Formation and Repair of Cisplatin-induced Adducts to DNA in Cultured Normal and Repair-deficient Human Fibroblasts

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ABSTRACT

The formation and repair of cisplatin [cis-PtCl$_2$(NH$_3$)$_2$] adducts in the DNA of cultured normal and repair-deficient human fibroblasts are presented in relation to cell survival after cisplatin treatment. Directly after treatment with cisplatin, in normal (MB), Fanconi's anemia (FA), and xeroderma pigmentosum (XP) fibroblasts four platinated products are found. The major adduct is cisplatin bound to two neighboring guanines, Pt-GG (62–75%). A less abundant product is cisplatin bound to an AG sequence (Pt-AG). Binding to two guanines separated by one or more bases or to two guanines in opposite DNA strands (together measured as G-Pt-G) and cisplatin bound monofunctionally to guanine (Pt-G) are also found in small amounts. The distribution of the four products is similar to that found previously, in vitro systems as well as in living cells. Directly after cisplatin treatment, the removal of cisplatin-DNA adducts is fast in normal and FA fibroblasts, whereas in XP fibroblasts adduct removal proceeds slowly throughout the repair period studied. Both FA and XP fibroblasts are extremely sensitive to cisplatin with regard to cell killing. For FA fibroblasts this sensitivity may be attributed to the fact that in these cells initially more DNA-adducts are formed than in normal fibroblasts, and/or to their known deficiency in the repair of DNA interstrand cross-links. For XP fibroblasts this sensitivity may be caused by their deficiency in the fast repair process, known as excision repair.

INTRODUCTION

Cisplatin [cis-PtCl$_2$(NH$_3$)$_2$] is an antitumor drug, widely used in the treatment of testicular (1, 2) and ovarian (3) cancers. Since the discovery of the antineoplastic activity of this compound (4), much research has been done to elucidate its working mechanism (for reviews see References 5 and 6), which is not fully understood yet. Inside the cell, cisplatin is known to react with DNA, RNA, and proteins. Consensus exists about DNA being the essential target for the antitumor activity (7). In recent years (8–10) much attention has been focused on the detection of products resulting from the reaction between cisplatin and DNA. The major adduct appeared to be an intrastrand cross-link, i.e., cisplatin coordinated to GpG sequences in the DNA. Smaller amounts were detected of cisplatin cross-linked to ApG and of cisplatin linking two guanines separated by one or more bases [the GuPic (Xp)G adduct]. Also, cisplatin bound monofunctionally to guanine, thought to be the intermediate in the formation of the bifunctional products (8, 11), was found. A product formed in very small quantities is the DNA-DNA interstrand cross-link (12, 13). In the presence of proteins, DNA-protein cross-links can also be formed (14, 15).

The role of the various DNA adducts in cytotoxicity and antitumor activity is still uncertain. The monofunctional adducts and the DNA-protein cross-links do not seem to be important in this respect (14, 16), whereas conflicting data have been published on a possible correlation between the DNA interstrand cross-links and the cytotoxicity of cisplatin (14–20). It is generally assumed that an adduct has to be rather persistent to cause cell killing. Therefore, the persistence of the different cisplatin-DNA adducts was studied in normal human fibroblasts as well as in fibroblast cell lines deficient in certain DNA repair processes, in an attempt to find correlation between the presence of specific lesions and the cytotoxicity of cisplatin.

The repair-deficient cells used were FA fibroblasts, which are very sensitive to cross-linking agents (21, 22), and XP fibroblasts, known to be deficient in excision repair (23, 24). Both are hypersensitive to cisplatin. The formation and repair of the DNA interstrand cross-link in relation to the survival of these three types of cells after cisplatin treatment have already been studied by Plooy et al. (17); the other cisplatin-DNA lesions were not included in that study, however.

MATERIALS AND METHODS

Compounds. Cisplatin was synthesized by an established method (25). Purity was confirmed by infrared spectroscopy and elemental analysis (Microanalytical Laboratory, University College, Dublin, Ireland).

Cell Lines. 82MB2 (MB) (provided by Dr. L. Roza, TNO Medical Biological Laboratory, Rijswijk, The Netherlands) is a cell line originating from a healthy person. XP26RO (XP) is a xeroderma pigmentosum cell line, classified in complementation group A, described by Kleijer et al. (26). The FA cell line JaVo has been provided by Dr. F. Arwert (Free University, Amsterdam, The Netherlands). Cells were not used beyond 28 passages.

