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Evidence for lack of mitochondrial DNA repair following *cis*-dichlorodiammineplatinum treatment

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Summary. The purpose of this study was to determine whether *cis*-dichlorodiammineplatinum (cisplatin) causes mitochondrial DNA (mtDNA) damage. A specific and sensitive method for quantitation of damage to mtDNA was used, by which the physical forms of mtDNA (supercoiled, open circular and linear forms) were separated by gel electrophoresis. The DNA specificity was then obtained by hybridizing with a mtDNA probe. *In vitro* incubation of mtDNA with cisplatin showed that the drug did not induce any changes in the proportion of physical forms; similar results were obtained *in vivo*. Since cisplatin did not cause any strand scission in mtDNA but induces strand breaks in nuclear DNA, which is an indirect effect, a lack of repair for cisplatin-induced adducts in mtDNA is suggested.

Introduction

Cisplatin is one of the most widely used chemotherapeutic agents for several malignancies [15]. It damages DNA in a manner similar to that of alkylating agents by binding to two sites in the DNA [22] and inducing DNA inter- and intrastrand cross-links as well as DNA-protein cross-links. The cytotoxic and mutagenic effect of cisplatin correlates with cross-link formation [12, 24]. There is evidence that the primary biochemical lesion induced by cisplatin in cancer cells involves inhibition of DNA synthesis [6]. Inhibition of DNA template replication in mammalian cells has also been shown to occur [7].

Drug-induced damage to nuclear DNA has been assayed by the alkaline elution technique, which determines DNA strand breaks [23]. Cisplatin has been shown to cause strand breaks in nuclear DNA [10]; since it does not do so directly, such lesions are possibly due to endonucleases or topoisomerases involved in repair [15].

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It is known that cisplatin causes damage to nuclear DNA, but little is known about the drug's effects on mitochondrial DNA (mtDNA), which is a supercoiled, circular duplex molecule of about 10^7 daltons [5] consisting of 16 kilobase pairs [1]. Unlike nuclear DNA, mtDNA is not associated with histones [17]. Since mtDNA is a covalently closed circle, strand breaks can be measured by a specific and sensitive method developed by Singh et al. [21], which detects the proportion of physical forms of mtDNA. In the present study we monitored the mtDNA damage caused by cisplatin both *in vitro* and *in vivo*.

Materials and methods

Animals and drugs. C57BL/6J male mice (12–16 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Me). Water and food were available ad libitum. *cis*-Dichlorodiammineplatinum was purchased from Sigma (St. Louis, Mo). Scheduled doses of cisplatin were prepared fresh daily, dissolved in physiological saline at a concentration of 1 mg/ml and given by intraperitoneal injection. All analytical reagents were purchased from Sigma (St. Louis, Mo) or Bethesda Research Laboratories (Gaithersburg, Md), unless otherwise specified.

Isolation of mitochondria and mtDNA. Mice were sacrificed by cervical dislocation and the kidneys were excised and weighed, then homogenized in ice-cold 0.25 *M* sucrose, 10 m*M* TRIS-Cl (pH 7.4) and 1 m*M* ethylenediaminetetraacetate (EDTA) buffer in a Wheaton glass homogenizer. Mitochondria were prepared by a modified differential centrifugation technique previously described by Pederson et al. [11]. Mitochondria were solubilized in 1% (w/v) sodium dodecyl sulfate (SDS), 1 m*M* EDTA and 100 µg/ml proteinase K at 65° C for 30 min. Mitochondrial DNA was extracted with 2.5 vol. phenol : chloroform : isoamyl alcohol (25 : 24 : 1, by vol.) saturated with 0.1 *M* TRIS (pH 7.6) and 0.2% (v/v) 2-mercaptoethanol. Equal volumes of chloroform and isoamyl alcohol (24 : 1) were then added. The DNA was precipitated with 2.5 vol. ice-cold 95% ethanol, washed with 70% ethanol and lyophilized. The final pellet was resuspended in 60 µl 10 m*M* TRIS (pH 7.4) and 1 m*M* EDTA buffer or water.

