—**Short Communications**—

Cisplatin May Induce Frataxin Expression

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Abstract

Cisplatin is a widely used drug in cancer chemotherapy and resistance to cisplatin is a major limitation for its successful application. Intracellular inactivation of cisplatin and detoxification of reactive oxygen species (ROS) by glutathione (a crucial cellular antioxidant) is a mechanism for cisplatin resistance. During cDNA microarray analyses of differential gene expression between a cisplatin-resistant A2780CP70 human ovarian carcinoma cell line and its parental A2780 cell line, we discovered that frataxin gene expression was frequently overexpressed in the cisplatin-resistant variant. Decreased expression of frataxin protein is associated with Friedreich's ataxia (FRDA) which is a neurodegenerative disease involving ROS-mediated cellular damage. Recent evidence suggests that frataxin might detoxify ROS via activation of glutathione peroxidase and elevation of thiols. To exploit potential involvement of frataxin gene in the development of resistance to cisplatin, we compared the levels of frataxin gene and protein in the cisplatin-resistant A2780CP70 ovarian carcinoma cell line and its parental A2780 cell line. We found that frataxin mRNA and protein expressions were elevated in the cisplatin-resistant cells. Our results suggest a potential role for cisplatin as an inducer of frataxin expression and implies that this gene may be a potential target for modulating the response to cisplatin. This is the first report showing an association between frataxin exprression and cisplatin resistance. (J Nippon Med Sch 2003; 70: 367–371)

Key words: Cisplatin, resistance, reactive oxygen species, glutathione, Friedreich's ataxia, FRDA, frataxin, ovarian carcinoma cell line A2780

Introduction

Cisplatin is a widely used chemotherapeutic agent against various types of malignant tumors.[1] Treatment of tumor cells with cisplatin provokes several responses including membrane peroxidation,[2] dysfunction of mitochondria,[3] inhibition of protein synthesis and DNA damage.[4] The cytotoxic action of cisplatin is thought to be mediated through the formation of platinum-DNA adducts, which inhibit DNA replication and/or transcription.[5] In addition, glutathione, the most abandant intracellular thiol and a critical cellular antioxidant, executes intracellular inactivation of cisplatin and detoxification of reactive oxygen species (ROS) as a mechanism for cisplatin resistance.[6]

During cDNA microarray analyses of differential gene expression between cisplatin-resistant A2780 CP70 human ovarian carcinoma cells and the parental A2780 cells, we discovered that frataxin gene expression was frequently overexpressed in the cisplatin-resistant variant (unpublished data). A decreased expression of frataxin protein leads to the

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development of Friedreich's ataxia (FRDA) which is an autosomal recessive neurodegenerative disease causing limb and gait ataxia and cardiomyopathy.[7] This disease is caused by the expansion of a GAA trinucleotide repeat located in the first intron of the frataxin gene, resulting in decreased levels of frataxin mRNA and protein.[8] Frataxin deficiency involves ROS-mediated cellular damage. Recent evidence suggests that frataxin might detoxify ROS via activation of glutathione peroxidase and elevation of thiols,[9] a mechanism similar to that utilized by cisplatin-resistant cells.

The purpose of this study was to exploit potential involvement of frataxin gene in the development of resistance to cisplatin that might implicate cisplatin as an inducer of frataxin expression. To assess this, we compared the levels of frataxin mRNA and protein in a human ovarian carcinoma cell line (A 2780) and its cisplatin-resistant variant (A2780CP70).

Materials and Methods

Chemicals and reagents. Cisplatin was obtained from Bristol-Myers Squibb K.K. Monoclonal antibody to frataxin (immunogen: TrpE-Frataxin full length) was purchased from Chemicon International, Temecula, CA. Other reagents including normal goat serum and alkaline phosphatase conjugated and FITC-labeled goat anti-mouse immunoglobulins (IgGs) were from Dako Japan. The NBT and BICP color development reagents were from Sigma, MO.

