Formation and Repair of DNA Interstrand Cross-Links in Relation to Cytotoxicity and Unscheduled DNA Synthesis Induced in Control and Mutant Human Cells Treated with cis-Diamminedichloroplatinum(II)$^1$

Adrie C. M. Plooy, Margriet van Dijk, Frits Berends, and Paul H. M. Lohman$^2$

Medical Biological Laboratory TNO, P. O. Box 45, 2280 AA Rijswijk, The Netherlands

ABSTRACT

A comparative study was performed with a variety of human cell lines on the effects of treatments with cis-diamminedichloroplatinum(II) (cisplatin) on cell survival and the induction of unscheduled DNA synthesis. In addition to control fibroblasts (Han, MB), cell lines defective in DNA repair were used [xeroderma pigmentosum, XP(A) and XP(F), and Fanconi’s anemia (FA)], as well as cells deficient in arylsulfatase A (mucolipidosis II, ML1 and ML2). Ultraviolet light and mitomycin C were included in this study as model DNA-damaging agents. Furthermore, induction of DNA interstrand cross-links by cisplatin and their repair were studied.

As for survival, only XP cells were abnormally sensitive to ultraviolet light, and only FA cells were abnormally sensitive to mitomycin C. To cisplatin, however, all mutants tested were more sensitive (2 to 5 times) than were normal cells. Unscheduled DNA synthesis induction by ultraviolet light was strong in all but the XP cells; the other two agents did not induce unscheduled DNA synthesis. Induction of DNA interstrand cross-links by cisplatin was linear with dose. Formation continued for up to 18 to 24 h after treatment. During this period, all cells but the ML mutants responded similarly. In ML cells, much fewer cross-links were induced, which were repaired rapidly. In FA cells, accumulation continued for at least 96 h; in the other cells, most of the cross-links had been removed after that period.

In the discussion, the cisplatin-induced DNA interstrand cross-links are proposed as an important potentially lethal lesion, in view of their persistence in the highly sensitive FA cells. Furthermore, the possible involvement of certain steps of the long-patch excision repair pathway in the removal of this lesion is considered. The sensitivity of ML cells to cisplatin is attributed to cytoplasmic effects, rather than to chromosomal damage.

INTRODUCTION

Platinum coordination compounds, especially cisplatin,$^3$ are very effective when applied in chemotherapy of a variety of human neoplasms (2, 25). Beside antitumor activity, cisplatin possesses genotoxic properties. Its reactivity toward DNA in cells is thought to be responsible for the antitumor effects of cisplatin (18, 27). Cisplatin is a bifunctional agent that can form various reaction products with DNA, such as cross-links between DNA and protein (9) and DNA inter- and intrastand cross-links (20, 33). In addition, monofunctional platinum-DNA adducts are formed, which are supposed to be an intermediate for bifunctional binding (19). The DNA interstrand cross-links probably are formed between 2 N-7 sites of guanines (20). Only recently, the detection of DNA interstrand cross-links induced by cisplatin in cells has become possible (1, 4, 22). To what extent the various cross-links are important for the antineoplastic and genotoxic action of cisplatin is not fully clarified yet, and in some cases conflicting results have been reported (21, 26, 30, 32, 34, 35).

Important information on the biological importance of certain DNA lesions can sometimes be obtained through the use of cells with a known deficiency in one of the systems for the repair of damaged DNA. Mutant cell lines of human origin that are relevant for the subject of this paper are cells isolated from XP patients and those derived from persons suffering from FA. XP cells are thought to be defective in one or more genes coding for proteins involved in excision repair (12, 14); they are extremely sensitive to UV irradiation (5). Several complementation groups have been identified with different UV sensitivities (5, 14, 31). XP cells of Complementation Group A are more sensitive to UV than are Group F XP cells, because they are more defective in their repair systems (13). Cells derived from FA patients are abnormally sensitive to cross-linking agents (6, 10, 29). FA cells are thought to be defective in DNA interstrand cross-link repair (28).

