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Programmed Cell Death in Response to Chemotherapeutic Agents in Human Germ Cell Tumour Lines

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Testicular germ cell tumours are amongst the most chemosensitive neoplasms both *in vivo* and *in vitro*. In the present study we demonstrate that following exposure to drugs used in chemotherapeutic treatment of testicular germ cell cancer tumour cells undergo death by apoptosis. Thus, after exposure of the GCT27 embryonal carcinoma cell line to cisplatin, we observed the degradation of DNA into oligonucleosomal fragments, which is a hallmark of apoptosis. Furthermore, light, fluorescence and electron microscopy reveal the presence of condensed abnormal shaped nuclear chromatin which is characteristic of apoptosis. Changes diagnostic of apoptosis were also observed following (a) cisplatin treatment of the GCT48 and Susa embryonal carcinoma cell lines and the GCT44 yolk sac tumour cell line and (b) etoposide treatment of the GCT27 and Susa cell lines. When the GCT27 cell line was treated with 15 μm cisplatin, apoptosis was first observed at 6–9 h and greater than 90% of cells were dead within 24 h. Apoptosis was not blocked when cisplatin-treated cells were incubated in the presence of cycloheximide, although this agent did cause a 4–6 h delay in the onset of cell death. In addition, we demonstrated that the GCT27 cell line can be induced to undergo apoptosis by exposure to low concentrations of the calcium ionophore, ionomycin. These observations show that germ cell tumours are remarkably sensitive to a range of agents that act by different mechanisms. They are triggered to undergo apoptosis rapidly by a mechanism that is not blocked by inhibitors of protein synthesis.

Key words: germ cell tumours, apoptosis, chemosensitivity
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INTRODUCTION

FOR MOST types of malignancy, the response rates and long term survival following chemotherapy are disappointing due to inherent or acquired drug resistance [1, 2]. In contrast, almost all primary and metastatic germ cell tumours can be cured using combination chemotherapy regimes which commonly involve treatment with drugs such as cisplatin, etoposide and bleomycin [3, 4]. Although the reason for this exceptional behaviour has not been established, the response to drug treatment can be reproduced *in vitro*, where it has been demonstrated that germ cell tumour lines are sensitive to a range of drugs that act by distinct mechanisms, including cisplatin, etoposide, bleomycin and radiation [5–7].

Many haemopoietic cell types and leukaemia and lymphoma cell lines have also been found to undergo cell death readily in response to a range of stimuli such as growth factor withdrawal, glucocorticoids, chemotherapy and irradiation [8–12]. Cell death in these cells is known to involve programmed cell death or apoptosis [8]. It has been argued that haemopoietic cells may be sensitive to a wide range of agents because they are primed to undergo apoptosis, simply requiring exposure to the agent to initiate this process [13, 14]. Frequent characteristics of this primed state include sensitivity to calcium ionophores such as ionomycin which induces cell death in 24 h or less with activation of the Ca^{2+}–Mg^{2+} dependent endonuclease associated with apoptosis. Alternatively, it has been proposed that for other less sensitive cell types, synthesis and organisation of the cellular components involved in apoptosis may be required before programmed cell death can proceed [9, 15, 16].

It is believed that haemopoietic cells are primed to undergo apoptosis because this process has a key role in controlling cell development. For example, thymocytes that bind to antigens within the thymus are triggered to undergo cell death by apoptosis, thus providing a mechanism for elimination of self-reactive T lymphocytes [11]. Recent studies have demonstrated that programmed cell death is also responsible for deletion of spermatogonia during spermatogenesis [17, 35], and is responsible for testicular atrophy following hypophysectomy [18]. These observations raise the possibility that germ cells may also

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be primed to undergo apoptosis and invites speculation that this predisposition to engage the cell death programme may explain the remarkable sensitivity of germ cell tumours to a wide range of chemotherapeutic agents.

As a first step in testing this hypothesis, we have examined whether germ cell tumours undergo apoptosis in response to drug treatment. Apoptosis is characterised by morphological changes, the most striking of which are the condensation of the chromatin and appearance of apoptotic bodies [8, 9, 15]. Degradation of the nuclear DNA into oligonucleosomal length fragments is also a biochemical marker [9, 19]. Accordingly, the techniques that we have used for visualising apoptosis include electron microscopy and fluorescence microscopy to examine cell morphology, and electrophoresis of DNA through agarose gels to detect the "ladder" of DNA fragments that is characteristic of apoptosis.

MATERIALS AND METHODS

Cell culture

Four germ cell tumour lines have been investigated; GCT27, GCT44, GCT48 supplied by Dr M Pera [20] and Susa supplied by Dr G Ross [21]. GCT27 was grown in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal calf serum (FCS). GCT44 and GCT48 were grown on a feeder layer of irradiated fibroblasts in DMEM plus 10% FCS with the addition of 2 mM glutamine and 0.2 mM hydrocortisone. Susa was grown in RPMI supplemented by 10% FCS. All lines were incubated at 37°C in 10% CO_2. Stocks were grown in 80 cm^2 Nunc flasks and test cell lines in 25 cm^2 Nunc flasks.

Drugs

Cisplatinum was obtained from Johnson Mathey, dissolved in normal saline and stored at −20°C. Etoposide (Bristol Myers, Middlesex, U.K.) was made fresh and diluted with phosphate buffered saline. Ionomycin (Sigma, St Louis, Missouri, U.S.A.) was dissolved in ethanol and diluted so that the ethanol concentration was less than 10^{-4} M. Cycloheximide (Sigma) was dissolved in DMSO and then diluted to a 0.4% DMSO working solution.

