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Apoptosis and c-*jun* induction by cisplatin in a human melanoma cell line and a drug-resistant daughter cell line

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Cisplatin resistance was developed in the human melanoma cell line RPMI8322 by repeated short-term exposures to cisplatin. The most resistant daughter cell line, RPMI8322/CDDP-300, was 4-fold resistant to cisplatin, and partially cross-resistant to carboplatin, melphalan and UV, but not to BCNU. RPMI8322/CDDP-300 cells showed less apoptosis after cisplatin than the parental cells. The cisplatin resistance was not paralleled by a similar reduction in cellular cisplatin accumulation or DNA cross-links in RPMI8322/CDDP-300 cells, and these cells exhibited no increase in cellular glutathione or in mRNA encoding the DNA excision repair proteins ERCC1 and XPB. Induction of c-*jun* mRNA by cisplatin was considerably lower in RPMI8322/CDDP-300 cells than in RPMI8322 cells, consistent with the possibility that c-*jun* induction may be involved in a pathway that triggers apoptosis after exposure to DNA damaging agents. However, c-*jun* induction is not necessary for apoptosis, since cisplatin also induced apoptosis in A14 rat embryo fibroblasts, cells in which the c-*jun* gene is deleted.

Key words: Apoptosis, cisplatin, c-*jun*, DNA damage, melanoma cells.

Introduction

The cytostatic drug cisplatin is an effective agent in the treatment of many kinds of human malignancies.[1] In some tumors, however, cisplatin has a limited activity. Thus, in malignant melanoma cisplatin as single drug therapy yields a response rate of only 15%.[2] While the reasons for resistance to clinical therapy with cisplatin are largely unknown, several mechanisms which contribute to resistance to cisplatin in cultured tumor cells have been described.[3,4] These include reduced intracellular cisplatin accumulation, increased amounts of glutathione (GSH) or elevated glutathione transferase activity, elevated levels of metallothioneins, reduced induction of DNA lesions and enhanced DNA repair. The cytotoxicity of cisplatin is directly related to the induction of DNA lesions, which mainly consist of DNA intrastrand cross-links.[5] Unrepaired DNA adducts are likely to be the cause of cisplatin-induced cell death and some studies suggest that increased repair of DNA lesions may play an important role in cisplatin resistance.[6–8]

It has been demonstrated that cisplatin can induce apoptosis in tumor cells. Apoptosis and necrosis are two modes of cell death which differ both morphologically and biochemically.[9] While necrosis is associated with cell swelling, rupture of membranes and dissolution of organized structure, apoptosis has little effect on organelle integrity, but is characterized by cell shrinkage and chromatin condensation. Necrosis results from loss of osmoregulation, with random DNA degradation by lysosomal enzymes at a late stage. In contrast, in apoptosis an unidentified endonuclease cleaves DNA at internucleosomal linker sites within the chromatin, which results in a characteristic DNA ladder pattern after gel electrophoresis.[10] However, the general occurrence of this internucleosomal DNA degradation during apoptosis in different cell types has not been established. It is therefore important to demonstrate the morphological markers of apoptosis while internucleosomal DNA cleavage may serve as supportive evidence.[11]

Exposure of cells to cisplatin as well as several other DNA-damaging agents including ionizing radiation, UV irradiation and cytotoxic drugs such

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as etoposide and 1-β-D-arabinofuranosylcytosin activates transcription of the c-*jun* immediate early response gene.[12–15] The c-*jun* gene product, c-Jun, is a major component of the transcription factor AP-1, which contains a highly conserved DNA binding domain shared by a family of mammalian transcription factors including Jun-B, Jun-D, c-Fos, Fos-B and Fra-1.[16] A conserved leucine zipper motif allows for dimerization between certain members of this family. These AP-1 dimers bind to a consensus sequence, the so called TPA responsive element (TRE), which has been identified in the promoter regions of several genes.[16] The consequences of activation of c-*jun* expression following DNA damage is presently unclear. Since c-*jun* expression occurs before and during periods of internucleosomal DNA cleavage characteristic of apoptosis,[15] it may be involved in the signal transduction pathway that initiates apoptosis.

To set up a clinically relevant *in vitro* model for acquired drug resistance, we established cisplatin resistant daughter cell lines *in vitro* by treating the human melanoma cell line RPMI8322 with short exposures to stepwise increasing concentrations of cisplatin. We have now compared several parameters, including drug accumulation, induction of DNA adducts, occurrence of apoptosis and c-*jun* induction in parental and cisplatin resistant daughter cells. To further explore the possible relationship between c-*jun* induction and apoptosis we have also studied the effects of cisplatin treatment on the A14 rat embryonal fibroblast cell line in which the c-*jun* gene is deleted.[17]

Material and methods

Drugs and chemicals

Cisplatin, *cis*-diammine[1,1-cyclobutane dicarboxylato-(2-)0,0′]platinum(II) (carboplatin) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) were generously provided by Bristol-Myers Squibb Laboratories (Syracuse, NY). Melphalan was obtained as a sterile powder from the Wellcome Foundation (London, UK). For drug treatments, cisplatin was dissolved in 50 µl dimethyl sulfoxide and diluted to the appropriate concentrations in cell culture medium without fetal calf serum (FCS). All other drugs were dissolved and diluted in cell culture medium without FCS. [methyl-^{14}C]thymidine (2.22 GBq/mmol, 1.85 MBq/ml) and [methyl-^{3}H]-thymidine (185 GBq/mmol, 37 MBq/ml) were obtained from the Radiochemical Center (Amersham, UK). Hoechst 33342 and propidium iodide were from Fluka (Buchs, Switzerland).