Cell lines. The human ovarian adenocarcinoma cell lines A2780 and A2780CP70 (obtained from Dr. Thomas C. Hamilton, Fox Chase Cancer Center, Philadelphia, PA) were maintained at 37℃ in a humidified incubator containing 5% CO_2, in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/m*l* penicillin, and 100 μg/m*l* streptomycin. A2780 is a cisplatin-sensitive cell line (IC_{50} 0.32 μg/m*l*) and A2780CP70 is a cisplatin-resistant cell line (IC_{50} 5.5 μg/m*l*).[10]

Semiquantitative RT-PCR Analysis. Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA concentration was determined by UV spectrophotometry. Aliquots of total cellular RNA (1.0 μg) were subjected to first-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and the cDNA was diluted five times with water. One μ*l* of the diluted cDNA was used for each PCR reaction. The PCR primer set used for frataxin was: forward, 5'-AGCATGTGGACTCTCGGGCGCC-3' and reverse, 5'-GCATCTTTTCCGGAATAGGCC-3'; the product size, 633bp. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in RT-PCR reaction with primers: forward, 5'-TCCACCACCCTGTTGCTGTA-3' and reverse, 5'-ACCACAGTCCATGCCATCAC-3'; the product size, 450 bp. PCR was carried out for 24 cycles consisting of heat denaturation (94℃ for 1 min), annealing (55℃ for 1 min), and extention (72℃ for 3 min). The PCR products were electrophoresed on 2% agarose gels containing 0.1 mg/m*l* of ethidium bromide, and the gels were photographed under ultraviolet illumination. Semiquantitative analysis was done by densitometric scanning based on the standard curves constructed for frataxin and GAPDH mRNAs. The analyses were performed using Molecular Imager FX and Quantity One Software Package (Bio-Rad Labs., Hercules, CA).

Western blot. Total protein extracts were prepared from cisplatin-resistant A2780CP70 cells and the parental A2780 cells by homogenization in 50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 5 mM EDTA, 150 mM KCl, 1 mM phenylmethylsulphonyl fluoride followed by sonication. Extracts were normalized for total protein content and used for Western blotting (50 μg protein/lane) with anti-frataxin antibody. After electrophoresis on SDS-PAGE and transfer to polyvinylidene difluoride (PVDF) membrane, the membrane was blocked with 5% skimmed milk in buffer [0.5 M NaCl, 10 mM Tris-HCl (pH 8.2), and 0.5 % Tween 20, 0.2% sodium azide] for 1 h at room temperature and then incubated overnight at 4℃ with 1:5000 diluted monoclonal anti-frataxin. The membrane was washed 3 times with the above buffer and after incubation with alkaline phosphatase conjugated goat anti-mouse IgGs, the reaction was developed with the NBT and BICP color development reagents. Western blot bands

were also semiquantified by densitometric scanning.

Immunofluorescence microscopy. Cells were grown in glass chamber slides and then fixed with 4% buffered paraformaldehyde, pH 7.4, for 1 h. Cells were washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min, washed again and incubated sequentially with 1: 10 normal goat serum for 15 min, 1: 200 mouse monoclonal anti-frataxin for 1 h, and 1: 40 FITC-labeled goat anti-mouse IgGs for 30 min. After washes in PBS, nuclei were stained with 4,6-diamino-2-phenylindole (1 μg/m*l* in PBS). Coverslips were mounted onto slides and the cells were observed under an immunofluorescence microscope.

Statistics

F-test was used to compare the mean intensities of frataxin mRNA and protein, and a P value less than 0.05 was considered as significant.

Results

Frataxin mRNA levels in cisplatin-resistant A2780CP70 cells and the parental A2780 cells were measured by a semiquantitative RT-PCR analysis. The mean intensity value of frataxin mRNA expression in cisplatin-resistant A2780CP70 cells was estimated to be 30.3 ± 16.7 and that in the parental A2780 cells was 7.0 ± 2.6 which were significantly different by the F-test ($F = 4.25$; $P < 0.00007$; **Fig. 1**). Relative frataxin protein contents were assessed by Western blot. The extracts prepared from cisplatin-resistant A2780CP70 cells and the parental A2780 cells were normalized for total protein content and used for Western blotting at 50 μg protein/lane with the anti-frataxin antibody. The mean intensity value of frataxin protein expression in A2780CP70 cells was estimated to be 14.18 ± 2.30 and that in the parental A2780 cells was 6.61 ± 1.05 which were significantly different ($F = 4.79$; $P = 0.03$; **Fig. 2**). To confirm the intracellular expression of frataxin in the two cell lines, immunofluorescence staining for frataxin was performed. An enhanced expression of frataxin protein was detected in cisplatin-resistant A2780CP70 ovarian carcinoma cells as compared to the parental A2780 cells, and the staining was appeared to be mainly associated with the mitochondria (**Fig. 3**).

