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cis-Diamminedichloroplatinum(II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines

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Abstract We have studied the effects of the chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (cisplatin) on three human ovarian carcinoma cell lines – one sensitive to the drug (CH1), one with acquired resistance (CH1cisR) and one with intrinsic resistance (SKOV-3). Previous work has shown that the 50% inhibitory concentrations (IC_{50} values) after a 2-h exposure to the drug are: CH1, 2.5 μ*M*; CH1cisR, 7.5 μ*M*; and SKOV-3, 33 μ*M*. Despite the variation in sensitivity, the amount of Pt bound to DNA and the rate of removal of Pt was similar for the three lines. There were significant differences in the rates of formation of DNA cross-links but these were not large enough to account for the high resistance of the SKOV-3 line. We have reported that in the L1210 murine leukaemia cell line there are two mechanisms of cisplatin-induced cell death – one of which involves apoptosis. In this paper, we report on an investigation into whether sensitivity to apoptosis played a role in the resistance of these ovarian lines towards cisplatin. After a 2-h incubation with the drug, cells from the three lines showed evidence of death through apoptosis. The cells detached from the culture dish in a time- and dose-dependent fashion. These cells morphologically were quite distinctive from the attached cells and showed changes in their chromatin structure indicative of apoptosis. Their DNA had not been degraded into oligonucleosomal fragments (200 bp and multiples thereof) but had been cut into larger fragments (30 kilobase pairs, kbp) of a size associated with chromatin domains (chromatin loops). At equitoxic doses of drug, the quantity of cells undergoing apoptosis was similar for the three cell lines. The most prominent effect on cell-cycle kinetics was a slowdown in S-phase transit during which the cells underwent apoptosis. Cells that successfully completed the S phase subsequently suffered a temporary G2 block. We propose that the sensitivity of these cell lines to cisplatin was governed by their ability to handle damage caused by platination of the DNA and that the major mechanism of cisplatin-induced cell death in all three cell lines was the induction of apoptosis.

Key words Apoptosis · Ovarian carcinoma · Cisplatin · Flow cytometry

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Introduction

cis-Dichlorodiammineplatinum(II) (cisplatin) is widely used in chemotherapy, particularly for teratomas and ovarian carcinomas [31, 61]. One of the problems encountered in the clinic is the development of drug resistance. Studies of cells in vitro have indicated that such resistance can have several causes [1, 12, 52]. Resistant cells may take up less cisplatin [28, 35]; some cells have an increased intracellular concentration of glutathione that can react with cisplatin, rendering it ineffective [24]; and resistant cells may have an increased capacity for repair of damage to their DNA [54]. It has been proposed that apoptosis might be an important and ubiquitous mode of cell death for cells treated with chemotherapeutic drugs [4, 14, 21]. Apoptosis has been observed in a variety of tumour cell lines after incubation with cisplatin [4, 16, 47, 48, 57] and these results raise the possibility that decreased

susceptibility to the induction of apoptosis might be another factor involved in drug resistance.

We have been investigating three cell lines derived from human ovarian carcinomas that show a wide variation in sensitivity to cisplatin – CH1 [23]; a resistant derivative, CH1cisR [28]; and an intrinsically resistant line, SKOV-3 [19]. Despite a variation in sensitivity of approximately 13 orders of magnitude, the level of platination of DNA and the rate of removal of Pt from the DNA showed only small differences [38–40]. There were significant differences in the rates of formation of DNA cross-links but these were not large enough to account for the high resistance of the SKOV-3 line [40]. We have shown that cisplatin kills CH1 cells by inducing apoptosis [47], as evidenced by the morphology of cells detaching from the surface of the culture vessel and by the induction of 30-kilobase pair (kbp) fragments of DNA – a feature of many apoptotic cells [7, 37, 59]. Since a failure to initiate apoptosis might account for the resistance to cisplatin of the other ovarian cell lines, we undertook a more detailed investigation of the effects of the drug on CH1, CH1cisR and SKOV-3 cell lines.

Materials and methods

Chemicals

Cisplatin and ammine dibutyratodichloro(cyclohexylamine)platinum(IV) (JM221) were supplied by the Johnson Matthey Technology Centre (Reading, Berks), and all other reagents were bought from Sigma Ltd. (Poole, Dorset).