Cell lines

Cisplatin-resistant sublines of the human melanoma cell line RPMI8322[18] were obtained by repeated 30 min exposures to increasing concentrations of cisplatin during a period of 3 years. Each cisplatin treatment was given at a dose estimated to kill the majority of cells. Between treatments cells were allowed to recover for 4–6 weeks. Since the parental RPMI8322 cells are themselves quite resistant to cisplatin, high drug doses were used, and the numbers at the end of designations of the resistant sublines RPMI8322/CDDP-80, RPMI8322/CDDP-240 and RPMI8322/CDDP-300 indicate the final cisplatin concentration in µM that the particular daughter cell line was exposed to. The rat embryo fibroblast cell line A14 in which the c-*jun* gene is deleted has been previously described.[17] These cell lines were cultured in Eagle's MEM with Earle's salts (Flow), supplemented with 2 mmol/l L-glutamine, 10% FCS, 125 IU/ml benzylpenicillin and 125 µg/ml streptomycin. L1210 mouse leukaemia cells were grown in RPMI 1640 medium (Flow), supplemented with 10% FCS, L-glutamine, benzylpenicillin and streptomycin.

Drug-induced cytotoxicity

Drug-induced cytotoxicity was measured by inhibition of colony formation. Appropriate numbers of cells were seeded into 6 cm diameter Petri dishes with 4 ml Eagle's MEM10% FCS and 2 mmol/l L-glutamine, and allowed to attach overnight. The cells were then exposed to various concentrations of drug for 30 min in medium without FCS. In each experiment triplicate dishes of drug-treated as well as untreated control cells were included. After removal of the drug, the cells were grown in fresh medium with 10% FCS and L-glutamine for 14 days to produce colonies. The dishes were then rinsed with phosphate buffered saline (PBS), fixed with absolute ethanol and stained with Giemsa. Colonies containing at least 50 cells were counted. The plating efficiency was calculated as the ratio of the number of colonies over the number of cells plated. For each drug dose the surviving fraction was calculated as the ratio of the mean plating efficiency in dishes containing drug-treated cells over the mean plating efficiency in control dishes with untreated cells.

Measurements of cellular GSH

Approximately 10^6 cells were precipitated with 5% trichloroacetic acid in 12.5 mM EDTA on ice for at least 15 min. The GSH content was determined by the method of Tietze[19] and related to the protein content of each cell sample.

Measurements of cellular cisplatin uptake

Approximately 50×10^6 cells were incubated with cisplatin in medium without FCS for 30 min, immediately harvested by scraping with a rubber policeman, spun down and washed once with PBS. The cell pellet was then lysed in 300 µl distilled H_2O by freeezing/thawing three times. The total platinum content of the cell lysate was measured by a Varian AA-1275 series atomic absorption spectrophotometer, equipped with a GAT-95 pyrolytic coated graphite tube atomizer. The instrumentation and conditions for the quantitative determination of intact cisplatin by liquid chromatography has been described elsewhere.[20] Briefly, the column used was a strong anionic exchanger (Nucleosil SA 5 µm, 200 × 4.6 mm I.D.) and a mobile phase composed of 0.125 M succinic acid adjusted to pH 5.2 with NaOH, and methanol (2 : 3, v/v). The post-column derivatization was performed in a packed-bed reactor using diethyldithiocarbamate as derivatization reagent. Absorbance was monitored at 344 nm. Results of measurements of total platinum and intact cisplatin were related to the protein content of cell lysates.

Drug-induced DNA interstrand cross-linking

The alkaline elution technique developed by Kohn and coworkers was used with minor modifications as described before.[21] Melanoma cells were labeled by growth in medium containing [^{14}C]thymidine (37–74 kBq/ml) overnight. L1210 cells were similarly labeled by [^{3}H]thymidine. After cisplatin treatment for 30 min in medium without FCS, the melanoma cells were incubated in drug-free medium containing 10% FCS for 6 h. Melanoma cells were then irradiated with 6 Gy and the L1210 cells with 3 Gy in ice-cold medium with 4% FCS using a Siemens Stabilopan orthovoltage X-ray machine. Melanoma cells and L1210 cells (1.2×10^6 of each) were collected on polycarbonate filters (pore size 2 µm, diameter 25 mm; Nucleopore, Pleasanton, CA), lysed with a 2% sarkosyl/0.02 mM EDTA solution (pH 10.0) and treated with 0.5 mg/ml proteinase K (Merck, Darmstadt, Germany) for 1 h. The DNA was then eluted with a tetraethyl-ammoniumhydroxide solution (pH 12.1) and collected in eight fractions. The radioactive label in each fraction as well as that remaining on the filter was analysed by scintillation counting. From the resulting elution curves the amounts of DNA interstrand cross-links were calculated, as previously described.[21]

RNA preparation and Northern blots

The day before drug treatment 10^6 cells were plated in 6 cm diameter Petri dishes. After a 40 min cisplatin treatment, cells were further incubated in drug-free culture medium. At different time points, total cellular RNA was isolated by the hot phenol extraction method, electrophoretically separated on 1% formaldehyde–agarose gels and transferred to nitrocellulose filters.[22] Hybridization was performed at 44°C using ^{32}P-labeled DNA probes. The c-*jun* cDNA probe was isolated from plasmid pAH119 obtained from Dr R Bravo.[23] Plasmids pE12-12 containing the *ERCC1* cDNA[24] and pCD1-1 with the *XPB* cDNA[25] were obtained from Dr JHJ Hoeijmakers. The β-actin cDNA insert was prepared from plasmid pAc18.1[26] and α-enolase cDNA from plasmid pH48.[27] Autoradiographs were obtained using Hyperfilm-MP from Amersham, (Amersham, UK). The autoradiographs were scanned using an LKB UltroScan XL Enhanced Laser Densitometer (Pharmacia Biotech, Sollentuna, Sweden) and analyzed using the LKB GelScan XL software package. The density of c-*jun* hybridization was normalized against β-actin expression.

