Redox Control of Resistance to *cis*-Diamminedichloroplatinum (II) (CDDP)

Protective Effect of Human Thioredoxin against CDDP-induced Cytotoxicity

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Abstract

Thioredoxin is a small ubiquitous protein with multiple biological functions, including cellular defense mechanisms against oxidative stress. In the present study, we investigated the role of human thioredoxin (hTRX) in the acquisition of cellular resistance to *cis*-diamminedichloroplatinum (II) (CDDP). The expression and activity of hTRX in Jurkat T cells was dose-dependently enhanced by exposure to CDDP, as determined by immunoblot analysis and insulin reducing assay. Furthermore, chloramphenicol acetyltransferase analysis using the hTRX promoter-reporter gene construct revealed that treatment of Jurkat cells with CDDP caused transcriptional activation of the hTRX gene, which might be mediated through increased generation of intracellular reactive oxygen intermediates. To examine the biological significance of hTRX induction, we established hTRX-overexpressing derivatives of L929 fibrosarcoma cells by stable transfection with the hTRX cDNA. The clones, which constitutively expressed the exogenous hTRX, displayed increased resistance to CDDP-induced cytotoxicity, compared with the control clones. After exposure to CDDP, the control cells showed a significant increase in the intracellular accumulation of peroxides, whereas the hTRX-transfected cells did not. Taken together, these results suggest that overexpressed hTRX is responsible for the development of cellular resistance to CDDP, possibly by scavenging intracellular toxic oxidants generated by this anticancer agent. (*J. Clin. Invest.* 1996. 97:2268–2276.) Key words: human thioredoxin • *cis*-diamminedichloroplatinum (II) • resistance • reactive oxygen intermediate • redox

Introduction

Thioredoxin (TRX)[1] is a small multifunctional and ubiquitous protein having a redox-active disulfide/dithiol within the conserved active site sequence: -Cys-Gly-Pro-Cys- (1, 2). Since its original isolation from *Escherichia coli* as a hydrogen donor for ribonucleotide reductase, the key enzyme in the biosynthesis of deoxyribonucleotides, TRX has been purified and characterized from a wide variety of prokaryotic and eukaryotic species (1, 2). Adult T cell leukemia–derived factor (ADF), which we originally defined as an IL-2 receptor α-chain/Tac inducer produced by human T cell lymphotropic virus-I–transformed T cells, has been identified as human thioredoxin (hTRX) (3–6). It has been suggested that hTRX has multiple biological functions and regulates different systems via thiol redox control, including the reduction of oxidized ribonucleotide reductase, the degradation of insulin, and the redox regulation of transcription factors (1, 2, 5, 7, 8). hTRX has also been shown to be a scavenger of reactive oxygen intermediates (ROI) (9), and recombinant hTRX (rTRX) has a protective activity against hydrogen peroxide (H_2O_2)- or TNFα-induced cytotoxicity, in which the generation of ROI seems to be involved (10, 11). Furthermore, hTRX has been reported to be a stress-inducible protein, whose expression is enhanced by a variety of oxidative stresses, including H_2O_2, x-ray, and ultraviolet exposure (5, 11, 12). These observations suggest that hTRX has an important role in the cellular responses against oxidative stress.

The tendency of tumors to express resistance to anticancer chemotherapeutic drugs has been a significant obstacle against successful treatment of cancer. *cis*-Diamminedichloroplatinum (II) (CDDP) is one of the most effective chemotherapeutic drugs for a variety of human malignancies, including ovarian, testicular, bladder, head and neck, esophageal, and small-cell lung cancers, and the development of resistance to this drug has restricted the efficacy of cancer treatment. Thus, it is particularly important to elucidate the mechanisms by which resistance to CDDP ocurrs and to develop a clinically useful means of overcoming the CDDP resistance in human cancer cells. Previous studies have suggested that cellular sensitivity against CDDP-induced cytotoxicity is modulated by several mechanisms, including accumulation of CDDP, DNA-repair processes, and inactivating factors, such as glutathione and metallothioneins (13, 14). In addition, several investigators have reported that administration of free radical scavengers or antioxidants, such as SOD or α-tocopherol, inhibits the CDDP-induced nephrotoxicity in in vivo animal models (15–17). Indeed, increased generation of ROI seems to be closely associated with CDDP-induced cytotoxicity (15–19).

hTRX has been shown to be highly expressed in a variety of human malignant cells, including adult T cell leukemia caused by human T cell lymphotropic virus–I, squamous cell

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1. *Abbreviations used in this paper:* ADF, adult T cell leukemia–derived factor; BHA, butylated hydroxyanisole; CAT, chloramphenicol acetyltransferase; CDDP, *cis*-diamminedichloroplatinum (II); DCFH, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; hTRX, human TRX; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide; ROI, reactive oxygen intermediates; rTRX, recombinant hTRX; TRX, thioredoxin.

carcinoma of the uterine cervix, hepatocellular carcinoma, lung carcinoma, and adenocarcinoma of the gastrointestinal tract (6, 20–22). In addition, it has been recently recognized that some CDDP-resistant human cancer cell lines have higher levels of hTRX expression (23, 24). Therefore, we have hypothesized that hTRX, which can function as an endogenous cellular redox buffer through its ROI-scavenging activity, may be an attractive candidate as a modulator of cellular sensitivity against CDDP. In the present study, we examined the role of hTRX in the acquisition of cellular resistance to CDDP-induced cytotoxicity. We report here that CDDP induces transcriptional activation of the hTRX gene, and that the hTRX-overexpressing cells display increased resistance to CDDP. Our findings strongly suggest that overexpressed hTRX induced by CDDP-mediated oxidative stress acts as an inhibitor of CDDP-induced cytotoxicity.

