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Original Research Communication

Secretion of Thioredoxin Enhances Cellular Resistance to *cis*-Diamminedichloroplatinum (II)

TETSURO SASADA,[1] HAJIME NAKAMURA,[1] SHUGO UEDA,[1] SATOSHI IWATA,[1] MASAYA UENO,[1] ARIMICHI TAKABAYASHI,[2] and JUNJI YODOI[1]

ABSTRACT

Thioredoxin (TRX) is a redox-active protein induced by a variety of stress and secreted from cells. Collecting evidence revealed that extracellular TRX shows cytokine- and chemokine-like activities. In the present study, we studied the role of secreted TRX on cellular resistance to *cis*-diamminedichloroplatinum (II) (CDDP). The CDDP-resistant variants of HeLa cells not only have enhanced expression of intracellular TRX, but also show increased secretion of TRX into the culture medium, compared to the parental cells. The CDDP-resistant cells also exhibit an enhanced L-cystine uptake capability, which results in a significant increase in the intracellular sulfhydryl content, including glutathione (GSH). Exogenous administration of recombinant TRX (rTRX) increases cellular resistance to CDDP and augments the L-cystine uptake in the parental HeLa cells. Moreover, depletion of L-cystine from the culture medium or combined treatment with L-cystine uptake inhibitors increases cellular sensitivity to CDDP in the CDDP-resistant cells. These findings suggest that secreted TRX may play an important role in the acquisition of cellular CDDP resistance through enhancement of the L-cystine uptake activity, and that the L-cystine transport system, as well as the TRX system, may be a novel therapeutic target in CDDP-resistant cancer cells. Antiox. Redox Signal. 2, 695–705.

INTRODUCTION

THIOREDOXIN (TRX) is a small ubiquitous protein having a redox-active disulfide/dithiol within the conserved active site sequence, -Cys-Gly-Pro-Cys- (Holmgren, 1985, 1989). Oxidized TRX with a disulfide on its active site is reduced by NADPH and TRX reductase, and reduced TRX can function as a general protein disulfide reductase (Holmgren, 1985, 1989). Human TRX, which was originally cloned as adult T-cell leukemia-derived factor (ADF) produced by human T-cell leukemia virus type I-transformed T cells (Tagaya *et al.*, 1989), has been reported to possess multiple biological functions and regulate different systems via thiol redox control, including the activation of several essential enzymes, the reduction of oxygen radicals, and the redox regulation of certain receptors and transcription factors, such as NF-κB, AP-1, and glucocorticoid receptor (Okamoto *et al.*, 1992; Hirota *et al.*, 1997; Nakamura *et al.*, 1997). In addition to these intracellular functions, TRX has been shown to be secreted by normal and transformed cells through a leaderless secretary pathway (Rubartelli *et al.*, 1992, 1995; Hori *et al.*, 1994) and to possess a variety of extracellular activities as an autocrine/paracrine factor (Wakasugi *et al.*, 1990; Matsuda *et al.*, 1991;

[1]Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan.
[2]Department of Surgery, Tazuke Kofukai Kitano Hospital Medical Institute, Osaka 530-0026, Japan.

Iwata *et al.*, 1994; Newman *et al.*, 1994; Nakamura *et al.*, 1994; Gasdaska *et al.*, 1995; Schenk *et al.*, 1996; Iwata *et al.*, 1997; Bertini *et al.*, 1999). For example, extracellular TRX can stimulate cellular proliferation (Wakasugi *et al.*, 1990; Gasdaska *et al.*, 1995), enhance the expression of various cytokines and cytokine receptors (Tagaya *et al.*, 1989; Schenk *et al.*, 1996), inhibit the human immunodeficiency virus (HIV) expression (Newman *et al.*, 1994), protect cells from oxidative stresses (Matsuda *et al.*, 1991; Nakamura *et al.*, 1994), and function as a unique chemoattractant in infection and inflammation (Bertini *et al.*, 1999). In addition, we have recently demonstrated that exogenously added rTRX enhances the L-cystine uptake activity, which is one of the limiting factors in the biosynthesis of intracellular thiols, and regulates cell activation and proliferation or apoptotic cell death in human lymphoid cells (Iwata *et al.*, 1994, 1997).

cis-Diamminedichloroplatinum (CDDP) is one of the most important chemotherapeutic agents in treating a variety of solid tumors, including ovarian, testicular, bladder, head and neck, esophageal, and small-cell lung cancers. However, the development of acquired resistance to this agent represents a significant problem in cancer therapy (Chu, 1994). Recently, we and others have demonstrated that the enhanced expression and activity of human TRX system, TRX and TRX reductase, is closely associated with cellular resistance to chemotherapeutic agents, such as CDDP (Yokomizo *et al.*, 1995; Kawahara *et al.*, 1996; Sasada *et al.*, 1996, 1999; Yamada *et al.*, 1996) and doxorubicin (Wang *et al.*, 1997). However, the molecular mechanisms by which TRX enhances CDDP-resistance have not been fully addressed. We report here that the CDDP-resistant HeLa cells show an enhanced secretion of TRX from cells and an increased L-cystine uptake activity, which might be mediated through the increased extracellular TRX as an autocrine/paracrine factor. Our findings strongly suggest that secreted TRX may play an important role in the acquisition of the cellular resistance to CDDP through enhanced L-cystine uptake activity, and that the L-cystine transport system, as well as the TRX system, may be a novel therapeutic target for CDDP-resistant cancer cells.