Cell survival

Cell survival was assayed by assessing the uptake of fluorescein diacetate (FDA) and propidium iodide (PI) by flow cytometry. FDA is non-fluorescent, but when taken up by living cells, it is metabolised by esterase to an active fluorescent form. Therefore, living cells take up and activate FDA whilst excluding PI. Dead cells take up both dyes but are unable to activate FDA [22]. This allows quantification of both living and dead cells by flow cytometry. To assess survival, cells were cultured as above with the addition of the test drug. At the appropriate time, the media was collected and cells washed with phosphate buffered saline (PBS). Adherent cells were than harvested by trypsinisation (using 0.1% trypsin/0.5% Versine). Trypsinised cells, the PBS wash and collected media were combinined and cells were collected by centrifugation at 2000 rpm for 5 min. The cell pellet was resuspended in DMEM and placed immediately on ice. Cells (1×10^6) were then incubated with 50 μl of 100 ng/ml FDA and 50 μl of 100 μg/ml PI for 10 min at room temperature. Analysis of the cells was performed on an Orthocytofluorograf 50H flow cytometer with an associated 2150 computer system, using a spectra-physics 2120 argon laser producing 200 mw at 388 nm. Forward and orthogonal light scattering was collected and green (520 nm) and red (>630 nm) fluorescence was displayed two-dimensionally with gating to include single cells and exclude clumps [22]. Each experiment was repeated at least once.

Assessment for DNA degradation

DNA degradation was assessed by agarose gel electrophoresis using the method as described by Smith and associates [23]. Approximately 1×10^6 cells were harvested as above, but washed initially in ice-cold 0.01 M Tris–HCl pH 8.2, then spun at 3000 rpm for 5 min in a Hereaus sepatech centrifuge. The supernatant was removed and the cell pellet was either analysed immediately or stored at −70°C for future analysis. Cells were resuspended in 20 μl of digestion buffer [0.5% (w/v) sodium lauroyl sarcinosinate, 10 mM EDTA, 50 mM Tris–HCl pH 8.0] and incubated at 50°C for 2 h with 1 μl of 20 mg/ml proteinase K. After 1 h, 2 μl of 10 mg/ml DNase-free RNase was added and incubation continued for a further 1 h. The temperature was then increased to 70°C and 10 μl of loading buffer [10 mM EDTA, 0.25% (w/v) Bromophenol Blue, 40% (w/v) sucrose and 1% (w/v) low gelling temperature agarose] was added. Thirty microliters were then loaded dry into a 2% (w/v) agarose gel with ethidium bromide (0.1 μg/ml) which was then run either overnight at 18 V or over 2 h at 80 V in TAE electrophoresis buffer. Results were compared to BAF3 cells following IL3 withdrawal (positive control) and cisplatinum treated fibroblasts (negative control).

Fluorescent microscopy

Cell morphology was assessed by fluorescence microscopy. Cells prepared as above were fixed overnight in ice-cold 70% ethanol. After centrifugation at 1200 rpm, the pellet was resuspended in 500 μl PBS and 100 μl of 1 mg/ml RNase. Two hundred microliters of 100 μg/ml PI was then added and the mixture was incubated overnight. An aliquot of cells was viewed using a Zeiss fluorescence microscope. Apoptotic cells were identified by the presence of nuclear chromatin condensation. For quantification of apoptosis, the numbers of cells with normal and apoptotic appearance were counted. At least 200 cells from each sample in a minimum of five high power fields were examined. Comparison was made with fibroblast control cells. The low proportion of clearly degraded cells observed in some experiments were excluded.

Light and electron microscopy

Cells were centrifuged and the pellets 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 Toluidine Blue. For electron microscopy, 0.1 μm sections were collected on to copper grids, double stained with uranyl acetate and lead citrate, and examined in a Philips CM10 transmission electron microscope.

Flow cytometry

Cells were prepared as for fluorescence microscopy. Analysis was performed on an Orthocytofluorograf 50H flow cytometer. Doublets were excluded by gating on a cytogram of peak red fluorescence versus area of red fluorescence before displaying 90° and forward light scatter and further gating to produce histogram of red fluorescence [22, 24]. Comparison was made to BAF3 and fibroblast controls. Results were checked by flow sorting and fluorescence microscopy.

RESULTS

Mechanism of cisplatin-induced cell death in GCT27 embryonal carcinoma cells

Following treatment with 15 μM cisplatin, greater than 90% of GCT27 cells had died within 24 h judged by their ability to take up PI and their failure to metabolise FDA (Figure 1). To assess whether cell death occurred by apoptosis, DNA from cells treated with cisplatin was subjected to electrophoresis on agarose gels. In these experiments the characteristic fragmentation of DNA into oligonucleosomal lengths, which is a hallmark of apoptosis, was first observed at 6–9 h after treatment (Figure 2). To confirm that cell death involved apoptosis, cisplatin-treated cells were examined by electron microscopy, by light microscopy and by fluorescence micropscopy following ethanol fixation and staining with PI. These techniques revealed the presence of cells with the condensed abnormal shaped nuclear chromatin that are characteristic of apoptosis (Figure 3).

Fluorescence microscopy proved to be a reproducible and convenient method for assessing the proportion of cells that had undergone apoptosis. Using this method, it could be demonstrated that the proportion of cells recognised as apoptotic increased with both time and dose (for example see Figure 4), whereas in control fibroblast cells <1% apoptotic cells were seen at all time points. Quantitative analysis of apoptosis showed the onset of apoptosis from 6–12 h after exposure to cisplatin, preceding the loss of viability by 4–6 h, as judged by the ability of cells to take up PI and their inability to metabolise FDA (Figure 4). When cells from the media and those remaining attached to the culture dish were examined separately, the apoptotic population were found mainly in the media, with most attached cells having normal nuclear morphology and viability (results not shown). At higher doses (>15 μM) and at longer time intervals (>24 h), the number of fragmented and degraded cells detected increased so making interpretation of the apoptotic proportion more difficult, but most of the remaining cells were found to be apoptotic and only a few isolated cells had a normal morphology.

