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Cisplatin-induced endoreduplication in CHO cells: DNA damage and inhibition of topoisomerase II

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Abstract

It has been proposed that polyploid cells that arise during a variety of pathological conditions and as a result of exposure to genotoxicants, typically in the liver, become aneuploid through genetic instability. Aneuploidy contributes to, or even drives, tumour development. We have assessed the capacity of the drug cisplatin, one of the most commonly used compounds for the treatment of malignancies, to induce endoreduplication, a particular type of polyploidy, in cultured Chinese hamster AA8 cells. Taking into account that any interference with DNA topoisomerase II (topo II) function leads to endoreduplication, we have found that treatment of the cells with this platinum compound results in a dose-dependent inhibition of the catalytic activity of the enzyme. These observations are discussed on the basis of a possible dual action of cisplatin leading to a combined negative effect on normal segregation of chromosomes. On the one hand, through the drug capacity to efficiently inhibiting the catalytic activity of topo II itself and, on the other hand, as a consequence of changes in DNA such as base modifications and cross-links that result from cisplatin treatment, likely leading to a lack of recognition/binding of DNA by the enzyme. These observations support a model in which the involvement of topo II in different pathways leading to induced endoreduplication has been proposed, and seem to bear significance as to the possible origin of the development of secondary tumours as a result of cisplatin treatment of primary malignancies.

Keywords: Cell-cycle; Endoreduplication; Genetic instability; DNA topoisomerase II; DNA damage

1. Introduction

There is a growing body of evidence supporting the idea that genetic instability in polyploid cells might provide a pathway to aneuploidy thereby contributing to the development of cancer. *cis*-Diamminedichloroplatinum (II), commonly referred to as cisplatin or *cis*-DDP, is currently one of the most commonly used compounds for the treatment of a wide spectrum of human malignancies [1]. As a single agent or in combination, cisplatin is now the foundation of treatment for testicular, ovarian, bladder, cervical, head and neck, and small-cell and non-small-cell lung cancers [2]. A significant drawback, however, is that the compound also has important renal side effects including reduction of antioxidant plasma levels and generation of free radicals in normal cells [3,4]. At the molecular level, biological targets of cisplatin are many cellular components that have nucleophilic sites such as DNA, RNA, proteins, membrane phospho-

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lipids, cytoskeleton proteins, etc. [5]. Currently however, it is widely accepted that the cellular target for platinum complexes that is responsible for their antitumour effects is DNA. Cisplatin can form monoadducts with DNA, interstrand cross-links, intrastrand cross-links, and protein–DNA cross-links, such as glutathione–DNA cross-links [6]. During replication, or as a consequence of the processing of these lesions by the DNA repair machinery, potentially lethal DNA double-strand breaks (DSBs) do arise [7].

According to their catalytic mechanisms, two main classes of DNA topoisomerases (topos) have been described so far, namely topo I, that breaks and rejoins one DNA strand at a time, and topo II, that is able to do so with the two strands that make up duplex DNA. Given their molecular mechanisms of action, both types of enzymes are able to perform relaxation of supercoiled DNA which in turn relieves torsional tension generated during replication and transcription.

Contrasting with topo I however, topo II is unique in that it is able to decatenate intertwined DNA molecules. This decatenating as well as unknotting activity of DNA topo II is essential for segregating replicated daughter chromosomes before anaphase [8–14]. Concerning the relationship between DNA nucleotide sequence and topo II, though it is generally agreed upon that the former plays a role in enzyme function, the rules that determine the nucleic acid specificity of topo II are as yet far from being completely elucidated. It has been reported that topo II cleaves DNA at preferred sequences within its recognition/binding sites, but there is no report on high specificity [15–18].

An interesting endpoint of choice to assess any possible interference with topo II in chromosome segregation is endoreduplication, that consist of two consecutive rounds of DNA replication without intervening mitosis, yielding metaphases showing diplochromosomes, made up of four chromatids instead of the normal two [14,19]. We have recently found that endoreduplication is readily induced in AA8 Chinese hamster cells treated for two consecutive cell cycles with different halogenated nucleosides, namely 5-chlorodeoxyuridine (CldU), 5-iododeoxyuridine (IdU), and 5-bromodeoxyuridine (BrdU) [20]. The frequency of endoreduplicated cells was highest for CldU, intermediate for IdU and lowest for BrdU. Besides, the frequency of cells showing diplochromosomes paralleled the relative percentage established concerning the halogenated pyrimidine:deoxythymidine incorporation into DNA. Similar results on the production of endoreduplication have been recently reported by us when instead of halogenated nucleosides we used the demethylating drug 5-azacytidine (5-azaC) to induce an extensive hypomethylation in DNA [21].