MATERIALS AND METHODS

Reagents

CDDP, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), NADPH, glutathione (GSH) (reduced form), glutathione reductase (yeast), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide (MTT), L-homocysteic acid, and DL-α-aminoadipic acid were obtained from Sigma Chemical Co. (St. Louis, MO). L-Methionine, L-cystine, fetal calf serum (FCS), and L-methionine- and L-cystine-free Dulbecco's modified Eagle medium (DMEM) were from Life Technologies Inc. (Grand Island, NY). Anti-human TRX monoclonal antibodies (mAbs) (ADF-11 and ADF-21 mAbs) were established and provided by Fujirebio Inc. (Tokyo, Japan) (Kogaki *et al.*, 1996). rTRX was produced and provided by Basic Research Laboratory, Ajinomoto Co. Inc. (Kawasaki, Japan), according to the method described previously (Mitsui *et al.*, 1992). All other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated.

Cell culture

HeLa, a human cervical carcinoma cell line, and its CDDP-resistant variants were maintained in DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) 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 CDDP-resistant variants were established by continuous exposure of HeLa cells to escalating doses of CDDP over a period of 9 months. The acquired-resistance clones, HeLa-CP5 and HeLa-CP7, were isolated after continuous exposure to 5 and 7 μ*M* CDDP, respectively (Sasada *et al.*, 1999). Prior to the start of experiments, the CDDP-resistant clones were grown continuously in the medium without CDDP for at least 1 month.

For cell culture with rTRX, after cells were cultured in 100 μl of FCS-free DMEM in the presence of the indicated doses of rTRX and CDDP for 6 hr, 100 μl of DMEM with 20% FCS was added and cells were cultured for an additional 42 hr. For cell culture in the L-cystine-depleted medium, L-methionine- and L-cystine-

free DMEM was supplemented with 30 μg/ml L-methionine. As a control, L-methionine- and L-cystine-free DMEM supplemented with both 30 μg/ml L-methionine and 48 μg/ml L-cystine were used. FCS, which was dialyzed against 40-fold volume of PBS twice, was used in this experiment (Iwata *et al.*, 1994, 1997).

Determination of the concentration of TRX in the culture medium

The concentration of TRX in the culture supernatant was determined by sandwich enzyme-linked immunosorbent assay (ELISA) with two anti-human TRX mAbs (ADF-11 and ADF-21 mAbs) recognizing non-overlapped epitopes of human TRX, according to the method previously described (Kogaki *et al.*, 1996; Nakamura *et al.*, 1996). Cells (4×10^5 cells/well), plated in a six-well flat-bottomed plate and cultured overnight, were washed with phosphate-buffered saline (PBS) twice, and then cultured in 1 ml of DMEM containing 10% FCS and antibiotics in a humidified atmosphere of 5% CO_2/95% air at 37°C for 6 hr. The culture medium was harvested, followed by centrifugation, and the supernatant was assayed by sandwich ELISA. The standard curve for rTRX (5–320 ng/ml) was used for the determination of TRX concentration. To show the amount of TRX excreted from cells, the value in the untreated control medium was subtracted from that in the culture medium, in which the cells had been incubated for 6 hr.

Determination of the L-cystine and L-glutamate uptake activity

The activity of L-cystine and L-glutamate uptake was determined, according to the method previously described by Bannai *et al.* with minor modification (Iwata *et al.*, 1994, 1997). In brief, cells (4×10^5 cells/well), plated in a six-well flat-bottomed plate and cultured overnight, were washed three times in the prewarmed uptake medium (Dulbecco's PBS containing 0.5 m*M* $MgCl_2$, 0.9 m*M* $CaCl_2$, and 5.6 m*M* D-glucose, pH 7.4), and cultured in the prewarmed uptake medium containing 0.5 μCi/ml ^{14}C-L-cystine or ^{14}C-L-glutamate (the final concentration is around 3 μ*M*) at 37°C for 60 min. After washing with ice-cold PBS three times, cells were lysed by the addition of 500 μl of 0.5 *N* NaOH. The cell extracts (100 μl) were used for the determination of the radioactivity, and protein concentration was assayed by the Lowry method with a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Determination of intracellular sulfhydryl content and total GSH

