Biochemical Pharmacology, Vol. 57, pp. 545–548, 1999.

ISSN 0006-2952/99/$–see front matter
PII S006-2952(98)00328-1

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SHORT COMMUNICATION

Reduced Sensitivity of HeLa Cells to *cis*-Platinum by Simultaneous Overexpression of Copper, Zinc-Superoxide Dismutase and Catalase

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ABSTRACT. The overexpression of catalase or Cu,Zn-superoxide dismutase (Cu,Zn-SOD) did not affect the sensitivity of HeLa cells to *cis*-platinum. However, the cytotoxicity of *cis*-platinum was depressed significantly by the simultaneous overexpression of catalase and Cu,Zn-SOD. We concluded that *cis*-platinum accelerated the generation of superoxide anion in the cells, and the superoxide anion produced was converted into H_2O by the cooperative roles of catalase and Cu,Zn-SOD. BIOCHEM PHARMACOL **57**;5:545–548, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *cis*-platinum; resistance; reactive oxygen species; HeLa cells; catalase; superoxide dismutase

The enhancement of lipid peroxidation by *cis*-platinum has been observed by several investigators [1–4]. Reactive oxygen species are believed to be initiators of peroxidative damage. *cis*-Platinum inhibits the activity of antioxidant enzymes such as catalase and superoxide dismutase, both *in vivo* and *in vitro* [5–8]. The protective effect of antioxidant agents against the toxicity of *cis*-platinum has also been reported [2, 5, 6, 9–13]. These experimental results suggest the possibility that an increase in the formation of reactive oxygen species may be one of the causes of the toxicity of *cis*-platinum. However, very little direct evidence supporting the involvement of reactive oxygen species in the cytotoxicity of *cis*-platinum has been presented. In the present study, to investigate the role of reactive oxygen species in the cytotoxicity of *cis*-platinum, we established cell lines that constitutively expressed exogenous genes for antioxidant enzymes such as catalase and Cu,Zn-SOD** and examined the sensitivity of these transfected cells to *cis*-platinum.

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***Abbreviations*: SOD, superoxide dismutase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified minimum essential medium; and FBS, fetal bovine serum.

Received 19 May 1998; accepted 21 September 1998.

MATERIALS AND METHODS

Chemicals

cis-Platinum was supplied by the Nippon Kayaku Co., Ltd. and was dissolved in saline. Other chemicals were purchased from Wako Pure Chemical Industries.

Gene Transfection

HeLa S3 cells were maintained in DMEM containing 10% FBS and kanamycin (60 μg/mL). Plasmid pcDL-SRα 296 containing SRα promoter [14] was used as an expression vector. cDNA of mouse catalase or mouse Cu,Zn-SOD, obtained by cloning from the BALB/c mouse liver cDNA library, was inserted downstream of the SRα promoter region of pcDL-SRα 296. Exponentially growing HeLa-S3 cells were cotransfected with each expression vector and pSV2-gpt containing a gene for xanthine-guanine phosphoribosyl transferase (Ecogpt), using the calcium phosphate precipitation method [15]. After 12 hr of incubation, the cells were rinsed, refed with growth medium, and then incubated at 37° for 24 hr. Then 1×10^6 cells were distributed to each of five 10-cm dishes, and the transfectants were selected by cultivation for 10 days in medium containing 10% FBS, xanthine sodium salt (250 μg/mL), hypoxanthine (15 μg/mL), adenine (25 μg/mL), L-glutamine (150 μg/mL), thymidine (10 μg/mL), and mycophenolic acid (10 μg/mL) [16]. Then, mycophenolic acid-resistant clones were isolated and screened to obtain clones

overexpressing transfected genes by limiting dilution and determining the enzyme activities. The clones that overexpressed the gene for catalase or Cu,Zn-SOD were designated HeLa/CL 12 and HeLa/CL 47 or HeLa/Cu,ZnSOD 10 and HeLa/Cu,ZnSOD 11, respectively. To establish clones simultaneously overexpressing genes for catalase and Cu,Zn-SOD, cDNA of Cu,Zn-SOD was introduced into HeLa/CL 12 cells with pSV2-neo [17] in the same way as above, and the clones resistant to G418 (200 μg/mL) were selected.

Determination of Enzyme Activity

Enzyme activities in the lysate of the cells were determined spectrophotometrically. Catalase activity was determined by the method of Johansson and Borg [18]. SOD activity was measured as the percent inhibition of superoxide formation induced by the xanthine–xanthine oxidase system [19]. Cu,Zn-SOD activity was estimated by subtraction of Mn-SOD activity measured in the presence of 5 mM potassium cyanide from total SOD activity measured without potassium cyanide. The concentration of metallothionein was determined using a ^{203}Hg-binding assay [20]. The GSH concentration was determined by the method of Tanaka-Kagawa *et al.* [21], using high-performance liquid chromatography. The protein concentration was estimated according to the method of Bradford [22].