Detection of mtDNA. mtDNA was separated by electrophoresis on 0.7% agarose gels in 0.89 m*M* TRIS, 0.89 m*M* borate and 10 m*M* EDTA buffer at 100 V for 3 h. Following depurination in 0.25 *M* HCl for 20 min, denaturation in 1.5 *M* NaCl and 0.5 *M* NaOH for 1 h and neutralization in 1 *M* TRISCl (pH 8.0) and 1.5 *M* NaCl for 40–60 min, South-

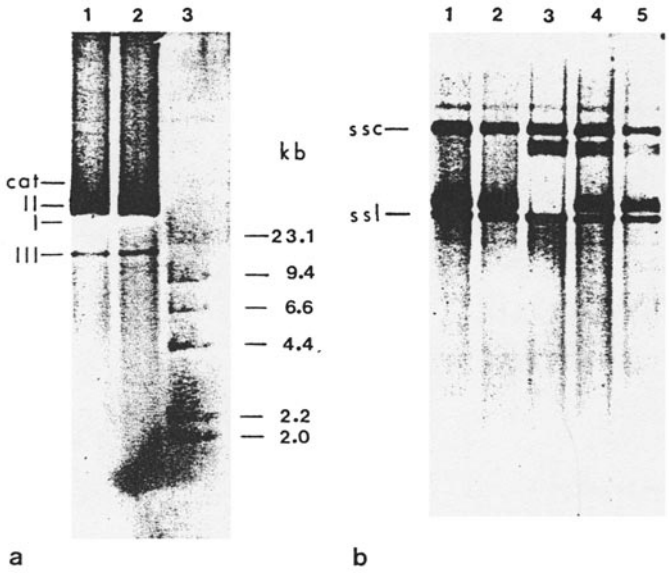

Fig. 1. Comparison of mitochondrial DNA with marker and assignment of mitochondrial DNA forms following gel electrophoresis. a *Lanes 1, 2:* isolated mouse (C57BL/6J) kidney mitochondrial DNA. *Lane 3:* lambda phage DNA digested with Hind III (the 560-base-pair fragment was not visible). b *Lanes 1, 2:* heat-denatured mouse (C57BL/6J) kidney mitochondrial DNA (*lane 1*, 90° C for 5 min; *lane 2*, 90° C for 2 min). *Lanes 3–5:* alkali-denatured mouse (C57BL/6J) kidney mitochondrial DNA (0.2 *N* NaOH, 0.10 *N* NaOH and 0.05 *N* NaOH, respectively). Forms of mitochondrial DNA: *I*, intact circular; *II*, single-nicked circular; *III*, double-stranded linear; *ssc*, single-stranded circular; *ssl*, single-stranded linear; *cat*, catenates

Table 1. Percentage of distribution of mitochondrial DNA forms following *in vitro* incubation with cisplatin

Treatment	DNA form: Form I+II	Form III
Control	89.6 ± 1.7	10.4 ± 1.6
2 µg/ml cisplatin	87.5 ± 5.1	12.5 ± 8.5
10 µg/ml cisplatin	89.1 ± 2.3	10.9 ± 2.5
50 µg/ml cisplatin	92.7 ± 1.3	7.3 ± 1.1

Mouse (C57BL/6J) kidney mitochondrial DNA was incubated with cisplatin. Quantitation of hybridized DNA, represented as the percentage of total mitochondrial DNA in each form, was done using densitometric analysis. Results are presented as the mean ± SE of 6 determinations. Statistical analysis revealed no significant difference between observations ($P > 0.05$)
Form I, intact circular; Form II, single-nicked circular; Form III, double-stranded linear

ern blots were prepared. The mtDNA probe consisted of the entire mouse-liver mitochondrial genome with the plasmid pSP64 as the vector, cloned into *Escherichia coli* HB101 (provided by Dr. W. Hauswirth, Department of Immunology and Medical Microbiology, University of Florida). Following hybridization, filters were washed twice at room temperature in 2 × SSC containing 0.1% SDS (w/v) for 3-min intervals. Filters were exposed to X-OMAT AR Kodak X-ray film.

