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# Overexpression of thioredoxin does not confer resistance to cisplatin in transfected human ovarian and colon cancer cell lines

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**Abstract** *Purpose*: We have previously reported increased expression of thioredoxin (TRX) in cell lines with both acquired and intrinsic cisplatin (cDDP) resistance. We found that the expression levels of TRX correlate with cellular resistance to the drug. The purpose of this study was to elucidate whether TRX induces cDDP resistance in the absence of other intracellular changes. *Methods*: We developed cell lines stably expressing high levels of TRX by transfection of human ovarian cancer A2780 and colon cancer HT-29, and examined their sensitivity to cDDP. *Results*: The TRX transfectants expressed two- to threefold more TRX with corresponding activities than the parental cells or mock transfectants. TRX-transfected HT-29 cells expressed higher levels of TRX than cDDP-resistant variant cells. Both TRX-transfected A2780 and HT-29 cells showed no resistance to cDDP. Though TRX-transfected A2780 cells showed 1.8-fold increased resistance to $H_2O_2$, resistance to adriamycin and mitomycin C, which generate oxygen radicals, was not observed in the transfectants. *Conclusions*: These results suggest that TRX may be necessary but insufficient to induce resistance against cDDP as well as other chemotherapeutic drugs.

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## Introduction

Thioredoxin (TRX) is a redox-active protein with relative molecular mass of 13 000 Da widely distributed in tissues and organs [8]. The TRX system, consisting of TRX, TRX reductase and NADPH, has a major intracellular reducing capacity similar to the glutathione (GSH)-mediated system [9]. Reduced TRX has oxygen radical-scavenging and protein-refolding activities in vitro [20], and the expression of TRX is induced by various stresses, including $H_2O_2$ [10, 22], ischemia-reperfusion injury [23, 31], and viral infection [5, 29]. These properties suggest that TRX plays a protective role in the cell, especially against oxidative stress. Notably, it has been reported that TRX protects endothelial cells [22] and neurons [10] from the actions of $H_2O_2$ and human leukemia U937 cells from the cytotoxic action of tumor necrosis factor (TNF) mediated by reactive oxygen species (ROS) [15, 18].

We have previously reported [33] that TRX expression is increased in cell lines with both acquired and intrinsic cisplatin (cDDP) resistance and that the correlation between TRX and cDDP resistance is equivalent to the correlation between GSH and cDDP resistance. To elucidate the role of TRX in cDDP resistance in the absence of other intracellular changes, we established cell lines by transfection that stably expressed high levels of TRX and examined their sensitivity to chemotherapeutic drugs, including cDDP.

## Materials and methods

### Chemicals

cDDP was obtained from Bristol Myers Squibb Co., Tokyo, adriamycin and mitomycin C were from Kyowa Hakko Kogyo Co.,

Tokyo, camptothecin was from Yakult Co., Tokyo, and vincristine was from Eli Lilly Japan Co., Kobe. 5-Fluorouracil was purchased from Wako Pure Chemical Industries, Osaka. Mouse monoclonal antibody against human TRX [13] and recombinant human TRX were gifts from the Basic Research Laboratory, Ajinomoto Co., Kawasaki. TRX reductase from human placenta was a gift from Prof. J. Yodoi, Kyoto University.

### Cell lines

A2780, a human ovarian cancer cell line, was recloned before transfection by limiting dilution to avoid the possible effects of clonal heterogeneity. HT-29T9 is a clonal derivative of the human colon cancer cell line HT-29 as described previously [30]. HT/DDP, a cDDP-resistant variant of HT-29, had been established and characterized previously [33]. All cell lines were cultured in RPMI-1640 medium supplemented with 5% heat inactivated fetal bovine serum and 100 μg/ml kanamycin, and maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### Plasmids