Morphological analysis of apoptotic cells

Hoechst 33342/propidium iodide double staining was used to study cell morphology at different times after cisplatin treatment. Cells were replated (10^6 cells per 6 cm diameter Petri dish) 1 day before drug treatment. After a 40 min cisplatin treatment, cells were incubated in drug-free medium for different times. Hoechst 33342 (1 µg/ml) was then added to the medium and cells were incubated for 20 min at 37°C. Cells were then collected by trypsinization and pellets were resuspended in medium containing Hoechst 33342. Just before viewing in the fluorescence microscope 1–5 µg/ml propidium iodide was added. Hoechst 33342 dye passes through the plasma membrane and binds to AT-rich

regions of DNA. When bound to DNA the dye fluoresces blue and thus visualizes the chromatin. Propidium iodide, which gives a red fluorescence, is a vital staining dye which only enters dead cells who have lost their membrane integrity. The morphology of the cells was examined using an Orthoplan Fluorescence Microscope equipped with an automatic camera (Orthomat, Germany) and a B2 long pass filter (Leitz, Germany). The percentage of apoptotic and dead cells (cells showing characteristic chromatin condensation, nuclear or cellular fragmentation with or without loss of membrane integrity) was determined by counting at least 500 cells in each sample.

Analysis of DNA cleavage

Cells (10^6) were washed with PBS and lysed in 20 µl of 50 mM Tris–HCl (pH 8.0), 0.5% sodium dodecyl sulfate, 10 mM EDTA, and 50 µg/ml proteinase K. After incubation at 37°C for 10 h, 10 µl of 0.5 mg/ml RNase A was added and the cell material was incubated for an additional 10 h. The samples were mixed with 10 µl of 10 mM EDTA (pH 8.0) containing 1% (w/v) low-melting-point agarose, 0.25% bromophenol blue and 40% sucrose at 70°C. The DNA was separated in 2% agarose gels containing 0.5 µg/ml ethidium bromide and visualized by UV transillumination.

Results

Cisplatin sensitivity of cell lines

The relative sensitivities of the parental and daughter cell lines to cisplatin were assayed by inhibition of colony formation. The cell lines that had been repeatedly exposed to increasing concentrations of cisplatin showed resistance to the drug (Figure 1). Based on comparisons of D_{50} concentrations, the most resistant subline RPMI8322/CDDP-300 was 4-fold less sensitive than the parental cell line, and showed varying levels of cross-resistance to carboplatin, melphalan and UV, but not to BCNU (Table 1). RPMI8322/CDDP-300 cells retained most of their resistance during prolonged growth in normal medium without cisplatin: after 8 months the cells were still 3-fold resistant to cisplatin compared to the parental cell line (data not shown).

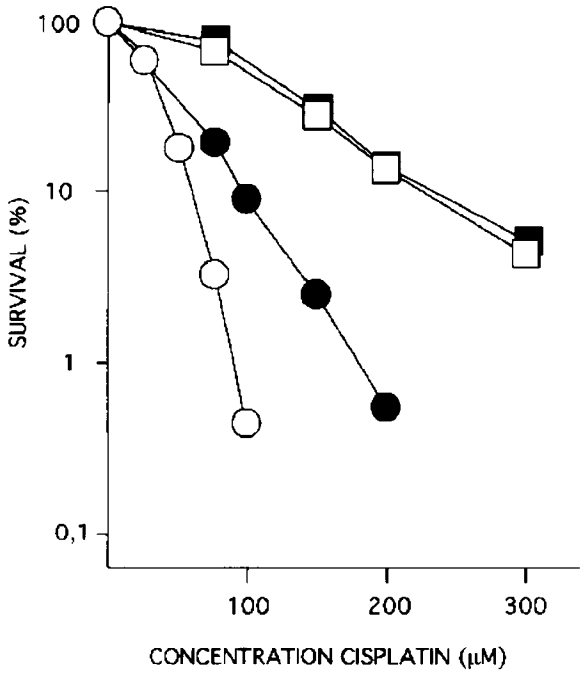

Figure 1. Cytotoxicity of cisplatin to RPMI8322 (○), RPMI8322/CDDP-80 (●), RPMI8322/CDDP-240 (□), and RPMI8322/CDDP-300 cells (■) measured by inhibition of colony formation.

Table 1. Sensitivity of RPMI8322 and RPMI8322/CDDP-300 cells to cytostatic drugs and UV, expressed as IC_{50} values (the dose that reduces colony formation to 50%); the right column shows ratios between RPMI8322 and RPMI8322/CDDP-300 cells.

Agent	RPMI8322	RPMI8322/CDDP-300	Ratio
Cisplatin[a]	27.2 ± 5.0[c]	105.5 ± 15.2[c]	4.0 ± 1.0[c]
Carboplatin	70.3[d]	265.4[d]	3.8
Melphalan	9.5[d]	16.0[d]	1.7
BCNU	14.1[d]	13.4[d]	1.0
UV[b]	6.7[d]	10.9[d]	1.6

[a]Drug concentrations are listed in µM.
[b]UV doses are listed in J/m^2.
[c]Mean ± SD (n = 4), $P < 0.001$ (Student's t-test).
[d]Results of single experiments with triplicate dishes in each.

Cellular GSH, cisplatin uptake and DNA interstrand cross-linking.

Since increased GSH content has been described as one mechanism of cisplatin resistance, the GSH levels in parental and cisplatin-resistant cells were analysed. The GSH content of RPMI8322/CDDP-300 cells was not significantly higher than the parental cells (Table 2).

Since cisplatin-resistant cells frequently show reduced drug uptake, the cellular accumulation of cisplatin was investigated. For this purpose we used two techniques: the intracellular amounts of intact cisplatin was estimated by liquid chromatography and the total levels of intracellular platinum by atomic absorption spectroscopy. Both methods yielded almost identical results: after exposure to 200 or 400 μM cisplatin the drug accumulation in RPMI84322/CDDP-300 cells was approximately 35 and 15% lower than in the parental cells, respectively (Table 2).