Table 2. Percentage of distribution of mitochondrial DNA forms following *in vitro* incubation with cisplatin and alkali

Treatment	DNA form: ssl	Form I	ssc
Control	35.0 ± 2.0	40.0 ± 3.1	25.0 ± 3.0
2 µg/ml cisplatin	34.5 ± 1.0	37.5 ± 4.0	28.5 ± 4.5
10 µg/ml cisplatin	43.0 ± 8.0	32.6 ± 6.0	25.0 ± 3.0
50 µg/ml cisplatin	35.2 ± 2.5	34.5 ± 4.6	30.3 ± 2.4

Mouse (C57BL/6J) kidney mitochondrial DNA was incubated with cisplatin followed by alkali treatment. Quantitation of hybridized DNA is represented as the percentage of total mitochondrial DNA in each form. Results are presented as the mean ± SE of 6 determinations. Statistical analysis revealed no significant difference between observations ($P > 0.05$)
ssl, single-stranded linear; Form I, intact circular; ssc, single-stranded circular

In vitro and in vivo cisplatin-induced mtDNA. For *in vitro* studies, renal mtDNA isolated from control mice was incubated directly with cisplatin (2, 10 and 50 µg/ml) for 2 h at 37° C. For *in vivo* studies, renal mtDNA was isolated from C_{57}BL/6J mice pretreated with 10, 20 and 40 mg/kg cisplatin. Following DNA isolation, a dye solution containing 30% glycerol, 0.25% bromophenol blue and 1 m*M* EDTA was added to the samples for electrophoresis. Alkali was added to the mtDNA samples 30 min prior to gel electrophoresis. The mtDNA forms were detected by Southern blot hybridization.

Assignment of mtDNA forms. Forms of mtDNA were identified according to Lim and Neims [8] as follows: I, intact circular; II, single-nicked circles; III, double-stranded linear; ssl, single-stranded linear; ssc, single-stranded circular; cat, catenates (Fig. 1). Quantitation of hybridized DNA in the different forms was carried out by scanning an autoradiograph of the blot with a video densitometer (Biorad) connected to an HP3392A integrator. The percentage of total mtDNA in each form was represented as the percentage of area in arbitrary units.

Results

In vitro effects of cisplatin on mtDNA

Comparison of the effects of cisplatin on the percentage of mtDNA forms with that in controls are shown in Table 1. In controls, 10.4% existed as double-stranded linear forms (form III) and 89.6%, as circular and single-nicked forms (forms I and II, respectively). With increasing doses of cisplatin, the percentage of total mtDNA occurring in different forms did not vary significantly from that in controls ($P > 0.05$). With alkali treatment (Table 2), 35% of the mtDNA in controls occurred as single-stranded linear forms and 40% and 25%, as intact circular and single-stranded circular forms, respectively. Similarly, the data showed that the relative percentage of the various mtDNA forms was not significantly different from control values ($P > 0.05$).

Table 3. Percentage of total kidney mitochondrial DNA in the single-stranded circular form following *in vivo* cisplatin and alkali administration