Eukaryotic expression vector for human TRX/ADF, pcDSRαADF [29], was a gift from Prof. J. Yodoi. The expression vector pOPI3-CAT (a constituent of LacSwitch Inducible Mammalian Expression System) was obtained from Stratagene (La Jolla, Calif.). The expression vector pSV2bsr for the blasticidin S resistance gene [11] was purchased from Funakoshi Co. (Tokyo). The vector pOPSRα was constructed by exchanging the RSV promoter of pOPI3 for the SRα promoter of pcDSRαADF according to the manufacturer's instructions (Stratagene). The TRX expression vector pOPSRαTRX and antisense-directed vector pOPSRαASTRX were obtained as follows. A DNA fragment encompassing the entire coding region of TRX was amplified by polymerase chain reaction (PCR) from pcDSRαADF. The 5′ primer contained a complete *Not*I site and 18 bases annealing to the sequence surrounding the start codon of TRX (5′-GCG GCC GCC AAG ATG GTG AAG CAG-3′). The 3′ primer contained an *Eco*RI site and 15 bases annealing to the 3′ end of the TRX coding sequence (5′-GAA TTC ATG ATT AGA CTA AT-3′). The PCR-amplified fragment was cloned into pCR™II (Invitrogen, San Diego, Calif.) and sequenced. The TRX coding fragment with *Not*I sites at both ends (pCR™II has a *Not*I site 31bp downstream of the insertion site for PCR products) was cloned into the *Not*I site of pOPSRα.

The vector pHr14E3 that contains the 3′-portion of human ribosomal DNA was provided by Japanese Cancer Research Resources Bank.

### Transfection of TRX gene

All plasmids were purified with QIAGEN-tips (QIAGEN, Chatsworth, calif.).

For expression of TRX in A2780 cells, 2 μg *Eco*RI-digested pSV2bsr and 40 μg *Sca*I-digested pcDSRαADF or pcDSRα (plasmid without TRX cDNA) were cotransfected into $4 \times 10^6$ A2780 cells by electroporation at 230 V and 960 μF) using a Gene Pulser (Bio-Rad, Hercules, Calif.). After a 48-h incubation at 37 °C, 5 μg/ml blasticidin S (Funakoshi) was added to the culture medium. Resistant clones were picked from culture dishes after a 3-week selection and were maintained in the presence of blasticidin S.

For transfection into HT-29T9 cells, 20 μg pOPSRαTRX or pOPSRαASTRX linearized with *Sca*I were introduced into $4 \times 10^6$ HT-29T9 cells by electroporation at 220 V and 960 μF. After a 48-h incubation, 400 μg/ml geneticin (Sigma, St. Louis, Mo.) was added to the culture medium. Resistant clones were picked after a 3-week selection and were cultured with geneticin.

### Immunoblot analysis

Whole cell lysates were prepared and subjected to immunoblot analysis using the mouse antihuman TRX antibody as described previously [33].

### Enzyme assays

Sonicated cell lysates were prepared as described previously [33].

The activity of TRX was assayed according to the method described by Kitaoka et al. [13] with some modifications. The assay mixture contained 100 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 0.22 m*M* NADPH, 160 μ*M* bovine insulin and 15 mU/ml TRX reductase from human placenta. A 10 μl aliquot of the cell lysate was 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 an absorbance 340 nm.

The activity of TRX reductase in the cell lysates was assayed in a similar way using 90 μg/ml recombinant human TRX instead of TRX reductase in the above-mentioned assay mixture [33]. The enzyme activities in the transfectants are expressed as milliunits per milligram protein as previously described [33].

### Southern blot hybridization analysis

Purified cellular DNAs were digested with *Eco*RI, and the DNA digests were subjected to electrophoresis in 0.7% agarose gels. The DNAs were transferred to Nytran (Schleicher & Schuell, Dasse, Germany). The probe for the TRX gene was amplified by PCR as described above and was labeled with [α-$^{32}$P] dCTP using a Multiprime DNA labeling system (Amersham Japan, Tokyo). After an overnight hybridization, the washed membranes were exposed to Kodak X-OMAT AR film at −70 °C.

### Northern blot hybridization analysis

Total cellular RNAs were isolated as described by Peppel and Baglioni [24]. The RNAs were subjected to electrophoresis in denaturing formaldehyde/agarose gels [25]. After transfer to Nytran, the blots were analyzed as described above. To estimate the variation in RNA loading of the gel, the blots were stripped and reprobed with a $^{32}$P-labeled BamHI fragment of pHr14E3 that hybridizes 28S rRNA.

### Growth inhibition assay

A 72-h MTT assay was performed as described previously [33] to determine the $IC_{50}$ values of the cytotoxic agents for the transfectants.

### Colony-forming assay

The colony-forming assay was performed as described previously [16]. The experiments were carried out in triplicate and repeated at least three times.

