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Cellular sensitization to cisplatin and carboplatin with decreased removal of platinum-DNA adduct by glucose-regulated stress

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Abstract *Purpose*: Stress conditions, such as glucose starvation and hypoxia, that induce glucose-regulated proteins (GRPs) in cells, are seen in most solid tumors. These conditions have been shown to cause cellular resistance to multiple anticancer drugs, such as etoposide, doxorubicin, and camptothecin. We examined the effect of the GRP-inducing conditions on cellular sensitivity to cisplatin and carboplatin, which are widely used drugs against solid tumors. *Methods*: We generated the GRP-inducing culture conditions by exposing cells to 2-deoxyglucose (2DG), calcium ionophore A23187 and tunicamycin, and examined cellular sensitivity to cisplatin and carboplatin under these conditions. We next measured platinum accumulation and DNA-bound platinum in 2DG-stressed cells after cisplatin exposure. *Results*: The GRP-inducing stress conditions led to cellular sensitization to cisplatin and carboplatin. This sensitization was reversible, as the cellular sensitivity returned to normal levels 12 h after removal of 2DG. Platinum accumulation and DNA-bound platinum that were found immediately after exposure to cisplatin for 1 h were slightly increased in 2DG-stressed cells as compared with nonstressed cells. After a drug-free recovery incubation of 8 h, the DNA-bound platinum in the nonstressed cells was reduced by 33% while the amount in the 2DG-stressed cells was sustained at the initial levels. *Conclusions*: These results indicated that the decreased removal of platinum-DNA adducts was associated with increased sensitivity to cisplatin and carboplatin in the stressed cells. The sensitization of cancer cells under the GRP-inducing stress conditions would explain, in part, the clinical potency of platinum drugs against solid tumors.

Key words Glucose-regulated stress · Cisplatin · Carboplatin · Chemosensitization · DNA platination

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Introduction

Solid tumors are relatively resistant to treatment with anticancer drugs. In addition to limited drug access because of decreased blood flow, cellular drug resistance mediated by stress conditions can lead to resistance in solid tumors. The stress conditions that induce glucose-regulated proteins (GRPs), a major family of stress proteins, are glucose starvation, hypoxia and low pH, all of which are normally observed in solid tumors [19, 21, 25]. Indeed, induction of GRP has been observed during the growth of a murine RIF tumor mass [2, 20]. GRP-inducing conditions have been shown to cause cellular resistance to drugs such as etoposide, doxorubicin, camptothecin, and vincristine [5, 9, 14, 23, 27]. Thus, these conditions could be an important mechanism for drug resistance in solid tumors.

Cisplatin is one of the most widely used drugs for the treatment of malignant solid tumors. Cisplatin and

its analog carboplatin are effective against solid tumors, especially head and neck [7], testicular [1], and ovarian [16] cancers. Because of the drug's clinical importance, its mechanism of action is of interest as a subject of study. Most evidence indicates that the adduct formation with DNA is responsible for the antitumor activity of cisplatin and its analogs [18]. Supporting this is the finding that there is a decrease in the platinum-DNA adducts in cisplatin-resistant cancer cells. This type of cisplatin resistance has been explained by multiple mechanisms, including decreased platinum accumulation in cells [15, 17], increased detoxification by elevated glutathione (GSH) and metallothionein levels [8, 12], and enhanced platinum-DNA removal [11, 13, 17]. However, it is still unclear why cisplatin is more effective against solid tumors than other antitumor agents.

In this study, we examined the effect of GRP-inducing conditions on cellular sensitivity to cisplatin and carboplatin. When ovarian cancer RTSG cells were cultured under stress conditions produced by 2-deoxy-D-glucose (2DG), GRPs were induced in the cells. However, the sensitivity of stressed cells, versus nonstressed cells, to cisplatin and carboplatin was unexpectedly enhanced. The other chemical stressors, A23187 and tunicamycin, also caused sensitivity to the drugs. We found that the 2DG-stressed cells had a decreased capacity to remove platinum-DNA adducts. These results demonstrate that GRP-inducing conditions sensitize solid tumor cells to cisplatin and carboplatin in sharp contrast to other drugs. These observations indicate a potential benefit of 2DG in platinum-based chemotherapy.

Materials and methods

Chemicals

Cisplatin and carboplatin were generous gifts from Bristol Myers Squibb, Tokyo, Japan. Etoposide was a gift from Nippon Kayaku Co., Tokyo, Japan. 2DG, calcium ionophore A23187, and tunicamycin were purchased from Wako Pure Chemical Industries, Osaka, Japan. All other reagents were of analytical grade.