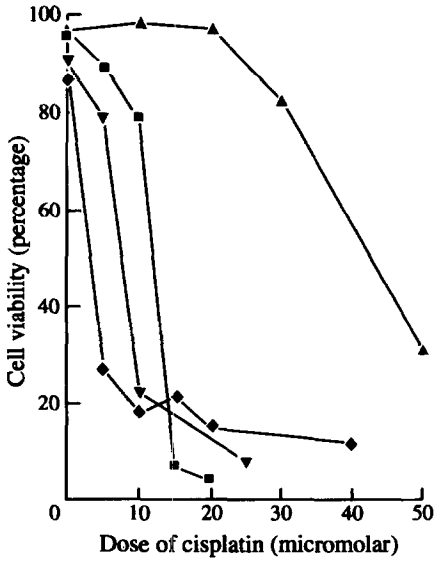

Figure 1. Viability of germ cell tumour lines 24 h after treatment with cisplatin as judged by the combined ability of cells to exclude PI and to metabolise FDA. The cell lines examined were the GCT27 embryonal carcinoma line (■), the GCT48 embryonal carcinoma line (◆), the Susa embryonal carcinoma line (▼) and the GCT44 yolk sac tumour line (▲).

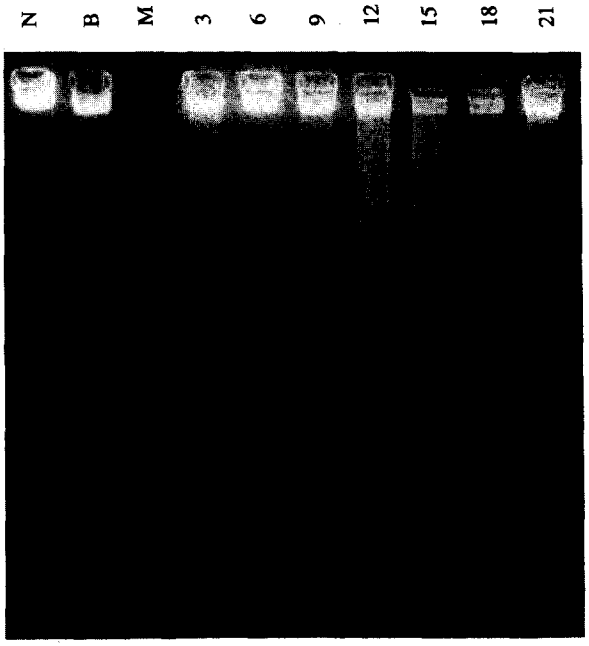

Figure 2. Agarose gel electrophoresis of total cellular DNA from GCT27 cells treated with 15 μM cisplatin for 0–21 h. Oligonucleosomal DNA fragment lengths, which are characteristic of apoptosis, are visible from 9 h, reaching maximum intensity at 15 h. Numbers refer to hours of treatment. N = no treatment. B = DNA from BAF3 leukaemia cells 15 h after withdrawal of IL3, included as a positive control. M = Hae III fragments used as size markers.

Apoptotic cells can, in some cases, be visualised using flow cytometric methods by the appearance of a sub-G1 peak in a DNA histogram. The sub-G1 peak is believed to be derived from cells that undergo apoptosis in the G1 phase of the cell cycle, and the apparent decrease in DNA content presumably results from partial digestion or changes in PI binding, with changes in conformation of the nuclear DNA during apoptosis [24].

When this approach was used to examine GCT27 cells that had been treated with cisplatin, the sub-G1 peak was only seen over a limited time period (15–18 h; Figure 5a), and was relatively small compared to that observed for control lymphoma cells (Figure 5b). At earlier time points no distinct additional peaks were observed although the G1 peak was noted to be broader than for control cells. To determine where the apoptotic cells appeared on the DNA histogram we examined fractions of cells with different DNA contents by fluorescence microscopy. This revealed that the most apoptotic cells were found to the left of the G1 peak (Figure 6). Apoptotic and normal cells could, however, be separated by a protocol dependent on light scatter rather than DNA content [22, 25; Figure 7].

Response of GCT48, Susa and GCT44 germ cell tumour lines to cisplatin treatment

The GCT48 and Susa embryonal carcinoma cell lines showed similar sensitivity to treatment with cisplatin to GCT27. Cisplatin of 10 μM and 5 μM caused death of greater than 70% of cells at 24 h of the Susa and GCT48 cell lines, respectively (Figure 1). A yolk sac cell line, GCT44, also responded to cisplatin but was less sensitive than the embryonal carcinoma cell lines, although still more sensitive than fibroblasts. Electron microscopy and fluorescence microscopy established that, for