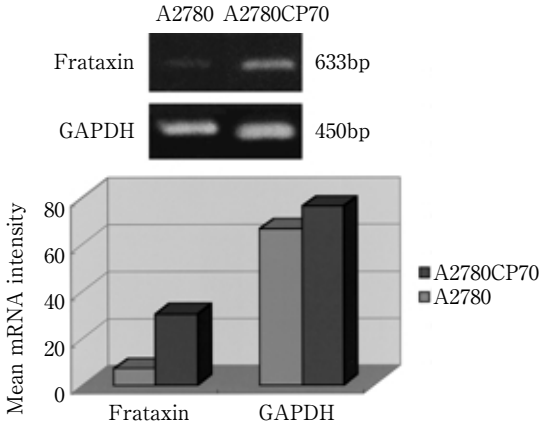

Fig. 1 Semiquantitative RT-PCR analysis of frataxin mRNA. The mean intensity value of frataxin mRNA expression in cisplatin-resistant A2780CP70 cells was significantly higher than that in the parental A2780 cells ($P = 0.00007$). GAPDH was used as an internal control.

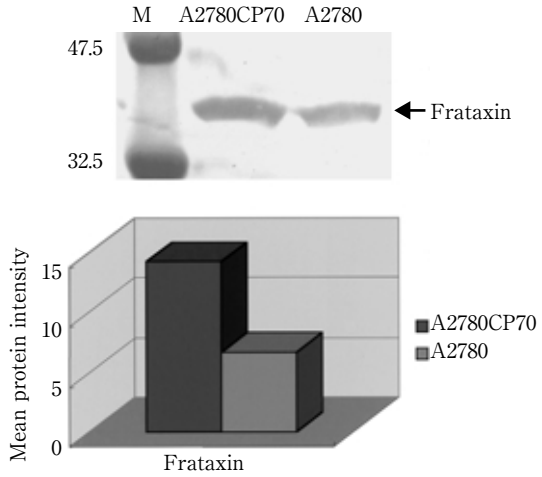

Fig. 2 Relative frataxin protein contents were assessed by Western blot at 50 μg protein/lane. The mean intensity value of frataxin protein expression in A2780CP70 cells was significantly higher than that in the parental A2780 ($P = 0.03$).

Discussion

The major finding of our study was the discovery that expression of frataxin mRNA and protein was increased in cisplatin-resistant ovarian carcinoma cells. This is the first report that shows an

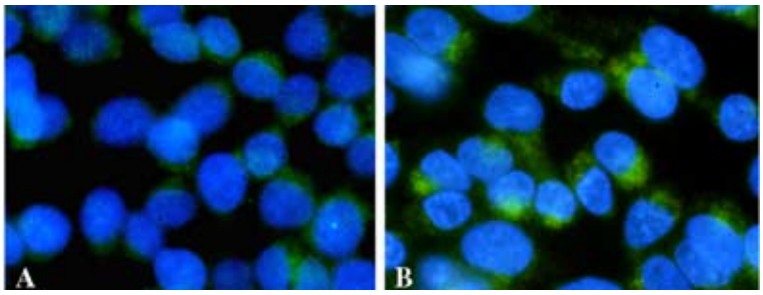

Fig. 3 Intracellular localization of frataxin was examined by immunofluorescence staining. There was an enhanced expression of frataxin protein in cisplatin-resistant A2780CP70 ovarian carcinoma cells (B) as compared to the parental A2780 cells (A). The staining appeared to be mainly associated with the mitochondria.

association between frataxin expression and cisplatin resistance which suggests cisplatin as an inducer of frataxin gene. Although the mechanism(s) underlyng this effect is yet to be defined, several lines of evidence suggest a protective role for it against cellular damage.