## Results

### Isolation of clones overexpressing TRX

The expression of TRX in the blasticidin S- or geneticin-resistant clones (A2780 and HT-29, respectively) was determined by immunoblotting. Of 30 independent blasticidin S-resistant clones of the transfected A2780 cells examined, 2 clones, designated A/TRX12 and

A/TRX19, expressed levels of TRX about twice those of mock-transfectants A/01 and A/07 (Fig. 1A). In the transfected HT-29 cells, of the 60 clones examined, 5 clones expressed high levels of TRX (Fig. 1B). HT/TRX25 and HT/TRX28, the clones with the highest expression of TRX, were preferentially used for further analysis. Notably, HT/TRX28 cells expressed a higher level of TRX than HT/DDP cells. Of the antisense TRX cDNA-transfected HT-29 cells, 15 clones were also examined for TRX expression, but none demonstrated a marked decrease in TRX. Two clones designated HT/AS11 and HT/AS13 were used as negative controls. After confirming TRX expression by immunoblotting twice, the TRX-transfected cells were grown to $5 \times 10^7$ cells and stored at −80 °C to minimize the passaging of the cells before characterization. We performed the cytotoxicity assays within 1 month of thawing the cells, and no decrease in TRX expression was observed by immunoblotting in these thawed cells (date not shown).

The activity of TRX in the transfectants was examined using an insulin reduction assay using NADPH and TRX reductase (Table 1). The assay revealed that insulin-reducing activity of the transfectants was elevated in accordance with the amount of TRX detected by immunoblotting, confirming that the increased TRX was functional in these clones. Unlike TRX, the activity of TRX reductase was not increased in the TRX transfectants (Table 1).

The integration of exogenous TRX gene into the genomes of the transfectants was checked by Southern blot analysis (Fig. 2). A/TRX12 and A/TRX19 showed band intensities at about 6 kb and 15 kb, respectively, which were not observed in the wildtype A2780 or the mock transfectants, A/01 and A/07. HT/TRX25 and HT/TRX28 showed strong signals at 2.3 kb, consistent with the *Eco*RI fragment of pOPSR$\alpha$TRX. Another blot of the DNAs digested with *Bam*HI instead of *Eco*RI revealed that HT/TRX25 and HT/TRX28 were different

**Table 1** Activities of TRX and TRX reductase in TRX-transfected A2780 and HT-29 Cells. Values are means ± SD determined in triplicate expressed as mU/mg protein. Values in parentheses are relative enzyme activity ratios as compared with the corresponding parental cells

| Cells | TRX | | TRX reductase | |
|---|---|---|---|---|
| A2780 | 2.4 ± 0.2 | (1) | 11.1 ± 2.0 | (1) |
| A/01 | 1.9 ± 0.3 | (0.79) | 12.4 ± 1.1 | (1.1) |
| A/07 | 2.3 ± 0.3 | (0.96) | 11.3 ± 3.0 | (1.0) |
| A/TRX12 | 4.4 ± 0.6* | (1.8) | 9.3 ± 3.4 | (0.84) |
| A/TRX19 | 4.3 ± 0.5* | (1.8) | 11.7 ± 1.5 | (1.1) |
| HT-29T9 | 2.8 ± 0.2 | (1) | 19.0 ± 2.2 | (1) |
| HT/AS11 | 2.2 ± 0.5 | (0.79) | 16.0 ± 1.8 | (0.84) |
| HT/AS13 | 2.0 ± 0.4 | (0.71) | 10.8 ± 2.3 | (0.57) |
| HT/TRX25 | 6.0 ± 0.3* | (2.1) | 17.0 ± 4.4 | (0.89) |
| HT/TRX28 | 8.1 ± 0.3* | (2.9) | 12.7 ± 2.0 | (0.67) |
| HT/DDP | 5.5 ± 0.3* | (2.0) | 31.1 ± 6.3 | (1.6) |

*$P < 0.005$, Student's *t*-test, vs corresponding parental and mock-transfected cells

clones from each other (data not shown). HT/AS11 and HT/AS13 also showed additional band intensities representing antisense TRX cDNA. Northern blot analysis (Fig. 3) revealed additional TRX transcripts in the TRX transfectants, confirming the functional expression of the vector-derived TRX gene. Transcripts derived from the antisense TRX cDNA were not detected in either HT/AS11 or HT/AS13.