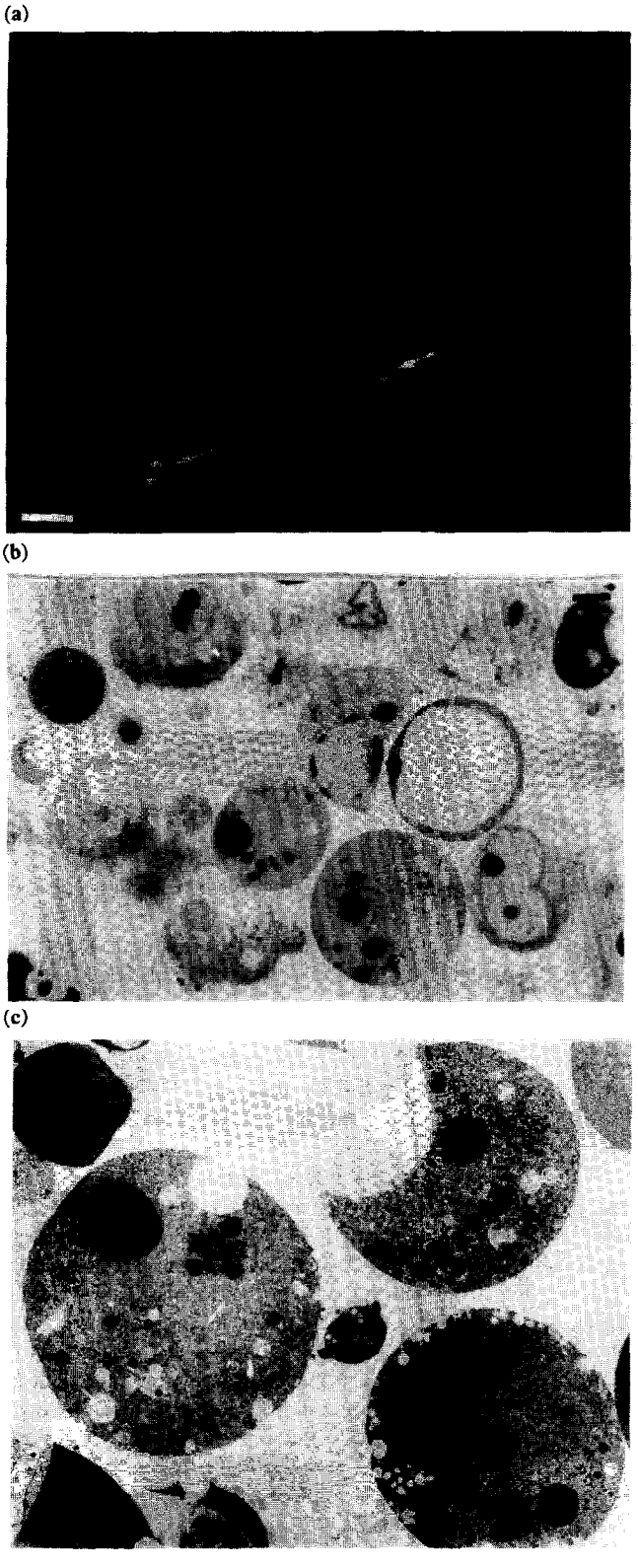

Figure 3. Appearance of GCT27 cells 18 h following treatment with 15 μM cisplatin. (a) Fluorescence microscopy. Apoptotic cells are indicated by arrows (× 700; scale bar = 14 μm). (b) Light microscopy (× 1500; scale bar = 6.7 μm). (c) Electron microscopy (× 3600; scale bar = 2.8 μm).

each cell line, cell death involved apoptosis (results not shown). Apoptosis was largely seen in GCT44 only at higher doses of cisplatin (30–50 μM) as expected from its greater resistance to chemotherapy. When considered together with the studies on GCT27 cells these results show that apoptosis is a common

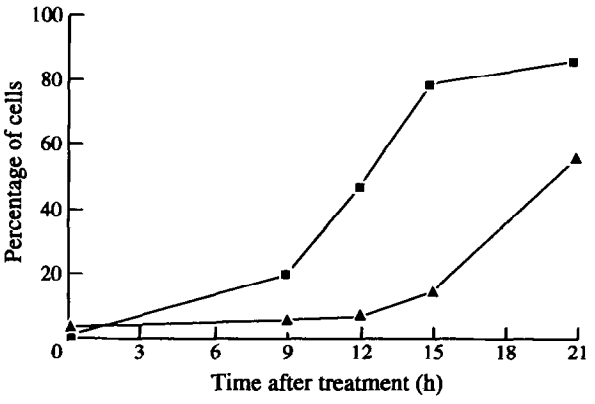

Figure 4. Time course of apoptosis (■) and cell death (▲) in GCT27 cells following treatment by 15 μM cisplatin. The proportion of apoptotic cells was assessed by fluorescence microscopy, and the proportion of viable cells assessed by ability to exclude PI and to metabolise FDA.

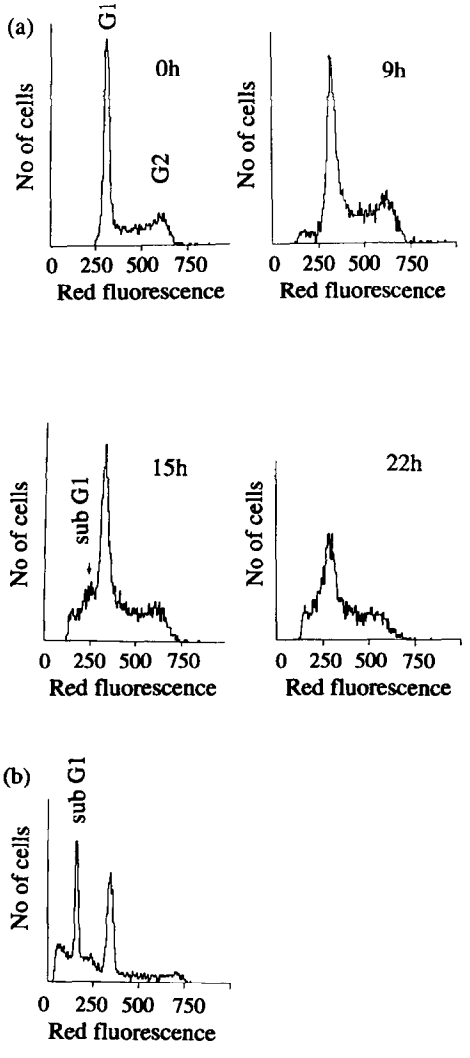

Figure 5. (a) DNA histograms of GCT27 cells at 0, 9, 15 and 22 h following treatment by 15 μm cisplatin. DNA content is proportional to PI fluorescence. A broader G1 peak is seen at 22 h after treatment and a small sub-G1 peak is seen at 15 h post treatment. (b) DNA histogram of BAF3 cells following IL3 withdrawal.