Cell Culture, Treatment, and Survival. Conditions were essentially as described by Plooy et al. (17). Culture media: DMEM (Flow Laboratories, Irvine, Scotland) + 10% FCS (Flow) for MB cells; equal volumes of DMEM and F10 (Flow) + 15% FCS for XP and FA cells; both with penicillin and streptomycin. Media kept for longer than 2 weeks contained 1-glutamine (1 mM; BDH, Poole, UK). Subculturing: every fourth day, 1:3 or 1:4 (MB), 1:2 (XP, FA), after washing with PBS (8.1 mM NaHPO$_4$, 1.5 mM KH$_2$PO$_4$, 0.14 M NaCl, 2.6 mM KCl). For cisplatin treatments cells were grown in plastic Petri dishes (15 cm; Greiner, Nuringen, FRG), to confluence for at least 2 days to prevent DNA replication during posttreatment incubations. Cisplatin was freshly dissolved in the dark, in medium without FCS to prevent reaction with proteins. Cells were washed with PBS before and after treatment.

In survival experiments, feeder layers were used of trypsinized cells irradiated with 40 Gy of 60Co-y-rays; 10-ml portions with 3,5 × 10$^3$ cells/ml were plated in 9-cm Petri dishes. Cells to be treated were trypsinized in 1 ml 0.25% trypsin, diluted to 5 × 10$^5$ cells/ml and, per dose point, 4 ml was added to a 6-cm Petri dish. The next day the cells were washed, treated with cisplatin, washed, and trypsinized. After dilution to about 75 colony-forming cells per 2 ml, the suspensions were added to the feeder layers (5 dishes per dose point). Cells were incubated at 37°C for 18 days without disturbance. Then, the cells were stained with 1% méthylène blue and the colonies were counted. Survival experiments were performed at least twice.

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2 To whom requests for reprints should be sent, at TNO Medical Biological Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands.

3 The abbreviations used are: FA, Fanconi's anemia; Pt-GG, cis-Pt(NH$_3$)$_2$(G); Pt-Ag, cis-Pt(NH$_3$)$_2$(ApG); G-Pt-G, cis-Pt(NH$_3$)$_2$(d(GMP)); Pt-G, Pt(NH$_3$)$_2$(d(GMP)); XP, xeroderma pigmentosum; MB, control human cell line; FCS, fetal calf serum; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium.

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DNA Isolation. Cells were scraped from the Petri dishes with a rubber policeman, collected by centrifugation, and either used right away or stored at −20°C. Cell pellets obtained from 8 to 10 dishes (collected immediately after cisplatin treatment) or from 13 to 17 dishes (collected after a repair period) were suspended in 5 ml 10 mM Tris-HCl/1 mM EDTA, pH 7.8, to which NH4HCO3 was added immediately before use (final concentration, 0.1 M) for the inactivation of unbound and mono- or bifunctionally bound cisplatin (11). Then the cells were lysed and DNA was isolated after protein extractions (phenol and chloroform/isoamyl alcohol), RNA digestion and alcohol precipitation (27).

Enzymatic Degradation of the DNA. The DNA samples were digested to yield unmodified mononucleotides and platinum-containing (di)nucleotides as described previously (8). To prevent interference during the adduct measurements, the digested samples were incubated with proteinase K (0.5 mg/ml for 2 h at 37°C) to destroy any remaining nuclease activity, followed by heat inactivation (5 min, 100°C) of the proteinase K.

Anion-Exchange Chromatography. Unmodified nucleotides and platinum-containing (di)nucleotides in the digestion mixture were separated at pH 8.8 on an anion-exchange column (MonoQ, Pharmacia), after adjustment of the pH of the mixture with 1 M Tris. The elution was performed with a NaCl gradient from 65 to 125 mM in 12.5 mM Tris-HCl according to Reference 8, with the modifications described in Reference 27. Fractions (0.25 ml) were collected and screened for the presence of Pt-DNA digestion products, with immunochemical techniques (see below). The DNA content of the chromatographed samples was measured from the absorbance at 260 nm.

Enzyme-linked Immunosorbent Assay. For the quantitation of the Pt-containing products in the column eluate, the ELISA was used in a competitive mode, essentially as described (27, 28). Three different antisera were used for the detection of the adducts (27); antiserum W101 for the detection of Pt-GG and G-Pt-G, antiserum 3/65 for the detection of Pt-AG, and 3/43 for the detection of Pt-G. Column-eluate fractions were incubated, in 4 dilutions differing by a factor of 75 between the extremes, with a standard amount of the relevant antiserum. Subsequently, the fraction unbound antibodies was determined in an ELISA, by binding to cisplatin-treated DNA adsorbed to the wall of microtiter plates followed by detection with enzyme-conjugated “second” antibodies. From the four results, the dilution removing 50% of the antibodies was computed (ID50). Under the same conditions, known quantities of reference compound were carried through the procedure, resulting in a calibration graph which allowed conversion of the ID50 into the actual amount of adduct. Each eluate sample was measured in at least two independent competitive ELISAs. For details, see References 27 and 28.