Cytotoxicity Measurements

The sensitivity of the transfected cells to *cis*-platinum was determined by the MTT assay. The transfected cells (1×10^4 cells/well) were suspended in DMEM containing 10% FBS and kanamycin (60 μg/mL), and 100-μL aliquots were dispensed into each well of a 96-well plate, and cultured for 24 hr at 37°. Then these cells were incubated for 20 hr in the presence of various concentrations of *cis*-platinum. After incubation, 10 μL of MTT (5 mg/mL) was added, and the cells were incubated for an additional 2 hr. The resultant formazan product was solubilized in 100 μL of 20% SDS solution containing 50% *N,N*-dimethylformamide (pH 4.7), and the concentration was measured spectrophotometrically at 550 nm.

RESULTS AND DISCUSSION

Catalase has been known to decompose hydrogen peroxide [23]. Cu,Zn-SOD is an enzyme that dismutates superoxide anion into oxygen and hydrogen peroxide [24]. In the present study, we established clones of HeLa cells that overexpress catalase (HeLa/CL 12 and HeLa/CL 47), Cu,Zn-SOD (HeLa/Cu,ZnSOD 10 and HeLa/Cu,ZnSOD 11), or both of them (HeLa/CLSOD 8 and HeLa/CLSOD 22) by a stable transfection method. Growth rates of these clonal derivative lines determined by doubling time were not significantly different from the parent HeLa cells. As shown in Table 1, each selected transfectant manifested higher activity of catalase, Cu,Zn-SOD, or both of them. The overexpression of these genes was also confirmed by increased levels of each mRNA determined by northern blotting analysis (data not shown). Figure 1 shows the sensitivity of these clones to *cis*-platinum. Overexpression of catalase or Cu,Zn-SOD did not affect the sensitivity of HeLa cells to the toxicity of *cis*-platinum. However, HeLa cell derivatives that overexpressed both catalase and Cu,Zn-SOD exhibited marked resistance to *cis*-platinum.

TABLE 1. Activity of catalase and Cu,Zn-SOD in transfected HeLa cells

Cell strain	Catalase (HCHO formed μmol/min/mg protein)	Cu,Zn-SOD (units/mg/protein)
HeLa	0.59 ± 0.18 (1.0)	126 ± 18 (1.0)
HeLa/gpt	0.74 ± 0.19 (1.3)	132 ± 11 (1.0)
HeLa/CL 12	2.88 ± 0.02 (4.9)	152 ± 12 (1.2)
HeLa/CL 47	2.14 ± 0.41 (3.6)	146 ± 26 (1.2)
HeLa/Cu,ZnSOD 10	0.53 ± 0.07 (0.9)	288 ± 30 (2.3)
HeLa/Cu,ZnSOD 11	0.82 ± 0.17 (1.4)	430 ± 44 (3.4)
HeLa/CLSOD 8	2.19 ± 0.16 (3.7)	280 ± 17 (2.2)
HeLa/CLSOD 22	1.85 ± 0.31 (3.1)	255 ± 29 (1.8)

HeLa cells were transfected wth cDNA of either catalase (HeLa/CL 12 and 47), or Cu,Zn-SOD (HeLa/Cu,ZnSOD 10 and 11) alone or in combination (HeLa/CLSOD 8 and 22). HeLa/gpt is a control clone which transfected with empty vector and pSV2-gpt. Each value represents the mean ± S.D. (N = 3).

Numbers in parentheses indicate relative activities.

An increase in the cellular concentration of metallothionein [25] or glutathione [26, 27] has been reported to result in resistance of tumor cells to *cis*-platinum. Boogaard *et al.* [28] suggested that peroxidation may contribute to nephrotoxicity induced by *cis*-platinum, and the antioxidant properties [29] of metallothionein are responsible for the reduction of *cis*-platinum toxicity. The concentrations of metallothionein and GSH in each transfected HeLa cell line used in this study were not higher than those of the parent HeLa cells (data not shown). The difference in uptake of platinum by tumor cells during 1 hr after the addition of *cis*-platinum has also been demonstrated to be involved in acquired resistance to *cis*-platinum [30]. Therefore, we determined platinum concentrations in the transfected HeLa cell lines from 1 to 8 hr after the addition of *cis*-platinum by flameless atomic absorption spectrophotometry. No significant decrease in the accumulation of platinum in comparison with that of the parent HeLa cells was observed in any of the transfected HeLa cell lines examined (data not shown).