Total and protein sulfhydryl contents were determined using a modification of the Ellman method (Sedlak and Lindsay, 1968; Teicher *et al.*, 1987). Cells (1×10^6 cells) were lysed in 500 μl of 0.02 *M* EDTA, pH 4.7. For determination of total sulfhydryl content, 200 μl of cell lysate was mixed with 600 μl of 0.2 *M* Tris buffer, pH 8.2, followed by addition of 40 μl of 0.02 *M* DTNB in methanol. After bringing the sample volume to 1.2 ml with methanol, the color was allowed to develop for 30 min at room temperature and the absorbance was read at 412 nm. A value for the molecular absorptivity of 13,600 M^{-1} cm^{-1} was used for calculation. For determination of nonprotein sulfhydryl content, 250 μl of cell lysate was treated with 250 μl of 10% trichloroacetic acid for 15 min, followed by centrifugation. The supernatant (400 μl) was mixed with 800 μl of 0.4 *M* Tris buffer, pH 8.9, and 20 μl of 0.01 M DTNB in methanol. The color was allowed to develop for 30 min at room temperature and the absorbance was read at 412 nm. Protein-sulfhydryl content was determined from the difference between the total sulfhydryl content and nonprotein sulfhydryl content. Intracellular total GSH content was determined by the enzymatic recycling assay (Anderson, 1985; Sato *et al.*, 1995). Briefly, cells (1×10^6 cells) were lysed in 100 μl of PBS containing 5% sulfosalicylic acid and homogenized by repeated freezing and thawing. After centrifugation the acid-soluble supernatant was assayed for total GSH content in the reaction buffer (100 m*M* sodium phosphate buffer, 5 m*M* EDTA, pH 7.5, 0.3 m*M* NADPH, 0.6 m*M* DTNB, 0.1 U/ml glutathione reductase). The reaction was followed by recording the increase in absorbance at 412 nm at 30-sec intervals at 25°C. The total GSH content of samples was determined by the standard curve for known amounts of GSH. Protein concentration

was measured by the Bradford method using a Bio-Rad Protein Assay (Bio-Rad Laboratories).

Cytotoxicity assay

Cytotoxicity was assessed by MTT assay. For MTT assay, cells (5×10^3 cells/well), plated in a 96-well flat-bottomed plate and cultured overnight, were incubated in 100 μl of culture medium containing the indicated doses of agents. After 44 hr of culture at 37°C, 10 μl of MTT (5 μg/ml) was added to the wells, followed by incubation for an additional 4 hr. 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 model 3550; Bio-Rad Laboratories).

Statistics

Significant differences were determined by Student's *t*-test.

RESULTS

Enhanced secretion of TRX in the CDDP-resistant HeLa cells

To study mechanisms by which the acquisition of cellular resistance to CDDP occurs, we used CDDP-resistant variants of the HeLa cell line, HeLa-CP5 and HeLa-CP7, that were isolated after continuous exposure to 5 and 7 μM CDDP, respectively (Sasada *et al.*, 1999). Recently, we have demonstrated that HeLa-CP5 and HeLa-CP7 cells were about 2.8- and 5.3-fold resistant to CDDP and showed enhanced expression of TRX protein and mRNA, compared to the parental cells (Sasada *et al.*, 1999). In addition, an insulin reducing assay revealed that HeLa-CP5 and HeLa-CP7 cells exhibited 2.1- and 3.0-fold increase in TRX activity over the parental cells, respectively (Sasada *et al.*, 1999). Because TRX has been reported to be actively secreted from cells through a leaderless secretory pathway (Rubartelli *et al.*, 1992, 1995; Hori *et al.*, 1994), we examined the concentration of extracellular TRX in the culture medium in the parental and CDDP-resistant cells. As shown in Table 1, the TRX concentration in the culture medium in the resistant cells was significantly higher than that in the parental cells (HeLa vs. HeLa-CP5, $p < 0.0005$; HeLa vs. HeLa-CP7, $p < 0.005$), suggesting that overexpressed TRX in the CDDP-resistant cells is actively secreted to the extracellular space.

