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Heterogeneous repair of platinum–DNA adducts by protein extracts from mammalian tissues

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Abstract—*Cis*-diamminedichloroplatinum (II) (cisplatin) is a mainstay of human cancer chemotherapy. In addition to its antitumour effects however, cisplatin is toxic to normal tissues, causing dose-limiting nephrotoxicity and neurotoxicity. On the other hand, myelosuppression is uncommon. In the light of data suggesting a role for DNA repair mechanisms as determinants of cellular cisplatin sensitivity, we postulated that varying DNA repair capacities between tissues could explain the patterns of organ toxicity seen in clinical practice. Using a novel cell-free assay of repair of cisplatin–DNA adducts, we find that the DNA repair capacity of protein extracts from different tissues varies significantly in our assay, but does not directly correlate with the organ toxicity profile of cisplatin.

Key words: cisplatinum; DNA repair; cell-free assay

The platinum coordination complex *cis*-diamminedichloroplatinum (II) (cisplatin*) is one of the most frequently used anti-cancer agents in clinical practice. Cisplatin is curative in germ cell tumours of the testis, and forms the mainstay of therapy in carcinoma of the ovary, bladder, lung, and head and neck. In addition to its antitumour effects, cisplatin is toxic to normal tissues. The predominant organ toxicity is nephrotoxicity, necessitating the routine use of saline diuresis in the clinic [1]. Other significant, often treatment-limiting toxicities include neurotoxicity, most frequently manifest as either a distal sensory neuropathy or as high frequency hearing loss [2]. Unlike most other useful cytotoxic agents however, cisplatin is relatively sparing of the bone marrow, with clinically significant myelosuppression being modest at usual clinical doses [3]. The biochemical bases of the heterogeneous end-organ toxicity of cisplatin are largely unknown.

Cisplatin exerts its cytotoxic effect by interaction with cellular DNA, forming a variety of cisplatin–DNA adducts. The most frequent is the G–G intrastrand adduct, but interstrand and other less frequent intrastrand adducts are also produced [4]. The cellular response to cisplatin is to remove these adducts via the nucleotide excision repair process. This process involves recognition of cisplatin–DNA adducts, single strand incision on either side of the lesion, removal of the damaged strand, followed by resynthesis and ligation of a complementary strand [4]. A number of studies suggest that variations in the efficiency with which cells repair cisplatin–DNA adducts may determine cisplatin sensitivity [5–8]. If this postulate is correct, then varying repair capacities between tissues could explain the patterns of organ toxicity seen in clinical practice. Furthermore, the cisplatin sensitivity of tumours may reflect that of the tissue from which they derive.

The recent development of cell-free assays of DNA repair pathways offers the potential to explore the DNA repair capacity of mammalian tissues [9]. In these assays, protein extracts from the cells of interest are reacted with DNA substrates containing defined DNA damage, and the extent to which the damaged DNA is repaired by the extract is quantitated. Given the potential role of DNA repair mechanisms in determining cisplatin sensitivity, we have investigated the application of a cell-free assay of nucleotide excision repair of platinated DNA [4] using protein extracts from direct biopsies of rat and human tissues. We find that protein extracts prepared from normal rodent and human tissues express platinum-specific DNA repair activity in our assay. The basal repair capacity of extracts from different tissues varies significantly in our assay, but does not directly correlate with the end-organ toxicity profile of cisplatin.

Materials and Methods

Tissue samples. Healthy Wistar rats were killed, and multiple tissues collected at necropsy, snap frozen in liquid nitrogen, and stored at −80°. Normal human ovary was obtained at laparotomy from a patient undergoing extensive pelvic surgery for carcinoma of the cervix. The tissue was snap frozen in theatre, and stored in liquid nitrogen for 2 days before extract preparation.

Cell lines. The SV40 transformed human fibroblast line 1BR.3N HeLa, and the Chinese hamster ovary line CHO K1, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Both lines were maintained in RPMI 1640 with 10% foetal calf serum. These cell lines are regularly tested and shown to be free of mycoplasma contamination.