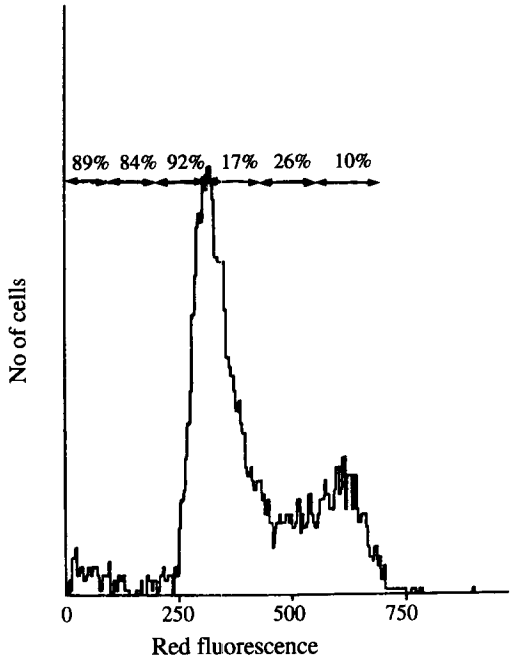

Figure 6. Proportion of apoptotic cells according to position in DNA histogram. Cells were flow sorted according to position in DNA histogram. The percentage of cells undergoing apoptosis was measured by fluorescence microscopy.

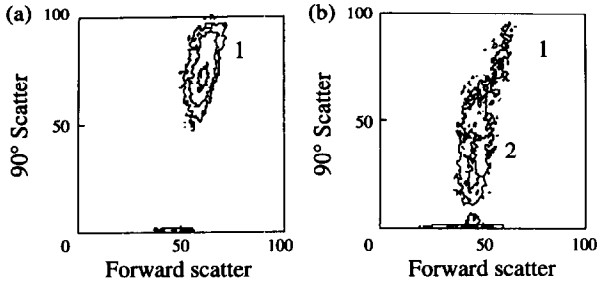

Figure 7. Analysis of PI staining by light scatter (see Materials and Methods). Control GCT27 cells (a) and GCT27 cells 18 h following treatment with 15 μM cisplatin (b). The areas of normal cells (1) and apoptotic cells (2) are shown.

mechanism of death in germ cell tumours following treatment with cisplatin.

Apoptosis of germ cell tumour cell lines in response to treatment with etoposide

Cisplatin exerts its biological effects by binding covalently to and cross-linking DNA. In contrast, etoposide, another drug commonly used for treating germ cell tumours, acts by inhibiting topoisomersase II, an enzyme involved in controlling the degree of coiling of DNA [1, 26]. It was therefore of interest to establish whether etoposide could also trigger germ cells to undergo apoptosis. Our studies established that Susa cells were more sensitive to treatment with etoposide than GCT27 cells, which were in turn more sensitive to treatment than GCT48 and GCT44 cells (Table 1). For GCT44, GCT48 and GCT27 cells 7, 17 and 24% cell death, respectively, was observed 24 h after exposure to 20 μg/ml etoposide. In comparison, around 75% of Susa cells were dead after 24 h with only 10 μg/ml etoposide. For each cell line, electron microscopy and fluorescence microscopy were used to establish that cell death occurred by apoptosis.

Table 1. Apoptosis following treatment of germ cell tumour lines with etoposide

Dose of etoposide (μg/ml)	Cell line*			
	GCT27	Susa	GCT44	GCT48
0	1.5%	3%	1%	0.5%
5	10%	66%	-	-
10	15%	77%	7%	11%
20	24%	-	7%	17%

*The percentage of cells undergoing apoptosis was assessed by fluorescence microscopy 24 h after treatment (average of at least two experiments).

Apoptosis of GCT27 cells in response to treatment with ionomycin

Increased calcium levels have been shown to stimulate endonuclease activation both in whole cells and isolated nuclei. [15]. This process can be blocked by calcium chelaters and stimulated by calcium ionophores in sensitive cell types such as thymocytes [27] and Burkitt's lymphoma cells [28]. This is thought to be due either to direct activation of Ca^{2+}–Mg^{2+} dependent endonuclease or by Ca^{2+} acting as a second messenger in cooperation with protein kinase C [29]. The results of ionomycin treatment of GCT27 are shown in Figure 8. Following exposure to 5 μg/ml ionomcyin, 85% of cells were dead after 24 h. Electron microscopy revealed the morphological features that are characteristic of apoptosis. These results establish that germ cell lines can be induced to undergo apoptosis in response to treatment with low concentrations of Ca^{2+} ionophore.