Normal cellular metabolism is associated with the production of reactive oxygen species (ROS) by mitochondria, and consequently, damage to DNA and proteins[11,12] which under certain conditions induces apoptosis and necrosis. Mitochondria are dependent on glutathione to detoxify ROS and prevent oxidative damage.[13] ROS-mediated cell damage has been associated with both necrosis and apoptosis.[14] Excessive formation of ROS as well as depletion of cellular antioxidants resulted in apoptosis.[15] ROS has been shown to be associated with death receptor-mediated apoptotic pathways such as by modulating the expression of Fas or Fas ligand.[16] These data suggest that ROS damage induces cell death.

In ovarian cancer cells, multiple mechanisms of cisplatin resistance have been proposed, including decreased drug accumulation, increased drug inactivation, enhanced repair of platinum-DNA damage, and tolerance to DNA damage.[17] A mechanism by which cisplatin exerts its cytotoxicity is through the generation of ROS.[18] On the other hand, intracellular inactivation of cisplatin by glutathione has been proposed as a mechanism of cisplatin resistance.[6] Glutathione is the most abundant intracellular thiol and acts as a crucial cellular antioxidant.[19] The reduction-oxidation (redox) state of a cell is largely determined by the balance between generated ROS and endogenous expression of thiol buffers such as glutathione.[20] Glutathione is necessary for resistance to oxidative stress through detoxification of ROS. It can also detoxify many endogenous toxins, including cisplatin, through the formation of glutathione adducts.[6,21] Inhibition of intracellular glutathione by buthionine sulfoximine in cancer cell lines was shown to increase cisplatin sensitivity, but induction of glutathione production by *N*-acetyl cysteine led to increased cisplatin resistance.[22] In addition, the glutathione content of tumor cells has been correlated with cisplatin resistance.[23] These results indicate that glutathione may protect cells from ROS damage.

Increasing evidence suggests a role for frataxin in promoting cellular defense against ROS[24] and that frataxin might detoxify ROS.[25] Mutation in frataxin gene leading to the deceased expression of frataxin conferred cellular sensitivity to oxidant stress which was rescued by chelators of iron and calcium and inhibitors of apoptosis.[24] In a mouse model, transgenic overexpression of human frataxin increased cellular antioxidant defense via activation of glutathione peroxidase and elevation of reduced thiols.[9] These data suggest that frataxin may activate glutathione to protect cells from ROS damage. Taken together, these observations demonstrate that glutathione shares a role in both mechanisms of frataxin function and cisplatin resistance. It is likely that repeated oxidative stress during acquisition of resistance to cisplatin might have regulated the glutathione level at a higher threshold to cope with the cellular ROS detoxification need. To accomplish this task, the frataxin gene might have been recruited.

In summary, we hypothesize that in cisplatin-resistant A2780CP70 ovarian carcinoma cells, increased frataxin expression may promote resistance to cisplatin through mechanisms that protect the cells from ROS damage. Additionally, as recent studies have shown that chemotherapeutic agents including cisplatin may reduce expanded triplet repeats during DNA damage and subsequent repair which may delay the onset and reduce the severity of disease[26] and because FRDA is a disease with expanded triplet repeats, we are currently studying the effect of cisplatin on frataxin expression in fibroblast cell cultures from FRDA patients. Our present study suggest that further investigation on frataxin function in relation to the cellular redox balance and cisplatin chemoresistance is warranted.