To determine if the resistance of the RPMI8322/CDDP-300 subline is caused by reduced induction of cisplatin–DNA adducts, the alkaline elution technique was applied to assess DNA interstrand cross-linking after cisplatin treatment. Although DNA interstrand cross-linking represents only approximately 1–5% of the total DNA damage by cisplatin,[5,28] we have previously shown that interstrand cross-linking is well correlated to the levels of the major cisplatin–DNA adducts in human melanoma cell lines and may therefore be used as an indicator of the total levels of cisplatin-induced DNA damage.[21] DNA interstrand cross-linking was analyzed 6 h after cisplatin treatment, since we have previously shown that maximum DNA interstrand cross-linking in RPMI8322 cells is seen 6–12 h after cisplatin treatment.[29] After exposure to 200–400 μM cisplatin, the levels of DNA interstrand cross-links were 30–32% lower in the RPMI8322/CDDP-300 subline than in the parental cell line; the difference is not statistically significant (Table 2). Thus, while the slight reduction in cisplatin uptake and DNA adducts may contribute to the reduced drug sensitivity of RPMI8322/CDDP-300 cells, these minor differences are not of a sufficient magnitude to explain the 4-fold cisplatin resistance of these cells.

Expression of nucleotide excision repair genes

Since cisplatin resistance has been related to increased repair of DNA adducts in some cases, we investigated the levels of mRNA encoding ERCC1 and XPB, two proteins involved in nucleotide excision repair, the repair pathway which removes platinum adducts from DNA. Analyses by Northern blot showed no significant differences in *ERCC1* and *XPB* mRNA levels in parental RPMI8322 cells and cisplatin resistant daughter cell lines (data not shown).

TABLE 2. Cellular concentrations of GSH, as well as content of platinum, intact cisplatin and DNA interstrand cross-linking following exposure to 200 and 400 μM cisplatin; the right column shows ratios between RPMI8322/CDDP-300 and RPMI8322 cells.

	RPMI8322	RPMI8322/CDDP-300	Ratio
GSH[a]	50.4 ± 6.6	53.7 ± 4.5	1.08 (NS)
Platinum[b]			
200 μM	7.4; 7.8	4.9; 5.1	0.66
400 μM	12.0; 14.2	10.9; 11.3	0.85
Cisplatin[b]			
200 μM	5.5; 5.7	3.6; 3.6	0.64
400 μM	10.2; 12.5	9.2; 9.5	0.83
Cross-links[c]			
200 μM	0.50 ± 0.17	0.36 ± 0.14	0.72 (NS)
400 μM	0.94 ± 0.31	0.64 ± 0.15	0.68 (NS)

[a]nmol/mg protein, mean values ± SEM (n = 4).
[b] nmol/mg protein, figures show results of two separate experiments, mean values were used for calculation of ratios.
[c]Gy-equivalents, mean values ± SEM (n = 5).
NS, difference not statistically significant using Student's t-test.

