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Differential effects of *all-trans*-retinoic acid, docosahexaenoic acid, and hexadecylphosphocholine on cisplatin-induced cytotoxicity and apoptosis in a cisplantin-sensitive and resistant human embryonal carcinoma cell line

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Abstract Apart from modulation of tumor-cell drug sensitivity, induction of differentiation might be an alternative in the treatment of tumors resistant to cytotoxic drugs. In this report the capacity to induce differentiation and to modulate the cis-diamminedichloroplatinum(II) (CDDP) sensitivity of *all-trans*-retinoic acid (RA), docosahexaenoic acid (DCHA), and hexadecylphosphocholine (HePC) is examined in human germ-cell tumor cell lines. In the embryonal carcinoma cell line Tera-2 and its 3.7-fold CDDP-resistant subline Tera2-CP, we evaluated the effects of 96 h of pretreatment with RA (0.1 μ*M*), DCHA (23 μ*M*), and HePC (25 μ*M*) on differenctiation induction and on CDDP-induced cytotoxicity, DNA platination (4-h incubation), and apoptosis (continuous incubation). Without drug treatment, Tera2-CP showed less apoptosis than Tera-2. Pretreatment with RA decreased the cytotoxicity and apoptosis induced by CDDP without resulting in decreased DNA platination and increased differentiation in both cell lines. DCHA enhanced CDDP-induced cytotoxicity and apoptosis and did not affect the embryonal character of either cell line. HePC did not affect CDDP cytotoxicity or differentiation in either cell lines. Effects of the modulators on differentiation and on CDDP-induced cytotoxicity, DNA platination, and apoptosis did not differ between Tera-2 and Tera2-CP. RA can be applied for differentiation induction in CDDP-resistant germ-cell tumor models. However, in this model, RA reduced the apoptotic susceptibility. DCHA potentiated CDDP cytotoxicity in vitro; its in vivo modulatory capacity in germ-cell tumor cells deserves further study.

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Abbreviations *RA all-trans*-retinoic acid · *CDDP cis*-diamminedichloroplatinum(II) · *DCHA* docosahexaenoic acid · *EC* embryonal carcinoma · *HePC* hexadecyl phosphocholine · *NTera2/D1* Tera-2 · *NTera2/D1-CP* Tera2-CP · *PBS* phosphate-buffered saline (0.14 *M* NaCl, 2.7 m*M* Kcl, 6.4 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, pH 7.4)

Introduction

Patients with advanced nonseminomatous testicular germ-cell tumors treated with *cis*-diamminedichloroplatinum(II) (CDDP)-containing chemotherapy regimens have a 70–80% chance to be cured [20]. However, treatment in the remaining patients fails, usually due to the development of drug resistance by the tumor. The development of methods by which such resistance is circumvented has considerable clinical relevance. Nonseminomatous testicular germ-cell tumors are often capable of spontaneous differentiation. Therefore, the induction of terminal differentiation could be a way to reduce surviving malignant residues. On the other hand, the cellular mechanisms underlying resistance might be modulated by combination with other, often noncytotoxic drugs. Resistance to CDDP can be due to a variety of mechanisms, including reduced drug accumulation and increased detoxification of CDDP in the cellular cytoplasm. In the cell nucleus, decreased DNA accessibility and increased DNA repair can play a role [2, 18]. The latter can be the result of increased amounts of repair enzymes [18,31]. The net effect of all these systems is often reduced platinum-DNA adduct formation and, thus, decreased cytotoxicity, as platinum-DNA interactions are considered to be the main cytotoxic lesions induced by CDDP [30]. Apart from resistance mechanisms directly related to CDDP, cells might have a different propensity to undergo programmed cell death,

so-called apoptosis [9, 12]. This process is probably triggered by the reduction of proliferative potential and an inhibition of cell-cycle progression, a phenomenon also observed after CDDP-induced DNA damage [5, 14, 27].

In the present study the human embryonal carcinoma (EC) cell line NTera2/D1 (Tera-2) [4] and its CDDP-resistant subline NTera2/D1-CP (Tera2-CP) were chosen as a model [39]. Tera-2 is capable of spontaneous [4] as well as *all-trans*-retinoic acid (RA)-induced [1] differentiation. We attempted in this setting to modulate the in vitro-acquired resistance of Tera2-CP with the agents RA, docosahexaenoic acid (DCHA), and hexadecylphosphocholine (HePC). The rationale for modulation was 2-fold, namely, establishment of the capacity to induce differentiation and of the capacity to potentiate CDDP cytotoxicity. Potentiation of CDDP cytotoxicity by RA has been reported in a murine EC cell line, combined with differentiation induction [15], and in human ovarian carcinoma [7] and teratocarcinoma cell lines [19]. DCHA and HePC are agents that affect membrane composition and cell signal transduction [13, 36, 41], whereas the induction of differentiation of myeloid leukemic cell lines by HePC has also been described [6]. Previously, the potentiation of CDDP cytotoxicity in a CDDP-resistant small-cell lung-carcinoma cell line by DCHA and by transduction modulation had been reported [17, 37]. In the present study the effects of the above-mentioned modulators on the differentiation state, CDDP-induced cytotoxicity, and apoptosis in the Tera-2/Tera2-CP model were evaluated.