Methods

Reagents. CDDP, butylated hydroxyanisole (BHA), NADPH, insulin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, FCS, and G418 were from Life Technologies Inc. (Grand Island, NY). DME was supplied from Nissui Pharmaceutical Co. (Tokyo, Japan). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes, Inc. (Eugene, OR). rTRX was produced and provided by Ajinomoto Co. Inc., Basic Research Laboratory (Kawasaki, Japan), according to the method described previously (9). Anti-hTRX mAb (ADF-11 mAb; mouse IgG1), established by immunizing BALB/c mice with rTRX, was provided by Fujirebio Inc. (Tokyo, Japan). This mAb can specifically recognize hTRX, but not mouse TRX, determined by immunoblot analysis with recombinant human and mouse TRX (Sasada, T., K. Hirota, and J. Yodoi, unpublished observations). Human TRX reductase was purified from term-delivery human placenta and its activity (units per milliliter) was determined by insulin reducing assay as described (25). All other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated.

Cell culture. Jurkat, a human T cell line, was maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), in a humidified atmosphere of 5% CO_2/95% air at 37°C. L929, a murine fibrosarcoma cell line, and its transfected derivatives were cultured in DME supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2/95% air at 37°C. The transfected derivatives of L929 cells were continuously maintained in the presence of 200 μg/ml G418.

Immunoblot analysis for hTRX protein. The expression of hTRX protein was determined by immunoblot analysis using anti-hTRX mAb (ADF-11 mAb). Cells were washed with ice-cold PBS three times, and then lysed with a solubilizing solution (0.5% NP-40, 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM PMSF, 0.111 U/ml aprotinin, and 0.02% NaN_3) on ice for 30 min. The extracts were cleared by centrifugation. Equal amounts of protein (5 or 10 μg protein/lane), estimated by the Bradford method using a protein assay (Bio-Rad Laboratories, Hercules, CA), were subjected to electrophoresis on a 15% SDS-polyacrylamide gel, and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). After blocking with 10% FCS and 10% skim milk in PBS containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with anti-hTRX mAb (ADF-11 mAb), followed by peroxidase-linked anti–mouse Ig (Amersham Intl., Little Chalfont, UK). Detection by chemiluminescence was performed with an ECL Western blot detection kit (Amersham Intl.) according to the supplier's recommendation.

Determination of TRX and TRX reductase activity. The activity of TRX and TRX reductase in cells was determined by insulin reducing assay, according to the method previously described by Holmgren and co-workers, with slight modification (26, 27). Mammalian TRX reductase has been reported to have a broad substrate specificity and reduce TRXs from different species (26, 28), and we have also found that human TRX reductase can react with recombinant mouse TRX as efficiently as recombinant human TRX (Sasada, T., K. Hirota, and J. Yodoi, unpublished observations). Cells were washed with ice-cold PBS three times, and then homogenized by repeated freezing and thawing. The protein concentration of cell extracts was measured by the Bradford method. For determination of the TRX activity, equal amounts of total cellular protein (20 μg for the parental and transfected L929 cells or 40 μg for Jurkat cells) in a volume of 8 μl were preincubated with 2 μl of the DTT activation buffer (0.1 M Tris-Cl (pH 7.5), 2 mM EDTA containing 1 mg/ml BSA and 2 mM DTT) at 37°C for 15 min, to reduce and activate TRX, which might contain aggregated material with inter- or intramolecular disulfides (27). The preincubated cell extracts were mixed with 110 μl of the reaction mixture (0.1 M Tris-Cl (pH 7.5), 2 mM EDTA, 0.2 mM NADPH, 0.4 U/ml human TRX reductase, 140 μM insulin) and incubated at 25°C. The reaction was followed by recording the decrease in absorbance at 340 nm, which reflected the oxidation of NADPH, at 30-s intervals. As a control for other oxidoreductases consuming NADPH in the crude cell extracts, the preincubated extracts were mixed with the reaction mixture containing everything except insulin and treated the same way as all other samples. From the change in absorbance of each sample was subtracted the change in absorbance of the respective control sample lacking insulin. The calculated values were compared to the standard curve for rTRX (100–1,200 ng/ml) to obtain quantitative determinations of the absolute amounts of TRX. For determination of the TRX reductase activity, equal amounts of total cellular protein (5 μg for the parental and transfected L929 cells) in a volume of 10 μl were mixed with 110 μl of the reaction mixture (0.1 M Tris-Cl (pH 7.5), 2 mM EDTA, 0.2 mM NADPH, 10 μM rTRX, and 140 μM insulin) and incubated at 25°C. The reaction was followed by recording the decrease in absorbance at 340 nm at 30-s intervals. As a control, the extracts were mixed with the reaction mixture containing everything except insulin and treated the same way as all other samples. From the change in absorbance of each sample was subtracted the change in absorbance of the respective control sample lacking insulin. The activity of TRX reductase (units per milligram protein) was calculated as micromoles of NADPH oxidized per minute in the extract (1 mg) from the relation $\Delta A_{340} \times 0.12 \times 200/6.2$ (25, 26).