Effect of cycloheximide on cisplatin-included apoptosis in GCT27 cells

To test whether programmed cell death in germ cell tumours lines is dependent on protein synthesis we examined the effect of cycloheximide on cisplatin-induced apoptosis. In control experiments we established that concentrations of up to 10 μg/ml cycloheximide could be used for 48 h without observable cytotoxicity of GCT27 cells. Following treatment with 15 μM

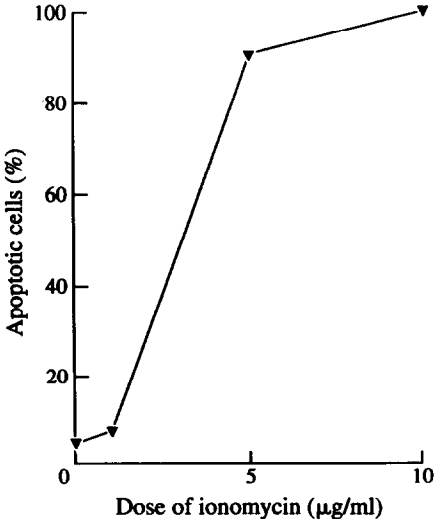

Figure 8. Apoptosis of GCT27 cells following treatment with ionomycin for 24 h. The percentage of cells undergoing apoptosis was judged by fluorescence microscopy.

cisplatin, concentrations of between 1–10 μg/ml cycloheximide caused by short but reproducible delay in the onset of apoptosis (Figure 9) as judged by fluorescence microscopy. The delay was approximately 4 h at 1 μg/ml cycloheximide (Figure 9a) and approximately 6 h at 10 μg/ml of this inhibitor (Figure 9b).

DISCUSSION

In these studies we have established that the rapid cell death following treatment of the GCT27 cell line with the DNA damaging agent, cisplatin, occurs by apoptosis. Thus, we have shown that cell death is accompanied by fragmentation of DNA into oligonucleosomal lengths and that there are characteristic changes in morphology including nuclear condensation and the appearance of apoptotic bodies. In addition, it has been demonstrated that three additional germ cell lines, GCT44, GCT48 and Susa, also undergo apoptosis in response to treatment with cisplatin and that apoptosis can occur in response to treatment with the topoisomerase II inhibitor, etoposide. Apoptosis has also been induced when CHO cells [30] and JB1 hepatocellular cells [31] are treated with cisplatin. In the case of CHO cells, it has been established that cell death usually proceeds from the G2 phase of the cycle [30], although under certain circumstances and in other cell types (e.g. HL60), cell death can occur at all stages of the cell cycle [32]. In agreement with these observations, we found that the DNA content of apoptotic GCT27 cells is consistent with death throughout the cell cycle (Figure 6).

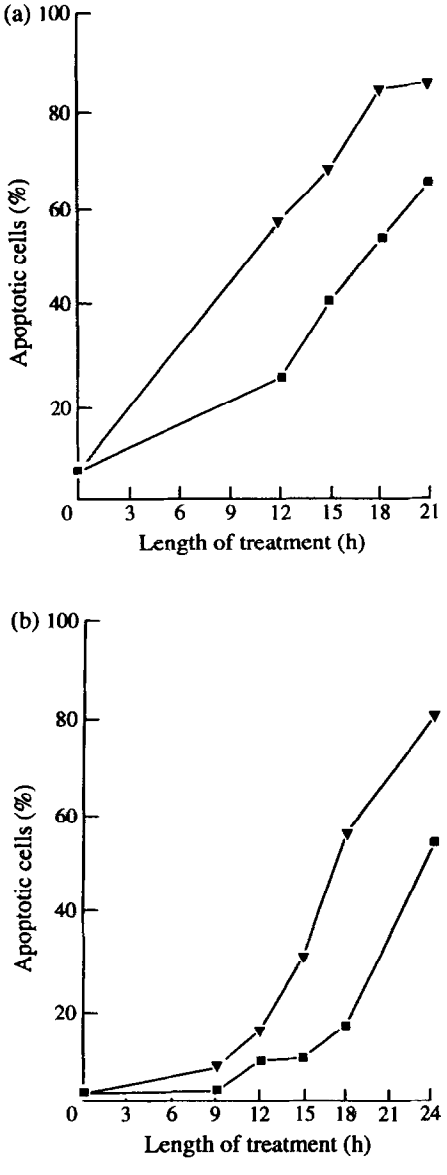

Figure 9. The effect of cycloheximide on the rate of apoptosis after treatment of GCT27 cells with 15 μm cisplatin. (a) In experiment 1, cells treated with cisplatin were incubated either in the absence (▼) or presence (■) of 1 μg/ml cycloheximide. (b) In experiment 2, cells treated with cisplatin were incubated either in the absence (▼) or presence (■) of 10 μg/ml cycloheximide.

Testicular germ cell tumours are amongst the most chemosensitive and radiosensitive neoplasms. Indeed, whereas most malignant tumours are incurable, with multiagent or even high dose therapy, the majority of testicular germ cell tumours can be cured even when advanced [4]. Although much is known about mechanisms of drug resistance, the reason why selected groups of tumours, which include germ cell tumours, some paediatric sarcomas and lymphomas, are inherently more sensitive to chemotherapy remains obscure. Differences in cell cycle kinetics, tumour microenvironment, DNA damage induction and repair have so far failed to fully explain the phenomena [1, 2, 33]. For example, recent evidence suggests that resistance is not due to increased ability of cells to mutate to a drug resistant genotype since mutation rates of the HGPRT locus were similar in chemosensitive germ cell tumours and a relatively resistant bladder carcinoma line [34].