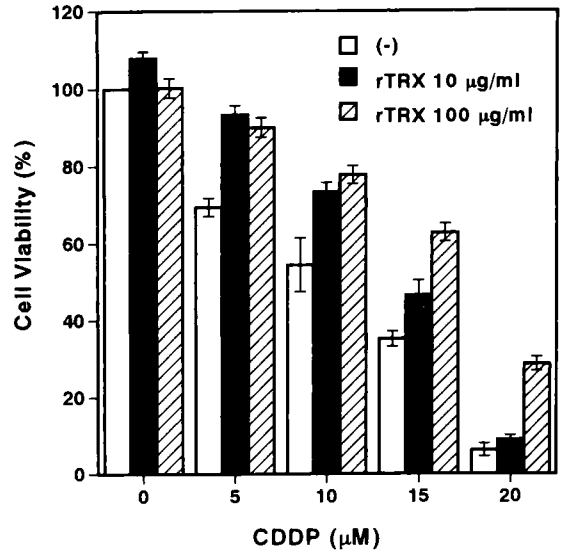

FIG. 1. The effect of rTRX on the CDDP-induced cytotoxicity in HeLa cells. After HeLa cells (5×10^3 cells) were cultured in 100 μl of FCS-free medium with the indicated doses of CDDP in the absence (open bars) or presence of 10 μg/ml (solid bars) and 100 μg/ml (hatched bars) rTRX for 6 hr, 100 μl of medium with 20% FCS was added and the cells were cultured for an additional 42 hr. Cell viability was determined by MTT assay. Data are expressed as the percentage of the absorbance obtained from the untreated cells. Mean and SD of triplicated cultures are shown. The data are representative of three similar results.

TABLE 1. EXTRACELLULAR TRX IN THE PARENTAL AND CDDP-RESISTANT HELA CELLS

	Extracellular TRX (ng/ml)
HeLa	0.61 ± 0.56
HeLa-CP5	7.05 ± 0.69**
HeLa-CP7	10.38 ± 1.57*

The concentrations of extracellular TRX in the culture supernatant were determined by sandwich ELISA, after HeLa, HeLa-CP5, or HeLa-CP7 cells (4×10^5 cells/well) were cultured in 1 ml of culture medium at 37°C for 6 hr. Results are the means and SD of three separate experiments. HeLa-CP5 and HeLa-CP7 cells show a significant increase in the concentration of extracellular TRX compared to the parental HeLa cells.

*$p < 0.005$; **$p < 0.0005$ vs. HeLa cells).

Decreased cellular sensitivity to CDDP by exogenous administration of rTRX

To investigate the importance of extracellular TRX in CDDP resistance, we examined the effects of exogenously administered rTRX on cellular sensitivity to CDDP in the parental HeLa cells. As shown in Fig. 1, exogenous administration of rTRX decreased the cellular sensitivity to CDDP, suggesting that increase in extracellular secreted TRX may be responsible for the development of CDDP resistance.

Enhanced L-cystine uptake activity in the CDDP-resistant cells

Previously, we have demonstrated that extracellular TRX can enhance the cellular activity of L-cystine uptake and regulate the intracellular thiol metabolism in human lymphoid cells (Iwata *et al.*, 1994, 1997). Therefore, we investigated the L-cystine uptake activity and the levels of intracellular thiols in the CDDP-resistant cells. As shown in Table 2, HeLa-CP5 and HeLa-CP7 cells showed a significant increase in the L-cystine uptake activity over the parental cells (HeLa vs. HeLa-CP5, $p < 0.005$; HeLa vs. HeLa-CP7, $p < 0.0005$), whereas they displayed no significant differences in the uptake activity of L-glutamate, an amino acid that is known to share the transport system x_c^- with L-cystine (Bannai and Kitamura, 1980; Bannai, 1984). Because the activity of L-cystine uptake can be significantly enhanced by the administration of rTRX in the parental HeLa cells in a dose-dependent manner (Fig. 2), enhanced L-cystine uptake activity in the CDDP-resistant cells may be attributed to the increase in extracellular TRX. The levels of intracellular L-cystine/cysteine content are thought to affect the biosynthesis of intracellular thiol compounds, such as GSH (Miura *et al.*, 1992; Iwata *et al.*, 1994, 1997; Meier and Issels, 1995). Therefore, we next determined whether the levels of intracellular sulfhydryl groups and total GSH were affected in the CDDP-resistant cells, which exhibited increased L-cystine uptake activity. As shown in Table 3, HeLa-CP5 and HeLa-CP7 cells showed a significant increase in the concentration of intracellular total sulfhydryl groups over the parental cells (HeLa vs. HeLa-CP5, $p < 0.05$; HeLa vs. HeLa-CP7, $p < 0.005$), which was well correlated with the increase in L-cystine uptake activity. In addition, although the concentrations of intracellular protein sulfhydryl groups were not significantly different between the parental and