Interestingly, cisplatin inhibits bacterial DNA gyrase [22] and has also recently been shown to strongly inhibit the decatenation and relaxation activity of isolated human DNA topo IIα through a mechanism different from poisoning the enzyme in a stabilized covalent topo IIα–DNA intermediate (“cleavable complex”) [23,24]. It was concluded that cisplatin may exert some of its potent cell growth inhibitory and antitumor activity by inhibition of topo IIα through reaction with critical enzyme sulphydril groups and/or forming DNA adducts that render the DNA substrate refractory to topo IIα [23,24]. This latter possible mechanism of interaction of cisplatin with DNA should represent another example of the importance of an unaltered DNA sequence for efficient recognition/binding by topo II to perform its function, as shown by us using halogen substitution of DNA [20].

Taking into account that, as reported recently by us [25–27] as well as previously by others [14,28], misfunction of DNA topo II leads to endoreduplication, in the present investigation we have assessed the possible influence of cisplatin on chromosome segregation in AA8 Chinese hamster ovary fibroblasts. On the one hand, the drug appears as able of inhibiting the catalytic activity of topo II itself and, on the other hand, changes in DNA that result from cisplatin treatment are likely leading to a lack of recognition/binding of DNA by the enzyme. According to our expectations, we have found that treatment of the cells with this platinum compound results in a dose-dependent induction of endoreduplication. These observations are discussed on the basis of a possible dual action of cisplatin leading to a combined negative effect on normal segregation of chromosomes.

2. Materials and methods

2.1. Chemicals

A stock solution of 10 mg/ml of cisplatin (Sigma) was prepared in DMSO and kept in 100 μl vials at −20 °C until use. Just before an experiment, a vial was thawed and then diluted in complete medium in order to obtain the final concentration of cisplatin desired.

2.2. Cell culture

Chinese hamster ovary AA8 cells were grown as monolayers in McCoy’s 5A medium with 10% fetal calf serum, 2 mM L-glutamine, and the antibiotics penicillin (50 U/ml) and strep-

tomycin (50 μg/ml). Cells were cultured at 37 °C in 5% CO_2 in air.

2.3. Sulforhodamine B (SRB) assay for cytotoxicity

AA8 cells in exponential growth phase were harvested using trypsin-EDTA (Gibco BRL), and resuspended in medium. They were seeded in 96-well microtitre plates (Nunc) at a density of 5×10^3 cells/100 μl and allowed for 24 h to attach. Then, they were incubated further for 48 h in the presence of different doses of cisplatin, ranging from 1.0 to 90 μg/ml.

Following the recommendations of the National Cancer Institute (USA), the analysis of cytotoxic effects induced by ACLA was determined using a cell growth assay, the SRB assay, as described previously [29,30]. Briefly, 50 μl/well of cold 50% trichloroacetic acid (TCA) (final concentration 10%) was added to the culture and incubated at 4 °C for 1 h, to precipitate the proteins and fix the cells. The supernatant was then discarded, and the plates were washed five times with deionized water and air-dried. The cells were then stained with 100 μl/well of 0.4% SRB dissolved in 1% acetic acid for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid, and then the plates were air-dried. The stained protein was solubilised in 100 μl/well of 10 mM unbuffered Tris base by shaking. The optical density was read at 492 nm using a microtitre plate reader (ELISA). Each experiment on cytotoxicity of cisplatin was performed in triplicate.

2.4. Preparation of nuclear extracts

Topo II activity in nuclear extracts from AA8 cells was obtained as described by Heartlein et al. [31]. Approximately 1×10^7 cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris–HCl pH 7.5, 0.05 M $MgCl_2$ and 1% Triton X-100 and thoroughly vortexed to lyse the cells. Nuclear pellets were obtained by centrifugation at 1800 × *g* (Eppendorf centrifuge), for 5 min at 4 °C. Nuclei were then washed in 1 ml of nucleus wash buffer (5×10^{-3} M potassium phosphate buffer, pH 7.5, 10^{-3} M phenylmethyl sulfonyl fluoride (PMSF), 10^{-3} M β-mercaptoethanol and 0.5×10^{-3} M dithiothreitol (DTT)). The nuclei were then pelleted as described above and resuspended in 50 μl of nucleus wash buffer, and 50 μl of 4×10^{-3} M EDTA was added. Following incubation at 0 °C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, 20×10^{-3} M Tris-HCl pH 7.5, 10^{-2} M β-mercaptoethanol and 10^{-3} M PMSF. Following an 15 min incubation at 0 °C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50×10^{-3} M Tris–HCl pH 7.5, 10^{-2} M β-mercaptoethanol, and 10^{-3} M PMSF was added. The suspension was incubated for a further 40 min period at 0 °C. The supernatant from a 30 min centrifugation at 11,200 × *g* at 4 °C was then collected. Total protein concentration in each extract [32] was determined in a Beckman DU-64 spectrophotometer by the Bio-Rad protein assay (Bio-Rad Laboratories) and the extracts were kept at −80 °C for no longer than a month.