Transfection and chloramphenicol acetyltransferase (CAT) assay. Transient transfections of the reporter plasmid were performed by electroporation using a gene pulser (Bio-Rad Laboratories). Jurkat cells (5×10^6 cells) were suspended in 300 μl of culture medium (RPMI 1640 with 10% FCS) with 5 μg of the reporter plasmid (phTRX-CAT), in which the hTRX promoter region ranging from −2629 to +28 was subcloned into the pCAT-Basic vector (Promega Corp., Madison, WI), and then subjected to electroporation. 24 h after the transfection, the cells were treated with agents (CDDP and/or BHA) for an additional 24 h, and cell extracts were prepared by three freeze–thaw cycles in 100 μl of 0.25 M Tris-HCl, pH 7.8. For the measurement of CAT activity, equal amounts of total cellular protein (70 μg), estimated by a protein assay, were incubated in the reaction mixture at 37°C for 2 h, and reaction products were analyzed by TLC followed by autoradiography (29). Quantification of CAT activity (percent conversion) was performed, based on the measurement of radioactive signals using an image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Flow cytometric estimation of intracellular redox state. The intracellular redox state was estimated by levels of intracellular peroxides, which were monitored by flow cytometric analysis with DCFH-DA (30). DCFH-DA is a nonpolar compound that readily diffuses into cells, where it is converted into a nonfluorescent polar derivative,
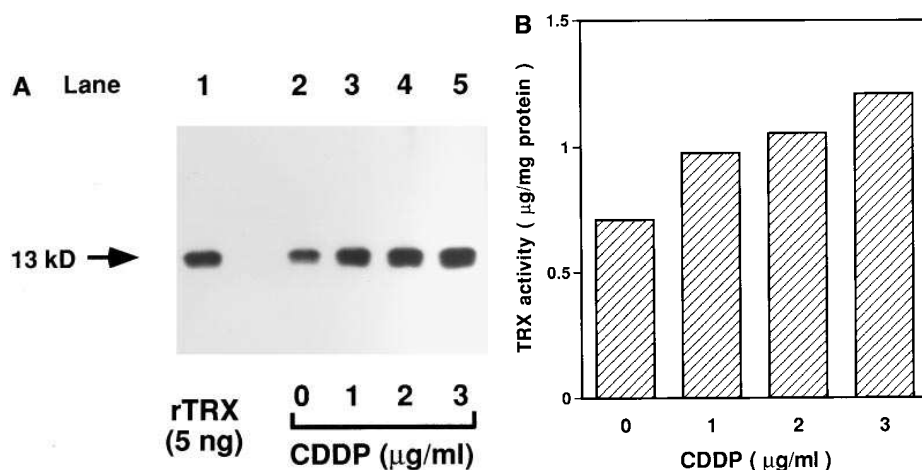
Figure 1. Effect of CDDP treatment on the expression (*A*) and activity (*B*) of hTRX in Jurkat cells. (*A*) The cell lysates (5 μg/lane), obtained from Jurkat cells treated with the indicated doses of CDDP for 48 h, were electrophoresed and subjected to immunoblot analysis with anti-hTRX mAb (ADF-11 mAb). Lane *1*, rTRX 5 ng (a positive control); lane *2*, untreated Jurkat cells; lanes *3–5*, Jurkat cells treated with 1 μg/ml (lane *3*), 2 μg/ml (lane *4*), or 3 μg/ml (lane *5*) CDDP. hTRX migrates at M_r 13,000. (*B*) The TRX activity in the cell extracts (40 μg), obtained from Jurkat cells treated with the indicated doses of CDDP for 48 h, was determined by insulin reducing assay as described in Methods. The values obtained from this assay were compared to the standard curve for rTRX (100–1,200 ng/ml) and data were expressed as the absolute amount of TRX per milligram of total protein (micrograms per milligram protein). Results are means of duplicate determinations of a single experiment and are representative of three similar experiments.

2′,7′-dichlorofluorescin (DCFH), by cellular esterase. DCFH is membrane impermeable and rapidly oxidizes to a highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF), in the presence of intracellular H_2O_2 and/or other peroxides. Thus, cellular fluorescence intensity is directly proportional to levels of intracellular peroxides and can be monitored by flow cytometry. For assays, 2×10^5 cells were treated with agents (CDDP and/or BHA) for the indicated time, and then the culture medium was replaced with freshly prepared medium containing 5 μM DCFH-DA. After 30 min of incubation at 37°C, the fluorescence intensity was measured by flow cytometry (Cytoron; Ortho Diagnostic Systems K.K., Tokyo, Japan).

Establishment of hTRX-transfected clones of L929 cells. Exponentially growing L929 cells, plated at a density of 10^5 cells/60-mm dish 1 d before transfection, were cotransfected with 20 μg of the hTRX expression vector (pcDSRαADF) (5) and 2 μg of pSV2-neo (31) using the DEAE-dextran method (29). After 72 h, G418 (200 μg/ml) was added to the transfected cells for selection. Then, G418-resistant clones were isolated and screened for expression of hTRX protein by immunoblotting with anti-hTRX mAb (ADF-11 mAb). Another population of L929 cells was similarly transfected with the control vector (pcDSRα) and pSV2-neo, and selected for G418 resistance to serve as the transfected controls.