Cell-free extracts. Frozen tissue specimens were pulverized in a pestle and mortar and the pulverized specimen then placed in hypotonic lysis buffer with subsequent extract preparation prepared essentially as described [9]. In the case of cell lines, approximately 10^9 cells growing in log phase were harvested, washed thoroughly in PBS at 4°, snap frozen in liquid N_2 and stored at −80°. Prior to extract making, the pellet was minced and placed in hypotonic lysis buffer as described [9]. For both cell lines and tumour biopsies, subsequent extract preparation involved, in brief, preparation of a cell lysate with a Dounce homogenizer, and centrifugation of the lysate at 100,000 *g* for 3 hr. After centrifugation, the pellet was discarded, and the cellular proteins precipitated by addition of ammonium sulphate (33% final concentration). The precipitated proteins were collected by centrifugation, and the resulting pellet dialysed for 12 hr against extract buffer. The small amount of precipitate formed during dialysis was removed by centrifugation and discarded. The final clear supernatant was referred to as the cell extract, and contained 10–35 mg/mL of protein. These extracts were then snap frozen on dry ice and stored at −80°.

Plasmid DNA. The 2958-bp plasmid pBluescript KS+

* Abbreviations: cisplatin, *cis*-diamminedichloroplatinum II; dGTP, deoxyguanosine triphosphate; dCTP, deoxycytidine triphosphate; dATP, deoxyadenosine triphosphate; CHO, Chinese hamster ovary cells.

(Stratagene) and the related 3740-bp plasmid pHM14 (kind gifts from Dr R. D. Wood, ICRF Laboratories, Clare Hall, U.K.) were grown in *E. coli* host strain JM 109 and then purified initially on cesium chloride, then by two sequential sucrose gradients [9]. Fractions containing only closed-circular forms were then collected and stored at −80° [9]. Purified pBluescript KS+ DNA was reacted *in vitro* with cisplatin so as to produce one cisplatin adduct per 100 nucleotides as previously described [4] and confirmed by flameless atomic absorption spectrophotometry.

In vitro *repair synthesis*. Reaction mixtures (50 μL) contained (final concentrations) 250 ng of unplatinated closed circular pHM14 and 250 ng of platinated pBluescript KS+, 45 mM HEPES/KOH (pH 7.8), 60 mM KCl, 7.4 mM $MgCl_2$, subscript, 0.9 mM dithiothreitol, 0.4 mM EDTA, 20 μM each dGTP, dCTP and TTP, 8 μM dATP, 74 kBq of [^{32}P]dATP (110 TBq/mmol), 2 mM ATP, 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3.4% glycerol, 18 mg bovine serum albumin, and 50–400 μg of protein extract [9]. Reaction mixtures were incubated for 20 min at 30° prior to the addition of DNA then incubated for 3 hr at 30°.

Quantitation of repair. Plasmid DNA was purified from the reaction mixtures as described [9], except that proteinase K digestion was carried out at 56° for 40 min before phenol extraction and ethanol precipitation of the DNA. The plasmids were linearized with HindIII and electrophoresed overnight on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Incorporation of [^{32}P]dAMP into the DNA was quantified by autoradiography and by scintillation counting of the excised bands. Results were standardized for variation in DNA recovery by densitometric scanning of a photographic negative of the ethidium bromide-stained gel as described previously [9].

Results

Preparation of protein extracts. The method of protein preparation previously applied to cultured cells was found to be satisfactory for tissues, the only modification necessary being an initial pulverization step under liquid nitrogen. This pulverization typically doubled the protein yield obtained (when compared to Dounce homogenization alone). Typically 1 g of frozen tissue yields a mean of 5 mg of protein per gram of frozen tissue (range 2.6–9.3 mg). At each step of the extract preparation, extract tissues behaved in a similar manner to extracts from cultured cells performed in parallel.