Materials and methods

Chemicals

CDDP was obtained from Bristol-Myers Squibb (Weesp, The Netherlands). HePC was a gift from Asta Medica (Frankfurt, Germany). RA and DCHA were purchased from Sigma Chemical Co. (St. Louis, Mo.). RPMI 1640 medium and fetal calf serum were obtained from Life Technologies (Paisley, United Kingdom). Antibodies directed against neurofilaments and vimentin were obtained from Eurodiagnostics (Apeldoorn, The Netherlands); the antibodies AE1/AE3 against a broad spectrum of cytokeratins (CKs), from Boehringer Mannheim (Mannheim, Germany); and the antibodies against desmin and glial fibrilar acidic protein (GFAP), from Dako (Glostrup, Denmark). The antibodies against CK 5 and 8 (M102) and against CK 8, 18, and 19 (M5D3) were kindly provided by F. Raemakers (University of Maastricht, The Netherlands). Hoechst 33258 was obtained from Calbiochem (La Jolla, Calif.); fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibody, from Dako (Glostrup, Denmark); and Immunofluor mounting medium, from ICN Biomedicals (Costa Mesa, Calif.). Bovine serum albumin (BSA) was provided by CLB (Amsterdam, the Netherlands) and human AB Serum, by the blood bank of Groningen (Groningen, The Netherlands).

Cell lines

The EC cell line Tera-2 and its 3.7-fold CDDP-resistant subline Tera2-CP were used as a model. Tera-2 is an embryonal cell clone of a teratocarcinoma cell line that has the capacity to differentiate in vitro as well as in vivo [4]. Both cell lines grow as monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37 °C and consist of nearly homogeneous EC cell populations when kept at high cell density (a minimum of 1.5×10^6 cells in a 25-cm^2 flask). The doubling time is 14 h for both cell lines. Tera2-CP has an increased glutathione level, 1.5-fold increased glutathione S-transferase activity, and 1.4-fold increased glutathione S-transferase-π expression as compared with Tera-2. DNA topoisomerase I and II activities and c-myc RNA and protein expression are the same in both lines [39]. Platinum accumulation is equal in both lines, whereas platinum-DNA binding is lower in Tera2-CP as compared with Tera-2 [39].

Incubation with modulators

Incubation of both cell lines with RA, DCHA, and HePC, respectively, was carried out for 96 h, with a renewal of culture medium and modulator being done after 48 h. RA, DCHA, and HePC were not present in the culture medium during or after CDDP treatment for cytotoxicity, apoptosis, or DNA-platination determinations. For establishment of the optimal concentration of the three modulators, Tera-2 and Tera2-CP cells were pretreated with various concentrations of each modulator for 96 h as described above. Cells were then harvested and plated in a 96-well microculture plate without CDDP. The highest concentration of a modulator inducing less than 10% reduction of survival in the microculture tetrazolium (MTT) assay at day 4 was chosen for combination experiments.

The *differentiation state* of Tera-2 and Tera2-CP after incubation with RA, DCHA, or HePC was determined by cellular antigen expression using immunocytochemistry. For these determinations the following antibodies were used: anti-SSEA-1 [34], anti-SSEA-3 [32], TRA-1-60 and TRA-1-81 [3], anti-vimentin, anti-desmin, anti-neurofilaments, and anti-GFAP as well as antibodies against CKs. Antigen expression was quantified as the percentage of positive cells $\pm$ SD. Experiments were repeated at least three times.

For *cytotoxicity measurements* the MTT assay was used with a 4-h CDDP incubation, as this had previously turned out to be the most optimal scheme for modulation as compared with 1-h or continuous CDDP incubation [37]. For Tera-2 and Tera2-CP cultured with or without RA, DCHA, or HePC, 10^4 cells/well were incubated for 4 h with a range of CDDP concentrations in fresh medium without modulator and were washed three times with culture medium. After a 4-day culture period, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium was added and formazan production was measured as described previously [37]. Effects of the modulators on CDDP cytotoxicity were expressed relative to the CDDP concentration inhibiting the survival of Tera-2 or Tera2-CP by 50% (IC_{50}). Mean IC_{50} values were determined in three independent experiments performed in quadruplicate.

Apoptosis

For apoptosis measurement, 10^6 Tera-2 or Tera2-CP cells precultured with or without RA or DCHA were plated in tissue-culture petri dishes (6-cm diameter). CDDP treatment was performed by continuous incubation to avoid loss of detached cells during washing procedures. Cells were incubated with equitoxic concentrations of CDDP inducing 70% reduction in cell survival in the MTT assay, namely, 1 μM CDDP for Tera-2 and 5 μM CDDP for Tera2-CP. Acridine orange staining of unfixed cells was used to distinguish apoptotic from vital cells [11]. Stainings were performed at 22, 44, and 70 h after the start of the CDDP incubation. Results are expressed as the percentage of apoptotic cells in a culture. Apart from preincubation with 0.1 μM RA, causing $<10\%$ survival reduction, preincubation with 1 μM RA was also included.

Immunocytochemical detection of Pt-DNA adducts

Tera-2 and Tera2-CP pretreated with 0.1 μM RA for 96 h or control cells were incubated for 4 h with 16.5 μM CDDP. Subsequently, cells were washed and resuspended in fresh medium.