The present results appear to directly support the hypothesis that generation of reactive oxygen species is involved in the manifestation of *cis*-platinum cytotoxicity, although the toxicity of *cis*-platinum was protected by the simultaneous overexpression of catalase and Cu,Zn-SOD, but not by the overexpression of one of them. A significant decrease in the activities of Cu,Zn-SOD [5–8] has been observed in the kidneys of animals after the administration of *cis*-platinum, which induces characteristic renal toxicity. Reduction of Cu,Zn-SOD activities may induce a decline of

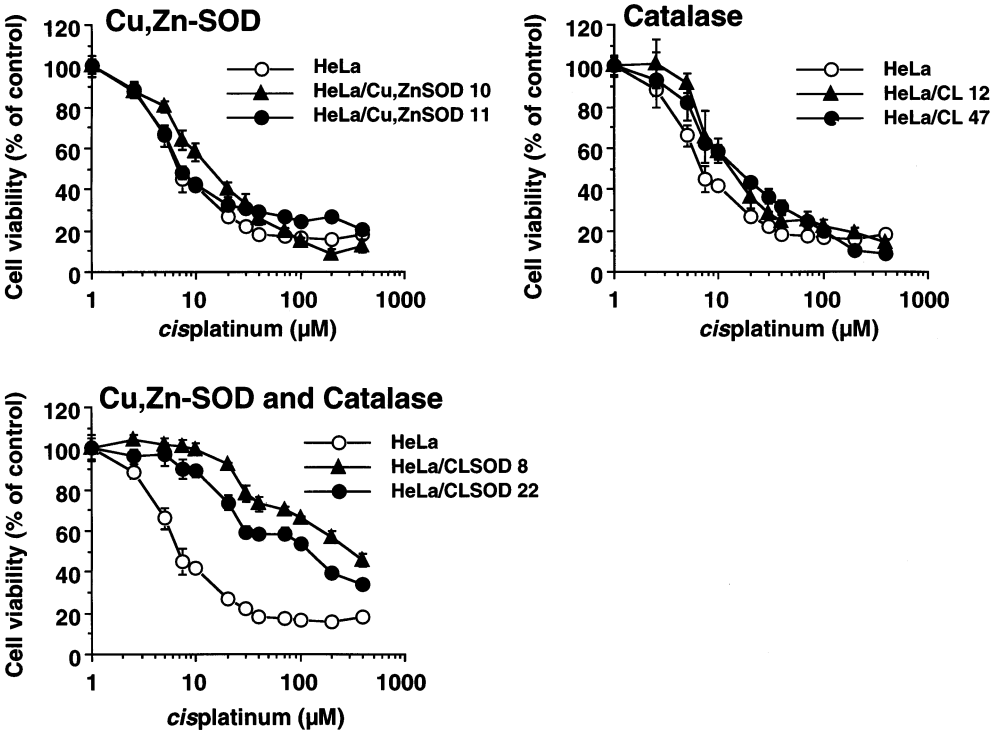

FIG. 1. Effect of *cis*-platinum on the cell viability of HeLa cells transfected with cDNA of either catalase, Cu,Zn-SOD, or both of them. The transfected HeLa cells (1×10^4) in 100 μL of medium were plated into each well of a 96-well plate and cultured at 37°. After a 24-hr incubation, these cells were further incubated for 20 hr in the presence of various concentrations of *cis*-platinum. Cell viability was determined by the MTT assay. Each point represents the mean ± SD (N = 3). The sensitivity of HeLa/gpt cells (see Table 1) to *cis*-platinum was not significantly different from that of parent HeLa cells.

the defense potency of cells against the toxicity of reactive oxygen species generated by *cis*-platinum and accelerate the manifestation of the toxic effects of this drug. In the present study, reduction of the sensitivity of cells to *cis*-platinum was observed only in HeLa cells transfected with cDNAs of catalase and Cu,Zn-SOD simultaneously. Cu,Zn-SOD catalyzes the dismutation of superoxide anion into hydrogen peroxide, one of the toxic reactive oxygen species, and catalase decomposes hydrogen peroxide into H_2O. It is possible to conclude that *cis*-platinum may accelerate the generation of superoxide anion and result in cell injury. In cells transfected with cDNA of Cu,Zn-SOD alone, hydrogen peroxide may be overproduced by the dismutation of the superoxide anion because of increased Cu,Zn-SOD activity and may cause cell injury. Amstad *et al.* [31] suggested that the balance of activities of catalase and Cu,Zn-SOD is more important for the overall sensitivity of cells to superoxide anion than the level of Cu,Zn-SOD alone. Therefore, we concluded that *cis*-platinum accelerated the generation of superoxide anion in the cells, and the superoxide anion produced was converted to H_2O by the harmonized cooperative roles of catalase and Cu,Zn-SOD.

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan

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