Numerous cell lines exist which were isolated from patients with diseases caused by a deficiency in factors other than DNA repair. Two of these cell lines (ML1, ML2) were included in this study. They were derived from ML patients and were shown to have an enzyme deficiency (aryl sulfatase A) resulting in intralysosomal storage of possibly harmful compounds (7). ML cell lines behave like control lines with regard to UV irradiation (31) and were used as such, also in the present study. These cells became more interesting, however, when they appeared to be oversensitive to cisplatin.

In the investigations presented here, the mutant cells and control human cells were compared with respect to the effects of cisplatin. Two model agents were included, UV as an inducer of interstrand cross-links on adjacent bases (pyrimidine dimers (8)) and mitomycin C, which induces predominantly DNA inter-

---

1 Supported by the Queen Wilhelmina Fund, The Netherlands, Project KWF-MBL 79-1.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: cisplatin, cis-diamminedichloroplatinum(II); UDS, unscheduled DNA synthesis; Han, MB, control human cell lines; XP, xeroderma pigmentosum; FA, Fanconi’s anemia; ML, mucolipidosis II; FCS, fetal calf serum; PBS, phosphate-buffered saline (8.1 μM Na2HPO4-1.5 μM KH2PO4-0.14 M NaCl-2.7 μM KCl); MEM, minimal essential medium; D0, dose increment which reduces survival to 37% of the original value; dThd, thymidine.

Received 11/6/84; revised 4/1/85; accepted 5/2/85.
MATERIALS AND METHODS

Cell Lines. XP2CA [XP(A)] and XP126Lo [XP(F)] are XP cell lines classified in Complementation Groups A and F, respectively (31); the FA cell line JaVo was kindly provided by Dr. F. Arwert (Free University, Amsterdam, The Netherlands); 79RD172 (MLT) and 75RD164 (ML2), both cell lines derived from ML patients, were kindly provided by Dr. A. Westerveld (Erasmus University, Rotterdam, The Netherlands); 82MB2 (MB), obtained from Dr. L. Roza (Medical Biological Laboratory TNO, Rijswijk, The Netherlands), and Han are cell lines originating from healthy persons. All cell lines were primary fibroblast cultures; the cells were not passed beyond the 20th passage.

Cell Culture. The fibroblasts were cultured in flasks (75-sq cm; Costar, Cambridge, MA) in Ham’s F-10 medium (Flow Laboratories, Irvine, Scotland), supplemented with 1 mm L-glutamine (BDH, Poole, United Kingdom), penicillin (100 units/ml; Gist-Brocades NV, Delft, The Netherlands), streptomycin (100 µg/ml; Gist-Brocades NV), and 15% FCS (Flow Laboratories) at 37°C in an incubator (Heraeus, Hanau, Federal Republic of Germany) with a humidified atmosphere of 95% air and 5% CO2. Cell handlings were performed in a laminar downflow system (Microflow Pathfinder, Ltd., Fleet, United Kingdom) under yellow light (filtered T.L. light; Philips, Eindhoven, The Netherlands; wavelength >525 nm). The cells were subcultured every week in 1:2 or 1:3 dilutions by trypsinization.

Cell Treatment. Cisplatin, synthesized by the group of Prof. Dr. J. Reedijk [State University, Leiden, The Netherlands (19)], was dissolved in F-10 medium containing 7.5% FCS just before use. The cells were subcultured every week in 1:2 or 1:3 dilutions by trypsinization.

Cell Survival. Cell survival experiments with human fibroblasts require feeder layers for the provision with necessary metabolites during the period when the inoculum is not yet self-sustaining. The same cell strain when treated with various dosages of UV, mitomycin C, or cisplatin is given in Chart 1. The survival of the various cell lines when treated with various dosages of UV, mitomycin C, or cisplatin is given in Chart 1. The
XP cells showed to be extremely sensitive to UV (Chart 1A), XP(A) more so than XP(F), which is consistent with the findings of Friedberg et al. (5) and Lehmann (13). XP(A) cells gave a completely linear survival curve, with a $D_0$ of 0.2 J/sq m, whereas in the curve for XP(F) cells ($D_0 = 0.6$ J/sq m), a shoulder was clearly present. Exposure of Han, MB, ML1, and FA cells to UV yielded survival curves with $D_0$ values which were not significantly different (MB, ML1, and FA, 1.8 J/sq m; Han, 2.2 J/sq m), but with different shoulder widths (absent for FA). The presence or absence of a shoulder and its shape are indicative for the mechanism of cell inactivation (e.g., one-hit/one-target or multitarget/mechanism, or presence or absence of repair of sublethal damage).