DNA repair synthesis by extracts from tissues. DNA repair synthesis carried out by protein extracts was assessed by monitoring the incorporation of short patches of nucleotides into platinated closed-circular plasmid DNA. A radioactively labelled deoxynucleotide was included in the reaction mixture so that synthesis can be detected by autoradiography after agarose gel electrophoresis of the reaction products, and quantified by measuring incorporation of radioactive material into damaged plasmid DNA. The relatively crude protein extracts employed contain a variety of DNA-modifying enzymes, some of which can radiolabel substrate DNA independent of the presence of DNA adducts (e.g. DNA polymerases). To control for labelling events which are not specific for repair of cisplatin adducts, a slightly larger undamaged plasmid was present in each reaction as an internal control. The subtracted difference in counts between the platinated plasmid (routinely pBS) and non-platinated control plasmid (routinely pHM) is the measure of platinum-specific repair events. In control experiments using platinated pHM and non-platinated pBS, identical results were obtained, indicating that the difference in counts between platinated and control plasmids is due to platinum-specific repair events.

The DNA repair activity of tissues and cell lines was examined at equal protein concentrations, and the results expressed as fentomoles of dAMP incorporated per 200 μg of protein extract as shown in Fig. 1. This protein concentration is on the lower half of the linear segment of the dose–response curve for our assay (unpublished data). Results were confirmed by testing at least three independent extracts from all tissue types examined. In Fig. 1, for each

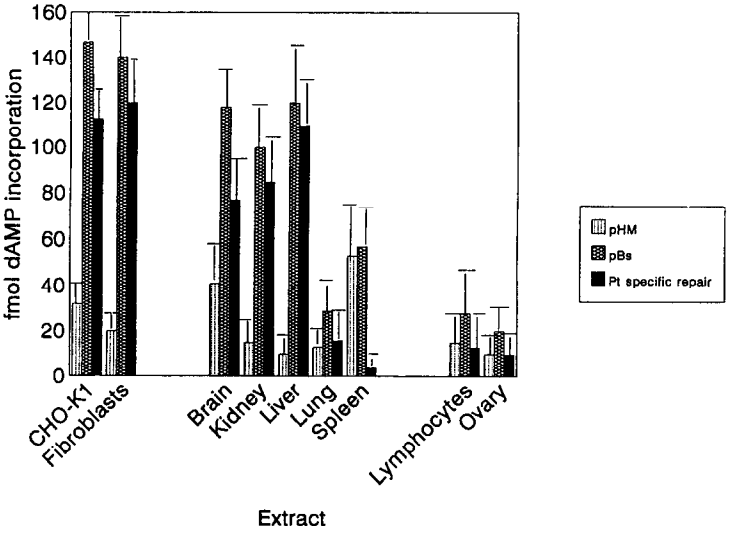

Fig. 1. The DNA repair activity (expressed as femtomoles of dAMP incorporated into DNA per 200 μg of protein extract) of cell lines and tissue extracts. For each extract dAMP incorporation into both non-platinated control pHM plasmid and cisplatin treated pBS plasmid are shown from a single representative experiment. The dark shaded column (mean values of three to five experiments) represents calculated platinum-specific repair events (i.e. background counts have been subtracted) expressed as fmol of dAMP incorporated per 200 μg of protein extract. Bars represent SE.

sample, dAMP incorporation into both control and platinated DNA from typical experiments are shown. In addition, the calculated platinum-specific repair events (i.e. by subtracting dAMP incorporation into control plasmid from that incorporated into platinated plasmid) is also shown in Fig. 1. The overall counts incorporated into platinated plasmid by tissue extracts were comparable to those produced by extracts from the cultured transformed human fibroblasts and CHO cell line. The "background" labelling of the non-platinated control DNA in tissue samples was in general comparably low as in cell line extracts, suggesting that the observed difference in signal intensity between tissues and cultured cells was due to reduction in platinum-specific repair events in tissue samples. The exception was the data obtained for extracts from spleen, in which background synthesis was always considerably higher than other tissues.

