td>
<td>161 ± 28</td>
<td>0.85</td>
<td></td>
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<tr>
<td>RMG-I</td>
<td>Untreated</td>
<td>2.7 ± 0.70</td>
<td>8.2</td>
<td>1.49 ± 0.09</td>
<td>10.9 ± 1.3</td>
<td>1.1</td>
<td>229 ± 9</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>RMG II</td>
<td>cDDP</td>
<td>3.2 ± 0.03</td>
<td>9.7</td>
<td>1.39 ± 0.17</td>
<td>8.7 ± 1.8</td>
<td>0.92</td>
<td>231 ± 13</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>RMU G</td>
<td>Untreated</td>
<td>3.7 ± 0.50</td>
<td>11</td>
<td>1.67 ± 0.24</td>
<td>15.5 ± 0.7</td>
<td>1.6</td>
<td>230 ± 7</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>RTSG</td>
<td>CPA, ADM, cDDP</td>
<td>4.0 ± 0.17</td>
<td>12</td>
<td>1.60 ± 0.39</td>
<td>12.2 ± 1.6</td>
<td>1.3</td>
<td>358 ± 25</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Thiotepa</td>
<td>1.5 ± 0.03</td>
<td>4.5</td>
<td>1.44 ± 0.3</td>
<td>7.5 ± 0.3</td>
<td>0.79</td>
<td>225 ± 16</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \)IC_{50} values of cDDP were determined by using a growth inhibition assay, as described in "Materials and Methods." The values are means ± SD of more than two independent experiments performed in triplicate.

\( ^{b} \)The level of TRX in each cell line was calculated as absorbance × band area of the cell line absorbance × band area of A2780 in the immunoblot film. The values are means ± SDs of three independent experiments.

\( ^{c} \)Means ± SDs of the triplicate.

Fig. 1 Immunoblot analysis of TRX in cDDP-resistant variants of St-4 and HT-29. Whole cell lysates from St-4 (Lane 1), St/DDP (Lane 2), St/R6 (Lane 3), St/R12 (Lane 4), HT-29 (Lane 5), HT/DDP (Lane 6) were prepared, and 20 μg of each cell lysate were subjected to immunoblot analysis.

Fig. 2 Immunoblot analysis of TRX in ovarian cancer cell lines. Whole cell lysates from A2780 (Lane 1), HAC2 (Lane 2), OVCAR-3 (Lane 3), OVCAR-4 (Lane 4), OVCAR-5 (Lane 5), OVCAR-8 (Lane 6), RMG-I (Lane 7), RMG-II (Lane 8), RTSG (Lane 9), SKOV-3 (Lane 10), and RMUG (Lane 11) were prepared, and 40 μg of each cell lysate were subjected to immunoblot analysis.
Correlation between TRX expression and sensitivity to cDDP of the ovarian cancer cell lines. IC$_{50}$ values were determined by using a 72-h growth inhibition assay, as described in "Materials and Methods." The level of TRX in each cell line was calculated as absorbance × band area of the cell line/absorbance × band area of A2780 in the immunoblot film. Points, mean; bars, SDs of more than two (for IC$_{50}$ of cDDP) or three (for TRX expression) independent experiments. Correlation coefficient (r) was determined between logarithms of IC$_{50}$ values and the levels of TRX, and the P value was determined using Student’s t test.

(r = 0.76, P = 0.007) was observed between IC$_{50}$ value of cDDP and the expression of TRX (Fig. 3). No correlation was observed between the expression of TRX, and the treatment of patients with cDDP before the cell lines were established.

TRX Reductase Activity and GSH Content of Ovarian Cancer Cells. TRX reductase activity of the 11 ovarian cancer cell lines was determined (Table 2). A weak correlation was observed between the activity and the IC$_{50}$ value of cDDP (r = 0.42, P = 0.20). TRX reductase activity correlated weakly but positively with the expression of TRX (r = 0.46, P = 0.16), suggesting the entire TRX system was up-regulated in cells with high levels of TRX.