2.5. Topoisomerase II activity in nuclear extracts

Topo II activity in nuclear extracts either untreated or incubated with different concentrations of cisplatin was assayed using an assay kit (TopoGen, Columbus OH, USA) based upon decatenation of kinetoplast DNA (kDNA). Reaction products were resolved using agarose gel electrophoresis of DNA. After incubation, 40 min at 37 °C, the samples were loaded onto 1% agarose gels and subjected to electrophoresis for 2.5 h at 100 V. Finally, gels were stained with 0.5 μg/ml ethidium bromide, destained (30 min) in distilled water and photographed under UV illumination. Densitometric analysis was performed using the software programme PCBAS version 2.08.

2.6. Induction of endoreduplication

Actively growing AA8 cells were cultured for 24 h in the presence of a wide range of different concentrations of the antitumour drug cisplatin (2, 5, 10, 20, 50, and 75 μg/ml) in the range of those shown as efficiently inhibiting topo II catalytic activity (see above). After treatment the cultures were thoroughly washed and maintained in fresh medium for 18 h to allow them to recover. Cultures that did not receive any treatment served as control. Colcemid (2×10^{-7} M) was finally added for 2 h 30′ to all the cultures for metaphase arrest. The flasks were gently shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 2 min (hypotonic treatment), fixed in methanol:acetic acid (3:1) and dropped onto clean glass microscope slides. The slides were stained with 3% Giemsa in phosphate buffer pH 6.8 and mounted in DPX. Two thousand metaphases per culture were counted and classified as normal or as made up of diplochromosomes. All the experiments were carried out in triplicate.

3. Results

3.1. Cytotoxicity of cisplatin

First, the cytotoxicity of cisplatin to AA8 Chinese hamster cells was determined by means of the SRB assay, which measures the protein production of the cell as a whole. Fig. 1 shows the results obtained when cells were treated with a range of concentrations of cisplatin. As can be seen, the SRB assay provided results that clearly indicate a dose-dependent cytotoxic effect of cisplatin on AA8 cells, more evident for concentrations of 20 μg/ml of cisplatin and higher, reaching a growth inhibition of roughly 50% when cells were treated with a dose of 90 μg/ml cisplatin (Fig. 1).

3.2. In vitro inhibition of topo II catalytic activity by cisplatin

The capacity of topo II to decatenate double-stranded catenated kinetoplast DNA (kDNA) was the endpoint

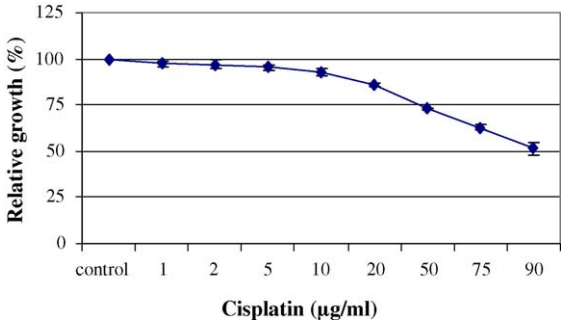

Fig. 1. Effectiveness of different concentrations of cisplatin to suppress cell growth in the Chinese hamster fibroblast cell line AA8 as shown by the SRB assay. Bars indicate standard deviation from three independent experiments. Statistical analysis (Student's *t*-test) shows a significant difference ($P < 0.01$) for doses of cisplatin of 5 μg/ml and higher as compared with control.

used to assess the possible inhibition of the catalytic activity of the nuclear enzyme by cisplatin. As can be seen in Fig. 2, in the absence of any drug treatment, topo II activity recovered in nuclear extracts from AA8 cells was able to efficiently decatenate the catenated DNA