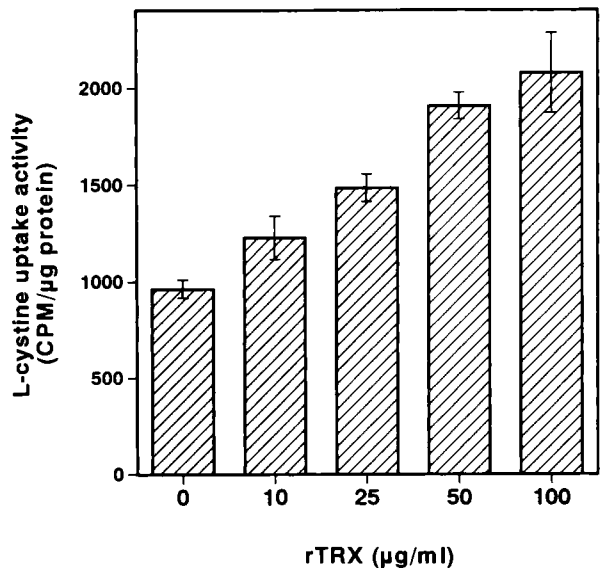

FIG. 2. The effect of rTRX on the L-cystine uptake activity in HeLa cells. HeLa cells (4×10^5 cells), cultured in a six-well flat-bottomed plate overnight, were incubated in the prewarmed uptake medium containing 0.5 μCi/ml ^{14}C-L-cystine (the final concentration is around 3 μM) in the absence or presence of the indicated doses of rTRX at 37°C for 60 min. The cell lysates were used for the determination of the radioactivity and protein concentration. Means and SD of three different experiments are shown. Treatment with rTRX results in a significant increase in the L-cystine uptake activity (untreated cells vs. 10 or 25 μg/ml rTRX-treated cells, $p < 0.05$; untreated cells vs. 50 or 100 μg/ml rTRX-treated cells, $p < 0.01$).

TABLE 2. L-CYSTINE AND L-GLUTAMATE UPTAKE ACTIVITY IN THE PARENTAL AND CDDP-RESISTANT HELA CELLS

	L-Cystine uptake (cpm/μg protein)	*L-Glutamate uptake (cpm/μg protein)*
HeLa	889.3 ± 40.4	5,029.2 ± 312.7
HeLa-CP5	1,266.1 ± 106.7*	5,296.1 ± 334.3
HeLa-CP7	1,384.5 ± 82.7**	4,921.6 ± 132.3

The activity of L-cystine and L-glutamate uptake was determined, according to the method previously described by Bannai *et al.* with minor modification (Iwata *et al.*, 1994, 1997), in HeLa, HeLa-CP5, or HeLa-CP7 cells. Results are the means and SD of five (L-cystine) or four (L-glutamate) separate experiments. HeLa-CP5 and HeLa-CP7 cells show a significant increase in the L-cystine uptake activity, but not in the L-glutamate uptake activity, compared to the parental HeLa cells.

*$p < 0.005$; **$p < 0.0005$ vs. HeLa cells.

CDDP-resistant cells, the concentrations of intracellular total GSH were significantly increased 1.3- and 2.3-fold for HeLa-CP5 and HeLa-CP7 cells compared to the parental cells (HeLa vs. HeLa-CP5, $p < 0.005$; HeLa vs. HeLa-CP7, $p < 0.0005$) (Table 3). These findings suggested that increased extracellular TRX results in an enhanced uptake activity of L-cystine, which may lead to enrichment of intracellular thiol compounds including GSH, in the CDDP-resistant cells.

Increased sensitivity to CDDP by the inhibition of L-cystine uptake in the CDDP-resistant cells

It seems likely that enhanced activity of L-cystine uptake, which increases intracellular sulfhydryl groups, is responsible for the development of CDDP resistance, because increase in intracellular thiol compounds, including GSH and protein thiols, has been reported to be associated with CDDP resistance in some human cell lines (Andrews *et al.*, 1987; Teicher *et al.*, 1987; Kelley *et al.*, 1988; Meijer *et al.*, 1990; Godwin *et al.*, 1992; Chu, 1994). Therefore, to investigate the significance of the L-cystine uptake capability in the sensitivity to CDDP, we first examined the effect of L-cystine depletion from the culture medium on cellular sensitivity to CDDP in the CDDP-resistant cells. As shown in Fig. 3, A and B, L-cystine depletion from medium drastically enhanced the cellular sensitivity to CDDP in the HeLa-CP5 and HeLa-CP7 cells. We next determined the effect of L-cystine uptake inhibitors, L-homocysteic acid and DL-α-aminoadipic acid, on CDDP resistance. L-Homocysteic acid and DL-α-aminoadipic acid are anionic amino acids that share the transport system x_c^- with L-cystine, and are known to inhibit the L-cystine uptake competitively (Bannai and Ishii, 1982; Miura *et al.*, 1992). As reported previously in other human cell lines (Bannai and Ishii, 1982; Miura *et al.*, 1992), we confirmed that these anionic amino acids strongly decreased the L-cystine uptake in the parental and CDDP-resistant HeLa cells (Table 4). Combined treatment with these amino acids and CDDP significantly reduced the resistance to CDDP in HeLa-CP7 cells (Fig. 4) and HeLa-CP5 cells (data not shown). These results suggested that the L-cystine uptake capability may be one of the critical determinants for the cellular sensitivity to CDDP, and that resistance to CDDP may be, at least partly, explained by the enhanced activity of L-cystine uptake.