Cytospin slides were prepared from one part immediately ($t = 0$) and from the other part after another 22 h at 37 °C ($t = 22$). Slides were air-dried, fixed in cold (−20 °C) methanol for 10 min followed by cold (−20 °C) acetone for 2 min, air-dried again, and stored at −20 °C until immunostaining. For immunocytochemical detection of Pt-DNA adducts, Gpt, a polyclonal antibody against platinated DNA capable of detecting the main Pt-containing intrastrand cross-links, the Pt-GG adducts, and the interstrand cross-links was used [24]. Upon staining the slides were dried, washed with PBS, and treated for 30 min with 1% human AB serum and 1% BSA to block nonspecific antibody binding, which was followed by an overnight treatment with Gpt (1:6) at room temperature. After the washing with PBS the presence of platinated DNA was visualized by incubation with an FITC label and the slides were counterstained for DNA detection with Hoechst 33258. An antifade mounting medium was applied and slides were stored at 4 °C in darkness until image analysis. Double-fluorescence microscopy image analysis was used to quantify DNA platination, with Hoechst 33258 fluorescence serving to locate the nuclei and FITC fluorescence serving to measure the immunosignal. At least 100 nuclei/slide were processed. The immunosignal was expressed as the median FITC surface fluorescence of the nuclei.

Statistical analysis

For statistical analysis the paired or unpaired Student's *t*-test or, for survival curves, the Mann-Whitney *U*-test for multiple unpaired samples was used. Differences were considered significant when P values were < 0.05.

Results

The concentrations of modulators resulting in less than 10% survival reduction in the MTT assay at day 4 were 0.1 μ*M* for RA, 23 μ*M* for DCHA, and 25 μ*M* for HePC. These concentrations were used for differentiation-induction, cytotoxicity, apoptosis, and DNA-platination measurements. Cellular antigen expression of Tera-2 and Tera2-CP cultured for 4 days in medium without modulators was clearly indicative of an embryonal phenotype; cultures predominantly consisted of cells that were positive for SSEA-3, a globoseries glycolipid antigen specific for human EC cells, and negative for SSEA-1, a fucosylated lactosamine antigen and early marker of differentiation [4]. The embryonal markers TRA1-60 and TRA1-81 were positive in all the cells, whereas desmin and GFAP, markers of muscle cells and astrocytes, respectively, were not detected. Cytokeratins (CKS) are epithelial markers, whereby CK 8 and 18 are mainly found in the least differentiated epithelial cells. AE1-3 positivity was found in only 9% of the cells, but the more specific antibodies recognizing CK 5 and 8 and CK 8, 18 and 19 were slightly positive in all the cells. Incubation of Tera-2 or Tera2-CP with 0.1 μ*M* RA for 4 days resulted in alterations indicative of neural-epithelial differentiation in both cell lines (Table 1), namely, a decrease in the embryonal antigens SSEA-3, TRA-1-60, and TRA-1-81 and a change in CK expression combined with an increase in SSEA-1 and neurofilament expression. Incubation for 4 days with DCHA or HePC did not induce any differentiation.

The effect of preincubation with RA, DCHA, and HePC on the IC_{50} values recorded for CDDP in Tera-2 and Tera2-CP after a 4-h CDDP incubation is shown in Table 2. IC_{50s} values achieved without modulator were 1.8 ± 0.2 μ*M* CDDP for Tera-2 and 7.0 ± 2.4 μ*M* CDDP for Tera2-CP. For determination of the effects of RA and DCHA, survival curves generated for Tera-2 and Tera2-CP cells incubated with CDDP in the presence or absence of pretreatment with one of the agents are shown in Fig. 1. Tera-2 and Tera2-CP could be sensitized 1.4-and 1.3-fold to CDDP by DCHA pretreatment, whereas the cell lines became 2.4- and 1.7-fold less sensitive to CDDP after RA preincubation of cells. Preincubation with HePC did not affect the CDDP sensitivity of either cell line. Therefore, HePC was not tested further in the apoptosis measurements.

The percentages of apoptotic cells detected in Tera-2 and Tera2-CP in the presence or absence of DCHA or RA pretreatment plus or minus continuous incubation with CDDP are shown in Fig 2. Without CDDP treatment, Tera2-CP showed less apoptosis than did Tera-2 in untreated controls (at $t = 44$ h, $P < 0.005$, and at $t = 70$ h, $P < 0.0005$), in DCHA-pretreated cells (at $t = 44$ h, $P < 0.0005$, and at $t = 70$ h, $P < 0.05$), and in 0.1 μM RA-pretreated cells (at $t = 22$ h, $P < 0.025$). Without CDDP treatment, DCHA and RA increased the precentage of apoptotic cells in a culture as compared with untreated cultures of Tera-2 (at $t = 22$ h: DCHA, $P < 0.025$; 0.1 μ*M* RA, $P < 0.025$; 1 μ*M* RA,

Table 1 Differentiation state of Tera-2 and Tera2-CP based on the expression of antigens.