RESULTS

Fig. 1 shows the survival for the three fibroblast cell lines after 1-h treatment with various concentrations of cisplatin. The XP and FA cells were found to be extremely sensitive to cisplatin, in agreement with earlier observations (17, 22, 29). In the semilogarithmic plot linear survival graphs were obtained, with a small shoulder in the case of MB and XP cells. From the slopes of the linear part of the graphs the Do values were calculated (Do = dose increment reducing survival to 37% of the original value). These were: MB 3.1, XP 0.8, and FA 0.7 μg cisplatin per ml, in satisfactory agreement with values published by other investigators (17, 22, 29).

In the cisplatin-treated fibroblasts the amounts of the various cisplatin-DNA adducts were determined. To this end, DNA was isolated and enzymatically digested to yield a mixture of mononucleotides and cisplatin-containing (di)nucleotides, which were separated by column chromatography. The cisplatin-containing products were identified by their elution behavior and their interaction with specific antibodies, which were used for sensitive immunochromatographic quantitation. Fig. 2 shows the elution pattern obtained with digested DNA from MB cells that had been exposed to 50 μg cisplatin/ml for 1 h. In addition to the unmodified mononucleotides (dCMP, dAMP, TMP, and dGMP) as detected by their UV absorbance. The amounts of adducts in the collected fractions were measured by using the relevant antibodies in the competitive ELISA. I, Pt-GG; II, G-Pt-G; III, Pt-AG; IV, Pt-G.
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Fig. 3. The amounts of the various cisplatin-DNA digestion products per microgram DNA from normal human (MB) fibroblasts as a function of the exposure concentration (exposure was for 60 min at 37°C). Points, mean (± range) of two independent experiments, each involving duplicate ELISAs. •, Pt-GG; O, Pt-AG; □, G-Pt-G; □, Pt-G.

Table 1 Relative amounts (%) of the various cisplatin-DNA adducts in DNA isolated immediately after cell treatment at 37°C

<table>
<thead>
<tr>
<th>Cisplatin treatment</th>
<th>Adduct</th>
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<tbody>
<tr>
<td>Cell strain</td>
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<tr>
<td>Time (min)</td>
<td>Dose (µg/ml)</td>
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<tr>
<td>MB 60</td>
<td>10</td>
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<tr>
<td>MB 60</td>
<td>25</td>
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<td>MB 60</td>
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<td>MB 70</td>
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<tr>
<td>XP 70</td>
<td>19</td>
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<td>FA 70</td>
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*G-Pt-G originates from both intrastrand Gp(Xp)nG adducts and interstrand cross-links.

formed in MB cells upon treatment with various concentrations of cisplatin are shown. All adducts show a roughly linear increase with dose. At the three concentrations investigated the main product is Pt-GG (70–75%), while G-Pt-G and Pt-AG occur at frequencies of 9–19%. The amount of monofunctionally bound cisplatin is very low (2–3%). The relative amounts of the identified adducts as determined in the various experiments have been summarized in Table 1. In the studies on the repair of cisplatin-DNA adducts in human fibroblasts the repair-deficient XP and FA cell lines were included. The cells were all treated with 19 µg cisplatin/ml for 70 min at 37°C. Part of the cells were harvested immediately after treatment, and the remaining cells were provided with fresh medium and allowed a repair period of either 4.5 or 22 h. DNA was isolated from the cells and after digestion and chromatography the cisplatin-containing products were quantitated with the ELISA.

Fig. 4 shows the amounts of the different cisplatin-DNA adducts present in the DNAs of the three cell types at t = 0, 4.5, and 22 h after the cisplatin treatment. Immediately after treatment in FA fibroblasts more adducts were present than in normal and XP cells, i.e., the Pt-GG and Pt-AG levels were approximately 50% higher. The distribution of the four adducts in the mutant cell lines was roughly the same as that found for the wild-type fibroblasts (see Table 1). For all three cell lines the data show decreasing amounts of the cisplatin products after longer repair periods. To compare the kinetics of adduct removal in the three cell lines, the amounts of adducts remaining after a repair period were calculated relative to the amount initially induced (t = 0). Fig. 5 shows the removal of the major adduct, Pt-GG, in a semilogarithmic plot. Removal of the adducts in the XP cells differs substantially from that in MB and FA cells. In XP cells the number of adducts per µg DNA decreases slowly during the whole period studied, in a first-order manner, whereas in MB and FA cells a fast repair is seen during the initial period after treatment, followed by a slower removal of the adducts.

DISCUSSION

In this study an attempt has been made to correlate the different sensitivities to cisplatin of cultured normal and repair-deficient human fibroblasts (Fig. 1) with the formation and/or removal of DNA adducts after cisplatin treatment. The results demonstrate that in the DNA of the fibroblasts investigated the...
same four Pt-DNA products are formed upon treatment with cisplatin (Figs. 2 and 4) as were found in in vitro-treated DNA (8–10, 30, 31), and in DNA of cisplatin-exposed bacteria (32), CHO cells (33), and human white blood cells (27).