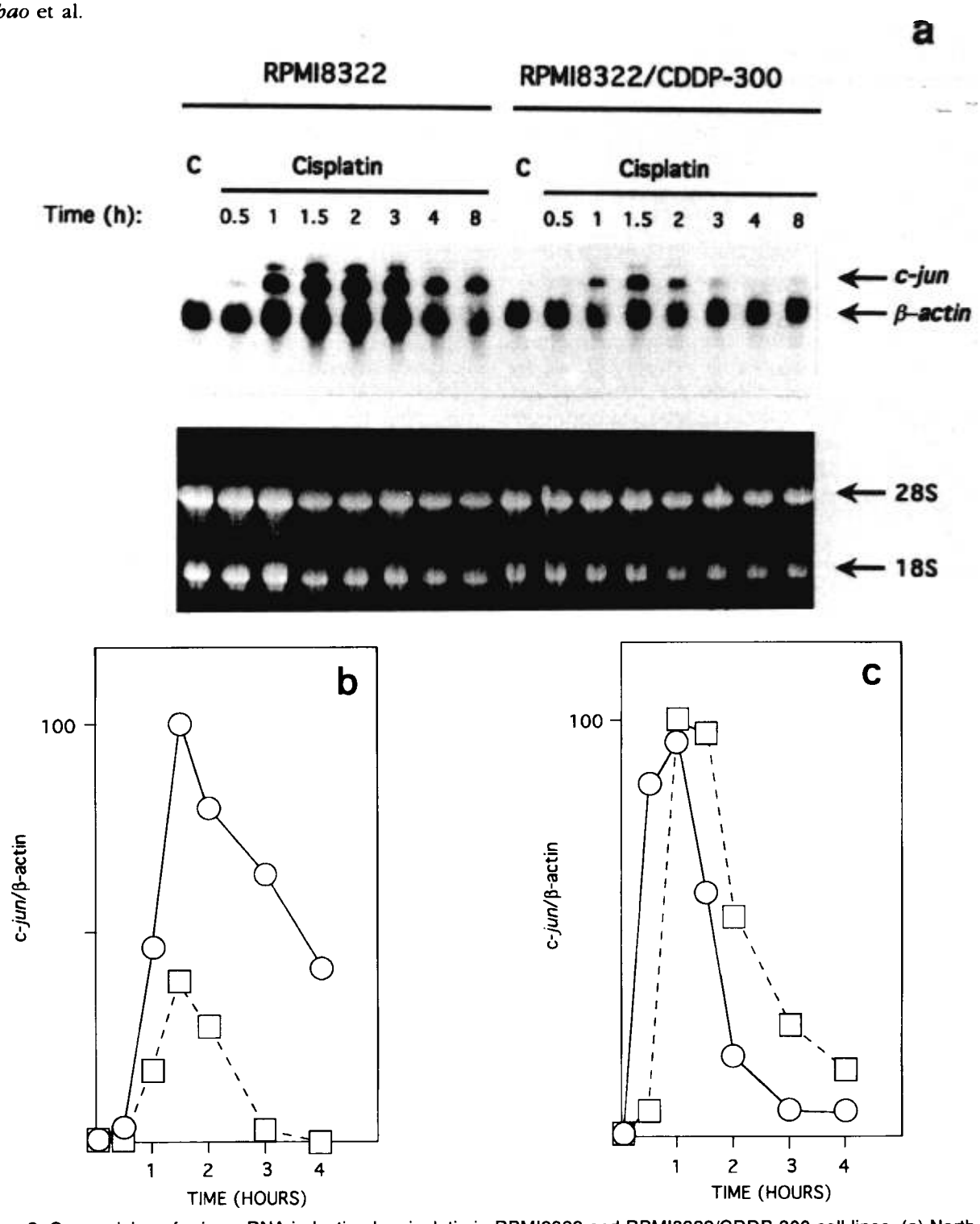

Figure 2. Comparision of c-*jun* mRNA induction by cisplatin in RPMI8322 and RPMI8322/CDDP-300 cell lines. (a) Northern blot analysis of total cellular RNA isolated from two cell lines after 600 μM cisplatin treatment for 40 min and incubation in drug-free medium for the indicated times. Hybridization was performed using ^{32}P-labeled c-*jun* and *β-actin* cDNA probe. The bottom panel shows a photograph of the ethidium bromide stained gel. (b) The bands in the autoradiograph were quantitated by densitometry, and relative amounts of c-*jun* mRNA after normalization to β-actin bands in RPMI8322 (○) and RPMI8322/CDDP-300 cells (□) are shown (the ratio of c-*jun* over *β-actin* band intensities at the time of maximum c-*jun* induction was given a value of 100). Values for the 8 h time point were excluded since there was a reduction in *β-actin* mRNA in RPMI8322 cells, possibly as a result of the cytotoxic effect of cisplatin treatment, which would lead to an overestimate of the amount of c-*jun* mRNA. (c) Relative c-*jun* mRNA induction following treatment of cells with equitoxic concentrations of cisplatin: 70 μM in RPMI8322 cells and 300 μM in RPMI8322/CDDP-300 cells, symbols as in (b).

Induction of c-*jun* expression by cisplatin

The levels of c-*jun* mRNA were determined by Northern blot analysis at different time points after a 30 min treatment of cells with 600 μM cisplatin. Very low levels of c-*jun* mRNA were present in untreated cells as shown in Figure 2. In the sensitive parental cell line a marked increase of c-*jun* mRNA was observed following cisplatin exposure. Maximum expression was seen 1.5 h after drug treatment and a considerable increase in mRNA remained 8 h following drug exposure. The resistant RPMI8322/CDDP-300 subline showed a much smaller increase of c-*jun* mRNA and 4 h after cisplatin treatment the amount of c-*jun* mRNA had returned to the level in untreated cells (Figure 2b). When cells were treated with equitoxic concentrations of cisplatin (RPMI8322 cells 70 μM and RPMI8322/CDDP-300 cells 300 μM), which cause a reduction in colony formation to approximately 5%, the induction of c-*jun* mRNA was very similar in RPMI8322 and RPMI8322/CDDP-300 cells (Figure 2c)

