Detection of Proteins that Recognize Platinum-modified DNA Using Gel Mobility Shift Assay

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Using a gel mobility shift assay, we have identified proteins, in the nuclear extracts of a human lung cancer cell line, that recognize *cis*-diamminedichloroplatinum(II) (*cis*-DDP, CDDP)-modified DNA. A 158-base-pair double-stranded DNA fragment, derived from pBR322 plasmid DNA, was modified by either CDDP, tetrachloro(*dl-trans*)-1,2-diaminocyclohexaneplatinum(IV) (tetraplatin) or *trans*-DDP (the stereoisomer of CDDP and clinically ineffective). These platinum drug-modified probes were incubated with nuclear extracts and analyzed by gel mobility shift assay. Proteins in the extracts selectively recognized the clinically active platinum-modified DNA fragment. No binding to the *trans*-DDP-modified DNA fragment was observed. These proteins may play a role in the cytotoxicity or in a DNA repair process.

Key words: Cisplatin — Transplatin — Tetraplatin — DNA-binding protein — Gel mobility shift assay

cis-Diamminedichloroplatinum(II) (*cis*-DDP, CDDP²) is a key drug in the treatment of lung cancer. The development of resistance to CDDP is one of the major obstacles to the improvement of lung cancer chemotherapy and to the therapy of many other types of human cancers. It is essential to elucidate the mechanism(s) of CDDP resistance, not only for the improvement of present chemotherapy, but also for the development of new anticancer agents, especially new platinum derivatives. Various mechanisms of CDDP resistance have been described, including decreased intracellular drug accumulation, increased scavenging nucleophiles and increased repair of damaged DNA.¹⁻⁵ To date, no proteins have been characterized that might be responsible for enhanced DNA repair. There has been one report that a UV-damaged DNA-binding protein was elevated in CDDP-resistant human cancer cell lines.⁶ In the present study, we describe the use of a gel mobility shift assay to detect cellular factors that bind to platinum-modified DNA fragments.

For the gel mobility shift assays, the DNA probe used in all experiments was a 158-base-pair *Hind* III/*Eco* RV fragment derived from pBR322 plasmid DNA. The DNA fragment, referred to as P158, was gel purified, and

5'-end-labeled with Klenow fragment, using [α -³²P]-dCTP (3,000 Ci/mmol; NEN Research Products). The CDDP-modified DNA probe was prepared as previously described.^{7,8} Briefly, 5 ng of end-labeled P158 was incubated with 7.2 ng of CDDP (Sigma) in 25 μ l of 3 mM sodium chloride, 1 mM sodium phosphate (pH 7.4) in the dark at 37°C for 18 h. *trans*-DDP- and tetraplatin-modified probes were constructed by incubating P158 with each platinum drug at the same molar concentration as used for CDDP.

Nuclear extracts were from non-small cell lung cancer cell line, PC-9 (donated by Prof. Hayata, Tokyo Medical College) prepared using a small-scale procedure as previously described.⁹ Protein (1 μ g) from nuclear extracts was incubated with approximately 0.2 ng (4000-8000 cpm) of the ³²P-labeled DNA probes in the presence of 1 μ g of poly (dI-dC) · poly (dI-dC) (Pharmacia) in a final volume of 25 μ l. Binding reactions and gel electrophoresis were performed by a modification of the methods described previously.^{10,11} Incubations were carried out at room temperature for 30 min in 5% glycerol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 1 mM dithiothreitol. The resulting reaction mixtures were resolved in a low-ionic strength 4% polyacrylamide gel (acrylamide:bisacrylamide weight ratio of 30:1) containing 25 mM Tris, 190 mM glycine, and 1 mM EDTA. The gel was preelectrophoresed for 30 min at 11 V/cm. Electrophoresis was carried out at 11 V/cm for 90 min at room temperature. The gel was then dried and autoradiographed for two days at -80°C with an intensifying screen.

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² The abbreviations used are: CDDP and *cis*-DDP, *cis*-diamminedichloroplatinum(II); tetraplatin, tetrachloro(*dl-trans*)-1,2-diaminocyclohexaneplatinum(IV); Pt-stDNA, unlabeled CDDP-modified salmon testis DNA; st-DNA, unlabeled, unmodified salmon testis DNA.

Fig. 1. Identification of proteins which bind to CDDP-modified DNA. A DNA probe (P158) 5'-end-labeled with [α - 32 P]dCTP, either unmodified (P158*) or CDDP-modified (CDDP-P158*), was used. P158* was incubated in the absence (-) (lane 1) or presence (+) (lane 2) of nuclear extract from PC-9 human non-small cell lung cancer cells. CDDP-P158* was incubated in the absence (-) (lane 3) or presence (+) (lane 4) of the same extract. Binding to CDDP-P158* can be competed with by increasing levels of unlabeled CDDP-modified salmon testis DNA (Pt-stDNA) (lane 5, 1 ng; lane 6, 10 ng; lane 7, 100 ng). However, no competition occurred using unmodified salmon testis DNA (stDNA) (lanes 8-10) or CDDP itself (lanes 11-13). The binding activity was sensitive to treatment with 50 µg of proteinase K (lane 14) or 0.1% SDS (lane 15), but was resistant to 50 µg/ml RNase A (lane 16).