After treatment with mitomycin C, none of the various cell lines showed a survival curve with a shoulder (Chart 1B). From the results, it is evident that only FA cells are abnormally sensitive to this compound. Because XP cells behave like control cells, their repair deficiencies do not seem to affect the repair of mitomycin C-induced damage.

Chart 1C gives the survival curves after cisplatin treatment. Almost all cell lines reacted differently to this agent. Usually, the shoulder in the survival curve is very small or absent. Only Han cells, which showed the broadest shoulder after exposure to UV, display a broad shoulder after treatment with cisplatin. The other normal cell line, MB, has about the same $D_0$ (5.2 and 4.8 $\mu$M for Han and MB, respectively) but has no shoulder. Both ML cell lines showed a distinctly higher sensitivity to cisplatin ($D_0 = 2.3$ and 2.8 $\mu$M for ML1 and ML2, respectively). This was rather surprising, because ML1 was considered as a control human cell line with respect to genotoxic treatments. The FA line and, in particular, the 2 XP cell lines appeared to be highly sensitive to cisplatin ($D_0 = 1.5, 1.1$, and 0.9 $\mu$M for FA, XP(F) and XP(A), respectively). A survey of the relative sensitivities of the various cell strains for the 3 agents used is given in Table 1.

In order to determine the ability of the various cell lines to perform repair synthesis, measurements of the induction of UDS as a result of treatment with UV, mitomycin C, or cisplatin were carried out by means of autoradiography. This method is thought to detect mainly long-patch excision repair, in which stretches of 35 to 100 nucleotides are excised and resynthesized. Other repair reactions not involving (substantial) DNA synthesis are not picked up with this method or with low sensitivity (short-patch repair). The results of the UDS measurements are given in Chart 2. As expected, UV irradiation (Chart 2A) strongly induced UDS in FA, ML1, and Han cells, although the level of repair synthesis in FA cells is lower than that in the other 2 cell lines. With regard to UDS induction, ML1 cells were comparable to control cell lines, which is in agreement with their normal survival after UV irradiation (Chart 1A). XP(A) cells did not show UDS after UV irradiation, which confirms earlier results (see Ref. 31).

Mitomycin C did not induce UDS in FA, Han, or ML1 cells (Chart 2B); XP cells were not included in this experiment. Apparently, mitomycin C-induced lesions are not repaired via a mechanism involving UDS.

In Table 1, the relative sensitivities of 3 cell lines to UV, mitomycin C, and cisplatin are given. Table 1 shows that the mutant cell lines are more sensitive to UV than the wild-type cells, with the exception of FA cells, which are only slightly more sensitive. The relative sensitivity to mitomycin C is highest for XP cells, and the least for ML1 cells. For cisplatin, the relative sensitivities are similar to those for UV and mitomycin C, with the exception of FA cells, which are again only slightly more sensitive.

Chart 2C shows the results obtained after treatment with...
EFFECTS OF CISPLATIN ON CULTURED HUMAN CELLS

Cisplatin. None of the cell lines tested showed a significant level of UDS, with the possible exception of FA cells at a very high (and toxic) dose of cisplatin (300 μM).