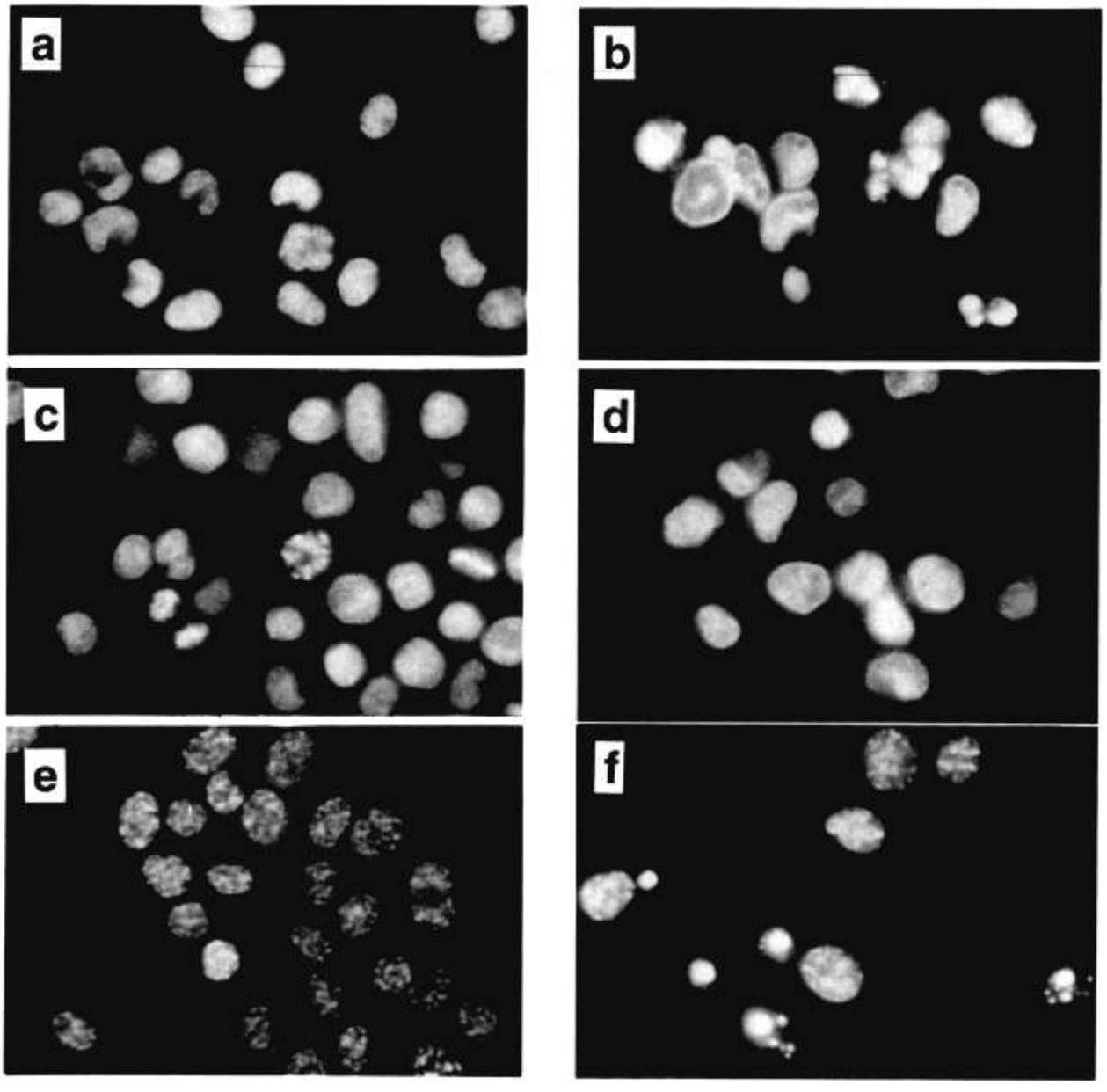

Figure 3. Analysis of apoptosis by Hoechst 33342/propidium iodide double staining of cells 24 h after cisplatin treatment. RPMI8322 cells: untreated (a) and treated with 600 μM cisplatin (b). RPMI8322/CDDP-300 cells: untreated (c) and treated with 600 μM cisplatin (d). A14 cells: untreated (e) and treated with 300 μM cisplatin (f).

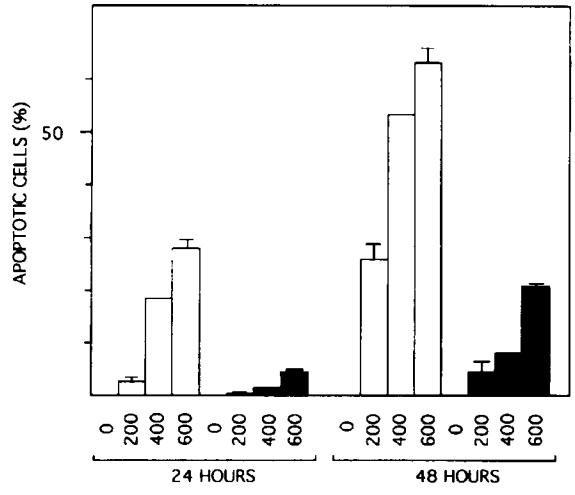

Figure 4. Cisplatin induced apoptosis in RPMI8322 cells (□) and RPMI8322/CDDP-300 cells (■). Cells were stained with Hoechst 33342/propidium iodide and analyzed by fluorescence microscopy after 24 and 48 h growth in drug-free medium following a 40 min cisplatin treatment. The percentage of apoptotic cells was determined by counting at least 500 cells in each sample (error bars: SEM).

Morphological analysis of apoptotic cells

Morphological studies of RPMI8322 and RPMI8322/CDDP-300 melanoma cell lines and the A14 rat embryo fibroblast cell line were performed after cisplatin treatment using the Hoechst 33342/propidium iodide double staining method. Figure 3 shows some typical views of cells after staining. The morphology of intact cells is shown in the control samples (Figure 3a, c and e). Typical apoptotic cells show condensed chromatin and fragmented cell nuclei (Figure 3b, d and f). Cell fragments (apoptotic bodies), which contain condensed chromatin, are also formed. When the cells die, they lose their ability to exclude propidium iodide and thus appear red, and many of the apoptotic bodies have also lost membrane integrity and appear red (Figure 3b, d and f). A14 rat embryo fibroblasts do not express c-*jun* mRNA due to a homozygous deletion of the c-*jun* gene.[17] Despite the lack of c-*jun* genes, A14 cells also show clear morphological changes characteristic of apoptosis after cisplatin treatment (Figure 3f).

To determine the extent of apoptosis the percentage of apoptotic and dead cells in the population was calculated at different time points after cisplatin treatment. As shown in Figure 4, a clear difference in