As an alternative hypothesis, it has been suggested that differences in chemosensitivity between cell types is not due to cellular damage *per se* but to differences in the response of the cells to damage. In such a model, "sensitive" cells are more likely to trigger programmed cell death in response to cellular damage than are "resistant" cells [13]. Such a model is supported by the well recognised finding of extensive and easily induced programmed cell death in some leukaemias and in some lymphoid tumours, malignancies distinguished by chemo- and radiosensitivity, as well as in their tissues of origin [9, 15]. There is already evidence that programmed cell death is responsible for the deletion of spermatogonia during spermatogenesis [17, 35] and is responsible for testicular atrophy following hypophysectomy [18]. Our observation that germ cell tumours undergo apoptosis in response to treatment with chemotherapeutic agents is also consistent with this model. In addition, the rapid time course of apoptosis and the observation that apoptosis can be induced by low doses of the calcium ionophore, ionomycin, suggests that germ cells may already be primed to undergo programmed cell death. That sensitivity is an inherent property of the cell of origin is supported by the observation that human germ cells are very sensitive both to irradiation and chemotherapy such that even a modest dose of irradiation or alkylating chemotherapy can cause irreversible sterility [36].

The effect of cycloheximide differs considerably between cell lines. For instance, Barry and associates [30] found that for CHO cells, the onset of apoptosis following cisplatin treatment was delayed by 24–48 h by cycloheximide, while it completely blocks apoptosis in cisplatin-treated JB1 hepatoma cells [31]. Similarly, protein synthesis inhibition blocks apoptosis in many other cell types, including gut and bone marrow cells treated by chemotherapy, glucocorticoid treated thymocytes [19] and growth factor deprived neuroblastoma and erythroid progenitor cell lines [37, 38]. However, in other cell types, cycloheximide either has no effect, such as in monophages [39] and cytotoxic T

cells [40], or stimulates apoptosis, for example, in HL60 cells [41], normal lymphocytes and B-CLL cell lines [10]. Wyllie and associates [14] suggested that such cells are primed and contain all the necessary enzymes for onset of apoptosis, while non-primed cells need to synthesise the enzymes after triggering. Our results seem to be intermediate between the two. Apoptosis was delayed by a few hours, but less than that reported for some other cell lines [30, 31]. Why this should be is unclear. It is possible that the doses of cycloheximide used were not sufficient to fully block protein synthesis, although higher doses were toxic to cells, and the dose used is similar to those used to substantially delay apoptosis in other cell lines. Alternatively, it is possible that all the necessary enzymes for apoptosis were present and cycloheximide simply showed progression of cells to the sensitive G2 phase of cell cycle. Further studies would be required to distinguish between these possibilities.

In conculsion, we have established that cell death following treatment with cisplatin and etoposide occurs by apoptosis. In addition, the rapid time course of cell death following drug treatment, the induction of apoptosis following exposure to low concentrations of ionomycin, and the relatively limited effect of cycloheximide treatment demonstrate that germ cell tumours can readily be triggered to undergo apoptosis by a mechanism which is not blocked by inhibition of protein synthesis.