Cytotoxicity assay. Cytotoxicity was assessed by trypan blue dye exclusion and MTT assay. For MTT assay, L929 cells (5×10^3 cells/well), plated in a 96-well flat-bottom plate and cultured overnight, were incubated in 100 μl of culture medium containing the indicated doses of agents (CDDP and/or BHA). After 20 h of culture at 37°C, 10 μl of MTT (5 mg/ml) was added to the wells, followed by incubation for an additional 4 h. The resulting formazan product was solubilized in 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) and measured spectrophotometrically at 595 nm on an ELISA reader (microplate reader 3550; Bio-Rad Laboratories).
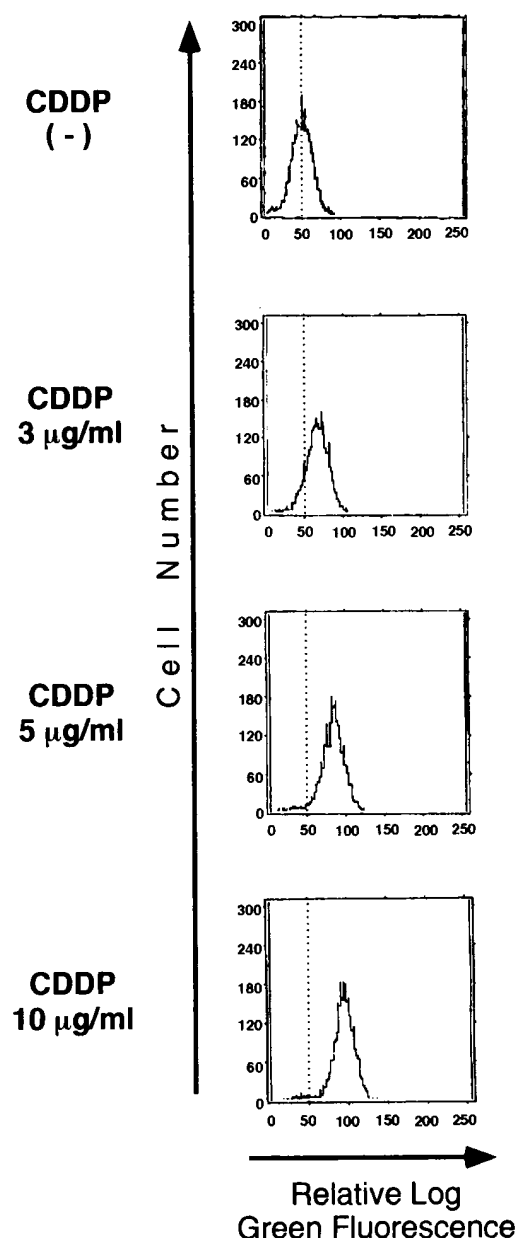
Results

Upregulation of hTRX expression and activity in the Jurkat human T cell line after exposure to CDDP. We first determined whether treatment with CDDP increased the expression levels of hTRX protein in the Jurkat human T cell line. As shown in Fig. 1 *A*, immunoblot analysis demonstrated that the expression of hTRX protein was enhanced after exposure to CDDP (1, 2, and 3 μg/ml) for 48 h. Moreover, insulin reducing assay showed that the hTRX activity was dose-dependently in-

creased by treatment with 1, 2, and 3 μg/ml CDDP for 48 h in Jurkat cells (Fig. 1 *B*). We next examined the effect of CDDP on the activity of hTRX promoter using CAT assay. Jurkat cells were transfected with phTRX-CAT, which contained the hTRX 5′-flanking region fused to the structural gene coding for CAT, and were treated with CDDP for 24 h. Treatment with 1, 2, and 3 μg/ml CDDP resulted in a 1.6-, 2.7-, and 5.2-fold increase of CAT activity, respectively (Fig. 2). These data indicated that treatment of Jurkat cells with CDDP caused transcriptional activation of the hTRX gene.

Involvement of ROI in the CDDP-induced activation of hTRX gene expression. It has been suspected that increased generation of intracellular ROI is related to the biological effects of CDDP (15–19). In fact, as shown in Fig. 3, Jurkat cells treated with CDDP displayed a dose-dependent increase in the intracellular accumulation of peroxides, estimated by flow cytometric analysis with DCFH-DA. In addition, our previous
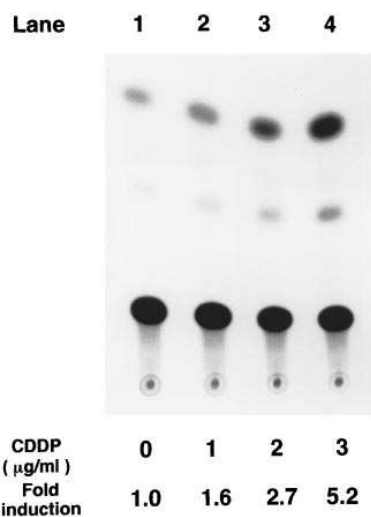

Figure 2. Effect of CDDP treatment on the activity of hTRX promoter in Jurkat cells. Jurkat cells (5×10^6 cells) were transfected with 5 μg of phTRX-CAT, which contained the hTRX 5′-flanking region (from −2629 to +28) fused to the structural gene coding for CAT. After 24 h of transfection, the cells were treated with the indicated doses of CDDP for the following 24 h. Equal amounts of the cell extracts (70 μg) were used for the measurement of CAT activity. Lane *1*, untreated Jurkat cells; lanes *2–4*, Jurkat cells treated with 1 μg/ml (lane *2*), 2 μg/ml (lane *3*), or 3 μg/ml (lane *4*) CDDP after transfection.

CDDP (-)

CDDP 3 μg/ml

CDDP 5 μg/ml

CDDP 10 μg/ml

Cell Number

Relative Log Green Fluorescence

Figure 3. Effect of CDDP treatment on the fluorescence distribution of DCFH oxidation in Jurkat cells. Jurkat cells (2×10^5 cells) were cultured for 6 h in the absence or presence of the indicated doses of CDDP (3, 5, or 10 μg/ml), and then the culture medium was replaced with freshly prepared medium containing 5 μM DCFH-DA. After 30 min of incubation at 37°C, fluorescence intensity was measured by flow cytometry.

studies demonstrated that the expression of hTRX was induced by various oxidative stimuli, including H_2O_2, x-ray, and ultraviolet exposure (5, 11, 12). Therefore, to determine whether ROI are involved in the CDDP-induced overexpression of hTRX, we examined the effect of an antioxidant, BHA, on the CDDP-induced transactivation of hTRX promoter. BHA is a phenolic, lipid-soluble, chain-breaking antioxidant, and has been shown to inhibit an intracellular signaling pathway regulated by ROI, such as nuclear factor-κB activation (32, 33). As shown in Fig. 4, CAT assay with the hTRX promoter–reporter gene construct (phTRX-CAT) demonstrated that the CDDP-
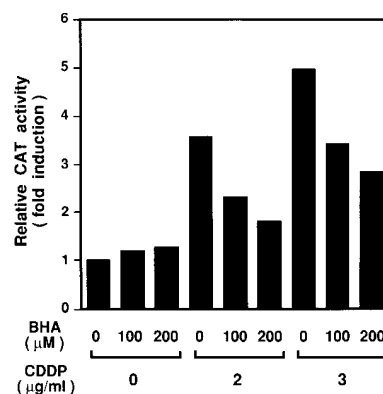