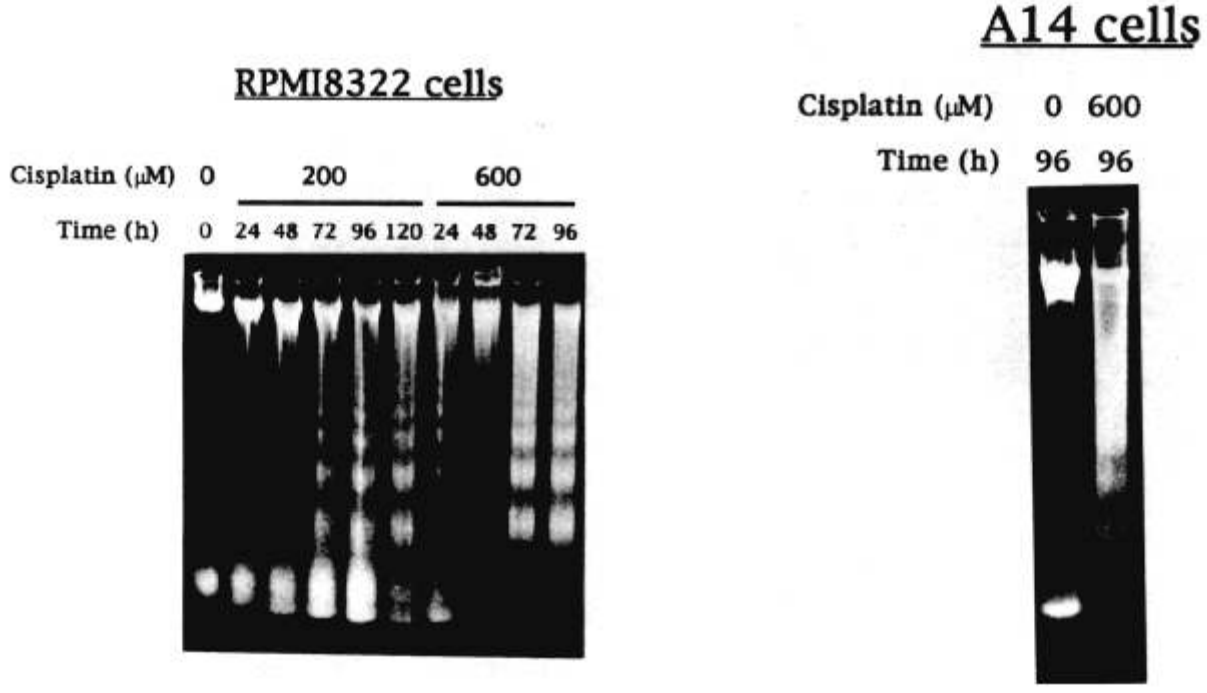

Figure 5. Fragmentation of nuclear DNA in RPMI8322 (left panel) and A14 cells (right panel) after cisplatin treatment for 40 min and incubation in drug-free medium for the indicated times. DNA was separated in 2% agarose gels containing 0.5 μg/ml ethidium bromide and visualized by UV transillumination.

the extent of cisplatin-induced apoptosis was observed between the RPMI8322 and RPMI8322/CDDP-300 cell lines.

DNA cleavage

The occurrence of internucleosomal DNA cleavage after cisplatin treatment was demonstrated by agarose gel electrophoresis (Figure 5). When DNA from RPMI8322 cells was analyzed 24–120 h after treatment for 40 min with 200 μM cisplatin, typical DNA ladder patterns were present. Treatment with 600 μM cisplatin gave more pronounced DNA ladders after 24–48 h. In the case of RPMI8322/CDDP-300 cells no DNA ladders were seen after exposure to 200 μM cisplatin. Only at 72–120 h after exposure to 600 μM cisplatin could weak DNA ladder patterns be seen (data not shown), consistent with the morphological evidence of less apoptosis in the RPMI8322/CDDP-300 cells than in the parental cell line. Cisplatin treatment of A14 rat embryo fibroblasts resulted in the typical pattern of DNA cleavage despite the lack of c-*jun* genes in these cells.

Discussion

We subjected a human melanoma cell line to repeated short exposures to increasing doses of the chemotherapeutic drug cisplatin. This protocol resembles clinical chemotherapy more closely than a continuous exposure to low drug concentrations and thus might provide a more relevant *in vitro* model of acquired drug resistance. The treatments with escalating doses of cisplatin resulted in the development of daughter lines with increasing resistance to cisplatin. A 3-fold increase in cisplatin resistance has been reported in a human ovarian cancer cell line established from a patient with acquired cisplatin resistance.[30] Thus, the approximately 4-fold increase in resistance of RPMI8322/CDDP-300 cells is probably of a similar magnitude as that which occurs when tumors acquire resistance to clinical chemotherapy with cisplatin. However, the 4-fold resistance of RPMI8322/CDDP-300 cells obtained by comparing IC_{50} concentrations is a minimum estimate, since due to the divergence of the survival curves (Figure 1), the difference in survival between the two cell lines was larger at the higher cisplatin doses which were used for investigations of drug accumulation and DNA adducts.

Previous studies indicate that the phenomenon of acquired cisplatin resistance in human cell lines may be a multifactorial process which can arise as a combined function of reduced drug uptake, increased cytosolic drug inactivation and enhanced DNA repair.[3,4,6,7] To investigate the possibility of increased cisplatin inactivation in the resistant subline, the levels of GSH were compared in RPMI8322 and RPMI8322/CDDP-300 cells, but no significant difference was found. The resistant cells showed a moderate decrease in cisplatin accumulation, whether measured as total cellular platinum content or intact cisplatin, as well as a similar reduction in DNA interstrand cross-links. While the moderately reduced cisplatin accumulation, which results in lower levels of cisplatin adducts, probably contributes to drug resistance, it is insufficient to explain the 4-fold cisplatin resistance of RPMI8322/CDDP-300 cells.

The RPMI8322/CDDP-300 cells exhibit partial cross-resistance to carboplatin, melphalan and UV irradiation. These agents all cause bulky adducts in DNA that are repaired by nucleotide excision repair.[31] We compared the expression of two of the repair enzymes, *ERCC1* and *XPB*, involved in this repair pathway. The implicaton of *ERCC1* in resistance to cisplatin is supported by the recent demonstration that cisplatin-resistant ovarian and bladder cancer cell lines have higher levels of *ERCC1* mRNA compared with cisplatin sensitive testis tumor cell lines.[32] In addition, increased levels of *ERCC1* expression has been found in tumor biopsies from ovarian cancer patients who are resistant to platinum-based chemotherapy, compared with samples from sensitive tumors.[33] No major differences were found in the levels of *ERCC1* or *XPB* mRNA in RPMI8322 cells and cisplatin-resistant daughter cell lines. This finding does not rule out that increased repair of cisplatin–DNA adducts may contribute to the resistance of RPMI8322/CDDP-300 cells and this possibility will be further examined.

No cross-resistance was seen to BCNU, an antineoplastic drug which causes DNA-adducts which are removed by a specific repair protein, O^6-methylguanine-DNA methyltransferase.[34] RPMI8322 cells lack this repair protein and are relatively sensitive to BCNU (Egyházi, unpublished results).