References

1. Cohen SM, Lippard SJ: Cisplatin: from DNA damage to cancer chemotherapy. Prog Nucleic Acis Res Mol Biol 2001; 67: 93–130.
2. Sugiyama S, Hayakawa M, Kato T, Hanaki Y, Shimizu K, Ozawa T: Adverse effects of anti-tumor drug, cisplatin, on rat kidney mitochondria: disturbances in glutathione peroxidase activity. Biochem Biophys Res Commun 1989; 159: 1121–1127.
3. Brady HR, Kone BC, Stromski ME, Zeidel ML, Giebisch G, Gullans SR: Mitochondrial injury: an early event in cisplatin toxicity to renal proximal tubules. Am J Physiol 1990; 258: F1181–F1187.
4. Kharbanda S, Ren R, Pandey P, Shafman TD, Feller SM, Weichselbaum RP, Kufe DW: Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. Nature (Lond) 1995; 376: 785–788.
5. Zamble DB, Lippard SJ: Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem Sci 1995; 20: 435–439.
6. Ishikawa T, Ali-Osman F: Glutathione-associated *cis*-diamminedinechloroplatinum (II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. J Biol Chem 1993; 268: 20116–20125.
7. Harding AE: Friedreich's ataxia: a clinical and genetic study of 90 families with an analysis of early diagnostic criteria and intrafamilial clustering of clinical features. Brain 1981; 104: 589–620.
8. Montermini L, Richter A, Morgan K, Justice CM, Julien D, Castellotti B, Mercier J, Poirier J, Capozzoli F, Bouchard JP, et al: Phenotypic variability in Friedreich's ataxia: role of the associated GAA triplet repeat expansion. Ann Neurol 1997; 41: 675–682.
9. Shoichet SA, Baumer AT, Stamenkovic D, Saucer H, Pfeiffer AFH, Kahn CR, Muller-Wieland D, Richter C, Ristow M: Frataxin promotes antioxidant defense in a thiol-dependent manner resulting in diminished malignant transformation in vitro. Hum Mol Genet 2002; 11: 815–821.
10. Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME: High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathiopne synthasis. Proc Natl Acad Sci USA 1992; 89: 3070–3074.
11. Croteau D, Bohr V: Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem 1997; 272: 25409–25412.
12. Berlett S, Stadtman: Protein oxidation in ageing, disease and oxidative stress. J Biol Chem 1997; 272: 20313–20316.
13. Davis W Jr, Ronai Z, Tew KD: Cellular thiols and reactive oxygen species in drug-induced apoptosis. J Pharmacol Exp Ther 2001; 296: 1–6.
14. Matsushima H, Yoneyama K, Ohishi K, Hishida A: The role of oxygen free radicals in cisplatin-induced acute renal failure in rats. J Lab Clin Med 1998; 131: 518–526.
15. Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y: Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions. Oncogene 1996; 12: 2045–2050.
16. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ: Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 1993; 75: 241–251.
17. Suhara T, Fukuo K, Sugimoto T, Morimoto S, Nakahashi T, Hata S, Shimizu M, Ogihara T: Hydrogen oeroxide induces up-regulation of Fas in human endothelial cells. J Immunol 1998; 160: 4042–4047.
18. Auersperg N, Edelson MI, Mok SC, Johanson SW, Hamilton TC: The biology of ovarian cancer. Semin Oncol 1998; 21: 281–304.
19. Schroder CP, Godwin AK, O'Dwyer PJ, Tew KD, Hamilton TC, Ozols RF: Glutathione and drug resistance. Cancer Investig 1996; 14: 158–168.
20. Davis W Jr, Ronai Z, Tew KD: Cellular thiols and reactive oxygen species in drug-induced apoptosis. J Pharmacol Exp Ther 2001; 296: 1–6.
21. Meister A: Glutathione metabolism and its selective modification. J Biol Chem 1988; 263: 17205–17208.
22. Yang Z, Faustino PJ, Andrews PA, Monastra R, Rasmussen AA, Ellison CD, Cullen KJ: Decreased cispatin/DNA adduct formation is associated with cispatin resistance in human head and neck cancer cell lines. Cancer Chemother Pharmacol 2000; 46: 255–2262.
23. Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME: High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathiopne synthasis. Proc Natl Acad Sci USA 1992; 89: 3070–3074.
24. Wong A, Yang J, Cavadini P, Gellera C, Lonnerdal B, Taroni F, Cortopassi G: The Friedreich's ataxia mutation confers cellular sensitivity to oxidant stress which is rescued by chelators of iron and calcium and inhibitors of apoptosis. Hum Mol Genet 1999; 8: 425–430.
25. Schulz JB, Dehmer T, Schols L, Mende H, Hardt C, Vorgerd M, Burk K, Matson W, Dichgans J, Beal MF, Bogdanov MB: Oxidative stress in patients with Friedreich's ataxia. Neurology 2000; 55: 1719–1721.
26. Hashem VI, and Sinden RR: Chemotherapeutically induced deletion of expanted triplet repeats. Mutat Res 2002; 508: 107–119.

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