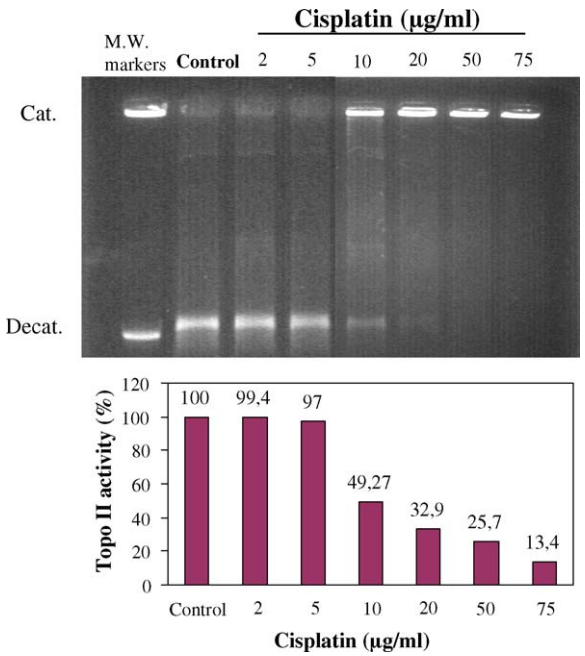

Fig. 2. Inhibition of the topo II catalytic activity by cisplatin. Nuclear extracts were obtained as described in Section 2 and their ability to decatenate catenated kinetoplast DNA after incubation with different doses of cisplatin ranging from 2 to 75 μg/ml was assayed by DNA gel electrophoresis. Below, the respective densitometric profiles are shown. The dose-dependent loss of topo II catalytic activity was in most cases significant as compared with non-treated controls ($P < 0.001$; Student's *t*-test), with the only exception of the lowest dose of cisplatin assayed (2 μg/ml).

substrate as shown by the release of closed minicircles. When the possible inhibition exerted by increasing concentrations of the platinum compound, ranging from 2.0 to 75 μg/ml, was tested, the observation was a dose-dependent inhibitory effect, as shown by a progressive increase in the amount of catenated DNA substrate remaining in the wells (Fig. 2). This observation on the inhibition of topo II catalytic activity present in our CHO cells nuclear extracts is in good agreement with that reported recently by Hasinoff et al. [23,24] using isolated human DNA topo II. According to these results, different intervals of doses were selected by us for the experiments aimed to assess any effect of the compound on chromosome segregation.

3.3. Cisplatin induces endoreduplication

Once established that cisplatin, at the doses tested by us, is capable of efficiently inhibiting topo II catalytic activity as shown by a loss in the yield of decatenation of catenated kDNA in AA8 Chinese hamster ovary cells, we analyzed the possible influence of the antitumour drug on normal chromosome segregation. The endpoint chosen by us to assess missegregation leading to aberrant mitosis was endoreduplication [14,19], typically visible as metaphases made up of diplochromosomes (Fig. 3).

Treatment with different doses of cisplatin took place for 24 h before allowing the cells to recover for an additional cell cycle during which endoreduplication (if any) might take place. As can be seen in Fig. 4, endoreduplication was effectively induced in a dose-dependent fashion at doses of cisplatin ranging from 2.0 to 20 μg/ml. At higher concentrations of the platinum compound, while the induction of a high yield of endoreduplication was still consistently observed, the dose-dependent relation-

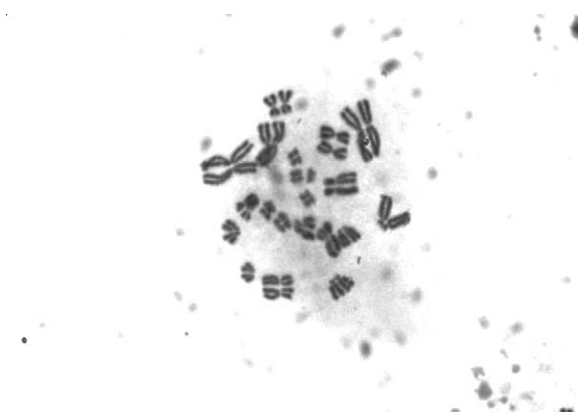

Fig. 3. Endoreduplicated AA8 Chinese hamster cell at metaphase showing the characteristic diplochromosomes after a treatment with the antitumour drug cisplatin (20 μg/ml).