Cells and drug treatment

The CH1, CH1cisR and SKOV-3 cell lines have been described previously [19, 23, 28]. Cells were grown as monolayers in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Imperial Laboratories, Andover, UK), 50 μg gentamicin/ml, 2.5 μg amphotericin B/ml, 2 m*M* L-glutamine, 10 μg insulin/ml and 0.5 μg hydrocortisone/ml in 10% CO_2/90% air. Under these conditions the doubling times for the cultures were: CH1, 14 h; CH1cisR, 15 h; and SKOV-3, 23 h. Cells were harvested as a suspension of single cells by incubation for 5 min with 0.25% trypsin, 0.02% ethylenediaminatetraacetic acid (EDTA).

Cisplatin was dissolved in 0.9% sterile saline immediately before use. Following exposure to cisplatin at different concentrations for 2 h, the cultures were washed once with medium and then incubated in fresh medium. Preliminary experiments showed that the effects of cisplatin were increased if the cell density was too low at the time of treatment (below 1000 cells mm^{-2} for the CH1 lines and below 500 cells mm^{-2} for SKOV-3). In the experiments reported herein, cisplatin was added to the cultures at a higher density when the cells covered approximately one-third of the culture dish.

We have previously found that the IC_{50} values (50% inhibitory concentration) after a 2-h exposure to the drug, as measured by the sulforhodamine B assay after 96 h, are: CH1, 2.5 μ*M*; CH1cisR, 7.5 μ*M*; and SKOV-3, 33 μ*M* [40]. The IC_{50} values measured under the same conditions by a clonogenic assay for CH1 and SKOV-3 are 0.8 and 11 μ*M*, respectively (L. R. Kelland, unpublished work). Although the IC_{50} values obtained by the sulforhodamine assay for cell killing of ovarian cells by cisplatin are usually higher than those given by a clonogenic assay, the two sets of values are highly correlated and give similar resistance [49].

DNA gel electrophoresis

Electrophoresis was performed on a horizontal 1% agarose gel for 2 h at 6 V/cm using TBE buffer (90 m*M* TRIS, 90 m*M* boric acid, 2 m*M* Na_2-EDTA, pH 8.0) as the running buffer as described previously [45]. For pulse-field gel electrophoresis (PFGE), cells were resuspended in low-melting-point agarose and the plugs of cells in agar were incubated at 37° C for 24 h in 10 m*M* TRIS, 0.5 *M* EDTA, 1% sodium lauryl sulfate and 1 mg proteinase K/ml (pH 8.0). Pulse-field gel – contour-clamped homogeneous electrophoresis (PFGE-CHEF) was performed using a CHEF system (Biorad Laboratories Ltd., Watford, England) equipped with a model 200/20 power supply and a Pulsewave 760 switcher. Horizontal gels of 1% agarose were run in half-strength TBE at 14° C at 200 V for 20 h with a pulse ramp of 20–80 s. Gels were stained with ethidium bromide. Two lanes always included DNA molecular-weight markers – *Saccharomyces cerevisiae* chromosomes in one lane and markers from λ phage in the other. The λ markers consisted of Hind III fragments plus the undigested genome and concatamers; the relative amounts in the mixture had been adjusted to give a prominent band at 48.5 kbp (Sigma Ltd. catalogue).

Microscopy

Cells were centrifuged and the pellets were fixed in 2% glutaraldehyde in 0.05 *M* phosphate buffer, 0.05 *M* sucrose (pH 7.3) for 2 h at room temperature. Pellets were post-fixed for 1 h in 1% osmium tetroxide, dehydrated through a graded series of ethanols, infiltrated and embedded in Epon. For light microscopy, 1-μm sections were cut and stained with toluidene blue.

Flow cytometry

For cell-cycle analysis, approximately 10^6 cells were fixed in ice-cold 70% ethanol and stored at 4° C. After being washed cells were resuspended in 800 μl phosphate-buffered saline (PBS) and 100 μl propidium iodide (PI) solution (100 μg/ml), and 100 μl RNase solution (1 mg/ml) was added before incubation for 2 h at 37° C. Flow cytometry measurements were made on an Ortho Cytofluorograf 50H using a Spectra-Physics argon-ion laser with an output of 200 mW at 488 nm. Forward and orthogonally scattered light and red fluorescence (peak and integrated area) were recorded. Pulse shape analysis was performed to eliminate any cell clumps [41] and the data were gated on light scatter before the recording of a histogram of red (PI-DNA) fluorescence. Data, normally from 2×10^4 cells, were acquired and analysed on an Ortho 2150 computer system. The DNA histograms were transferred to an IBM-PC compatible computer and figures were prepared using our own software (written by one of the authors – M.G.O.). To compute the percentage of the phases of the cell cycle from a DNA histogram, we used our own software employing the Watson algorithm as modified by Ormerod et al. [44]. For the figures the frequency scale was adjusted to optimise the display of the data.