Figure 4. Effect of BHA on the CDDP-induced transactivation of hTRX promoter in Jurkat cells. Jurkat cells (5×10^6 cells) were transfected with 5 μg of phTRX-CAT. After 24 h of transfection, the cells were cultured for the following 24 h with no drug, BHA alone (100 μM or 200 μM), or CDDP (2 μg/ml or 3 μg/ml) in the absence or presence of BHA (100 μM or 200 μM). Equal amounts of the cell extracts (70 μg) were used for the measurement of CAT activity. Each bar represents the relative CAT activity (fold induction) in comparison with the untreated cells. This experiment was performed three times with similar results.

induced transactivation of hTRX promoter was dose-dependently inhibited in the presence of BHA (100 μM or 200 μM) in Jurkat cells. As expected, incubation of Jurkat cells with BHA decreased the intracellular accumulation of peroxides after treatment with CDDP (data not shown). These results indicated that the CDDP-induced activation of hTRX gene expression might be dependent on the increased generation of intracellular ROI.

Establishment of hTRX-overexpressing derivatives of the L929 murine fibrosarcoma cell line. To evaluate the biological significance of the hTRX induction by CDDP, we determined whether overexpression of hTRX affected the cellular sensitivity to CDDP. We established hTRX-overexpressing derivatives of the L929 murine fibrosarcoma cell line by stable transfection with the hTRX cDNA. The hTRX expression vector (pcDSRαADF) was introduced into L929 cells with the selectable pSV2-neo plasmid, and two clones with resistance to G418 (200 μg/ml) were selected. As a control, L929 cells transfected with the control vector (pcDSRα) and pSV2-neo were similarly selected for G418 resistance, and two clones were isolated. As shown in Fig. 5, the hTRX-transfected clones, TRX-8 and TRX-14, demonstrated a readily detectable expression of hTRX protein by immunoblot analysis with anti-hTRX mAb, whereas the parental L929 and control transfected cells, Neo-1 and Neo-5, did not. To evaluate the function of overexpressed hTRX protein, the TRX activity in the parental and transfected clones was analyzed by insulin reducing assay. The control transfected cells, Neo-1 and Neo-5, and the parental L929 cells displayed almost the same levels of TRX activity, which might reflect the activity of endogenous mouse TRX. In contrast, the hTRX-transfected cell lines TRX-8 and TRX-14 showed a 1.8- and 2.9-fold increase in TRX activity over the parental L929 cells, respectively. However, the parental and transfected L929 cells displayed no significant differences in the TRX reductase activity (Table I).

Effect of the overexpression of hTRX on CDDP-induced cytotoxicity. The parental and transfected L929 cells were examined by viable cell counts using trypan blue exclusion and MTT assay for sensitivity to cytotoxicity induced by continuous exposure (24 h) to CDDP. As shown in Fig. 6, the hTRX-
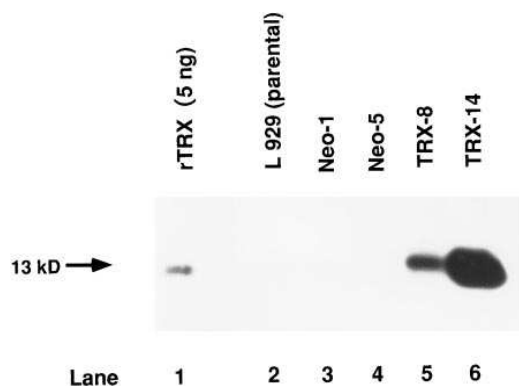
Figure 5. Expression of hTRX protein in the parental and transfected L929 cells. Cell lysates were prepared from the parental and transfected L929 cells. Equal amounts of the cell lysates (10 μg/lane) were electrophoresed and subjected to immunoblot analysis with anti-hTRX mAb (ADF-11 mAb). Lane *1*, rTRX 5 ng (a positive control); lane *2*, parental L929 cells; lane *3*, Neo-1 (control transfected cells); lane *4*, Neo-5 (control transfected cells); lane *5*, TRX-8 (hTRX-transfected cells); lane *6*, TRX-14 (hTRX-transfected cells). hTRX migrates at M_r 13,000.

transfected cells, TRX-8 and TRX-14, were more resistant to CDDP-induced cytotoxicity, compared to the parental L929 and control transfected cells, Neo-1 and Neo-5. Because hTRX has been shown to play a protective role against various oxidative stresses (10, 11) by functioning as a redox buffer, we estimated the intracellular redox state after CDDP treatment in the hTRX-transfected and control cells. As shown in Fig. 7, flow cytometric analysis with DCFH-DA demonstrated that exposure to CDDP caused a significant increase in the intracellular accumulation of peroxides in the parental L929 and control transfected cells, but not in the hTRX-transfected cells. Furthermore, we found that the CDDP-induced cytotoxicity decreased dose-dependently in the presence of an antioxidant, BHA, in the parental L929 cells (Fig. 8). This preventive effect of BHA on CDDP-induced cytotoxicity was also observed in another cell line, Jurkat, and was accompanied by a decreased accumulation of intracellular peroxides after CDDP treatment (data not shown). These results indicated that CDDP-induced cytotoxicity might be due, at least in part, to an increased generation of ROI and that hTRX might confer cellular resistance to CDDP by scavenging intracellular toxic oxidants generated by this agent.

