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Research paper

Cisplatin–DNA adducts and protein-bound platinum in blood of testicular cancer patients

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DNA adducts formed by cisplatin [*cis*-diamminedichloroplatinum(II)] were measured in blood samples from 48 testicular cancer patients treated in four centers in Europe during four to six cycles with cisplatin infusions on five successive days (total samples, 112). Total protein-bound platinum (Pt) in blood was also measured (total samples, 84). The mean on the main DNA adduct, *cis*-Pt(NH$_3$)$_2$d(pGpG) (Pt-GG), was 0.75 fmol/μg DNA [standard deviation (SD) = 0.66] on the first day of the first cycle, increased after the infusion at day 5 of the cycle (mean 1.74 fmol/μg DNA, SD = 0.90) and decreased on the following day (mean 1.09 fmol/μg DNA, SD = 0.62). In subsequent cycles, there was a tendency to an increase in the mean Pt-GG levels. The values of protein-bound Pt in blood showed little reduction between day 5 and 6 of each cycle, and a stable increase during the course of the therapy. Strong correlations were seen between day 1, 5 and 6 of the first cycle for both Pt-GG and protein-bound Pt in blood. A strong correlation ($r = 0.62$, $p < 0.001$, 69 pairs) was found between the levels of Pt-GG and protein-bound Pt. Only two patients relapsed during the follow-up; therefore, the analysis of the association between Pt-GG levels and response to therapy was not informative. The results of this study suggest that DNA adducts formed by cisplatin at the beginning of chemotherapy are predictive of values found during later days and cycles, and that the value of protein-bound Pt in blood is predictive of the value of DNA adducts.

Key words: Cisplatin, DNA adducts, protein-bound platinum, testicular cancer.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] is an antitumor drug widely used in the treatment of testicular, ovarian and bladder cancers. In particular, in combination with other drugs, it has become a standard drug used in the treatment of testicular cancer.[1] Cisplatin is a carcinogen in experimental animals[2] and cases of second neoplasms following combination therapies including cisplatin have been reported.[3–5]

The mechanism of antitumor activity of cisplatin and that of its possible carcinogenic activity are not fully understood, but they may be similar and have been associated with its binding to DNA.[6] After an initial monofunctional binding, cisplatin forms bifunctional adducts of which the intrastrand cross-link on two neighboring guanines, the *cis*-Pt(NH$_3$)$_2$d(pGpG) adduct (Pt-GG), comprises about 60–70% of the total DNA platination.[7,8] This adduct can be determined immunochemically following chromatography of digested DNA samples.[9]

The monitoring of DNA adduct formation in white blood cells (WBC) of testicular cancer patients may contribute to the adjustment of the dose of cisplatin during ongoing chemotherapy courses (e.g. when a patient forms an inadequate adduct level during the first course). In addition, as cisplatin is likely to induce secondary neoplasms,[2] its monitoring may contribute to the elucidation of the carcinogenic process in humans.[10]

The measurement of cisplatin–DNA adducts is a very laborious and time-consuming process, due to the low amounts of platinum (Pt) bound to DNA. Most of the cisplatin is bound to the blood proteins,

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and that Pt concentration can be determined relatively fast and easily with atomic absorption spectroscopy. Monitoring of protein-bound Pt would therefore be an attractive alternative for the DNA adduct measurements and it may be worthwile to investigate the correlation between protein-bound Pt in blood and DNA adduct levels in the WBC. As established by Mustonen *et al.*,[11] the majority of the administered cisplatin is already bound to the blood proteins during the infusion period. Therefore, a fair estimation of the amount of protein-bound Pt can be obtained by measuring the total protein and Pt contents in blood samples collected after the end of the treatment.

Although these types of assays do not discriminate between the various types of blood proteins and WBC, the methods can be used for interindividual comparison of blood samples collected after the cessation of cisplatin administration.

This study was conducted in order to measure cisplatin–DNA adduct and protein-bound Pt levels from testicular cancer patients at different times of chemotherapy and to correlate these data with treatment outcome.

Materials and methods

Patients included in the study were diagnosed and treated during 1989–1993 in The Netherlands (Rotterdam and Leiden), Belgium (Antwerp) and Norway (Oslo). Most of them were included in clinical trials performed by the Genito-Urinary Group of the European Organization for Research and Treatment of Cancer (EORTC). Patients were diagnosed with metastatic testicular or extragonadal seminoma or teratoma and were treated with 20 mg/m^2 cisplatin daily for four to six cycles of 5 days, in combination with other drugs depending on the trial and the randomization arm.