Measurement of viability

PI (final concentration, 5 μg/ml) was added to a suspension of cells and the number of cells excluding the dye were scored under a fluorescent microscope. For some samples, viability was measured on the

flow cytometer by staining with fluorescein diacetate (10 ng/ml, 10 min at room temperature) followed by addition of PI (10 μg/ml) and recording of green versus red fluorescence as described previously [42]. In this assay, viable cells are green-positive and red-negative and dead cells are green-negative and red-positive.

Results

The numbers of cells remaining attached to the culture dish were reduced after incubation with cisplatin. The corresponding numbers of detached cells harvested from the culture medium increased with time and dose of drug (Figs. 1–3). CH1 and CH1cisR cells showed a high percentage of non-adherent cells by 24 and 48 h (Figs. 1, 2), whereas cultures of SKOV-3 cells had to be incubated for 48 h before appreciable numbers of detached cells were observed (Fig. 3).

The detached cells showed the morphological attributes of apoptotic cells (Fig. 4c, f, i). They were smaller and, in the CH1 and CH1cisR cells, the chromatin was condensed around the periphery of the

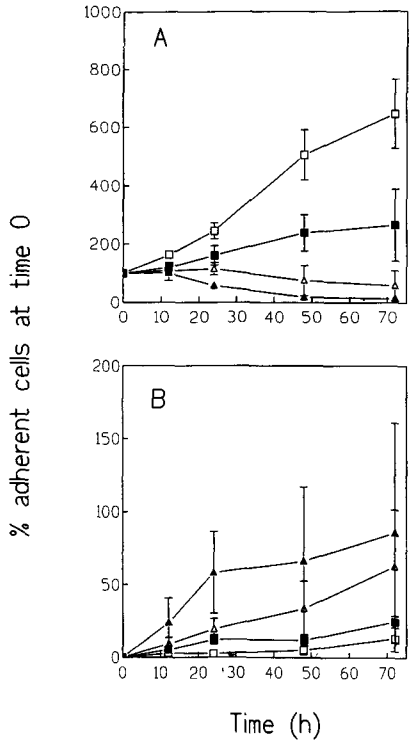

Fig. 1A, B Adherent (**A**), and non-adherent (**B**) CH1 cells at different times after treatment with cisplatin for 2 h (*white squares*, no cisplatin, *black squares*, 3 μ*M*, *cisplatin*, *white triangles* 10 μ*M* cisplatin, *black triangles* 25 μ*M* cisplatin). The *bars* represent the range of three separate experiments

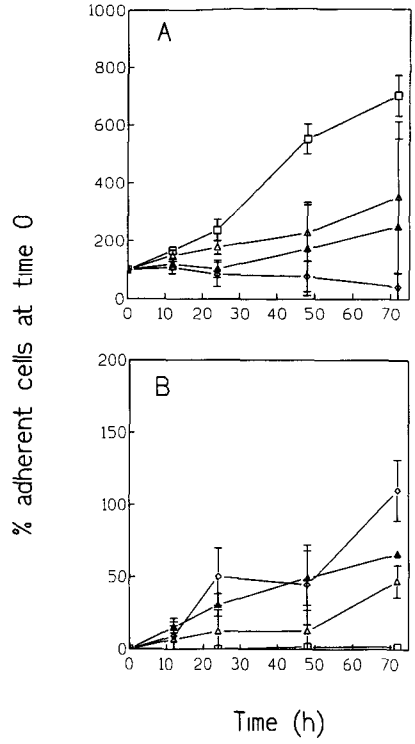

Fig. 2A, B Adherent (**A**) and no-adherent (**B**) CH1 cisR cells at different times after treatment with cisplatin for 2 h (*white squares* no cisplatin, *white triangles* 10 μ*M* cisplatin, *black trianlges* 25 μ*M* cisplatin, *white diamonds* 50 μ*M* cisplatin). The *bars* represent the range of three separate experiments