Cell lines

The human ovarian cancer cell line RTSG [24] was a generous gift from Professor Shiro Nozawa, Keio University, Tokyo, Japan. Cells were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml kanamycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell treatment and colony-forming ability assay

A colony-forming ability assay was performed essentially as described previously [27]. The stress conditions were produced by 2DG, A23187, or tunicamycin. Exponentially growing cells were treated with 10 m*M* 2DG, 1 μ*M* A23187, or 10 μg/ml tunicamycin for 24 h. For time-course experiments, cells were exposed to 2DG for 6 to 24 h. Release from stress experiments were performed by removing 2DG–containing medium and then incubating the cells in fresh medium. Following exposure to the above stress conditions, cisplatin or carboplatin was added directly to the cell culture at various concentrations. After a 1-h incubation, the cells were replated at an appropriate dilution in drug-free medium. After a 7-day incubation at 37 °C, the colonies were fixed and stained as described previously [22]. Relative survival was corrected for the plating efficiency of the control. The experiments were carried out in triplicate and repeated at least three times.

Immunoblot analysis

Expression of GRP78 was determined by immunoblot analysis using a mouse antihuman GRP78 monoclonal antibody (StressGen Biotechnologies, Victoria, BC, Canada), as described previously [27].

Measurement of intracellular accumulation of cisplatin

Cells were incubated with 10 m*M* 2DG for 24 h and then treated with 60 or 300 μ*M* cisplatin for 1 h. The cells were harvested and washed three times with ice-cold PBS (137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4). The cell pellets were lysed in 60 μl 0.1 *M* NaOH, and the protein content was determined using a Bio-Rad DC Protein Assay Kit. A 150-μl aliquot of 1 *M* HCl was added to 50 μl of the cell lysate, as described by Evans et al. [6]. The platinum content of the mixture was measured using a Hitachi Model Z-8000 flameless atomic absorption spectrophotometer. The platinum accumulation was determined in triplicate and is expressed as nanograms platinum per milligram protein.

Determination of platinum binding to genomic DNA

2DG-treated cells were exposed to 300 μ*M* cisplatin for 1 h. The cells were either harvested immediately or incubated in drug-free medium for 8 h. Genomic DNA of the cells was isolated using a QIAamp Blood Kit (QIAGEN, Chatworth, Calif.), and the ethanol-precipitated DNA was hydrolyzed in distilled water. The DNA content was measured by absorbance at 260 nm, and the platinum content binding to DNA was measured by flameless atomic absorption spectrometry as described above. The amount of platinum binding to DNA was assayed in triplicate and is expressed as nanograms platinum per milligram DNA.

Measurement of GSH content

Cells were harvested with PBS containing 5 m*M* EDTA and washed with ice-cold PBS twice. Cellular GSH content was assayed in triplicate using a GSH-400 assay kit (BIOXYTECH, Marne, France), and the protein content of the sample was assayed using a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, Calif.), as described previously [26]. Cellular GSH content is expressed as nanomoles per milligram protein.

Measurement of cellular ATP

ATP was extracted from monolayer-growing cells in a 12-well plate with 500 μl 1.5% (w/v) trichloroacetic acid. The ATP in a 20-μl aliquot was assayed using an ATP assay kit (Calbiochem-Novabiochem, La Jolla, Calif.) by measuring the luminescence with a TRI-CARB™ Liquid Scintillation Analyzer (Model 2500TR, Packard Instruments, Meriden, Ct.). After extraction of ATP, the cells were lysed in 200 μl 0.2 *M* NaOH. The ATP content was measured in triplicate and corrected by the cellular protein content, using a Bio-Rad DC Protein Assay Kit.

Results

Enhanced sensitivity to platinum-containing drugs caused by glucose-regulated stress conditions

RTSG cells were cultured for 24 h under 2DG stress conditions and then treated for 1 h with cisplatin. The cisplatin sensitivity was determined by a colony-forming ability assay (Fig. 1). 2DG stress enhanced the cisplatin sensitivity of RTSG cells in a dose-dependent manner. The same stress conditions induced resistance to etoposide in RTSG cells (data not shown), as has been observed in other cell lines [9, 27]. The reduction in colony-forming ability as a result of 2DG stress was negligible. Cisplatin sensitization caused by these stress conditions was also observed in other cell lines, including human epidermoid carcinoma A431, ovarian carcinoma A2780, and colon carcinoma HT-29 (data not shown).