DISCUSSION

A number of studies have demonstrated that intracellular and extracellular reduction/oxidation (redox) reactions are intimately involved in the control of various aspects of biological processes, including cell activation, cell proliferation, and cell death (Buttke and Sandstrom, 1994; Iwata *et al.*, 1994; Iwata *et al.*, 1997; Nakamura *et al.*, 1997). TRX, one of the most important molecules that control cellular redox state, is known to catalyze dithiol/disulfide exchange reaction in a variety of molecules, such as redox-sensitive enzymes and transcription factors, and regulate various biological pro-

TABLE 3. TOTAL AND PROTEIN SULFHYDRYL CONTENT AND GSH IN THE PARENTAL AND CDDP-RESISTANT HeLa CELLS

	Total sulfhydryl (nmol/mg protein)	*Protein sulfhydryl (nmol/mg protein)*	*GSH (nmol/mg protein)*
HeLa	239.6 ± 24.7	157.9 ± 5.7	107.4 ± 15.7
HeLa-CP5	345.4 ± 41.1*	189.8 ± 20.0	141.2 ± 12.1*
HeLa-CP7	404.1 ± 17.9**	171.3 ± 9.2	246.6 ± 15.8***

Total and protein sulfhydryl contents were determined using a modification of the Ellman method. Intracellular total GSH was determined by the enzymatic recycling assay. Results are expressed as the means and SD of three separate experiments. HeLa-CP5 and HeLa-CP7 cells show a significant increase in total sulfhydryl contents and GSH compared to the parental HeLa cells.

*$p < 0.05$; **$p < 0.005$ ***$p < 0.0005$ vs. HeLa cells. The differences of protein sulfhydryl contents were not significant between the parental and CDDP-resistant cells.

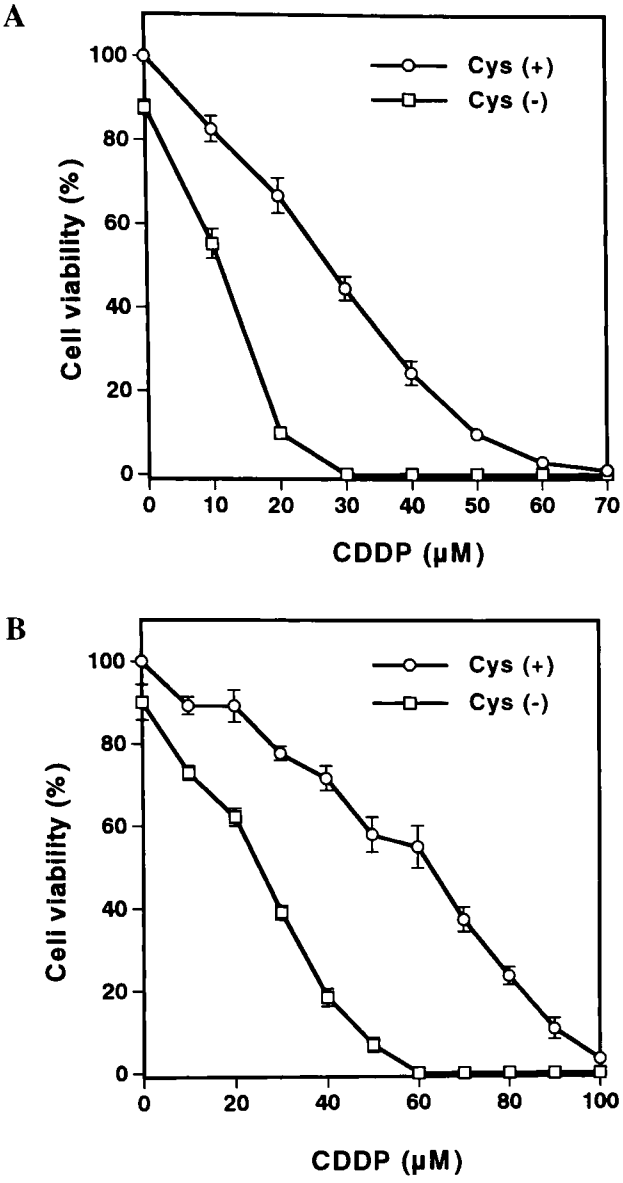

FIG. 3. The effect of L-cystine-depletion on the CDDP-induced cytotoxicity in the CDDP-resistant HeLa cells. HeLa-CP5 (**A**) and HeLa-CP7 (**B**) cells were cultured with the indicated doses of CDDP for 48 hr in L-cystine (Cys)-supplemented or -depleted DMEM. Cell viability was determined by MTT assay. Data are expressed as the percentage of the absorbance obtained from the untreated cells in DMEM supplemented with L-cystine. Mean and SD of triplicated cultures are shown. Results are representative of four similar experiments.

cesses (Nakamura *et al.*, 1997). Recently, we and others have reported that the expression levels of TRX are well correlated with cellular resistance to CDDP in several human cell lines (Yokomizo *et al.*, 1995; Sasada *et al.*, 1996, 1999; Yamada *et al.*, 1996) and surgically resected specimens (Kawahara *et al.*, 1996). In the present study, we tried to investigate the precise molecular mechanisms by which TRX enhances CDDP resistance. We have shown that the CDDP-resistant HeLa cells show an enhanced secretion of TRX from cells and an increased L-cystine uptake activity, which might be mediated through the increased extracellular TRX as an autocrine/paracrine factor. Our findings strongly indicate that secretion of TRX may play an important role in the acquisition of the cellular resistance to CDDP through enhanced L-cystine uptake activity.