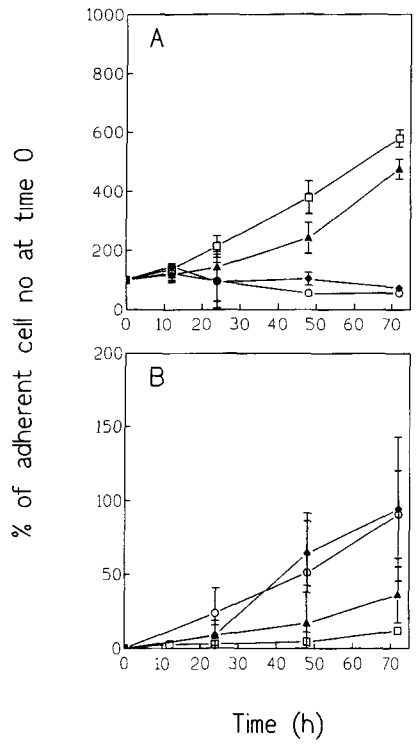

Fig. 3A, B Adherent (**A**) and non-adherent (**B**) SKOV-3 cells at different times after treatment with cisplatin for 2 h (*white squares* no cisplatin, *black triangles* 25 μ*M* cisplatin, *black diamonds* 50 μ*M* cisplatin, *wite circles* 200 μ*M* cisplatin). The *bars* represent the range of two separate experiments

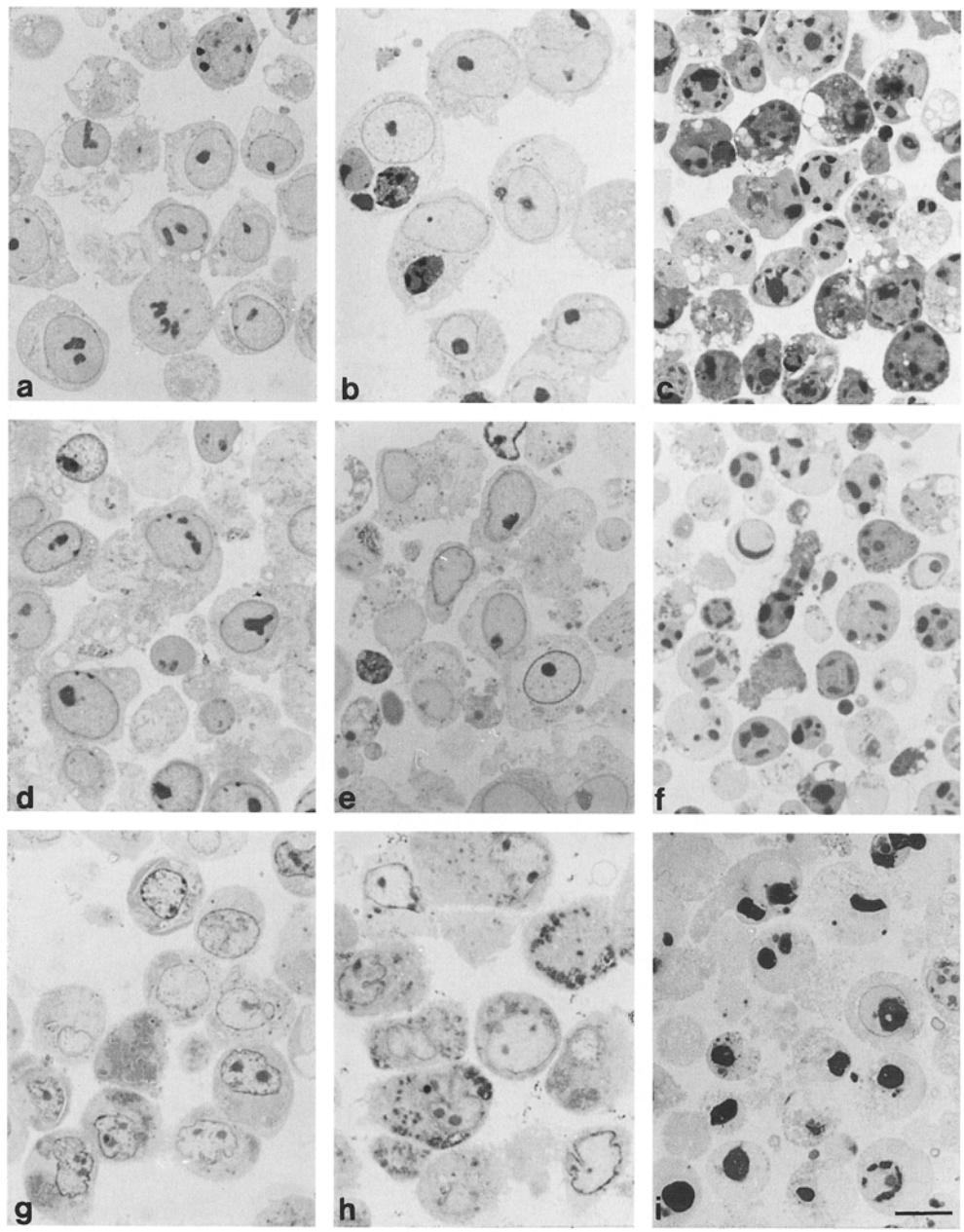

Fig. 4a–i Light micrographs of cells after incubation with cisplatin for 2 h. **a–c** CH1 cells at 24 h after incubation with 10 μ*M* cisplatin. **d–f** CH1cisR cells at 24 h after incubation with 25 μ*M* cisplatin. **g–i** SKOV-3 cells at 48 h after incubation with 75 μ*M* cisplatin. **a, d, g** No drug, adherent cells, **b, e, h** cisplatin-treated, adherent cells. **c, f, i** cisplatin-treated, non-adherent cells. *Bar* 10 μm