We next examined other chemical stressors that induce GRPs. Immunoblot analysis revealed that 24-h treatment with 1 μ*M* A23187 or 10 μg/ml tunicamycin induced GRP78 expression equivalent to that following treatment with 10 m*M* 2DG (Fig. 2). The reduction in colony-forming ability by A23187 was negligible while tunicamycin reduced that ability by about 40%. As shown in Fig. 3, all three stressors sensitized RTSG cells to both cisplatin and carboplatin.

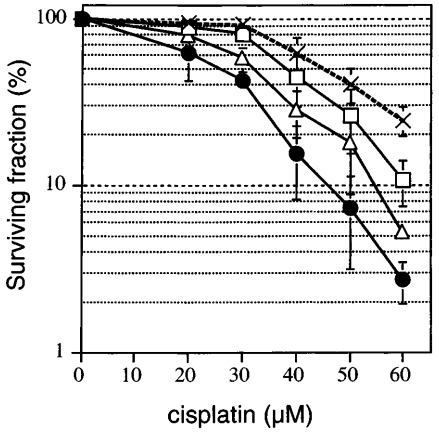

Fig. 1 Cellular sensitivity to cisplatin treated with 2DG. Cells were treated for 24 h without drug (×) or with 2 m*M* (□), 5 m*M* (△), or 10 m*M* (●) 2DG. Subsequently, cells were exposed to cisplatin at various concentrations for 1 h. Points are means of more than three independent determinations; *bars* ± SE

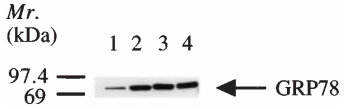

Fig. 2 Induction of GRP78 by chemical stressors. Cells were treated for 24 h without drug (*lane 1*), or with 10 m*M* 2DG (*lane 2*), 1 μ*M* A23187 (*lane 3*), or 10 μg/ml tunicamycin (*lane 4*). A 20-μg sample of each cell lysate was subjected to immunoblot analysis

Correlation between the sensitization to cisplatin and the stressed state of RTSG cells

For further characterization of the stress-induced cisplatin sensitization, we used 2DG as a representative stressor because it proved to be the most effective and the least toxic. We assessed the sensitization kinetics to cisplatin under the 2DG stress conditions (Fig. 4A). Cisplatin sensitization was detectable after a 6-h exposure to 2DG and was increased during longer exposures. Exposures for 12 and 24 h comparably sensitized RTSG cells to cisplatin. The development of cisplatin sensitization was well correlated with induction of GRP78 (Fig. 4B).

When RTSG cells stressed by the 24-h exposure to 2DG were released from the stress conditions, cisplatin sensitization decayed; cellular sensitivity to the drug returned to normal levels within 12 h (Fig. 5A). Similar decay after removal of stress conditions has been shown in stress-induced resistance to etoposide [9, 27].

When RTSG cells were first treated with cisplatin for 1 h and then with 2DG for 24 h, their survival from cisplatin was not affected by the 2DG (Fig. 5B). These results strongly suggest that cells under stress conditions become sensitized to cisplatin.

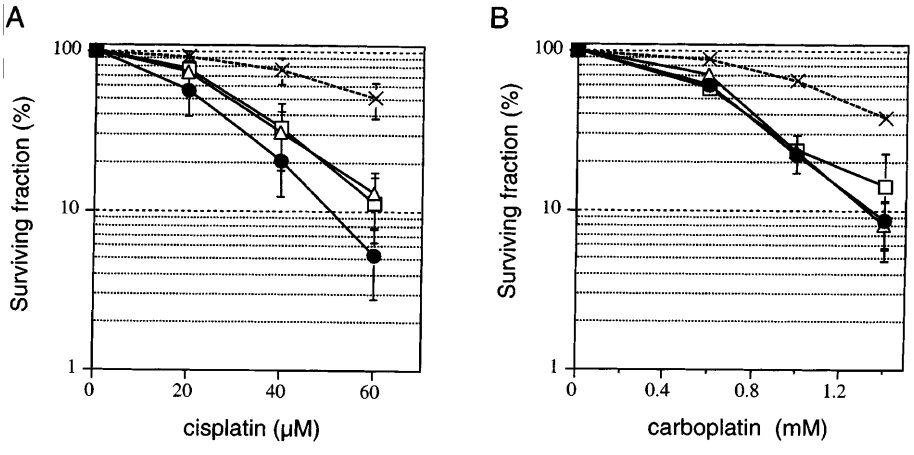

Fig. 3A, B Enhancement of cellular sensitivity to cisplatin (**A**) and carboplatin (**B**) by GRP-inducing stressors. Cells were treated for 24 h without drug (×) or with 10 m*M* 2DG (●), 1 μ*M* A23187 (△), or 10 μg/ml tunicamycin (□). Subsequently, cells were exposed to cisplatin (**A**) or carboplatin (**B**) at various concentrations for 1 h. Points are means of more than three independent determinations; *bars* ± SE