Discussion

CDDP remains one of the most effective chemotherapeutic agents for cancer therapy. However, its potential is limited by the presence of intrinsic or acquired resistance to this agent. Thus, it has been of particular importance to clarify the precise mechanisms by which resistance to CDDP arises and to develop efficient therapeutic strategies to overcome the resistance to CDDP treatment. Although the mechanisms resulting in cellular resistance to CDDP have been explained by several different factors, including reduced membrane permeability, enhanced drug detoxification, and increased efficiency of DNA repair processes (13, 14), they remain complex and as yet are not fully understood. In the present study, we have demonstrated that CDDP increases the expression of hTRX at

Table I. The TRX and TRX Reductase Activity in the Parental and Transfected L929 Cells

	TRX activity	TRX reductase activity
	μg/mg protein	*U/mg protein*
Parental L929	2.16	0.0209
Neo-1	2.08	0.0221
Neo-5	2.07	0.0205
TRX-8	3.85	0.0212
TRX-14	6.34	0.0220

The activity of TRX and TRX reductase in the parental and transfected L929 cells was determined by insulin reducing assay. For determination of the TRX activity, after preincubation with the DTT activation buffer, equal amounts of cell extracts (20 μg) were mixed with the reaction mixture (0.1 M Tris-Cl (pH 7.5), 2 mM EDTA, 0.2 mM NADPH, 0.4 U/ml human TRX reductase, 140 μM insulin) and incubated at 25°C, followed by recording the decrease in absorbance at 340 nm. As a control, the preincubated extracts were mixed with the reaction mixture containing everything except insulin. The obtained values were compared to the standard curve for rTRX (100–1,200 ng/ml). Data (micrograms per milligram protein) were expressed as the absolute amount of TRX per milligram of total protein. For determination of the TRX reductase activity, equal amounts of cell extracts (5 μg) were mixed with the reaction mixture (0.1 M Tris-Cl (pH 7.5), 2 mM EDTA, 0.2 mM NADPH, 10 μM rTRX, 140 μM insulin) and incubated at 25°C, followed by recording the decrease in absorbance at 340 nm. As a control, the extracts were mixed with the reaction mixture containing everything except insulin. Data (units per milligram protein) were calculated as micromoles of NADPH oxidized per min per milligram of total protein from the relation $\Delta A_{340} \times 0.12 \times 200/6.2$. Results are means of duplicate determinations of a single experiment and are representative of two similar experiments.
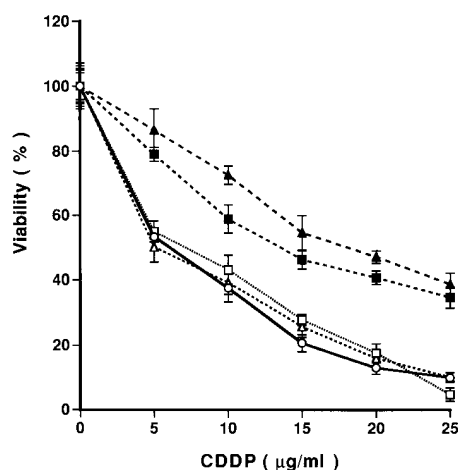
Figure 6. Effect of continuous exposure to CDDP on the cell viability of parental and transfected L929 cells. Cells (5×10^4/well) from each cell line (parental L929 (○), Neo-1 (□), Neo-5 (△), TRX-8 (■), and TRX-14 (▲)) were plated in a 24-well flat-bottom plate, cultured overnight, and then exposed to the indicated doses of CDDP for 24 h. Cell viability (percent viability) was determined by viable cell counts using trypan blue exclusion. Data were expressed as the percentage of the viable cell number without CDDP treatment. The means and SDs of triplicate cultures are shown. Cell viability assessed by MTT assay yielded similar results.
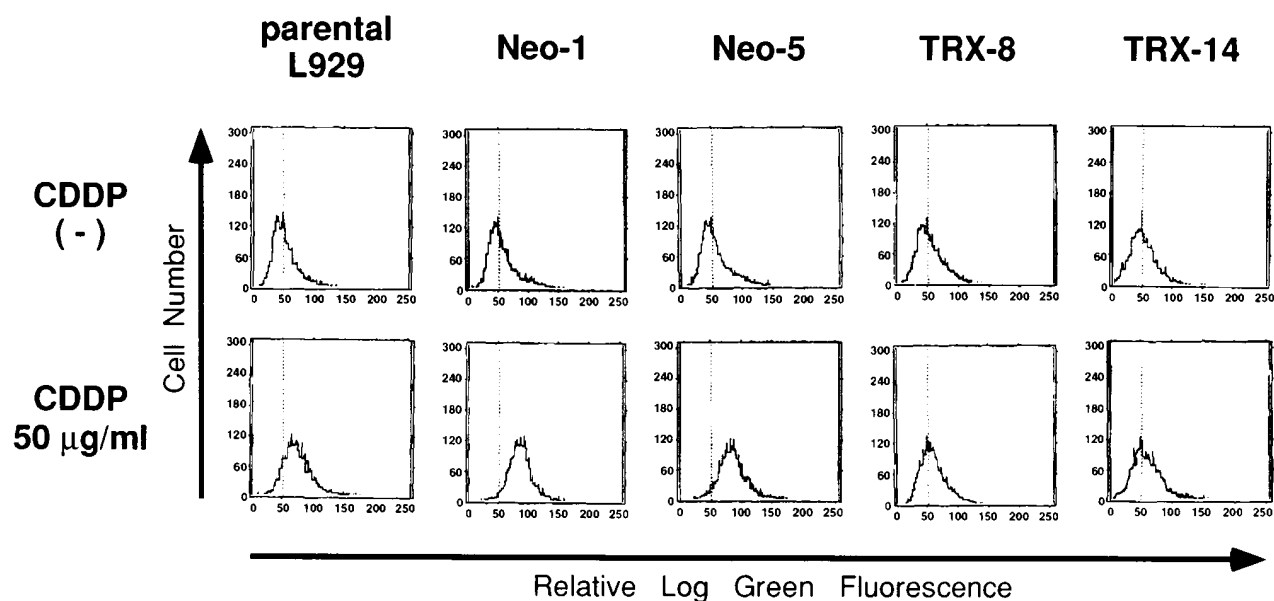
Figure 7. Effect of CDDP treatment on the fluorescence distribution of DCFH oxidation in the parental and transfected L929 cells. Cells (2×10^5 cells) from each cell line (*parental L929, Neo-1, Neo-5, TRX-8,* and *TRX-14*) were cultured for 6 h in the absence or presence of CDDP (50 µg/ml), and then the culture medium was replaced with freshly prepared medium containing 5 µM DCFH-DA. After 30 min of incubation at 37°C, fluorescence intensity was measured by flow cytometry.