	Percentage of ssc: Day 1	Day 2	Day 3	Day 4
Control	23.6 ± 13.0 ($n = 6$)	29.7 ± 5.4 ($n = 6$)	27.2 ± 7.6 ($n = 6$)	29.9 ± 5.9 ($n = 6$)
10 mg/kg cisplatin	19.5 ± 5.4 ($n = 6$)	20.7 ± 3.2 ($n = 6$)	14.4 ± 3.4 ($n = 6$)	26.2 ± 5.4 ($n = 6$)
20 mg/kg cisplatin	28.4 ± 5.4 ($n = 6$)	21.8 ± 10.0 ($n = 6$)	21.0 ± 5.1 ($n = 6$)	32.7 ± 2.6 ($n = 3$)
40 mg/kg cisplatin	23.0 ± 4.0 ($n = 6$)	18.8 ± 9.0 ($n = 4$)	20.5 ± 7.6 ($n = 4$)	20.0 ($n = 1$)

Cisplatin was injected intraperitoneally and the animals were sacrificed on days 1–4 post-treatment. Results are presented as the mean ± SE; numbers in parentheses represent the number of mice. Statistical analysis revealed no significant difference between observations ($P > 0.05$).
Forms of mitochondrial DNA (form identification based on [8]): ssl, single-stranded linear; ssc, single-stranded circular

In vivo effects of cisplatin on mtDNA

With alkali treatment, control mouse kidneys showed that approximately 17%–25% of their mtDNA occurred in the single-stranded circular form on various days of isolation (Table 3). With increasing doses of cisplatin and with time, the data showed that the percentage of total mtDNA occurring in the single-stranded circular form did not vary significantly from that in control animals. However, we have shown in previous studies [18] that a dose of 20 mg/kg causes nephrotoxicity in mice; furthermore, we found a high concentration of cisplatin in the mitochondrial fraction isolated from mouse kidneys. In a recent study [19], we demonstrated a decline in the total number of mitochondria in cisplatin-treated animals. Similar results were obtained from liver mtDNA of the pretreated animals (data not shown).

Discussion

It has been shown that nuclear DNA is repaired following cisplatin treatment [14]. Platinum is enzymatically excised from cellular DNA and, thus, a loss of platinum-bound adducts from cellular DNA is observed [14]. A mechanism exists for removing DNA-bound platinum adducts. Lesions are the result of the action of repair enzymes responding to damage [13]. We observed that cisplatin did not cause any strand breaks in mitochondrial DNA *in vitro*; this would imply that cisplatin does not directly cause DNA strand breaks.

In vivo studies show that with increasing doses of cisplatin, no mtDNA strand breakage occurred. However, Olinski [10] has observed cisplatin-induced strand breaks in nuclear DNA, although the drug does not cause such breaks by direct action [2]. The reason for these contradictory results is that nuclear DNA has repair endonucleases and topoisomerases that act to repair it at the region where it intercalates with the drug [16]. Since we did not observe any increase in cisplatin-induced mtDNA strand breakage, a lack of repair enzymes involved in removing drug adducts is implied. This provides further evidence that repair processes are deficient in mtDNA, as previously suggested by Clayton et al. [3].

Nuclear DNA strand breaks are determined by alkaline elution techniques that characterize drug-induced damage and measure the strand breaks [23]. mtDNA strand breaks were measured by a more sensitive technique [21] that measures damage directly. Damage to mtDNA has been observed with radiation and epichlorohydrine, which induces strand breaks [21]. Lim and Neims [8] have also observed mtDNA strand breaks with bleomycin treatment, which also directly causes mtDNA damage.

Morphological studies [4] have shown that cisplatin-induced damage occurs in the S-3 segment of the mouse proximal tubule. Subcellular distribution of platinum obtained from the kidney of C57BL/65 mice showed that the concentration of drug was much higher in the mitochondrial fraction than in the nuclear fraction [20]. Although no damage to mtDNA was observed in the present study, with cisplatin we have previously shown a decline in the total amount of mtDNA that was dose-dependent and time-related [9]. This is the first study to show the deleterious effect of an agent on DNA without the actual strand scission that has been reported in nuclear DNA.

In conclusion, studying mtDNA may be a better method of assessing drug-induced damage, since damage can be monitored directly. Thus, with this technique, agents that cause strand breaks can be differentiated from those that act as alkylating agents.

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