## Sensitivity of the TRX transfectants to cDDP

We assessed the sensitivity of the TRX transfectants to cDDP using a 72-h MTT growth inhibition assay. Neither A2780 nor HT-29 transfectants with high levels of TRX, including HT/TRX10, HT/TRX34 and HT/TRX62 (data not shown), showed resistance to

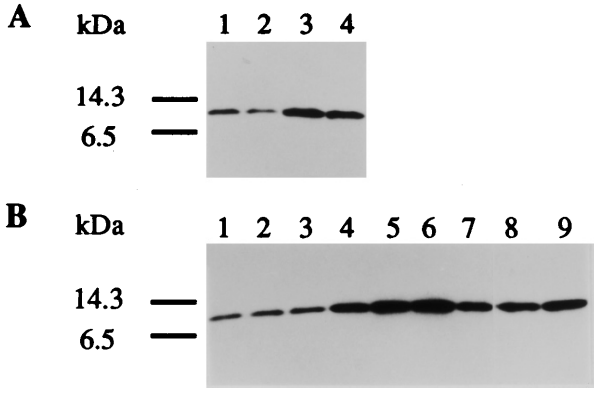


**Fig. 1A,B** Immunoblot analysis of TRX in transfected A2780 (**A**) and HT-29 (**B**) cells. Whole cell lysates were prepared as described in Materials and methods. **A** Lysates (40 μg) from A/01 (*lane 1*), A/07 (*lane 2*), A/TRX12 (*lane 3*) and A/TRX19 (*lane 4*). **B** Lysates (20 μg) from HT-29T9 (*lane 1*), HT/AS11 (*lane 2*), HT/AS13 (*lane 3*), HT/TRX10 (*lane 4*), HT/TRX25 (*lane 5*), HT/TRX28 (*lane 6*), HT/TRX34 (*lane 7*), HT.TRX62 (*lane 8*) and HT/DDP (*lane 9*)

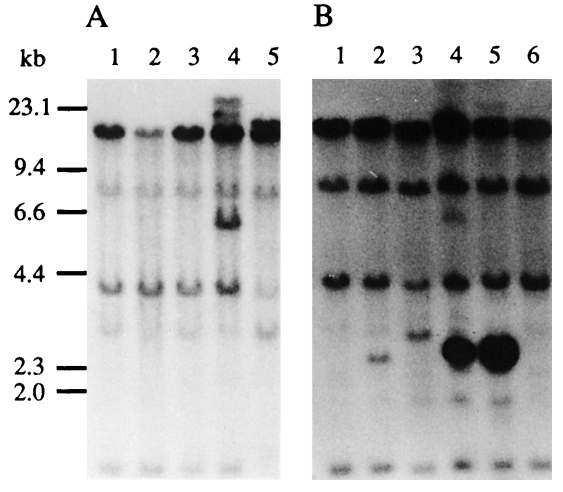


**Fig. 2A,B** Southern blot hybridization analysis of the TRX gene in transfected A2780 (**A**) and HT-29 (**B**) cells. The analysis was carried out on 10 μg of each DNA digest **A** *lane 1* A2780, *lane 2* A/01, *lane 3* A/07, *lane 4* A/TRX12, *lane 5* A/TRX19. **B** *lane 1* HT-29T9, *lane 2* HT/AS11, *lane 3* HT/AS13. *lane 4* HT/TRX25, *lane 5* HT/TRX28, *lane 6* HT/DDP

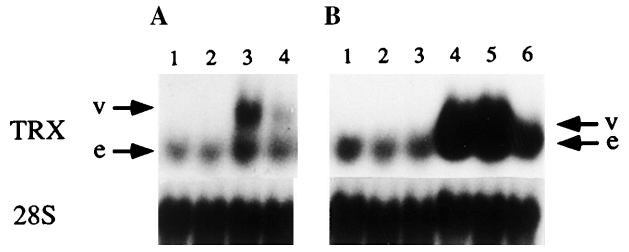


**Fig. 3A,B** Northern blot hybridization analysis of TRX and 28S rRNA in transfected A2780 (**A**) and HT-29 (**B**) cells. Each analysis was carried out on 20 μg of total RNA. **A** *lane 1* A/01, *lane 2* A/07, *lane 3* A/TRX12, *lane 4* A/TRX19. **B** *lane 1* HT-29T9, *lane 2* HT/AS11, *lane 3* HT/AS13, *lane 4* HT/TRX25, *lane 5* HT/TRX28, *lane 6* HT/DDP. Vector-derived TRX transcripts (*v*) were detected in the TRX transfectants in addition to endogenous transcripts (*e*)

cDDP (Fig. 4, Table 2). We also examined the colony-forming ability of the transfectants after a 1-h exposure of cDDP. Here again, no resistance was observed (Fig. 5).