the transcriptional level, and that overexpression of hTRX confers cellular resistance to CDDP-induced cytotoxicity. These observations indicate that hTRX may play an important role in the acquisition of CDDP resistance.

hTRX has been reported to be a stress-inducible protein, whose expression is enhanced by various oxidative stimuli. Previous studies have shown that the expression of hTRX is upregulated after exposure to H_2O_2, x-ray, or ultraviolet irradiation (5, 11, 12). Moreover, according to our recent observations, the hTRX promoter has a novel *cis*-acting regulatory element responsive for a variety of oxidative stimuli, such as H_2O_2, sulfhydryl-oxidizing agents, and ultraviolet irradiation (Taniguchi, Y., and J. Yodoi, manuscript in preparation). In the present study, we demonstrated that treatment with CDDP caused transcriptional activation of the hTRX gene in the Jurkat human T cell line. Furthermore, this activation was shown to be inhibited by an antioxidant, BHA, suggesting that CDDP enhanced the expression of hTRX through increased generation of ROI. In addition, CDDP has been reported to induce enhanced expression of several other genes, such as c-*jun*, c-*fos*, and HIV (34–36). Because these genes are also known to be transactivated by oxidative stress (37, 38), it is likely that ROI may function as a common signaling mediator in the modulation of gene expression after CDDP treatment.

There is growing evidence that the cytotoxic activity of CDDP is closely associated with increased generation of ROI. It has been reported that generation of ROI and subsequent peroxidation of membrane lipids are involved in CDDP-induced nephrotoxicity and that administration of free radical scavengers or antioxidants, such as SOD and α-tocopherol, protects the kidney from CDDP-induced toxicity in in vivo animal models (15–17). In addition, Sodhi and Gupta (18) have reported that murine macrophages treated with CDDP show increased production and release of H_2O_2 and superoxide anion in vitro. Masuda et al. (19) have demonstrated that the direct interaction of CDDP and DNA generates superoxide an-

ion and hydroxyl radical in a cell-free system. In the present study, we found that exposure to CDDP caused a dose-dependent increase in the intracellular accumulation of peroxides in murine L929 and human Jurkat T cells. Furthermore, we demonstrated that an antioxidant, BHA, inhibited the cytotoxic effect of CDDP in these cell lines, possibly by scavenging intracellular toxic oxidants. These observations indicate that increased production of intracellular ROI may, at least in part,
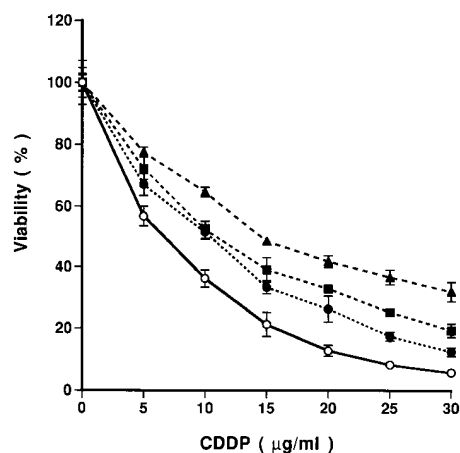

Figure 8. Effect of BHA on the cell viability of L929 cells after exposure to CDDP. L929 cells (5×10^3/well) were plated in a 96-well flat-bottom plate, cultured overnight, and then exposed to CDDP in the absence (○) or presence of BHA (50 µM [●], 100 µM[■], 150 µM[▲]). After 20 h of culture at 37°C, MTT was added to the wells, followed by incubation for an additional 4 h. The resulting formazan product was measured spectrophotometrically at 595 nm on an ELISA reader. Cell viability (percent viability) was expressed as the percentage of the OD value obtained from the cells without CDDP treatment. The means and SDs of triplicate cultures are shown.

be responsible for CDDP-induced cytotoxicity. Recently, attention has been directed to oxidative damage as a common mediator in chemotherapeutic drug-induced cell death, particularly as a signal in apoptotic cell death (39). For example, *bcl-2* gene product, which is known to prevent apoptosis by functioning as an antioxidant (40, 41), was reported to confer cellular resistance to CDDP (42, 43). This finding provides further support for the critical role of oxidative stress in CDDP-induced cytotoxicity.