For the determination of cisplatin–DNA adducts in WBC, and Pt and protein contents in whole blood, blood samples were collected at 15 min after the end of the cisplatin infusion at days 1 and 5, and at 20–24 h after the end of the last infusion at day 6. For most of the patients only two samples were taken: one at day 1 of the first cycle and one at day 6 of their last cycle of chemotherapy. Blood (20 ml) was collected by venipuncture in glass or plastic tubes containing anti-coagulant and stored at −70 C until analysis.

The methods for the quantification of the main cisplatin–DNA adduct, Pt-GG, in the WBC from frozen blood samples have been described elsewhere.[9] After thawing of the blood, 50 µl aliquots were removed from each tube for Pt and protein analysis (see below). Then, after lysis of the erythrocytes with NH_4Cl, the white blood cells were lysed in the presence of NH_4HCO_3 to prevent further reactions of cisplatin and of proteinase K to prevent enzymatic DNA digestion. After isolation, the DNA was enzymatically digested with DNase I and nuclease P1 to unmodified mononucleotides and Pt-containing (di)nucleotides. Then, the digestion mixture was chromatographed on the anion-exchange column Mono Q (Pharmacia, Uppsala, Sweden) followed by immunochemical determination (competitive ELISA) of the Pt-GG adducts in the collected column fractions. For this purpose a rabbit anti-serum raised against the hapten *cis*-$Pt(NH_3)_2$GuoGMP was used.[9] The DNA content in a sample was quantified from the peak area of the UV absorbance at 254 nm of the unmodified nucleotide GMP recorded during chromatography. Known amounts of digested DNA were used as reference. With this method adducts in DNA can be assayed down to about 0.02 fmol/µg DNA, depending on the amount of DNA analyzed.

To determine the Pt concentration in the blood samples, 50 µl aliquots of thawed blood were vigorously shaken with 100 µl 0.2% Triton X-100, followed by addition of 350 µl of water. The Pt contents in the clear solutions were determined by atomic absorption spectroscopy by use of a Perkin-Elmer model 4000 apparatus equipped with a HGA-500 graphite furnace and an AS-400 autosampling system. The drying of the samples was performed at 130 C, the ashing at 1550 C and the atomization at 2600 C. K_2PtCl_6 solutions were used for calibration.

The protein concentrations were determined with the BCA Protein Assay (Pierce, Rockford, IL) in the same Triton X-100-treated blood samples as described above for the Pt determinations. For the measurements 10 µl of 1:20 diluted Triton-treated blood samples was transferred in duplicate wells in polystyrene 96-well plates and incubated with 190 µl assay reagent at 37 C. The protein concentrations were calculated from the colorimetric data at 580 nm, using human hemoglobin as reference protein.

Follow-up of patients was conducted until the end of 1994. Information was collected on response to therapy, vital status and cause of death.

The statistical analyses were repeated after logarithmic transformation of the values of Pt-GG and protein-bound Pt, but the results were very similar to those obtained with original variables The statistical analysis was based on one-sided *t*-test, analysis of variance, and linear regression and correlation; it was conducted using the SAS package.[12]

Results

A total of 48 patients were included in the study, of whom 18 from Rotterdam, 16 from Antwerp, 12 from Leiden and two from Oslo. Their mean age was 28.6 years (SD = 7.2 years). A total of 112 blood samples were analyzed for the Pt-GG adduct and 84 samples for the protein-bound Pt levels.

Table 1 shows the mean and median DNA adduct levels at different days of treatment. The mean Pt-GG level increased between day 1 and 5 of the first cycle ($p < 0.01$). The samples taken at day 6, i.e. 1 day after the end of chemotherapy, had lower Pt-GG adduct level than the samples taken 15 min after the end of the infusion at day 5 ($p = 0.02$), but higher than the samples taken at day 1 ($p = 0.05$). This pattern was confirmed in subsequent cycles, although based on fewer samples, and is shown in Figure 1, that reports the values of Pt-GG on different days for blood samples from four patients. The highest level Pt-GG value, 3.64 fmol/μg DNA, was measured during cycle 6; the range of values on the first day of the first cycle was 0.11-3.55 fmol/μg DNA; similarly, a 10-fold range was found at day 5 (0.23 - 2.98 fmol/μg DNA) and at day 6 of the first cycle (0.26 - 2.66 fmol/μg DNA). The range was somewhat smaller for samples taken during cycles 4-6 (0.91-3.64 fmol/μg DNA). No correlation was found between Pt-GG level and age.