## Sensitivity of the TRX transfectants to other cytotoxic agents

We also assessed the sensitivity of the TRX transfectants to $H_2O_2$, adriamycin, camptothecin and mitomycin C using the MTT assay (Table 2). The TRX-transfected A2780 cells were 1.8-fold more resistant to $H_2O_2$ than the mock transfectants. In contrast, resistance to $H_2O_2$ was not observed in the TRX-transfected HT-29 cells and HT/DDP cells, both of which overexpressed TRX. The cDDP-resistant variant HT/DDP showed

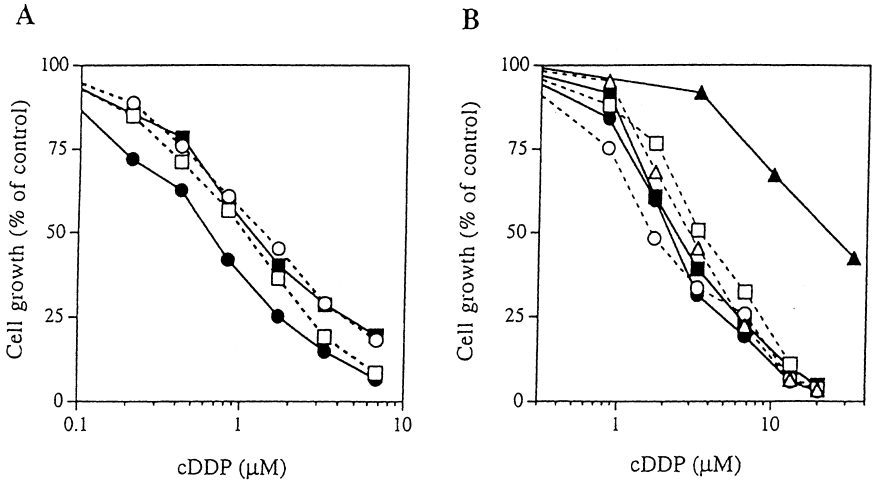


**Fig. 4A,B** Sensitivity to cDDP of the TRX-transfected A2780 (**A**) and HT-29 (**B**) cells determined using an MTT assay. **A** □ A/01, ○ A/07, ■ A/TRX12, ● A/TRX19. **B** △ HT-29T9, □ HT/AS11, ○ HT/AS13, ■ HT/TRX25, ● HT/TRX28, ▲ HT/DDP. Each point is the mean of three independent experiments performed in quadruplicate; SDs were less than 10%

**Table 2** Sensitivity of TRX-transfected A2780 and HT-29 cells to cytotoxic agents. The values are mean $IC_{50}$ values ± SD from three independent experiments. Where two experiments were carried out, the results of individual experiments are shown in parentheses

| Cells | cDDP (μ*M*) | $H_2O_2$ (μ*M*) | Adriamycin (n*M*) | Camptothecin (n*M*) | Mitomycin C (n*M*) |
|---|---|---|---|---|---|
| A/01 | 1.1 ± 0.1 | 13 ± 0.8 | 11 (9.2, 13) | 6.5 (5.2, 7.7) | 30 (30, 30) |
| A/07 | 1.4 ± 0.6 | 13 ± 1.5 | 12 (10, 13) | 12 (11, 13) | 77 (81, 72) |
| A/TRX12 | 1.1 ± 0.1 | 22 ± 1.6** | 11 (10, 11) | 7.2 (7.2, 7, 2) | 38 (33, 42) |
| A/TRX19 | 0.66 ± 0.08 | 22 ± 0.5** | 9.2 (7.4, 11) | 6.9 (6.6, 7.2) | 38 (39, 36) |
| HT-29T9 | 2.8 ± 0.1 | 22 (21, 22) | 86 (88, 83) | 17 (14, 19) | 320 (300, 330) |
| HT/AS11 | 3.4 ± 0.4 | 19 (22, 15) | 107 (130, 83) | 17 (20, 14) | 650 (750, 540) |
| HT/AS13 | 1.7 ± 0.2 | 16 (15, 16) | 68 (74, 61) | 7.8 (8.6, 6.9) | 330 (480, 170) |
| HT/TRX25 | 2.3 ± 0.5 | 17 (15, 18) | 106 (92, 120) | 12 (10, 14) | 530 (600, 450) |
| HT/TRX28 | 2.1 ± 0.1 | 20 (15, 24) | 97 (110, 83) | 16 (14, 17) | 300 (300, 300) |
| HT/DDP | 24 ± 9* | 12 (11, 13) | 220 (220, 220) | 51 (52, 49) | 1000 (1300, 690) |