The relative amounts of the four cisplatin-DNA digestion products indicate a strong preference for the induction of intrastrand cross-links on GpG sequences (62–75% of the DNA-bound cisplatin, see Table 1 and Figs. 2 and 4), in agreement with previous reports (8, 9).

The determinations of the various cisplatin-DNA adducts at different moments after exposure have not indicated a particular persistence of any of these. In the wild-type fibroblasts, all adducts show a similar repair pattern (Fig. 4), with a very fast initial removal of more than half of the adducts. After this initial period, which is completed within 4.5 h, hardly any significant removal appears to occur. This implies that at the end of the 22-h repair incubation, the intrastrand cross-link on neighboring GpG sequences still is the most abundant lesion, but this knowledge does not contribute on our insight of the possible harmfulness of this adduct. The possibility might be considered that the rapid reduction in the rate of adduct removal could be due to cytotoxic effects of the lethal treatment other than the damaging of DNA. There are no indications, however, that, e.g., cellular metabolism deteriorates already after a few hours. Also the observation that in similarly treated normal, XP and FA cells removal of interstrand cross-links continues beyond 72 h (17) does not support such an explanation. Moreover, rapid initial removal of Pt-GG (≤6h) followed by very slow repair was observed by Eastman et al. (34) in murine leukemia cells after treatments allowing more than 50% survival.

The persistence of the DNA interstrand cross-link, potentially a highly cytotoxic lesion, cannot be judged from our results, since these cross-links are determined together with an excess of cisplatin molecules linked to two nonadjacent guanines in the same strand. However, induction and removal of interstrand cross-links in eukaryotic cells have been studied, with different techniques, by other authors (14–17, 20, 35), who all reported a delayed formation followed by repair. For the same MB cells as used here, Plooy et al. (17) reported a maximal amount of these cross-links around 24 h after exposure and complete disappearance after 96 h. Therefore, cytotoxic and/or mutagenic effects of these lesions appear rather likely when they are induced in actively dividing cells.

The results obtained with the repair-deficient FA and XP fibroblasts do not provide direct information with respect to the identification of the cytotoxic lesion either. Both cell types are extremely sensitive to cisplatin, but this sensitivity cannot be related straightforwardly to the presence or persistence of one of the DNA adducts assayed. Nevertheless, with regard to the cisplatin-DNA adducts the two mutants show substantial differences, with the wild-type cells as well as one compared to the other. FA cells do not appear to be very different from MB cells in kinetics of the repair of the adducts studied, although the percentage removal of the Pt-GG adduct seems to be somewhat lower. Directly after cisplatin treatment, however, the FA cells contain about 50% more adducts. Together with the somewhat reduced removal, this results in residual adduct levels after the extended repair period that are about twice as high as in normal cells. This effect may contribute to the high sensitivity to cisplatin. On the other hand, the fact that FA cells are not capable at all of removing cisplatin-induced interstrand cross-links from the DNA (17) appears more likely to be the main cause of the highly enhanced sensitivity as compared to wild-type cells. In the cisplatin-sensitive XP cells about the same amounts of adducts are formed as in normal cells; however, the repair pattern is different from that in the normal and FA fibroblasts (Figs. 4 and 5). The disappearance of the various cisplatin-DNA adducts in XP cells is slow throughout the repair period; no fast initial repair is observed. Because XP cells are known to be defective in excision repair (23, 24), it is tempting to correlate the missing fast repair process with excision repair. XP cells, too, show less repair of interstrand cross-links than normal fibroblasts do, although this repair is better than that in FA cells (17). Possibly, this reduced repair is also a consequence of the excision repair deficiency, since it has been suggested that in mammalian cells the excision repair system provides part of the steps involved in the removal of DNA interstrand cross-links (36). XP cells are much more capable of removing these cross-links than FA cells, whereas they have about the same high sensitivity to cisplatin. It does not appear plausible, therefore, that partly defective cross-link repair is the predominant cause of the sensitivity of XP. Insufficient or erroneous repair of other lesions too, should be held responsible.

In summary, the present investigation has shown that the removal of the four cisplatin-DNA adducts studied proceeds with roughly the same kinetics in each of the three cell lines, but differences exist between the cell lines. In normal and FA cells a large proportion of the adducts is removed rapidly, within the first few hours after treatment, but further removal is very slow, if occurring. In XP cells the fast initial repair is missing, but a slow, gradual removal of the adducts is observed. It appears plausible to attribute the fast repair in MB and FA cells to the action of the system for excision repair, which is defective in XP. Our results have not identified a relatively persistent DNA adduct as the possible cause of cisplatin’s cytotoxicity. Probably, the equal hypersensitivity of the two mutant cell lines has to be ascribed, at least in part, to different lesions.

REFERENCES


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