hTRX can function not only as a hydrogen donor for various proteins, but also as a scavenger of ROI. rTRX has been shown to reduce ROI, such as H_2O_2 and hydroxyl radical, efficiently in vitro (9). Moreover, a family of TRX-dependent peroxide reductase has been recently identified from yeast and mammalian cells, which directly reduces H_2O_2 and various alkyl hydroperoxides with the use of hydrogens provided by the TRX system (44). These studies indicate that hTRX may play an important role in the cellular defense mechanisms against oxidative stress. In fact, rTRX has a protective activity against H_2O_2-induced cytotoxicity in murine endothelial cells (11). In addition, in a human histiocytic lymphoma cell line, pretreatment with rTRX can inhibit TNFα-induced cytotoxicity, in which the generation of ROI seems to be involved (10). In the present study, we demonstrated that the hTRX-transfected L929 cells showed reduced sensitivity to CDDP, and that they did not display a significant increase in the intracellular accumulation of peroxides after exposure to CDDP. Similar results were observed in the parental L929 cells treated with an antioxidant, BHA, suggesting that hTRX and BHA had a protective activity against CDDP-induced cytotoxicity through a common mechanism. Therefore, it is quite conceivable that endogenous hTRX has a protective role against CDDP-induced cytotoxicity, by scavenging toxic ROI generated by CDDP.

Besides the scavenging activity of intracellular ROI, however, other mechanisms may also contribute to the protective effect of hTRX against CDDP. CDDP is a potent electrophile that reacts with any nucleophiles, including the sulfhydryl groups (on proteins) and nucleophilic groups (on nucleic acids) (13, 45). Several studies have suggested that intracellular thiols, such as glutathione or metallothioneins, affect cellular sensitivity to CDDP, by covalently binding to CDDP and detoxifying it in the cytosol (13, 45–50). Similarly, it is likely that hTRX, a potent nucleophile with active sulfhydryl groups (1, 2), protects cells by directly intercepting reactive platinum complexes in the cytosol before they can react with other proteins or nucleic acids. Recently, we have reported that hTRX plays a critical role in the regulation of intracellular thiols, especially glutathione (51, 52). However, in the present study, the hTRX-mediated regulation of the glutathione system may not be attributed to cellular resistance to CDDP, because overexpression of hTRX did not result in a significant change of intracellular glutathione levels in L929 cells (data not shown). Alternatively, inactivation of hTRX by direct binding to CDDP may be one of the key processes in the induction of CDDP-mediated apoptotic cell death. hTRX seems to have crucial roles in the regulation of apoptosis, because it controls various cellular functions, including the redox regulation of several enzymes and transcription factors (1, 2, 5, 7, 8). In fact, we have demonstrated that oxidation and inactivation of intracellular protein thiols other than glutathione, such as hTRX, may be one of the critical processes in the induction of apoptosis in human lymphocytes treated with a sulfhydryl-specific oxidant, diamide (53).

Additionally, hTRX may be involved in the DNA replication or repair processes after CDDP exposure, because TRX is a hydrogen donor for ribonucleotide reductase, the essential enzyme for the biosynthesis of deoxyribonucleotides and DNA (1, 2). It was reported that ribonucleotide reductase from *E. coli* and mammalian cells was inhibited by CDDP in vitro (54, 55), and that inactivation of this enzyme had significant effects on the DNA repair processes after treatment with DNA-damaging chemotherapeutic agents (56, 57). Moreover, mammalian glutaredoxin, another hydrogen donor for ribonucleotide reductase (1, 2), was also shown to be inactivated by CDDP in vitro (58). Because the activity of ribonucleotide reductase depends on the generation of its active-site dithiol mediated by the TRX or glutaredoxin system (1, 2), overexpression of hTRX may enhance the efficiency of DNA replication or repair after CDDP treatment by providing required deoxyribonucleotides through regulation of the activity of this enzyme.

Although we could not find any significant differences in the TRX reductase activity between the control and hTRX-overexpressing L929 cells in the present study, the TRX reductase activity may also affect cellular sensitivity to CDDP. According to our preliminary observation, the enzyme activity of TRX reductase decreases after treatment with CDDP in cultured cells. Recent studies have shown that TRX reductase not only catalyzes the reduction of oxidized TRX by NADPH, but also reduces hydroperoxides directly or via activation of a selenium-dependent peroxidase in a cell-free system (59, 60). Moreover, inactivation of the disulfide reducing activity of TRX reductase, induced by an alkylating agent, has been reported to be accompanied by a large increase in NADPH oxidase activity, which will produce ROI and cause oxidative stress (61). Therefore, it may be possible that increased generation of intracellular ROI by CDDP exposure is attributed to impairment of the TRX system, especially caused by the inactivation of TRX reductase, and that overexpressed hTRX compensates for the decreased enzyme activity of TRX reductase to prevent alterations of the intracellular redox state. Further investigation is needed to clarify the role of TRX reductase in CDDP-induced cytotoxicity.

Recently, several investigators have found that some CDDP-resistant cell lines have higher levels of hTRX expression (23, 24). Moreover, it has been shown that hTRX-antisense transfectants of a human bladder cancer cell line display enhanced sensitivity against CDDP and other ROI-generating chemotherapeutic agents, such as doxorubicin, mitomycin C, and etoposide (24). Taken together with our results in this paper, these observations indicate the critical role of hTRX in the acquisition of CDDP resistance, although further studies are needed to elucidate the precise mechanism by which hTRX provides cellular resistance to CDDP. To validate our results, however, it remains to be determined whether the results obtained from these in vitro studies are also observed in in vivo animal models or clinical situations. Studies in animal models are now under way to clarify the critical role of hTRX in CDDP resistance in vivo. A variety of human cancer cells have been reported to show enhanced expression of hTRX, which may be induced by persistent exposure to various oxidative stresses (62). Therefore, the development of pharmacological or genetical methods to modulate the expression and/or

activity of hTRX may be a rational therapeutic strategy to overcome CDDP resistance in human cancer cells.

Acknowledgments

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