Mean and median values of protein-bound Pt are shown in Table 2: mean values increased within each cycle and in subsequent cycles, although they decreased somewhat between the end of each cycle and the beginning of the next one. These results are confirmed by the analysis of a subset of patients with repeated measurements (Table 2). The range of protein-bound Pt values at day 1 of the first cycle was 1.3-12.4 ng/mg protein, while it was 2.8-16.6 at day 6 of the same cycle and 9.1-16.5 in the samples taken during cycles 4-6. Protein-bound Pt levels were not correlated with age.

Correlation coefficients of Pt-GG values were calculated between different days of treatment. A strong correlation is found in cycle 1 between day 1 and day 5 (0.75, $p < 0.05$, nine patients) or day 6 (0.82, $p < 0.05$, 13 patients); the small number of patients with measurements available in subsequent cycles limits the interpretation of the comparison between days during the second and the third cycles.

Table 1. Levels of the DNA adducts Pt-GG (fmol/μg DNA)

Cycle	Day	Mean	SD	Median	Range	*N*
1	1	0.75	0.66	0.48	0.11–3.55	43
1	5	1.74	0.90	1.81	0.23–2.98	12
1	6	1.09	0.62	0.93	0.26–2.66	16
2	1	1.02	0.40	0.98	0.64–1.44	3
2	6	1.27	0.60	1.62	0.36–1.36	5
3	1	0.77	0.47	0.58	0.45–1.46	4
3	6	0.97	0.47	0.93	0.45–1.59	4
4–6	6	1.77	0.64	1.71	0.91–3.64	13

Table 2. Levels of protein-bound Pt (ng Pt/mg protein)

Cycle	Day	Mean	SD	Median	Range	*N*
1	1	2.9	2.1	2.3	1.28–12.44	31
1	5	6.4	2.0	6.5	4.16–8.61	4
1	6	6.7	4.4	5.1	2.83–16.63	9
2	1	4.5	1.5	4.1	2.98–7.00	5
2	6	6.9	1.9	6.8	4.45–9.86	5
3	1	5.7	1.0	5.5	4.73–7.06	4
3	6	8.5	3.4	9.7	4.69–11.14	3
4–6	6	12.3	2.2	11.7	9.15–16.53	12

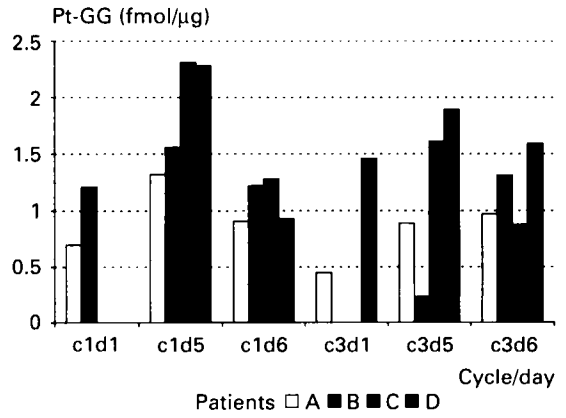

Figure 1. Levels of Pt-GG adducts in blood samples taken at different days during cycles 1 and 3 of four patients (A, B, C and D).

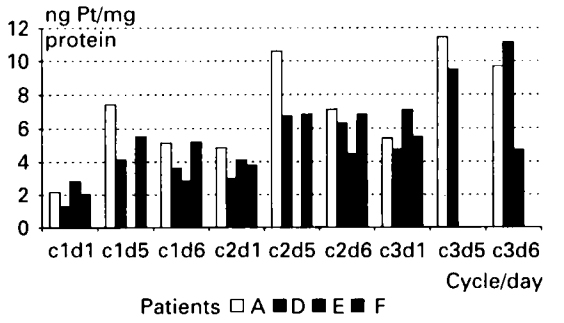

Figure 2. Levels of protein-bound Pt in blood samples taken at different days during cycles 1, 2 and 3 of four patients (A, D, E and F).