In the present study, we showed that administration of rTRX to culture medium increased cellular resistance to CDDP and enhanced the L-cystine uptake activity in the parental HeLa cells. The concentrations of TRX found in the culture medium seemed to be much lower than those of exogenously added rTRX required to increase CDDP resistance directly and enhance the L-cystine uptake activity. However, it seems possible that extracellular TRX can have a local effect in a microenvironment through a mechanism like autocrine or paracrine immediately after secreted from cells to the extracellular space, even if the concentrations in the medium are low. Recently, we and others have shown that the levels of TRX in serum are increased in the patients with malignancy, such as hepatocellular carcinoma (Miyazaki *et al.*, 1998), pancreatic cancer (Nakamura *et al.*, 2000), and adult T-cell leukemia (Y. Kitaoka, H. Nakamura, and J. Yodoi, unpublished observations). The TRX levels in serum may provide a novel tumor marker

TABLE 4. EFFECT OF L-HOMOCYSTEIC ACID ON L-CYSTINE UPTAKE ACTIVITY IN THE PARENTAL AND CDDP-RESISTANT HELA CELLS

	Untreated (cpm/μg protein)	*L-Homocysteic acid (cpm/μg protein)*
HeLa	889.3 ± 40.4	163.6 ± 2.1
HeLa-CP5	1,266.1 ± 106.7	159.1 ± 2.0
HeLa-CP7	1,384.5 ± 82.7	185.9 ± 3.8

The activity of L-cystine uptake was determined in the absence or presence of 2.5 m*M* L-homocysteic acid in HeLa, HeLa-CP5, or HeLa-CP7 cells. Results are the means and SD of five (untreated) or three (L-homocysteic acid-treated) separate experiments. DL-α-Aminoadipic acid showed similar inhibitory effects on the L-cystine uptake activity in the parental and CDDP-resistant cells.

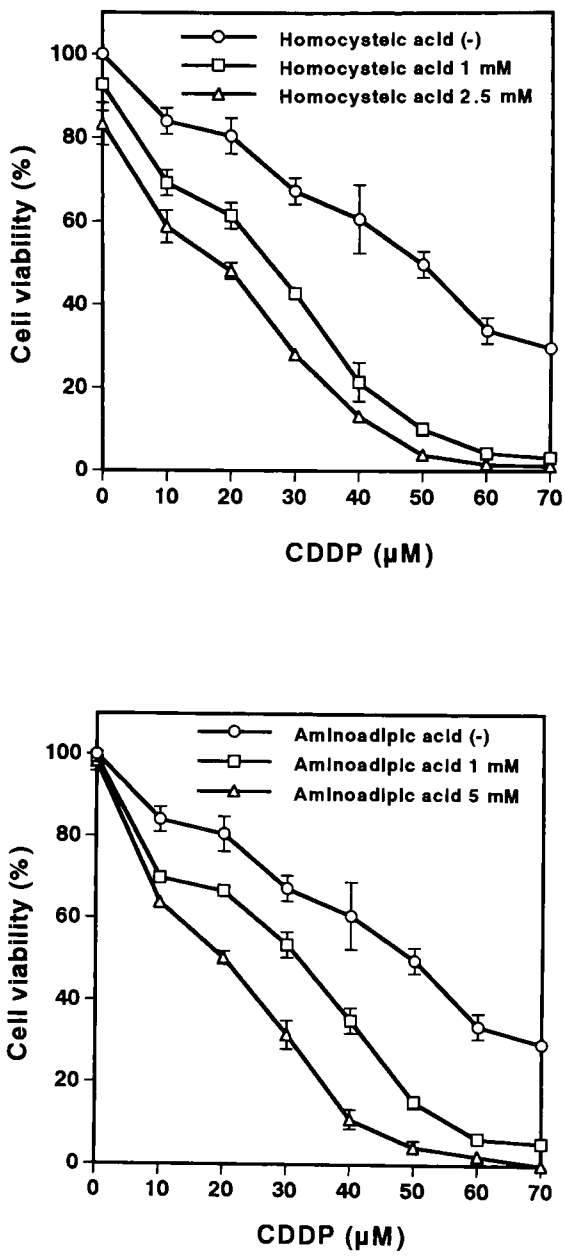

FIG. 4. The effect of L-cystine uptake inhibitors on the CDDP-induced cytotoxicity in the CDDP-resistant HeLa cells. (**A**) HeLa-CP7 cells were cultured with the indicated doses of CDDP in the absence or presence of 1 m*M* or 2.5 m*M* L-homocysteic acid for 48 hr, and cell viability was determined by MTT assay. Data are expressed as the percentage of the absorbance obtained from the untreated cells. Mean and SD of triplicated cultures are shown. Results are representative of three similar experiments. (**B**) HeLa-CP7 cells were cultured with the indicated doses of CDDP in the absence or presence of 1 m*M* or 5 m*M* DL-α-aminoadipic acid for 48 hr, and cell viability was determined by MTT assay. Data are expressed as the percentage of the absorbance obtained from the untreated cells. Mean and SD of triplicated cultures are shown. Results are representative of three similar experiments.