nucleus. In the SKOV-3 cells the chromatin was heavily condensed into a uniform mass (Fig. 4i). This difference was not caused by the larger dose of cisplatin used on these resistant cells since the same features were found in the small number of detached SKOV-3 cells that were harvested after incubation with 25 μ*M* cisplatin (data not shown).

At least 90% of the detached cells showed the morphological changes described above. Between 50% and 80% of the cells (depending on the dose of drug and the time of harvesting) had intact plasma membranes as revealed by the ability to exclude PI and to retain fluorescein as measured by flow cytometry [42]. Although DNA gel electrophoresis showed no evidence for internucleosomal degradation of the DNA of apoptotic cells [47] (Fig. 5), in all three cell lines the DNA was degraded into larger fragments of about 30-kbp length (Fig. 6); the estimate of the molecular weight of these fragments was made in relation to the 48.5-kbp fragment from λ phage. Figure 5 shows data obtained for one dose of drug. Similar results were obtained from cells incubated with different doses of cisplatin. Data for the CH1 cells are not shown as this result has previously been published [47]. Cells harvested after different periods of incubation also showed 30-kbp fragments on PFGE (data not shown).

The major effect of cisplatin on the cell cycle was an accumulation of cells in the S phase (Figs. 7–9). Any

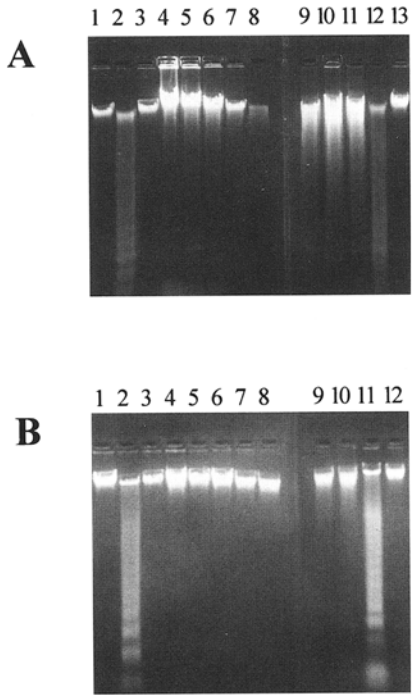

Fig. 5A, B Gel electrophoresis of adherent and non-adherent cells from ovarian cell lines after incubation for 2 h with cisplatin. **A** CH1cisR cells (*1* L1210 cells, no drug; *2* L1210 cells plus 2 μ*M* JM221, 4 h; *3* CH1cisR cells, no drug; 4–11 CH1cisR cells, 50 μ*M* cisplatin, 4–8 adherent cells; *4* time zero; *5* 12 h; *6* 24 h; *7* 48 h; *8* 72 h; *9–11* detached cells; *9* 24 h; *10* 48 h; *11* 72 h; *12* L1210 cells plus 2 μ*M* JM221, 4 h; *13* L1210 cells, no drug. **B** SKOV-3 cells (*1* L1210 cells no drug; *2* L1210 cells plus 2 μ*M* JM221, 4 h; *3* SKOV-3 cells, no drug; 4–10 SKOV-3 cells, 200 μ*M* cisplatin; 4–7 adherent cells; *4* 12 h; *5* 24 h; *6* 48 h; *7* 72 h; 8–10 detached cells; *8* 24 h; *9* 48 h; *10* 72 h; *11* L1210 cells plus 2 μ*M* JM221, 4 h; *12* L1210 cells, no drug). The lanes with the L1210 cells were included as a positive control to demonstrate the formation of ladders from apoptotic cells