Table 3. Correlation between Pt-GG adduct and protein-bound Pt levels on selected days

Cycle/day	r^a	*p* value	*N*
Any	0.62	0.0001	69
c1/d1	0.47	0.01	28
c1/d6	0.77	0.04	7
c4–6/d6	0.20	0.56	11

[a]Correlation coefficient.

The correlation was also strong for protein-bound Pt; e.g. between day 1 and 6 of the first cycle, the correlation coefficient was 0.96 ($p<0.01$, nine patients).

A strong positive correlation between Pt-GG and protein-bound Pt was present at each day of the first cycle; during cycles 2 and 3, the small number of measurements complicates the interpretation of the findings; in samples taken on day 6 of cycles 4–6 the correlation between Pt-GG and Pt was weaker than during the early stages of the therapy (Table 3). The correlation coefficient between all pairs of measurements was 0.62 ($p<0.001$, based on 69 pairs).

The follow-up was available for 41 patients and averaged 51 months; only two patients relapsed, one of whom died, during the follow-up. The Pt-GG and protein-bound Pt values of the deceased patient were close to the mean of the whole group (e.g. 1.23 fmol/μg DNA for DNA Pt-GG and 1.73 ng Pt/mg protein at day 1 of the first cycle). The Pt-GG value of the other relapsed patient at the first day of the first cycle was low (0.44 fmol/μg DNA).

Discussion

This study has the advantage to include a group of testicular cancer patients treated with the same amount of cisplatin, thus allowing the investigation of the role of host factors in the formation and repair of DNA adducts in WBC.

This study suggested a large interindividual variability in the level of Pt-GG adducts, of the order of 10-fold. The Pt-GG level increased between day 1 and 5 of the first cycle, and diminished between day 5 and 6, due to repair of the adducts. In subsequent cycles, little overall trend is shown, although the highest levels (and the highest mean) were measured during the late cycles. The ratio of the levels at days 5 and 6 to the levels at day 1 was similar in the different cycles. Our results are very similar to those found in a study conducted in the US on patients treated with either cisplatin (20 mg/m^5 for 5 days or 120 mg/m^2 once per cycle) or with carboplatin, in which a decrease was found in DNA adducts in subsequent cycles.[13] A previous study based on a smaller series of testicular and ovarian cancer patients reported a 20–70% decline (average 50%) in Pt-GG adducts between day 5 and 6 of the first or later cycles.[14] These results were confirmed by this investigation, in which the level of Pt-GG adducts measured at day 6 of the first cycle was lower than at day 5 of the same cycle (although higher than at day 1).

The pattern of levels of protein-bound Pt in blood at different points in time is different from the one found for DNA adducts. No decrease in the level was found between day 5 and 6 of each cycle, due to a lack of repair of protein adducts. The decrease between day 6 and 1 of a subsequent cycle has to be ascribed to the turnover of the serum proteins and the hemoglobin in red blood cells. As a result the protein-bound Pt levels increase throughout therapy.

A strong correlation was found in Pt-GG levels measured during day 1, 5 or 6 of the first cycle (the measurements were too sparse in other cycles to allow any conclusion): this suggests that measurement at one time point may be predictive of the level of adducts present at other times. A similar conclusion, although based on fewer measurements, can be drawn for protein-bound Pt levels.

An important result of this study is the comparison of the measurements of Pt-GG adducts and protein-bound Pt. There was a good correlation between the two series, in particular in samples taken on the first day of the first cycle. An important implication of these findings is that DNA adducts may not give substantially more information than the more easily detectable protein-Pt levels.

One of the aims of the study was to investigate the relationship of DNA adducts and protein-bound Pt with response to therapy: the fact that only two of the 48 patients included in this population relapsed during the follow-up prevents any meaningful conclusion. In a study of testicular cancer patients treated with different regimens, patients who achieved complete response (n = 28) had a lower mean DNA adduct level than patients who did not achieve such response (n = 8).[15] Different results were obtained in a study of 17 testicular cancer patients, in which the mean and median peak adduct levels were higher among the 12 patients in complete remission as compared to the four patients in partial remission.[16] A similar positive association between high DNA adducts formed by cisplatin and complete remission was found in studies of ovarian and breast cancer patients.[17–19] Other studies with testicular cancer patients with poorer prognosis as compared

to those included in the present study are needed to further clarify this issue.

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