Antibody	Indicating	RA-untreated cells[a] (%)	RA-treated cells[a] (%)
SSEA-1	Embryonic antigen	2 ± 2	12 ± 3[b]
SSEA-3	Embryonic antigen	100	4 ± 2[b]
TRA 1-60	Embryonic antigen	100	57 ± 16[b]
TRA 1-81	Embryonic antigen	100	23 ± 9[b]
MDesm	Desmin	0	0
MVim	Vimentin	50 ± 6	43 ± 15
MNF	Neurofilaments	3 ± 3	8 ± 2[b]
GFAP	Glial fibrilar acidic protein	0	0
M102	CK 5, 8	100	49 ± 17[b]
M5D3	CK 8, 18, 19	100	91 ± 15
AE1-3	CK 1–8, 10, 14–16, 19	9 ± 7	15 ± 4

[a] Antigen expression in Tera-2 and Tera2-CP did not differ; therefore, data for both cell lines are summarized in one column
[b] Significantly different from untreated cells

Table 2 Effect of pretreatment with RA, DCHA, or HePC on the cytotoxicity of a 4-h CDDP incubation of Tera-2 and Tera2-CP

Modulating agent	Tera-2		Tera2-CP	
	CDDP IC_{50} (μM)	MF[a]	CDDP IC_{50} (μM)	MF[a]
No modulator	1.8 ± 0.2	–	7.0 ± 2.4	–
RA (0.1 μM)	4.4 ± 2.5[b]	2.4	12 ± 2.2[c]	1.7
DCHA (23 μM)	1.2 ± 0.5[b]	0.7	5.5 ± 0.4[d]	0.8
HePC (25 μM)	1[e]	1	7.3[e]	1

[a] Modulation factor: the CDDP IC_{50} of modulator-treated cells relative to the CDDP IC_{50} of modulator-untreated cells
[b] $P < 0.025$ versus Tera-2
[c] $P < 0.025$ versus Tera2-CP
[d] $P < 0.05$ versus Tera2-CP
[e] $n = 2$, range for Tera-2 (1.7–2.0), for Tera2-CP (6.9-7.6)

$P < 0.005$). Also without CDDP treatment, in Tera2-CP the percentage of apoptotic cells was higher in RA-treated than in untreated cultures (at $t = 44$ h: 0.1 μM RA, $P < 0.025$; 1 μM RA, $P < 0.05$; at $t = 70$ h: 1 μM RA, $P < 0.005$). Incubation with equitoxic concentrations of CDDP revealed no difference in apoptosis induction between Tera-2 and Tera2-CP, independent of the pretreatment of cells. CDDP-induced apoptosis was higher in cells pretreated with DCHA than in cells treated with CDDP alone, for Tera-2 (at $t = 22$ h, $P < 0.0005$; at $t = 44$ h, $P < 0.0005$; at $t = 70$ h, $P < 0.050$) as well as for Tera2-CP (at $t = 22$ h, $P < 0.05$; at $t = 44$ h, $P < 0.025$). On the other hand, CDDP-induced apoptosis was decreased in cells pretreated with RA as compared with cells treated with CDDP alone, for Tera-2 (at $t = 44$ h, $P < 0.0025$) and Tera2-CP (at $t = 70$ h, $P < 0.025$). As the possible growth inhibition induced by RA was not considered a disturbing factor, pretreatment with 1 μM RA was included in this assay. At this higher RA concentration, CDDP-induced apoptosis was further reduced as compared with only-CDDP-treated cells in Tera-2 (at $t = 44$ h, $P < 0.0005$; at $t = 70$ h, $P < 0.0005$) and Tera2-CP (at $t = 22$ h, $P < 0.0025$; at $t = 44$ h, $P < 0.01$; at $t = 70$ h, $P < 0.0005$).

The protective effect of RA could be due to alterations at the level of DNA platination, as that is considered the main cytotoxic lesion of CDDP. Therefore, DNA platination with and without 0.1 μM RA pretreatment was studied in Tera-2 and Tera2-CP immediately after and at 22 h after CDDP incubation. Results are shown in Fig 3. All data are expressed relative to Tera-2 at $t = 0$; absolute measurements in Tera-2 at that time point were 9.8 ± 6.6 (median ± SD) median immunosignal ($n = 8$). At both time points, RA pretreatment led to an equal or higher level of DNA platination in Tera-2 and Tera2-CP (Tera2-CP versus Tera2-CP/RA, $P < 0.025$). Between Tera-2 and Tera2-CP, on difference was detected, and no decrease in Pt-DNA adducts was seen between $t = 0$ and $t = 22$ h.

Discussion

In the present study, CDDP-sensitive and resistant EC cells were pretreated in vitro with DCHA, RA, and HePC. Subsequently, the differentiation state, CDDP-induced cytotoxicity, and, after RA and DCHA pretreatment cell susceptibility to apoptosis were evaluated, whereas after RA treatment, DNA platination was also