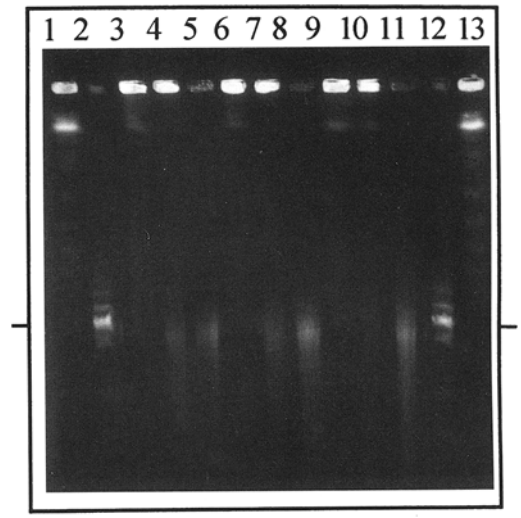

Fig. 6 Pulse-field gel electrophoresis of DNA from adherent and non-adherent cells from the three cell lines at 48 h after incubation for 2 h with cisplatin (*1, 13 Saccharomyces cerevisiae* chromosomes; *2, 12* fragments from λ phage; *3–5* CH1 cells; *3* no drug; *4* 25 μ*M* cisplatin, adherent cells; *5* 25 μ*M* cisplatin, non-adherent cells; *6–8* CH1cisR; *6* no drug; *7* 50 μ*M* cisplatin, adherent cells; *8* 50 μ*M* cisplatin, non-adherent cells; *9–11* SKOV-3 cells; *9* no drug; *10* 200 μ*M* cisplatin, adherent cells; *11* 200 μ*M* cisplatin, non-adherent cells). The *line* marks the band at 48.5 kbp (see Materials and methods)

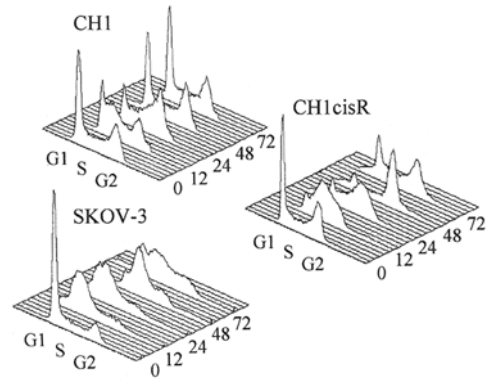

Fig. 7 DNA histograms of cells after a 2-h incubation with cisplatin showing changes with time in CH1 cells after incubation with 3 μ*M* cisplatin, CH1cisR cells after incubation with 10 μ*M* cisplatin and SKOV-3 cells after incubation with 40 μ*M* cisplatin. The *numbers* incidate the time (in h) after incubation with the drug (0, 12, 24, 24, 48, 72 h)

block in G2 was less apparent. The cells that became non-adherent as a result of drug treatment gave poorly resolved histograms that reflected those of the adherent cells (data not shown). There was no evidence in any of the histograms for a hypodiploid or 'sub-G1' peak indicative of DNA degradation associated with apoptosis ([11] and references cited therein).

Discussion

Apoptosis appeared to be the major mechanism of cell death from cisplatin in the three cell lines, despite the wide variation in chemosensitivity. The dying cells shrank, rounded up and detached from the culture dish – behaviour observed during apoptosis [15, 16, 25, 36]. The chromatin in the detached cells from the two CH1 lines was condensed on the nuclear membrane (Fig. 4). This appearance is typical of cells undergoing apoptosis [2, 63, 64] and is quite different from the features observed during necrosis [9, 29, 63]. Although the chromatin in the detached SKOV-3 cells had a different appearance, being heavily condensed throughout the nucleus, it was nonetheless remarkably dissimilar from the chromatin in the attached counterparts (Fig. 4). Furthermore, in the three cell lines the DNA was fragmented into 30-kbp fragments (Fig. 6) – a feature considered to be an early event in apoptosis [7, 37, 47, 59]. The DNA of apoptotic cells is often degraded into internucleosomal fragments whose lengths are multiples of 180 bp [3, 10, 62] (Fig. 4), but the absence of this feature does not preclude apoptotic death since this

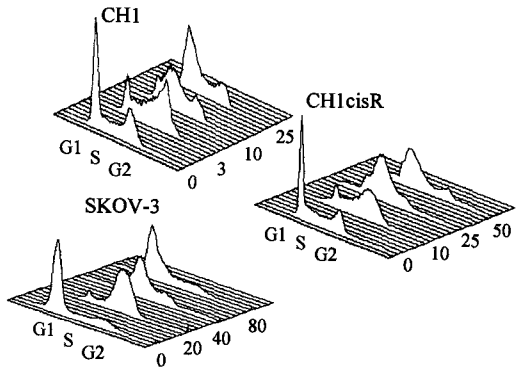

Fig. 8 DNA histograms of cells showing changes with drug dose at a fixed time after a 2-h incubation with cisplatin: in CH1 and CH1cisR cells after 24 h and in SKOV-3 cells after 48 h. The *numbers* indicate the dose of drug (in μM)