The DNA repair capacity of extracts from different tissue types varied considerably, with extracts from rat brain, liver and kidney being most active, whereas extracts from rat lung and spleen showed minimal platinum-specific repair. Extracts from unstimulated human peripheral blood lymphocytes and normal human ovary produced poor repair signals.

Discussion

In view of the comparative ease with which fibroblasts and lymphocytes can be obtained, the majority of studies of mammalian DNA repair mechanisms have been performed using cultured or immortalized fibroblast or lymphoid cell lines. Studies of these relatively accessible tissues have allowed the delineation of DNA repair pathways and their abnormalities. In rare inherited disorders such as xeroderma pigmentosum and ataxia telangiectasia, the severe defects in DNA repair seen in lymphoid or fibroblast cells reflect similar abnormalities in all body tissues [10]. However, in normal individuals the extent to which results from DNA repair assays on lymphoid or fibroblast cells can be extrapolated to other body tissues is less certain. For example, previous studies have demonstrated variable repair of UV induced lesions in different body tissues, [11] and also variable expression of DNA repair related proteins such as O^6-alkylguanine-DNA alkyltransferase [12, 13]. To date, no such data has been available for cisplatin-specific DNA repair pathways.

The advantage of the cell-free assay employed in this study is that it allows a direct functional assessment of the DNA repair protein machinery present in resting normal tissues without the need for growth factor stimulation or viral immortalization. The platinum-specific repair activity of extracts from tissues is low in comparison with the signals obtained using extracts from cultured cell lines. In light of previous studies suggesting that non-dividing cells contain lower levels of DNA repair related enzymes and repair damaged DNA slower than proliferating cells, the low levels of adduct repair we have observed may reflect, in part, the mitotic inactivity of the tissues studied [14, 15]. However, the differences between the tissues cannot be explained solely on this basis. Neural tissues are mitotically inactive, but in our assay produced relatively strong repair signals, indicating that functional platinum-specific repair activity shows tissue-specific variation.

In clinical practice, cisplatin toxicity is most evident as renal or neuro toxicity, with myelo toxicity being much less marked. We postulated that heterogeneous expression of DNA repair machinery could explain the observed tissue toxicities induced by cisplatin in the clinic. Our results, however, indicate that relatively high levels of repair activity is present in extracts from neural and kidney tissues, with low levels of repair activity expressed in non-stimulated lymphocytes. These results are obviously at variance with the clinical patterns of cisplatin toxicity. This result does not support a role for DNA repair mechanisms as a major determinant of the tissue toxicity of cisplatin. However, a role for DNA repair mechanisms is not entirely excluded, since it remains possible that repair of cisplatin–DNA adducts other than those measured in our assay may determine tissue toxicity. On the other hand, other factors may be more important. For example, studies employing immunohistochemistry or atomic absorption spectrophotometry demonstrate that following cisplatin treatment, platinum levels in normal tissues vary considerably [16, 17]. Moreover, at the cellular level other factors such as transmembrane transport, cytosolic quenching (e.g. by glutathione or metallothionein), or tolerance of DNA damage have been reported to play a role in cellular response to cisplatin *in vitro* [18]. Thus unexplored pharmacokinetic differences between tissues may play a role in heterogeneous tissue toxicities.

Potential variations in DNA repair pathways between different tissues such as we have observed may have significance over and above the response to clinically administered cytotoxics. For example, subtle alterations in DNA repair proteins may play a role in determining the organ specificity of mutagens and carcinogens. Such a mechanism has been suggested as the basis for development of hepatoma in some patients with chronic liver disease [19]. Moreover, the sensitivity of tumours could reflect that of the tissues from which they derive. It is of interest therefore that tumours of the brain and kidney are highly refractory to cisplatin chemotherapy, consistent with the data obtained in this study.

The biochemical determinants of cellular response to cisplatin treatment are complex. Although we have demonstrated heterogeneous DNA repair activities in mammalian tissues, the data does not support a major role for DNA repair as a determinant of the organ-specific toxicity of cisplatin.

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