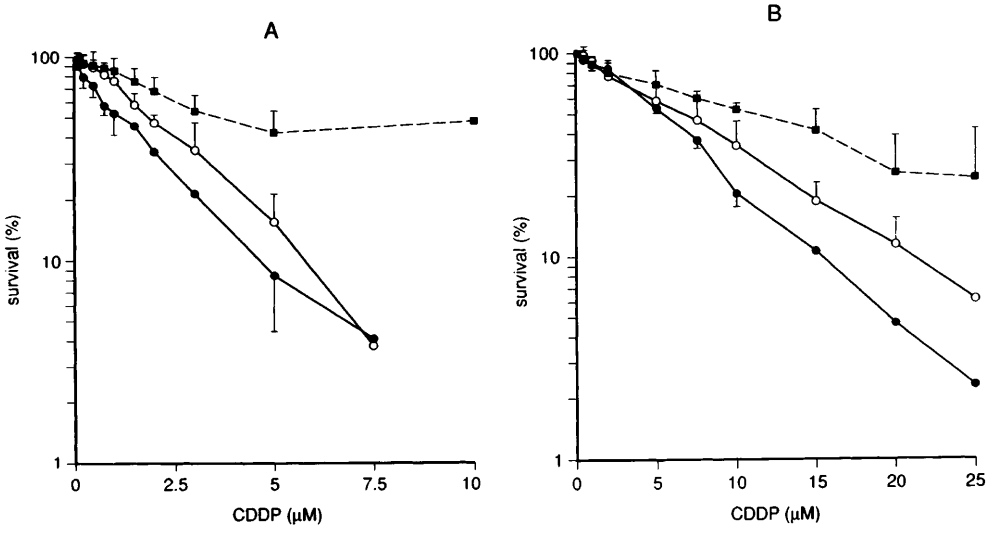

Fig. 1 Survival after a 4-h CDDP incubation of **(A)** Tera-2 and **(B)** Tera2-CP control (○–○), 96 h RA-pretreated (■–■), and 96 h DCHA-pretreated (●–●) cells as determined in the MTT assay after a 4-h CDDP incubation ($n = 3$). *Bars* SD. Curves of cells pretreated with DCMA were lower ($P < 0.001$ and $P < 0.002$) and with RA were higher ($P < 0.002$ and $P < 0.01$) than control curves of Tera 2 and Tera 2-CP, respectively

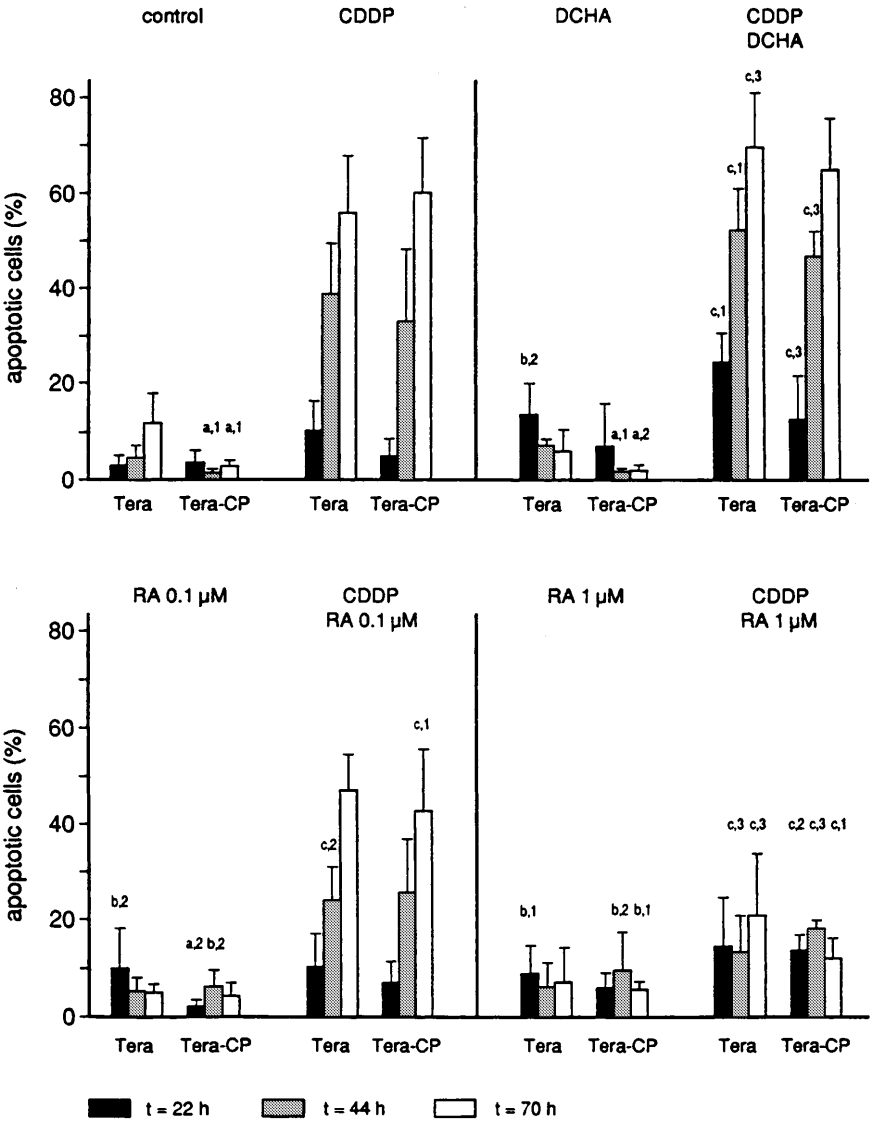

Fig. 2 Percentage of apoptotic cells found in Tera-2 and Tera2-CP controls and in cells pretreated for 96 h with RA or DCHA as indicated, as determined at $t = 22$ h, $t = 44$ h, and $t = 70$ h after the start of continuous CDDP incubation (Tera-2, 1 μM; Tera2-CP, 5 μM) by acridine orange staining of cells ($n = 3$–5). *Bars* SD. *a* Tera2-CP sample that is different from Tera-2 undergoing the same treatment at the same time point; $P < 0.005$ (*1*), $P < 0.05$ (*2*). *b* Sample treated with DCHA or RA that is different from Tera-2 or the Tera2-CP control; $P < 0.0005$ (*1*), $P < 0.05$ (*2*). *c* Sample treated with RA or DCHA plus CDDP that is different from Tera-2 or Tera2-CP treated with CDDP only; $P < 0.0005$ (*1*), $P < 0.005$ (*2*), $P < 0.05$ (*3*)

measured. The capacity of Tera-2 to differentiate in vitro upon RA treatment [4] made Tera-2/Tera2-CP a relevant model for the study of drug-induced modulation of CDDP sensitivity and induction of differentiation in human EC cells.