The GSH content of the 11 cell lines is listed in Table 2. As with TRX, positive correlation (r = 0.69, P = 0.019) was observed between GSH content and the IC$_{50}$ value of cDDP (Fig. 4).

DISCUSSION
In this study, we measured the expression of TRX in cDDP-resistant cell lines using immunoblot analysis. In S/or HT/DDP and HT/DDP with in vitro acquired resistance, we found expression of TRX increased over their parental cell lines. Such increased expression of TRX was also observed in the cDDP-resistant sublines of G3341 melanoma and MCF-7 breast cancer (28). Furthermore, in the 11 ovarian cancer cell lines that were not treated with cDDP in vitro, we observed a significant correlation between the IC$_{50}$ values of cDDP and the expression of TRX. It is noteworthy that neither cDDP sensitivity nor TRX expression was related to the chemotherapy (including cDDP) the patients received before the cell lines were established. This fact suggests that increased expression of TRX is associated not only with acquired but also with intrinsic cDDP resistance.

Some investigators have reported the correlation between the increased level of GSH and cDDP resistance in both acquired and intrinsic resistant cell lines (7, 29). This correlation was also observed in our system (Table 2). Notably, we show here that the correlation between cDDP resistance and TRX was comparable to the correlation between cDDP resistance and GSH.

cDDP treatment generates reactive oxygen species, which may play a role in the cytotoxicity of cDDP (30, 31). In this context, Spitz et al. (32) showed that resistance to oxidative stress was accompanied by resistance to cDDP. The hydrogen peroxide-resistant sublines were sensitized to cDDP by the treatment with buthionine sulfoximine, a potent inhibitor of GSH synthesis. By that observation, they concluded that resistance to cDDP by the hydrogen peroxide-resistant sublines was partially due to the elevated GSH level. But they also suggested other mechanisms of cDDP resistance observed in the cell lines because the hydrogen peroxide-resistant cells were more resistant to cDDP than the parent cells even after depletion of GSH caused by treatment with buthionine sulfoximine. In addition, other investigators reported incomplete or minimal effect of GSH depletion on sensitization to cDDP (33, 34).

TRX and GSH have several properties in common. Both are intracellular antioxidants, usually found in their reduced form by their specific reductases and NADPH (35, 36). TRX has protein-refolding activity (21), and GSH is a cofactor for protein disulfide isomerase (37). The TRX and GSH-glutaredoxin systems can serve as hydrogen donors to ribonucleotide reductase (38). Recently, the TRX system was shown to be an efficient electron donor to extracellular glutathione peroxidase, instead of
GSH, when the level of GSH is low (39). These facts suggest that the TRX system can be a partial substitute for GSH-mediated systems, particularly when GSH is depleted. It would be of interest to study the alterations of the TRX system and its influence on cDDP resistance under GSH depletion.

Reactive oxygen species are assumed to be one of the mediators of apoptosis, and radical scavengers, such as N-acetylcysteine that raises intracellular GSH level, are shown to inhibit apoptosis (40). Matsuda et al. (41) reported that TRX inhibited the cytotoxic effect of tumor necrosis factor on human monocytic leukemia U937 cells, which are known to undergo apoptosis by tumor necrosis factor (42). cDDP induces apoptosis in some cell lines (43, 44), and therefore it is possible that antioxidants, such as GSH and TRX, may mediate resistance to cDDP by blocking the pathway of apoptosis.

We have shown the correlation between the resistance to cDDP and the expression of TRX. This correlation is almost tantamount to that observed between cDDP resistance and GSH content; although the mechanisms of TRX to induce cDDP resistance remain to be proved.

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Thioredoxin in Cisplatin Resistance


Increased expression of thioredoxin/adult T-cell leukemia-derived factor in cisplatin-resistant human cancer cell lines.

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