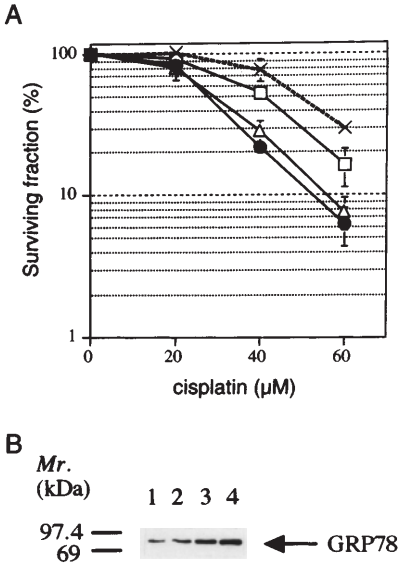

Fig. 4A, B Enhancement of cisplatin sensitivity and induction of GRP78 depending on the exposure time to 2DG. **A** Cells were treated with 10 m*M* 2DG for 0 h (×), 6 h (□), 12 h (△), or 24 h (●). Subsequently, cells were exposed to cisplatin at various concentrations for 1 h. Points are means of more than three independent determinations; *bars* ± SE. **B** Cells were treated with 10 m*M* 2DG for 0 h (*lane 1*), 6 h (*lane 2*), 12 h (*lane 3*), or 24 h (*lane 4*). A 20-μg sample of the cell lysate was subjected to immunoblot analysis with an anti-GRP78 antibody

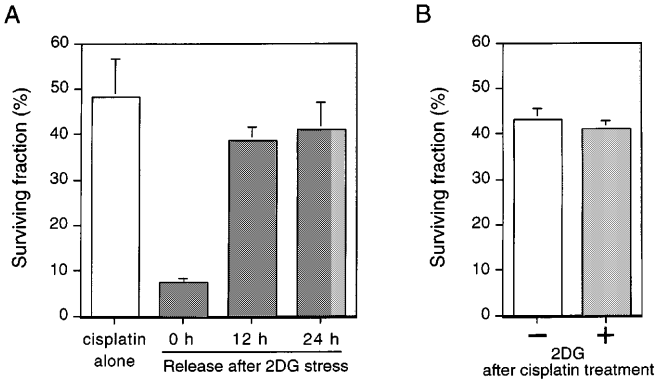

Fig. 5 **A** Recovery of cisplatin sensitivity after release from 2DG stress conditions. Cells were treated without or with 10 m*M* 2DG for 24 h and released from the stress condition by removal of 2DG. After the release, the cells were incubated in fresh medium for the indicated periods. Subsequently, the cells were exposed to 40 μ*M* cisplatin for 1 h. **B** No change in cisplatin sensitivity as a result of treatment with 2DG after exposure to cisplatin. Cells were first exposed to 40 μ*M* cisplatin for 1 h and subsequently treated without (−) or with 10 m*M* 2DG for 24 h (+). Surviving fractions (±SE of three independent determinations) were determined by colony formation

Decreased removal of platinum-DNA adducts in the 2DG-stressed cells

We next examined the mechanisms of cisplatin sensitization of cells under 2DG stress. As shown in Table 1, the intracellular accumulation of cisplatin (extracellular concentration 60 μ*M*) was almost equal between the nonstressed and the 2DG-stressed cells. However, a high dose of cisplatin (300 μ*M*), which increased the measurement sensitivity, revealed that cisplatin accumulation was slightly enhanced in the 2DG-stressed cells (not significant, $P = 0.09$). In agreement with this finding, the amount of platinum binding to genomic DNA immediately after cisplatin exposure was slightly increased in the 2DG-stressed cells ($P = 0.05$; Table 2). After an 8-h recovery incubation in drug-free medium, the DNA-bound platinum in the control cells was reduced by 33% (Table 2). In contrast, the DNA-bound platinum in the 2DG-stressed cells was sustained at the initial levels, indicating that the removal of platinum-DNA adducts was suppressed in the stressed cells.