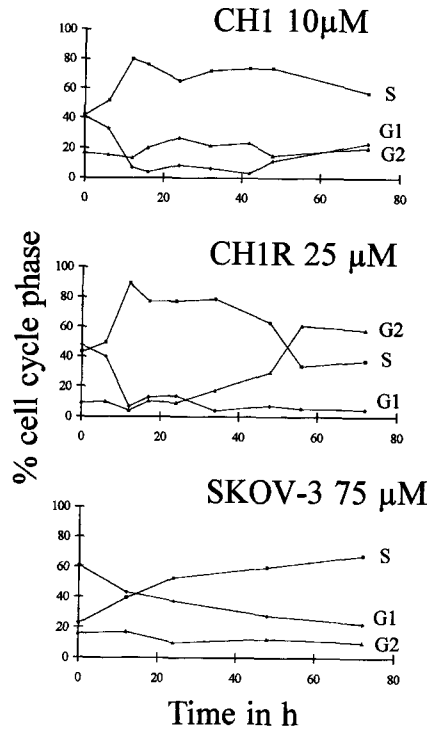

Fig. 9 Percentage of cells in the different phases of the cell cycle (G0/G1, S, G2/M) as obtained by computer analysis of DNA histograms. The cells were treated with cisplatin (at the doses indicated) for 2 h, washed and incubated for the periods shown (*Black diamonds G1, black squares S, black triangles G2*)

mode of degradation is not always observed [5, 8, 36, 47, 58]. We therefore conclude that all three cell lines died by apoptosis after incubation with sufficient concentrations of cisplatin.

We have previously shown that after the application of equimolar doses of cisplatin, the amount of platinum binding to the DNA does not differ significantly among the three cell lines [38–40]. These data suggest that extra-nuclear factors, such as the rate of uptake of drug and levels of intracellular glutathione, are not important. This conclusion is in agreement with the data of Shellard et al. [55], who found that although SKOV-3 had high levels of glutathione as compared with a more sensitive line (TR175), there was little difference in the amount of platinum bound to the DNA of the two lines.

When the cells were held in the G1 phase of the cell cycle by nutritional deprivation, there was also no difference in the subsequent rate of removal of Pt from the DNA [38–40]. Whereas the CH1cisR and SKOV-3 cells showed no difference in the formation and removal of DNA cross-links, there was a 2-fold increase in the number of cross-links observed in the CH1 cells as compared with the other two lines [40]. The latter effect could account for part of the difference in sensitivity between the CH1 and CH1cisR cells but not for the greater resistance of the SKOV-3 cells. Some caution has to be exercised in interpreting data on the overall loss of platinum from DNA since differences in the repair of a specific lesion may be masked [22]. Furthermore, repair rates are not uniform throughout the genome. Nucleotide excision repair can be related to transcription and this effect can lead to gene specific repair [51, 66].

The greater resistance of SKOV-3 cells may not be caused by the more efficient execution of a particular function (for instance, repair) but could also be produced by lack of a function. For example, cells lacking the appropriate methyl transferase are hypersensitive to agents that methylate DNA; death is caused by misfunction of the system for mismatch repair [26, 34]. In these cells a defect in the mismatch repair system creates tolerance to methylating agents. It has been reported that SKOV-3 cells show less extensive repair of cisplatin-generated lesions as compared with the more sensitive cell line TR175 [55], and a sensitive Chinese hamste ovary (CHO) cell line has been found to overexpress the nucleotide excision repair gene (ERCC-1) [6]. Cells can replicate on a DNA template damaged by cisplatin [20] and, at present, the only firm conclusion that can be drawn from the published data is that SKOV-3 cells can apparently tolerate higher levels of DNA damage and can replicate DNA on a damaged template.

Recently, attention has focused on the role of p53 in the response of cells to agents that react with DNA [30]. The p53 protein has a dual role. If the cells express wild-type p53, they exhibit an arrest in the G1 phase of the cell cycle after genotoxic damage [27]. It is hypothesised that the arrest allows DNA damage to be repaired before the cell attempts to replicate DNA. On the other hand, p53 plays a role in triggering an apoptotic pathway after ionising radiation and after incubation with a wide range of genotoxic drugs, including cisplatin [32, 33]. In some cells, over-expression of p53 can itself induce apoptosis [65]. It is unlikely that p53 plays an important role in determining the sensitivity of

the cells under study. The SKOV-3 cells are null for p53; they do not produce mRNA from the p53 gene [65]. The CH1 and CH1cisR cells are probably both heterozygous for p53 – one allele coding for wild-type p53 and the other, for a mutant form [60].