The induction and repair of one type of platinum-DNA adduct, namely, the DNA interstrand cross-link, were studied in more detail. For the cell lines ML1, MB, FA, and XP(A), the amount of cross-links present at various times after treatment with different dosages of cisplatin was determined (at 0, 12, 18, 24, 48, 72, and 96 h after treatment for most cell lines). In Chart 3, representative examples of dose-response curves (t = 0, 24, and 96 h) are given. In general, the results were consistent with a linear dose-response relationship. The effect of a posttreatment incubation on the number of interstrand cross-links is summarized in Chart 4A, which shows the results of time course experiments after treatments with 33 μM cisplatin. Immediately after treatment, the FA, XP, and normal cells contained about equal amounts of DNA interstrand cross-links (see also Chart 3A), whereas the ML1 cells were exceptional in showing much less induction of this lesion. In all cases, the formation of the cross-links continued for 18 to 24 h after termination of the treatment. Thereafter, a gradual decrease occurred, but not in FA cells where formation appeared to continue at a low rate; whereas in FA cells, induction appears to be normal, but repair does not seem to occur. For XP cells, the results suggest a slightly stronger induction and/or slower repair of cisplatin-induced interstrand cross-links than in normal cells, since all XP points fall above those of the MB cells (see also Chart 3). When the results after a repair period of 96 h are compared, it is evident that XP cells contain less cross-links than do FA cells but more than do MB cells. In general, the results obtained at other dosages of cisplatin confirmed this picture (see also Chart 3).

In order to see whether a direct relationship might exist between the induction (or persistence) of DNA interstrand cross-links and the cytotoxicity of cisplatin, a comparison was made...
EFFECTS OF CISPLATIN ON CULTURED HUMAN CELLS

Chart 4. Induction and repair of DNA interstrand cross-links in cells treated with cisplatin. A, the amount of cross-links per 10^9 molecular weight of DNA as function of the posttreatment incubation time, after treatment with 33 μM cisplatin for 1 h at 37°C. The data points were taken from experiments where the amount of cross-links in the cells after variable posttreatment incubation times were determined as a function of the dose of cisplatin; bars, SD. Data are mean values of at least 3 independent experiments ± SD. B, the amount of cross-links present in the cells after treatment with equitoxic doses of the drug (D1, resulting in 1% survival). The D1 values were read from the survival curves in Chart 1C. The data were extrapolated from the dose-response curves obtained after several posttreatment incubation times. Δ, XP(A) (D1 = 4.6 μM); +, FA (D1 = 7.5 μM); •, ML1 (D1 = 11.6 μM); □, MB (D1 = 26.0 μM).

at equal toxicity. To this end, the dose-response curves obtained after various posttreatment incubation times were used to calculate the number of cross-links present after treatments resulting in 1% survival of the cells; the corresponding dosages were obtained from the graphs in Chart 1C [D1, 11.6, 26.0, 7.5, and 4.6 μM for ML1, MB, FA, and XP(A), respectively]. (Extrapolation instead of direct measurement at D1 was preferred in view of the limited sensitivity of the cross-link assay.) The results are shown in Chart 4B. At equal toxicity levels, normal human fibroblasts (MB) appear to tolerate the induction of more interstrand cross-links than any of the other cell lines. The FA cells, known to be deficient in cross-link repair, tolerate less of these lesions according to this comparison. At moments late after treatment, however, the number of cross-links in FA cells surpasses that in the other, although monofunctional, alkylating agents (15).

DISCUSSION

The extreme sensitivity of both XP cell lines tested to cisplatin suggests that a substantial fraction of the platinum-DNA damage is removed via long-patch excision repair. This notion is refuted, however, by the observation that cisplatin does not induce UDS in any of the cell lines. To explain these seemingly contradictory observations, it might be postulated that in human cells the removal of cisplatin-induced DNA damage involves certain steps of the incision-excision repair pathways (those that are defective in XP cells of Complementation Group A and F) but does not result in the excision of long patches of DNA. Another possibility could be that the bulk of cisplatin-induced lesions is rather harmless and that only a small proportion is involved in lethality. If only lesions of the latter type are repaired via the long-patch excision pathway, it might remain unnoticed at the present sensitivity of the UDS assay.