Cellular GSH and ATP were determined (Table 3), and it was found that 2DG stress decreased the GSH and ATP levels. A23187 and tunicamycin led to a decrease in cellular ATP levels, although neither stressor altered cellular GSH levels. Some proposed mechanisms of cisplatin resistance, such as DNA repair [11, 13, 17] and drug efflux [10], are ATP-dependent. ATP diminution, therefore, may be involved in sensitization to cisplatin.

Table 1 Cellular accumulation of platinum after a 1-h exposure to cisplatin at the indicated doses in RTSG cells treated with 2DG. Values are means ± SD from triplicate experiments

Cisplatin (μ*M*)	Pt accumulation (ng Pt/mg protein)	
	Control	2DG (10 m*M*, 24 h)
60	280 ± 58	294 ± 13
300	1590 ± 193	1880 ± 110

Table 2 DNA platination after a 1-h exposure to 300 μ*M* cisplatin in RTSG cells treated with 2DG. Cells were harvested immediately (0) or 8 h (8) after exposure to cisplatin.Values are means ± SD from triplicate experiments

Drug-free incubation (h)	DNA platination (ng Pt/mg DNA)	
	Control	2DG (10 m*M*, 24 h)
0	57.6 ± 2.6	71.8 ± 6.7
8	38.4 ± 1.3	70.6 ± 1.0*

*$P = 0.00001$ vs control

Table 3 GSH and ATP content of RTSG cells treated with glucose-regulated stress inducers at the indicated doses for 24 h. Values are means ± SD from triplicate experiments

Treatment	GSH (nmol/mg protein)	ATP (nmol/mg protein)
Control	172 ± 4	52.8 ± 1.8
2DG (10 m*M*)	135 ± 7*	34.6 ± 2.0*
A23187 (1 μ*M*)	165 ± 1	43.6 ± 1.6*
Tunicamycin (10 μg/ml)	173 ± 6	38.0 ± 0.3*

*$P < 0.05$ vs control

Discussion

GRP-inducing stress conditions, such as glucose starvation and hypoxia, have been observed in solid tumors. In this study, we have shown that chemical stressors to induce GRP sensitized RTSG cells to cisplatin and carboplatin. Similar cisplatin sensitization was also observed in epidermoid cancer A431, ovarian cancer A2780, and colon cancer HT-29 cells (data not shown), suggesting that this phenomenon is not tumor- or cell-type-specific. It is generally understood that the same stress conditions cause resistance to multiple drugs. For example, resistance to topoisomerase I- and II-targeted drugs has been shown in several cell lines including A2780 and HT-29 [23, 27]. RTSG cells under stress also showed resistance to etoposide, a topoisomerase II-targeted drug (data not shown). This sharp contrast between cisplatin and the other drugs may account, in part, for the clinical potency of platinum drugs against solid tumors.

We also analyzed the mechanisms of cisplatin sensitization by using 2DG as a representative stressor. 2DG-stressed cells were found to have a decreased capacity to remove the platinum-DNA adducts. As a result, DNA-bound platinum was increased nearly twofold in these cells after a drug-free incubation of 8 h, although the difference in initial DNA-bound platinum was minimal (Table 2). On the basis of IC_{50} (50% inhibitory concentration) values for colony formation, the increased cellular sensitivity to cisplatin was 1.8- to 2.6-fold depending on the experiment (Figs. 1, 3 and 4). Therefore, the increased cellular sensitivity to cisplatin could be explained by decreased platinum adduct removal in the stressed cells. DNA-platinum adducts are known to be removed primarily by the nucleotide-excision repair pathway [3, 4], which requires ATP to accomplish the multistep processes. The GRP-inducing conditions, therefore, might suppress the DNA repair process through a decrease in cellular ATP levels (Table 3), although the precise mechanism of the decreased repair remains to be determined.

The data presented here have an implication for the therapeutic use of 2DG. Although solid tumors have regions stressed by hypoxia and glucose deprivation, they also have nonstressed regions [19, 21, 25]. We have shown that 2DG stress induces GRP and sensitizes cancer cells to cisplatin in vitro. Similarly, cancer cells in regions of solid tumors not under stress might be sensitized to cisplatin by 2DG. It will be of interest to examine 2DG, or related compounds, to determine their value as a chemosensitizer in vivo.

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