that correlates with the sensitivity to CDDP in cancer cells.

Intracellular transport of amino acids has been thought to be mediated by the specific systems acting on discrete amino acid groups as the substrate (Oxender and Christensen, 1963; Christensen, 1979). L-Cystine is transported through the system x_c^- that is also responsible for the intracellular uptake of other anionic amino acids such as L-glutamate, homocysteic acid, and aminoadipic acid (Bannai and Kitamura, 1980; Bannai and Ishii, 1982; Bannai, 1984; Miura *et al.*, 1992; Meier and Issels, 1995). In the present study, by the analysis of competitive inhibition with other amino acids, we suspect that the L-cystine uptake in the CDDP-resistant HeLa cells is mediated mainly through the system x_c^- (Table 4). However, the CDDP-resistant cells were found to have an enhanced uptake activity of L-cystine, but not of L-glutamate (Table 2), which shares the transport system x_c^- with L-cystine, suggesting that the uptake of L-cystine is selectively activated in these resistant cells. This finding suggests that increase in the L-cystine uptake capability in the CDDP-resistant cells seems not to be explained simply by the enhanced expression of the x_c^- transporter protein itself. Interestingly, in accordance with this finding, we found that extracellular administration of rTRX enhances the uptake activity of L-cystine, but not of L-glutamate (data not shown). These observations may support the critical role of extracellular TRX in the selective enhancement of L-cystine uptake activity in the CDDP-resistant cells.

Several studies demonstrated that oxidative stress induces an adaptive increase in the activity of system x_c^- to enhance L-cystine uptake and maintain cellular defense mechanisms against oxidative stress (Miura *et al.*, 1992; Sato *et al.*, 1998). Because TRX is known to be a stress-inducible protein, whose expression is enhanced by various types of oxidative stresses, *e.g.*, hydrogen peroxide, UV irradiation, and CDDP (Nakamura *et al.*, 1994; Sachi *et al.*, 1995; Sasada *et al.*, 1996; Taniguchi *et al.*, 1996), overexpressed TRX may be, at least in part, related to the adaptive increase in the activity of system x_c^- induced by oxidative stress.

Recent reports have indicated that TRX has several specific target molecules, such as NF-κB (Okamoto *et al.*, 1992), redox factor-1 (Hirota *et al.*, 1997; Ema *et al.*, 1999), and apoptosis signal-regulating kinase 1 (Saitoh *et al.*, 1998), and directly regulates their functional activities. Therefore, it seems likely that the L-cystine transporter is also one of the specific target molecules for TRX. Sato *et al.* (1999) have recently cloned the transport system x_c^-, which is composed of two distinct proteins, from activated mouse macrophages. Because both components of the transport system x_c^- are reported to have a conserved cysteine residue to form heterodimer through the disulfide bond (Sato *et al.*, 1999), it is an interesting possibility that the dithiol/sulfide formation by these cysteine residues is regulated by extracellular TRX. Further investigation is needed to address the molecular and functional association between TRX and the L-cystine transporter.

Recently, Gout *et al.* (1997) have also demonstrated that increased L-cystine uptake capability is associated with malignant progression of rat lymphoma cell lines. Although multiple mechanisms may be involved in cellular resistance to CDDP, our findings suggests that the L-cystine uptake capability regulated by secreted TRX is one of the important determinants for cellular sensitivity to CDDP. Therefore, the L-cystine transport system, as well as the TRX system, appears to provide a novel therapeutic target in CDDP-resistant cancer cells. Development of pharmacologic or genetic methods to modulate the activity of the TRX system and/or the L-cystine transporter may be a rational therapeutic strategy to overcome the CDDP resistance, which could be clinically applicable to cancer treatment.

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ABBREVIATIONS

CDDP, *cis*-Diamminedichloroplatinum (II); DMEM, Dulbecco's modified Eagle medium; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; FCS, fetal calf serum; GSH, glutathione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazorium bromide; rTRX, recombinant human thioredoxin; TRX, thioredoxin.

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Address reprint requests to:
Dr. J. Yodoi
Department of Biological Responses
Institute for Virus Research
Kyoto University
53 Kawahara-cho, Shogoin, Sakyo-ku
Kyoto 606-8507, Japan

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