It has been shown that cisplatin as well as other DNA damaging agents can induce apoptosis. Our present investigation confirms that cisplatin induces apoptosis, as determined both by morphological criteria and by the occurrence of internucleosomal DNA cleavage. The resistant RPMI8322/CDDP-300 subline showed considerably less apoptosis than the parental cell line. A previous study demonstra-

ted that cisplatin causes an induction of the immediate early response gene c-*jun*, a major component of the AP-1 transcription factor, and it was suggested that c-*jun* is involved in the signal transduction pathway that results in apoptosis.[15] We found that cisplatin causes an increase in c-*jun* mRNA which precedes the occurrence of apoptosis, consistent with the previous report. Furthermore, the lower levels of apoptosis after cisplatin treatment of the resistant RPMI8322/CDDP-300 subline was paralleled by a similar reduction in c-*jun* induction, in agreement with the possibility that c-*jun* may be a mediator of cisplatin induced apoptosis. Since Rubin *et al.*[15] had reported that HL-60 leukemia cells had to be treated for 3 h with 10 times the concentration of cisplatin which reduces colony formation to 10% in order to obtain induction of c-*jun* mRNA, we initially used a high cisplatin concentration (600 μM, approximately 10 times the concentration which reduces colony formation to 10%) to demonstrate induction of c-*jun* mRNA in RPMI8322 cells. However, we later found that considerably lower drug leves could cause c-*jun* mRNA induction (Figure 2c). This strengthens the hypotesis that c-*jun* may occur in tumors following the cisplatin doses used in clinical chemotherapy and that this might be related to the therapeutic effect obtained by apoptosis of tumor cells. Indeed, in a recent preliminary report induction of c-*jun* mRNA was observed in head-neck tumor biopsies from patients responding to cisplatin-based chemotherapy, but not in samples from resistant tumors.[35] It thus appears that induction of c-*jun* may be a determinant of clinical response to cisplatin treatment.

The mechanisms that cause c-*jun* induction in response to DNA damage are still unclear. It has been shown that stimulation of protein kinase C (PKC) by phorbol ester results in c-*jun* induction.[16] This induction is likely to be mediated by a combination of phosphorylation and dephosphorylation of pre-existing c-Jun molecules, resulting in increased binding and *trans*-activation of the c-*jun* promoter.[16] Cisplatin treatment results in activation of PKC in a human leukemia cell line.[15] Since PKC down-regulation or treatment with an inhibitor of PKC prevented c-*jun* induction following cisplatin, it is likely that c-*jun* induction by cisplatin is mediated by PKC in these cells. It was also reported that the TRE sequence was required for induction by cisplatin of a reporter plasmid carrying the c-*jun* promoter[15]. Further, indirect evidence for the involvement of PKC as a mediator of cisplatin-induced cell death comes from the demonstration that treatment with TPA can sensitize cells to cisplatin without increasing drug uptake.[36] Still, the capacity of DNA damaging agents to induce c-*jun* may not be mediated exclusively by PKC activation, since c-*jun* induction after UV is only partially dependent on PKC activity,[37] and has been reported to be mediated by activation of the the plasma membrane-associated Src tyrosine kinase, possibly as the result of lipid peroxidation.[38] Src activation then leads to c-*jun* induction mediated by the Ras signal transduction pathway. GSH is likely to be involved in the regulation of AP-1 activity, since it was demonstrated that depletion of cellular GSH results in elevated basal AP-1 binding activity as well as increased induction of AP-1 by several chemical agents.[39] There is, however, no indication that altered levels of GSH contribute to the decreased c-*jun* induction by cisplatin in RPMI8322/CDDP-300 cells (Table 2).

To further investigate the relationship between c-*jun* induction and apoptosis we used the rat embryo fibroblast cell line A14 in which the c-*jun* genes are deleted. Despite the absence of c-Jun, A14 cells showed the morphological changes associated with apoptosis as well as internucleosomal DNA cleavage after cisplatin treatment. Therefore, c-*jun* is not necessary for apoptosis after cisplatin treatment. However, this finding does not rule out the possibility that apoptosis induced by cisplatin is mediated by induction of AP-1, since the genes coding for other members of this family of transcription factors, such as *jun*-B, *jun*-D, c-*fos*, *fos*-B and *fra*-1 are present in A14 cells.[16] It is possible that induction of such genes by cisplatin may fulfil the function of the absent c-*jun* in these cells. This possibility is supported by the demonstration that cisplatin treatment can cause induction of c-*fos*.[15,40]

Several studies have shown that DNA damaging agents can cause apoptosis by inducing prolonged p53 stabilization.[41,42] However, functional p53 protein may not always be required for apoptosis following cisplatin exposure, since the RPMI8322 cells exhibit the increased levels of p53 protein characteristic of p53 dysfunction (Platz and Ring, personal communication).

The results we have presented indicate that the resistant melanoma subline differs from the sensitive parental cell line in mechanism(s) whereby cisplatin and possibly some other DNA damaging agents induce apoptosis. The reduced induction of c-*jun* mRNA after cisplatin treatment of RPMI8322/CDDP-300 cells is consistent with the possibility that an alteration in signal transduction may be related to the decreased occurrence of apoptosis in these cells. Further insights into the mechanisms of inducton of c-*jun* after cisplatin should be obtained by

experiments involving manipulation of the PKC- and Src-dependent signal transduction pathways.

Conclusion

We obtained a cisplatin-resistant daughter cell line by repeated exposure of a human melanoma cell line to increasing doses of cisplatin. The resistant daughter cell line was cross-resistant to carboplatin, melphalan and UV, but not to BCNU. The cisplatin resistance was not associated with increased cellular GSH or elevated levels of mRNA encoding the excision repair proteins ERCC1 and XPB. Although a slight reduction in cisplatin accumulation and induction of DNA adducts may contribute to the resistance of the daughter cell line, the differences are too small to explain the difference in cisplatin sensitivity. The cisplatin resistant daughter cells showed less apoptosis and reduced induction of c-*jun* mRNA after cisplatin treatment. This is consistent with the possibility that c-*jun* induction may be involved in a pathway that triggers apoptosis after exposure to DNA damaging agents, and that this mechanism may be altered in the resistant daughter cell line. However, cisplatin also induced apoptosis in A14 rat embryo fibroblasts, which lack c-*jun* genes. Induction of c-*jun* is thus not necessary for apoptosis.

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