Incubation with 0.1 μM RA induced differentiation in Tera2-CP. This showed that Tera2-CP had not lost its capacity to differentiate in vitro upon RA treatment during the process of resistance induction. Previously it had been shown that its capacity for spontaneous differentiation in vivo was retained [39]. These findings together indicate that differentiation induction can be a potentially useful tool in the treatment of CDDP-resistant germ-cell tumors. In the present study, neither HePC nor DCHA induced differentiation in Tera-2 or Tera2-CP, although HePC has been shown to be capable of differentiation induction in leukemic cell lines [6].

Pretreatment of Tera-2 and Tera2-CP with 0.1 μM RA decreased CDDP cytotoxicity, whereas 0.1 μM RA alone induced up to 10% cytotoxicity in the MTT assay. Oosterhuis et al. [26] had found that murine F9 and Tera-2 cells induced to differentiate in vitro by RA were as sensitive to CDDP as the EC cells from which they were derived. However, interpretation of these data is seriously hampered by the growth inhibition caused by the high RA concentrations used, a side effect that was avoided in the present study. In the murine EC cell line PCC4 [15], RA-induced differentiation was accompanied by potentiation of CDDP cytotoxicity. Moreover, although murine EC is considered to be a good model of human germ-cell tumors, effects of RA on murine and human EC cells, apart from morphological differentiation induction, are different (for a review, See Para et al. [28]). From the results of the present study it is also conceivable that RA has different effects on CDDP sensitivity in murine and human EC cell lines.

Changes in membrane phospholipid and/or fatty acid composition in CDDP-resistant cells as compared with their sensitive mother lines have been reported in CDDP-resistant cell lines with [22] and without [37]

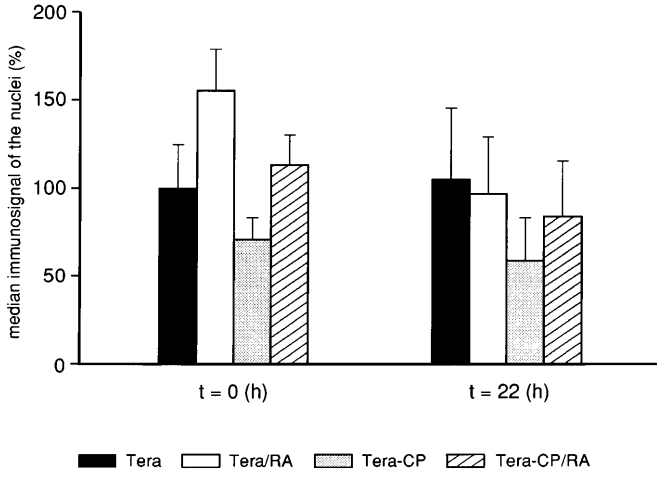

Fig. 3 DNA platination as measured with immunocytochemical detection and indicated as the median immunosignal relative to Tera-2 at t = 0 (h). Cells pretreated with RA (Tera-2/RA, Tera2-CP/RA) or not pretreated (Tera-2, Tera2-CP) were incubated with 16.5 μ*M* CDDP for 4 h. DNA platination was measured immediately after incubation (t = 0 h) or after another 22 h at 37 °C (t = 22 h; *n* = 3–8). *Bars* SEM. Significant differences: Tera2-CP at t = 0 versus Tera2-CP/RA at t = 0; $P < 0.025$

CDDP accumulation defects. Potentiation of the cytotoxicity of CDDP by incorporation of the polyunsaturated fatty acid DCHA has been observed in a CDDP-resistant small-cell lung carcinoma [37]. In the present study, DCHA potentiated CDDP cytotoxicity in both cell lines but did not reduce the resistance factor. It is therefore unlikely that in Tera2-CP a specific resistance-related mechanism would be affected by DCHA.

Enhancement of CDDP cytotoxicity with agents that inhibit protein kinase C have been reported, but effects were unpredictable and varied among different cell line models (for a review, See Timmer-Bosscha et al. [38]). HePC has inhibited protein kinase C activity in vitro [4, 13]. Modulation of CDDP cytotoxicity with HePC was not found in the Tera-2/Tera2-CP model. It is unlikely that this was caused by suboptimal HePC concentrations, as in other studies cytotoxicity of HePC was observed in the same concentration range [16]. As HePC neither induced differentiation nor increased CDDP cytotoxicity, it was not studied further.