The DNA histograms (Figs. 7–9) showed that the cells accumulated in the S phase of the cell cycle. This behaviour is consistent with a reduction in DNA synthesis and a consequential slowdown in transit through the S phase – an effect reported in other cells [13, 20, 46, 53, 56, 57]. The effect was strongly dose-dependent and was most marked in the SKOV-3 cells, which had the longest cell-cycle time. Although the dose of drug needed to block the cells in the S phase showed some relationship to sensitivity, the more resistant cells were capable of surviving a severer hold-up in the S phase. For example, at 24 h after the application of a dose of 10 μM cisplatin, the CH1 cells were in the early S phase, whereas the CH1cisR cells had progressed to the late S/G2 phase (Fig. 8). However, when equitoxic doses are examined (for example, 3 μM in CH1, 10 μM in CH1cisR and 40 μM in SKOV-3), it can be seen that the SKOV-3 cells took longer to progress through the S phase as compared with the two CH1 lines (Figs. 7, 9). This observation is consistent with the conclusion drawn above that the SKOV-3 cells are more capable of surviving replication on a damaged DNA template.

After incubation with concentrations of cisplatin equal to or in excess of $3 \times IC_{50}$, large numbers of cells became apoptotic at a time when the majority of the adherent cells were in the S phase (Fig. 9). At lower concentrations of drug, apoptotic cells were not observed in the CH1 or CH1cisR cells until a high percentage of them had reached the G2 phase and even divided (compare the 48- and 72-h time points in Fig. 7 with those in Fig. 2). From our present data we cannot draw any firm conclusion about the position in the cell cycle from which cells enter the apoptotic pathway after the application of concentrations of cisplatin lower than $3 \times IC_{50}$. Although it is known that apoptosis can occur from any phase of the cell cycle (for example, see [50]) and that apoptosis can be induced in quiescent (G0) thymocytes by glucocorticoids [15, 18, 62], it is of interest that proliferating immature thymocytes are more susceptible than quiescent thymocytes to the induction of apoptosis by cisplatin [17]. Our data are consistent with the hypothesis that cycling cells, particularly those in the S phase, are vulnerable to the induction of apoptosis by cisplatin.

The response to cisplatin of the ovarian cell lines contrasted with that of the murine lymphoma cell line L1210. We have shown that two modes of death are induced by cisplatin in these cells [48]. Concentrations of cisplatin in excess of $10 \times IC_{50}$ induced apoptosis; at lower concentrations of drug the cells underwent a slowdown in transit through the S phase and died while subsequently blocked in G2 [57] by a mechanism that probably did not involve the apoptotic pathway [48]. The failure to die during the S phase is unlikely to be caused by the resistance of L1210 cells to this mode of death since apoptosis can be induced by starvation (Fig. 5.6 in [43]), holding the cells in the plateau phase of growth (unpublished observation) and by the addition of caffeine while cisplatin-damaged cells are held up in S the phase [46].

After DNA has been damaged by cisplatin, there are probably two important points of decision. The first is while the cells are attempting to replicate their DNA on a damaged template, at which time they seem to be more susceptible to the induction of apoptosis. The other is while they are in a G2 block, during which the cell either divides, with an increased chance of survival, or enlarges and eventually dies – probably by necrosis [48].

The sensitivity of cells to cisplatin does not appear to be related to their susceptibility to one or the other of the two mechanisms of cell death. The L1210 and CH1 cells have similar sensitivities (IC_{50} values, 2.1 and 2.7 μM, respectively) but, at doses in the range of 3–10 IC_{50}, die through different mechanisms. At equitoxic doses all three ovarian lines died through apoptosis, despite the wide variation in their resistance to cisplatin.

In summary, our present data, taken together with our previously published work on these cell lines [40], are compatible with the following statements:

1. The major mechanism of cisplatin-induced cell death in the three human ovarian cell lines, CH1, CH1cisR and SKOV-3 is the induction of apoptosis.
2. Cisplatin causes a slowdown in S-phase transit and, at concentrations of drug sufficient to kill 80% or more of the cells, apoptosis is initiated while most of the cells are in this phase of the cell cycle.
3. The increased resistance of the SKOV-3 cell line is probably due to its ability to replicate DNA on a damaged template.

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