Fig. 2. Increase of modified-DNA-binding proteins in accordance with the increase of CDDP-induced modification. The 5'-end-labeled 158-base-pair DNA fragment (P158*), either unmodified (lane 1), modified with 1/10 molar concentration of CDDP (lane 2) or modified as in Fig. 1 (lane 3), was incubated in the absence (lane 1) or presence (lanes 2 and 3) of 1 µg of nuclear extract proteins from PC-9 cells.

The unmodified DNA fragment electrophoresed close to the bottom of the gel (Fig. 1, lane 1). When modified with CDDP, the mobility of the fragment was slightly decreased (Fig. 1, band F, lanes 3-16). Incubation with nuclear extract resulted in two intense bands with greater retardation in mobility (Fig. 1, bands B1 and B2, lane 4). No mobility shift was observed with unmodified P158 probe (P158*) or in the absence of the nuclear extract (Fig. 1, lanes 1-3). The bands of shifted mobility were sensitive to proteinase K and SDS treatments, but were resistant to RNase A treatment (Fig. 1, lanes 14-16). Thus, the bands appeared to be protein-DNA complexes and were not CDDP-mediated DNA-protein crosslinks.

The biochemical nature of these protein-DNA complexes was determined by competition assays (Fig. 1, lanes 5-13). Both unlabeled CDDP-modified P158 (CDDP-P158) (data not shown) and unlabeled CDDP-modified salmon testis double-stranded DNA (CDDP-

stDNA) competed for binding in band B2 in a dose-dependent manner (Fig. 1, lanes 5-7). However, unlabeled unmodified-P158 (data not shown), unlabeled unmodified-stDNA (Fig. 1, lanes 8-10) and CDDP itself (Fig. 1, lanes 11-13) could not compete for binding in band B2. As to band B1, the binding activity became weak only when we added 1000 ng of CDDP-stDNA as a competitor to the reaction mixture (data not shown). These results indicate that DNA-binding proteins can bind to a CDDP-modified DNA rather than to CDDP itself or to a particular DNA sequence and that there may be at least two modified DNA binding proteins (bands B1 and B2) with different DNA binding affinities. Similar work has been published during preparation of our manuscript.^{6,12} One of our proteins may be different from that of Chu and Chang.⁶

To characterize the CDDP-modified-DNA binding protein further, we modified the probe with a lower

Fig. 3. Selectivity of modified-DNA-binding proteins for clinically active platinum compounds. P158*, modified by either CDDP (*cis*-DDP) (lane 1), *trans*-DDP (lane 2) or tetraplatin (lane 3), was incubated in the presence (+) of 1 µg of nuclear extract proteins from PC-9 cells. All DNA probes were incubated with equimolar drug concentrations.

concentration of CDDP. This level of modification reduced the mobility shift of the probe alone in accordance with the known effect of CDDP on DNA migration. The binding activity in both bands B1 and B2 also decreased in accordance with the decrease of CDDP-induced modification (Fig. 2, lanes 2 and 3). When the probe was modified with an equimolar concentration of

trans-DDP (*trans*-DDP-P158*), no binding activity was found (Fig. 3, lane 2). This lack of binding activity was observed even when we modified the probe at a 10-fold higher concentration of *trans*-DDP (data not shown). These results suggest that CDDP-modified-DNA-binding proteins can discriminate the different DNA structural changes induced by platinum drugs. On the other hand, the same bands of shifted mobility occurred when we modified P-158 with either the clinically active CDDP or an active analogue, tetraplatin (a gift from the Upjohn Company, currently in phase II study in the United States).

Considering that the CDDP-modified-DNA-binding proteins did not bind to DNA that had been modified with the inactive platinum drug, *trans*-DDP, but did recognize active drug-modified DNA, these binding proteins may play some role in the platinum-induced cytotoxicity. Alternatively, these proteins may have some role in the repair process of CDDP-modified DNA, in other words, they may act in a manner analogous to the UvrA protein in *E. coli* that recognizes modified DNA. These proteins may be of value in screening for new platinum antitumor drugs. Further, we may be able to detect some critical change in expression of these modified-DNA-binding proteins between CDDP-sensitive and resistant cells. Our preliminary data show that the same modified-DNA-binding proteins exist in several human cancer cell lines (data not shown). It will be worthwhile to characterize these modified-DNA-binding proteins further to determine their potential role in CDDP cytotoxicity and CDDP resistance.

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