It has been found that a high apoptosis susceptibility is the possible mechanism underlying the extreme sensitivity of EC cells to cytotoxic drugs [8, 9] and that this is reduced in tumor cells derived from more differentiated tissues. It can be hypothesized that triggering might also be reduced upon differentiation of the EC cells. In addition, cell death due to chemotherapeutic drugs is not always directly correlated with drug-induced damage [12, 25]. In the present study the induction of apoptosis in Tera-2 and Tera2-CP was studied after treatment of cells with RA and DCHA and, subsequently, continuous CDDP incubation. If untreated, Tera2-CP cells showed less apoptosis than did Tera-2 cells. The modulators DCHA and 0.1 μ*M* RA induced less apoptosis in Tera2-CP than in Tera-2. The frequency of CDDP-induced apoptosis was the same in both lines after equitoxic CDDP treatment and after incubation with CDDP preceded by treatment with RA or DCHA. In both cell lines, RA treatment alone increased apoptosis as compared with untreated cells, whereas pretreatment with RA decreased apoptosis induced by CDDP. Other investigators have reported that RA and CDDP applied as single agents induce apoptosis in neuroblastoma cells by different pathways [29]. RA-induced apoptosis was compatible with apoptosis as the mode of death of differentiated leukemic cells [23, 33]. CDDP-induced apoptosis is likely to be due to the disturbance of cell-cycle progression [12] and has been found to be independent of the capacity of myeloid leukemic cells to differentiate [21]. The results of the present study suggested different, even antagonistic, pathways for apoptosis induction by RA and CDDP. DNA platination after RA incubation and subsequent CDDP treatment was measured as it was considered to represent the outcome of all parameters possibly affecting CDDP sensitivity before the final apoptosis induction. DNA platination was not decreased after pretreatment of the cell lines with RA. This, however, is not a common phenomenon. An unaltered level of CDDP-induced DNA damage was seen in a murine EC cell line in which RA pretreatment led to increased CDDP cytotoxicity [15], and in human ovarian carcinoma cell lines, treatment with RA increased CDDP sensitivity and DNA platination [7]. It seems that RA pretreatment of Tera-2 and Tera2-CP cells led to an alteration in the triggering of apoptosis by CDDP.

Apoptosis was increased by DCHA and by DCHA followed by CDDP in Tera-2 and Tera2-CP. The latter was most probably due to increased DNA damage caused by CDDP after pretreatment of the cell lines with DCHA, as has previously been found in a human small-cell lung-carcinoma cell line [37]. However, induction of apoptosis has also been found after incubation of stimulated, freshly isolated human lymphocytes with very-long-chain polyunsaturated fatty acids, such as DCHA [35], indicating that pretreatment with DCHA could also affect apoptosis-related processes. Potentiation of CDDP by DCHA in germ-cell tumors in vitro is thus achievable. The applicability of DCHA in vivo deserves further attention.

In conclusion, in the Tera-2/Tera2-CP model the reduced percentage of apoptotic cells detected in untreated Tera2-CP samples indicates that apoptosis susceptibility is altered in Tera-2 upon CDDP resistance induction. The finding that RA induced differentiation in both the CDDP-resistant and sensitive EC cell lines at a clinically achievable concentration [10, 40] justifies further study of the applicability of RA or RA analogues for the induction of differentiation in CDDP-resistant human germ-cell tumors. The observation that RA prohibited CDDP-induced apoptosis stresses the importance of adequate drug scheduling if these two agents are combined.