*$P < 0.05$, Student's *t*-test, vs HT-29T9 cells; **$P < 0.005$, Student's *t*-test, vs corresponding mock transfectants A/01 and A/07

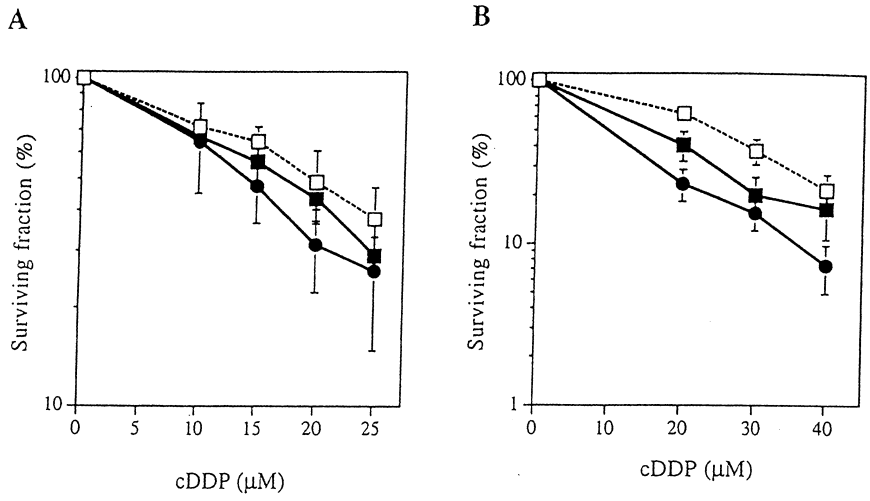


**Fig. 5A,B** Sensitivity to cDDP of TRX-transfected A2780 (**A**) and HT-29 (**B**) cells determined using a colony-forming assay. Cells were exposed to cDDP for 1 h. **A** □ A/01, ■ A/TRX12, ● A/TRX19. **B** □ HT/AS11, ■ HT/TRX25, ● HT/TRX28. Each point is the mean of more than three experiments *bars* SD

cross-resistance to adriamycin, camptothecin and mitomycin C, but no resistance to these drugs was observed in the TRX transfectants of A2780 and HT-29. Furthermore, the TRX transfectants did not differ from the mock transfectants in their sensitivity to 5-fluorouracil or vincristine (data not shown).

## Discussion

We established TRX-overexpressing A2780 and HT-29 cells by DNA transfection followed by selection with blasticidin S or geneticin, respectively. These cells had vector-derived TRX cDNAs integrated into their genome, they expressed vector-derived TRX mRNAs, they produced an increased amount of TRX, and they demonstrated increased TRX activities in their lysates. Furthermore, the TRX-transfected A2780 cells were more resistant to $H_2O_2$. This is compatible with previous reports that TRX protects cells from $H_2O_2$ [10, 22] and suggests that the transfected TRX was functional in the cells. Unexpectedly, resistance to $H_2O_2$ was not observed in the TRX-transfected HT-29 cells. Likewise, HT/DDP cells, which contained increased activities of both TRX and TRX reductase, were not more resistant to $H_2O_2$ than HT-29T9 cells. The parental HT-29 cell line itself is known to be resistant to ROS [32]. Therefore, intrinsically elevated activities of the radical-scavenging systems might be sufficient to scavenge $H_2O_2$, and thus conceal the effect of the TRX system on $H_2O_2$ resistance. In this way, overexpressed TRX did not contribute to $H_2O_2$ resistance in HT-29 sublines.