FA cells appear to be proficient in long-patch excision repair, because they react almost normally to UV irradiation (both in sensitivity and UDS induction). It has been assumed that FA cells are (partly) deficient in short-patch excision repair (6), which might explain their high sensitivity to mitomycin C if mitomycin C-induced damages were repaired via this pathway. FA cells are also abnormally sensitive to cisplatin. This would seem to indicate that the repair of damage induced by this agent requires the short-patch excision repair system that is defective in these cells, which is in conflict, however, with the fact that XP cells are highly sensitive for cisplatin but not for mitomycin C. It must be assumed, therefore, either that a variety of potentially lethal lesions is induced by cisplatin, some requiring short-patch excision repair and some removed via a pathway of which steps are defective in XP cells, or that the most important potentially lethal platinum-DNA adduct needs steps from both pathways for its removal. The latter situation might offer an explanation for the fact that only in FA cells a small but significant UDS induction by cisplatin was observed, if we furthermore assume that only a small amount of long-patch repair synthesis takes place, e.g., because...
of the limited number of the lesions involved. In that case, only in FA cells does UDS become noticeable, because the long-patch repair system continues to act on lesions which remain present since the steps resulting in complete removal are not performed.

The notion that removal of cisplatin-induced lesions in mammalian cells involves some repair synthesis of DNA, be it (very) limited, is supported by the results obtained with Chinese hamster ovary cells (23).

A model in which 2 types of excision repair are involved in the repair of DNA interstrand cross-links in mammalian cells has been proposed earlier, by Fujiwara et al. (6) and Cleaver (3). In this model, the 2 ends of the cross-link are regarded not to be equivalent and are supposed to be recognized by different enzymes. One arm of the cross-link would be unhooked by an enzyme involved in short-patch excision repair (defective in FA cells?) and the other arm would be removed by a long-patch excision repair enzyme (defective in XP cells?).

A plausible candidate for the potentially lethal platinum-DNA adduct appears to be the interstrand cross-link. It is a relatively rare lesion, because it amounts to not more than 1% of the total amount of platinum that has reacted with nuclear DNA (24). Furthermore, in the highly sensitive FA cells, in contrast to normal cells, this adduct remains unrepaired (Chart 4A). However, the fact that XP cells are even more sensitive to cisplatin seems to argue against the interstrand cross-links as the most important lesion, since in these cells removal of this adduct seems not to be blocked, merely to proceed somewhat less rapidly (Chart 4). This may be misleading. It appears conceivable that in XP cells repair of these cross-links does not go to completion. Possibly, only one arm of the interstrand connection is unhooked. After that, the lesion is no longer detected as a cross-link by the method used, but it remains present as a very bulky DNA adduct, which may be quite harmful for the cell if no further repair (long-patch excision) takes place. However, the possibility cannot be ruled that other lesions are responsible for the high cytotoxicity for XP cells.

ML cells behave like normal cells after exposure to UV or mitomycin C but are exceptional in their reaction to cisplatin. In our opinion, the increased sensitivity to this agent has nothing to do with adduct formation with nuclear DNA or with defective repair. The relatively low induction of interstrand cross-links (see Charts 3 and 4) suggests that, at the same external concentration of cisplatin, the nuclear DNA is less exposed in ML cells than in the other cells. This might indicate a hindered penetration of the compound into the nucleus. In view of the tendency of ML cells to accumulate certain compounds, it appears attractive to attribute both the reduced permeation into the nucleus and the high cytotoxicity of cisplatin to intralysosomal storage of this agent or its metabolites. This means that lethality should be due to cytoplasmic effects of the drug. It might be wondered whether a high level of adduct formation with mitochondrial DNA could occur and cause the death of the cells.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. A. Baan for his criticism during the preparation of the manuscript.

REFERENCES

EFFECTS OF CISPLATIN ON CULTURED HUMAN CELLS


Formation and Repair of DNA Interstrand Cross-Links in Relation to Cytotoxicity and Unscheduled DNA Synthesis Induced in Control and Mutant Human Cells Treated with cis-Diamminedichloroplatinum(II)

Adrie C. M. Plooy, Margriet van Dijk, Frits Berends, et al.


Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/45/9/4178

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.