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References

1. Andrews PW (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol 103: 285
2. Andrews PA, Howell SB (1990) Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells 2: 35
3. Andrews PW, Banting GS, Damjanov I, Araud D, Auner P (1984) Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. Hybridoma 3: 347
4. Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, Føgh J (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line tera-2. Differentiation in vivo and in vitro. J Lab Invest 50: 147
5. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40: 2353
6. Beckers T, Voegeli R, Hilgard P (1994) Molecular and cellular effects of hexadecylphosphocholine (miltefosine) in human myeloid leukaemic cell lines. Eur J Cancer 14: 2143
7. Caliaro MJ, Vitaux P, Lafon C, Lochon I, Néhmé A, Valette A, Canal P, Bugat R, Jozan S (1997) Multifactorial mechanism for the potentiation of cisplatin (CDDP) cytotoxicity by all-trans retinoic acid (ATRA) in human ovarian carcinoma cell lines. Br J Cancer 75: 333
8. Chresta CM, Hickman JA (1996) Oddball p53 in testicular tumors. Nature Med 2: 745
9. Chresta CM, Masters JRW, Hickman JA (1996) Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high Bax: Bcl-2 ratio. Cancer Res 56: 1834
10. Conley BA, Egorin MJ, Sridhara R, Finley R, Hemady R, Wu S, Tait NS, Van Echo DA (1997) Phase I clinical trial of all-trans retinoic acid with correlation of its pharmacokinetics and pharmacodynamics. Cancer Chemother Pharmacol 39: 291
11. Evans DL, Dive C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res 53: 2133
12. Evans DL, Tilby M, Dive C (1994) Differential sensitivity to the induction of apoptosis by cisplatin in proliferating and quiescent immature rat thymocytes is independent of the levels of drug accumulation and DNA adduct formation. Cancer Res 54: 1596
13. Geilen CC, Haase R, Buchner K, Wieder T, Hucho F, Reutter W (1991) The phospholipid analogue hexadecylphosphocholine inhibits protein kinase C in vitro and antagonises phorbol ester stimulated cell proliferation. Eur J Cancer 27: 1650
14. Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z (1993) The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res 53: 3186
15. Guchelaar HJ, Timmer-Bosscha H, Dam-Meiring A, Uges DRA, Oosterhuis JW, De Vries EGE, Mulder NH (1993) Enhancement of cisplatin and etoposide cytotoxicity after all-trans retinoic-acid-induced cellular differentiation of a murine embryonal carcinoma cell line. Int J Cancer 55: 442
16. Himmelmann AW, Danhauser-Riedl S, Steinhauser G, Busch R, Modest EJ, Noseda A, Rastetter J, Vogler WR, Berdel WE (1990) Cross-resistance pattern of cell lines selected for resistance towards different cytotoxic drugs to membrane-toxic phospholipids in vitro. Cancer Chemother Pharmacol 26: 437
17. Hofmann J, Doppler W, Jakob A, Maly K, Posch L, Uberall F, Grunicke HH (1988) Enhancement of the antiproliferative effect of *cis*-diamminedichloroplatinum(II) and nitrogen mustard by inhibitors of protein kinase C. Int J Cancer 42: 382
18. Kelly SL, Rozencweig M (1989) Resistance to platinum compounds: mechanisms and beyond. Eur J Cancer Clin Oncol 25: 1135
19. Le Ruppert KI, Masters JRW, Kneuchel R, Seegers S, Tainsky MA, Hofstaedter F, Buettner R (1992) The effect of all-trans retinoic acid on chemosensitivity of PA-1 human teratocarcinoma cells and its modulation by an activated *N-ras* oncogene. Int J Cancer 51: 646
20. Loehrer PJ, Einhorn LH (1984) Cisplatin. Ann Intern Med 100: 704
21. Lotem J, Sachs L (1993) Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukaemic cells. Cell Growth Differ 4: 41
22. Mann SC, Andrews PA, Howell SB (1988) Comparison of lipid content, surface membrane fluidity, and temperature dependence of *cis*-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. Anticancer Res 8: 1211
23. Martin SJ, Bradley JG, Cotter TG (1990) HL-60 cells induced to differentiate toward neutrophils subsequently die via apoptosis. Clin Exp Immunol 79: 448
24. Meijer C, De Vries EGE, Dam A, Wilkinson MHF, Hollema H, Hoekstra HJ, Mulder NH (1997) Immunocytochemical analysis of cisplatin-induced platinum-DNA adducts with double fluorescence video microscopy. Br J Cancer 76: 290
25. O'Connor PM, Wassermann K, Sarang M, Magrath I, Bohr VA, Kohn K (1991) Relationship between DNA cross-links, cell cycle, and apoptosis in Burkitt's lymphoma cell lines differing in sensitivity to nitrogen mustard. Cancer Res 51: 6550
26. Oosterhuis JW, Andrews PW, De Jong B (1986) Mechanisms of therapy-related differentiation in testicular germ cell tumours. In: McBrien DCH, Slater TF (eds) Biochemical mechanisms of platinum antitumour drugs. IRL, Oxford, p 65
27. Ormerod MG, O'Neill C, Robertson D, Kelland LR, Harrap KR (1996) *cis*-Diamminedichloroplatinum(II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines. Cancer Chemother Pharmacol 37: 463
28. Pera MF, Roach S, Elliss CJ (1990) Comparative biology of mouse and human embryonal carcinoma. Cancer Surv 9: 243
29. Piacentini M, Fesus L, Melino G (1993) Multiple cell cycle access to the apoptotic death program in human neuroblastoma cells. FEBS Lett 320: 150
30. Roberts JJ, Friedlos F (1987) Quantitative estimation of cisplatin-induced DNA interstrand cross-links and their repair in mammalian cells: relationship to toxicity. Pharmacol Ther 34: 215
31. Scanlon KJ, Kashani-Sabet M, Miyachi H, Sowers LC, Rossi J (1989) Molecular basis of cisplatin resistance in human carcinomas: model systems and patients. Anticancer Res 9: 1301
32. Shevinsky LH, Knowles BB, Damjanov I, Solter D (1982) Monoclonal antibody to murine embroys defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcimoma cells. Cell 30: 697
33. Solary E, Bertrand R, Kohn KW, Pommier Y (1993) Differential induction of apoptosis in undifferentiated and differentiated HL-60 cells by DNA topoisomerase I and II inhibitors. Blood 81: 1359
34. Solter D, Knowles BB (1978) Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci USA 75: 5565
35. Soyland E, Nenseter MS, Braathen L, Drevon CA (1993) Very long chain n-3 and n-6 polyunsaturated fatty acids inhibit proliferation of human T-lymphocytes in vitro. Eur J Clin Invest 23: 112
36. Stillwell W, Ehringer W, Jenski LJ (1993) Docosahexaenoic acid increases permeability of lipid vesicles and tumour cells. Lipids 28: 103
37. Timmer-Bosscha H, Hospers GAP, Meijer C, Mulder NH, Muskiet FAJ, Martini IA, Uges DRA, De Vries EGE (1989)

Influence of docosahexaenoic acid on cisplatin resistance in a human small cell lung cancer cell line. J Natl Cancer Inst 81: 1069
38. Timmer-Bosscha H, Mulder NH, De Vries EGE (1992) Modulation of *cis*-diamminedichloroplatinum(II) resistance: a review. Br J Cancer 66: 227
39. Timmer-Bosscha H, Timmer A, Meijer C, De Vries EGE, De Jong B, Oosterhuis JW, Mulder NH (1993) *cis*-Diamminedichloroplatinum(II) resistance in vitro and in vivo in human embryonal carcinoma cells. Cancer Res 53: 5707
40. Trump DL, Smith DC, Stiff D, Adedoyin A, Day R, Bahnson RR, Hofacker J, Branch RA (1997) A phase II trial of all-trans retinoic acid in hormone-refractory prostate cancer: a clinical trial with detailed pharmacokinetic analysis. Cancer Chemother Pharmacol 39: 349
41. Zheng B, Oihsi K, Shoji M, Eibl H, Berdel WE, Hadju J, Vogler WR, Kuo JF (1990) Inhibition of protein kinase C, (sodium plus potassium)-activated adenosine triphosphatase, and sodium pump by synthetic phospholipid analogues. Cancer Res 50: 3025