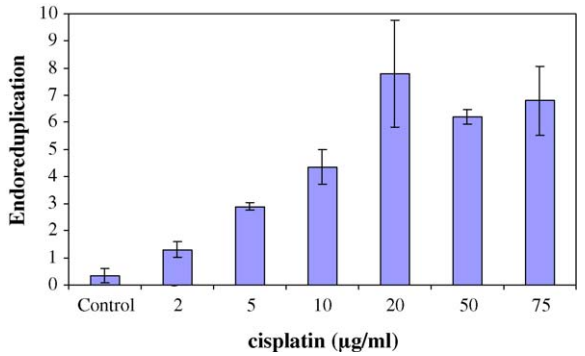

Fig. 4. Dose-dependent endoreduplication profile induced by different concentrations of cisplatin (μg/ml) in the range previously shown to inhibit efficiently the topo II catalytic activity in vitro. Bars represent standard deviations (S.D.) of the mean from three independent experiments.

ship was more variable, and was clearly influenced by a negative effect of the treatment on the rate of cell division.

4. Discussion

Many anticancer agents exert their cellular toxicity through DNA damage [6], mainly DNA double-strand breaks (DSB), and induction of apoptosis. The nuclear enzyme topo II, on the other hand, is also a target for a number of chemotherapeutic agents used in the treatment of cancer [33].

In order to study the sequence specificity of double-strand DNA cleavage by *Drosophila* topo II, the frequencies of the nucleotides and dinucleotides in the region near the site of phosphodiester bond breakage was analyzed [34] and revealed a nonrandom distribution. The consensus sequence derived was 5′GT.A/TAY ↓ ATT.AT..G3′ where a dot means no preferred nucleotide, and Y stands for pyrimidine [34]. On the other hand, DNase I footprint analysis has revealed that *Drosophila* topo II can protect a region in both strands of the duplex DNA, with the cleavage site located near the center of the protected region [17], and it has been proposed that the strong DNA cleavage sites of *Drosophila* topo II [34] likely correspond to specific DNA-binding sites of the enzyme [15,35].

Using a transcription assay [36] the interaction between topo II from calf thymus and DNA was also characterized. The conclusion was that topo II binds to a region of DNA located symmetrically around the enzyme-mediated cleavage site. Results from our laboratory have supported the idea that a conserved DNA sequence is necessary for topo II function in a correct segregation of fully replicated chromosomes during mitosis. Both halogen substitution in DNA for thymidine [20] and extensive DNA hypomethylation induced by 5-azacytidine [21] resulted in an abnormally high yield of endoreduplicated cells. Both modifications of DNA sequences have been shown recently by us to lower the production of stabilized cleavable complexes DNA–topo II by the enzyme poison m-AMSA [21,37] as a demonstration of the alteration of recognition/binding to DNA by the enzyme.

We have presented a model in a recent publication [27] on the involvement of topo II in the different pathways leading to induced endoreduplication. Based on this model, our present results on the induction of endoreduplication by cisplatin in CHO cells can be explained taking into account the likely dual effect of the anticancer drug. On the one hand, DNA is well known to be a major target of cisplatin either as a result of its direct action or indirectly, this later through the generation of reactive oxygen species [38–40]. Besides, cisplatin has been shown to directly inhibiting the catalytic activity of topo IIα [[23,24]; the present report] most likely through reaction of the platinum compound with critical sulphydril groups of the enzyme.

Anyway, regardless of the specific pathway through which the cells might become endoreduplicated, this form of polyploidy deserves discussion as to its possible implications for the development of malignancy as a whole [41,42]. There is a considerable amount of correlative evidence to indicate that polyploid (usually tetraploid) cells are formed in a variety of pathological conditions. In fact, tetraploid cells have been described during the development of some common tumours [43], and even a checkpoint has been recently reported that arrests and eliminates polyploid cells [44].

Our present data on the induction of endoreduplication by cisplatin raises the question as to the possible involvement of such a process of polyploidy in apoptotic cell death, i.e. the efficacy of the drug in tumour treatment and, on the other hand, the possible development of secondary cancers as a consequence of genetic instability caused by the therapy itself. As a matter of fact, a number of recent reports seem to lend support to this idea. For instance, the incidence of secondary leukaemia in patients treated with first line high-dose chemotherapy (HDCT) that included cisplatin has been reported [45]. Similar results on platinum compounds-related secondary acute myeloid leukaemia in patients with osteosarcoma have been also recently published [46]. A study on long-term sequelae after cisplatin-based chemotherapy for testicular cancer has concluded that the most serious complication in long-term survivors is the development of second, non-germ cell malignancies

[47]. Finally, a high incidence of the uncommon lesion cystic trophoblastic tumour has also been frequently observed in patients after cisplatin-based chemotherapy for testicular germ cells tumours [48].

In our opinion, monitoring the appearance of different forms of polyploidy in patients, as reported here for endoreduplication as a result of cisplatin treatment, might be a valuable procedure to evaluate the risk of developing secondary leukaemia and other malignancies as well.

Acknowledgements

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