We have previously shown that increased expression of TRX correlates with a cDDP-resistant phenotype in cell lines with resistance acquired in vitro and in intrinsically resistant ovarian cancer cell lines [33]. Yokomizo et al. have also demonstrated such increased expression of TRX in three cDDP-resistant cell lines [34]. Moreover, the TRX-antisense transfectants of human bladder cancer T24 cells show increased sensitivity to cDDP [34]. Recently, Sasada et al. have demonstrated increased resistance to cDDP-induced cytotoxicity in the TRX-transfected L929 mouse fibrosarcoma cell line [26]. These results strongly suggest the involvement of TRX in the mechanisms of cDDP resistance. However, in our present study, we observed no resistance to cDDP or other ROS-generating antineoplastic agents in two TRX-transfected cell lines examined by two different assays, an MTT assay with a 72-h continuous drug exposure and a colony-forming ability assay with a 1-h drug exposure.

It is interesting that the results of Sasada et al. [26] and our results using the TRX transfectants were quite different. In both experiments, the same promoter was used for TRX expression, nearly a threefold increase in TRX activities was obtained in the transfectants compared with the parental cells, and the activities of TRX reductase were unchanged. Consequently, the discrepancy in the results is probably due to the difference between the cell lines in which TRX was transfected.

The cytotoxicity of cDDP is believed to be due to DNA adduct formation, but the mechanism that links DNA damage to cell death is not fully understood [3]. Recent studies have shown that ROS contributes to the cytotoxicity of cDDP. Interaction of cDDP and DNA generates ROS in a cell-free system [17], and treatment with cDDP increases intracellular peroxides [26]. The cytotoxic activity by cDDP is inhibited by antioxidants and radical scavengers [6, 26, 28]. In this context, Sasada et al. [26] have emphasized that TRX protects the cells from cDDP through its radical-scavenging capacity [20]. ROS is regarded as a mediator of apoptosis [2] and antioxidants such as N-acetyl-L-cysteine [19] have been shown to inhibit apoptosis. Accordingly, the protective effect of radical scavengers against cDDP toxicity may be caused by blocking the apoptotic pathway. L929 cells used by Sasada et al. are susceptible to apoptosis by TNFα [19] and the antineoplastic drug etoposide [21]. Human monocytic leukemia U937 cells, in which TRX can show protective activity against TNF-induced cell death [18], are also susceptible to apoptosis [12]. In contrast, A2780 and HT-29 cells used in our experiments are relatively

insensitive to apoptosis induced by antineoplastic agents (Kataoka S, Chen Z, unpublished observations). The difference in the capacity of TRX to protect against cDDP cytotoxicity may be a consequence of the difference in cellular sensitivity to apoptosis.

Our present study showed that TRX alone could not induce resistance to cDDP and other ROS-generating antineoplastic drugs in two cell lines, A2780 and HT-29. However, as increased expression of TRX has been observed in many cDDP-resistant cell lines including a variant of HT-29, TRX is thought to play an important role in drug resistance. Various stresses that generate ROS induce the expression of TRX. TRX scavenges ROS [20], regenerates enzymes damaged by oxidative stress [4, 20], and influences gene expression through modifying NF$\kappa$B [7, 27] and AP-I [1, 27]. Through these functions, TRX may provide the "first aid" and "SOS signal" after oxidative stress and contribute ROS resistance at least in ROS-sensitive cell lines. In addition, TRX may inhibit the apoptotic pathway by scavenging ROS, a mediator of apoptosis. Decreased TRX would impair the response to oxidative stress, promote apoptosis, and result in increased sensitivity to ROS-generating antineoplastic drugs. However, the early and general response to oxidative stress and inhibition of apoptosis are not enough for some cell lines to survive the drug-induced damage.

Oxidative stress may not be the only mechanism by which ROS-generating antineoplastic drugs show cytotoxicity, and apoptosis may not be the only pathway of drug-induced cell death. More specific protection systems, such as drug-exporting pumps and DNA repair enzymes, must be required for inhibition of nonapoptotic cell death. Alternatively, increased TRX in cDDP-resistant cell lines may modulate the factors that directly induce drug resistance. For example, as in the proposed mechanism of cDDP resistance by GSH [14], stabilization of DNA repair enzymes by TRX may restore DNA repair activity, which is elevated in most cDDP-resistant cells [3].

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