1. Master JRW. Biochemical basis of resistance to chemotherapy. *Radiother Oncol* 1990, **19**, (4), 297–306.
2. Morrow CS, Cowan KH, Devita VT, Hellman S, Rosenberg SA, eds. Mechanisms of antineoplastic drug resistance. In *Cancer Principles and Practice of Oncology, 4th Edition*. Philadelphia, U.S.A., J.B. Lippincott, 1993, 340–348.
3. Rankin EM, Kaye SB. Principles of chemotherapy. In Sikora K, Halnan KE, eds. *Treatment of Cancer*. Philadelphia, U.S.A. Chapman & Hall, 1990, 127–146.
4. Horwich A (ed.) *Testicular Cancer: Investigation and Management*. London, Chapman and Hall Medical, 1991.
5. Walker MC, Parris CN, Masters JRW. Differential sensitivity of human testicular and bladder human cell lines to chemotherapeutic agents. *J Natl Cancer Inst* 1986, **79**, 213–216.
6. Masters JRW, Osborne EJ, Harris D, Walker MC, Parris CN. Hypersensitivity of testis tumours to anticancer agents. *Proc Am Assoc Cancer Res* 1990, **31**, 368.
7. Kelland LR, Mistry P, Abel G, Freidlos F, Loh SY, Roberts JJ & Harrap KR. Establishment and characterisation of an *in vitro* model of acquired resistance to cisplatin in a human non seminomatous germ cell line. *Cancer Res* 1992, **52**, 1710–1716.
8. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980, **68**, 251–306.
9. Fesus L, Davies PJA, Piacentini M. Apoptosis: molecular mechanisms in programmed cell death. *Eur J Cell Biol* 1991, **56**, 170–177.
10. Collins RJ, Harmon BV, Souvlis T, Pope JH, Kerr JHR. Effects of cycloheximide on B chronic lymphocytic leukaemia and normal lymphocytes *in vitro*: induction of apoptosis. *Br J Cancer* 1991, **64**, 518–522.
11. McCarthy NJ, Smith CA, Williams GT. Apoptosis in the development of the immune system: growth factors, clonal selection and BCL-2. *Cancer Metast Rev* 1992, **11**, 157–178.
12. Williams GT, Smith CA, McCarthy NJ, Grimes EA. Apoptosis: final control point in cell biology. *Trends Cell Biol*, **2**, 263–267.
13. Dive C, Hickman JA. Drug target interactions: only the first step in commitment to a programmed cell death? *Br J Cancer* 1991, **64**, 192–196.
14. Wyllie AH, Arends MJ, Morris RE, Walker SM, Evans G. The apoptosis endonuclease and its regulation. *Semin Immun* 1992, **4**, 389–397.
15. Wyllie AH. Apoptosis and regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer Metastasis Rev* 1992, **11**, 95–103.
16. Wyllie AH. Apoptosis. *Br J Cancer* 1993, **67**, 205–208.
17. Allan DJ, Harman BV, Roberts SA. Spermatogonal apoptosis has 3 morphologically recognisable phases and shows no circadian Rhythm during normal spermatogenesis in the rat. *Cell Prolif* 1992, **25**, 241–250.
18. Tapanainon JS, Tilly JL, Vihkro KK, Hsueh AJ. Hormonal control of apoptotic cell death in the testis: gonadotrophins and androgens as testicular cell survival factors. *Mol Endocrinol* 1993, **7**, (5), 643–650.
19. Wyllie AH. Glucocorticoid-induced thymocytic apoptosis is associated with endogenous activation. *Nature* 1980, **284**, 555–556.
20. Pera MF, Blascolafita MJ, Mills J. Cultured stem cells from human testicular teratoma the nature of human embryonal carcinoma and its comparison with two types of yolk sac carcinoma. *Int J Cancer* 1987, **40**, 334–343.
21. Hogan B, Fellous M, Avner P, Jacob F. Isolation of a human teratoma cell line which expresses F9 antigen. *Nature* 1977, **270**, 515–518.
22. Ormerod MG, ed. *Flow Cytometry: A Practical Approach*. Oxford, IRL Press, 1990.
23. Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJT. Antibodies to the CD3/T cell receptor complex induce death by apoptosis in immature T cells in thymic culture. *Nature* 1989, **337**, 181–184.
24. Ormerod MG, Collins MKL, Rodriguez-Tarduchy G., *et al.* Apoptosis in interleukin-3 dependent haemopoietic cells: Quantification of two flow cytometric methods. *J Immunol Methods* 1992, **153**, 57–62.
25. Zamai L, Falcieri E, Zauli G, Gataldi A, Vitale M. Optimal detection of apoptosis by flow cytometry depends on cell morphology. *Cytometry* 1993, **14**, 891–897.
26. Devita VT. Principles of chemotherapy. In Devita VT, Hellman S, Rosenberg SA, eds. *Cancer: Principles and Practice of Oncology, 4th Edition*. J.B. Lippincott, 1993, 276–292.
27. McConkey DJ, Hartzell P, Nicotera AP, Orrenius S. Calcium activated DNA fragmentation kills immature thymocytes. *FASEB* 1989, **3**, 1843–1849.
28. Liu YJ, Mason DY, Johnson GD, *et al.* Germinal centre cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur J Immunol* 1991, **21**, (8), 1905–1910.
29. Eastman A, Barry MA. The origins of DNA breaks: A consequence of DNA damage, DNA repair, or apoptosis? *Cancer Invest* 1992, **10**, 229–240.
30. Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer agents, drugs, toxins and hyperthermia. *Biochem Pharmacol* 1990, **40**, (10), 2353–2362.
31. Evans DL, Dive C. Effects of cisplatin on induction of apoptosis in proliferating hepatoma cells and non proliferating immature thymocytes. *Cancer Res* 1993, **53**, 2133–2139.
32. Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* 1993, **53**, 3186–3192.
33. Xiao C, Masters JRW. Spectrum of differential sensitivity *in vitro* of a testicular germ cell tumours. *Br J Cancer* 1988, **58**, 230.
34. Parris CN, Walker CM, Masters JRW, Artlett CF. Inherent sensitivity and induced resistance to chemotherapeutic drugs and irradiation in human cancer cell lines. Relationship to mutation frequencies. *Cancer Res* 1990, **50**, 7513–7518.
35. Allan DJ, Harman B, Fern JFR. Cell death in spermatogenesis. In Potter CS, ed. *Perspectives on Mammalian Cell Death*. London, Oxford University Press, 1987, 229–258.
36. Sherins RJ. Gondadal dysfunction. In DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer Principles and Practice of Oncology, 4th Edition*. Philadelphia, U.S.A. J.B. Lippincott, 1993, 2395–2408.
37. Linnik MD, Hatfield MD, Swope MD, Athmid NK. Induction of programmed cell death in a dorsal root ganglia X neuroblastoma cell line. *J Neurobiol* 1993, **24**, 433–446.
38. Kelley LL, Kowry MJ, Bondurant MC. Regulation of programmed death in erythroid progenitor cells by erythropoietin: effects of calcium and of protein and RNA synthesis. *J Cell Physiol* 1992, **151**, (3), 487–496.
39. Waring P. DNA Fragmentation induced in macrophages by gliotoxin does not require protein synthesis and is preceded by raised inositol phosphate levels. *J Biol Chem* 1990, **265**, 14476–14480.
40. Duke RC, Chervenak R, Cohen JJ. Endogenous endonuclease induced DNA fragmentation an early event in cell mediated cytolysis. *Proc Natl Acad Sci USA* 1983, **80**, 6361–6365.
41. Martin SJ, Lennon SV, Bonham AM, Cotter TE. Induction of

apoptosis (programmed cell death) in human leukaemia HL60 cells by inhibition at RNA or protein synthesis. *J Immun* 1990, 145, 1859–1867.

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Temozolomide Reduces the Metastatic Potential of Lewis Lung Carcinoma (3LL) in Mice: Role of α-6 Integrin Phosphorylation

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The involvement of protein kinase c (PKC) in the mechanism underlying the antimetastatic properties of triazenes was studied in C57BL/6 mice bearing Lewis lung carcinoma (3LL). *In vivo* and *in vitro* treatment with temozolomide, an *in-vitro* active analogue of dacarbazine, or calphostin c produced a concentration-dependent reduction of spontaneous and artificial metastases. Both agents reduced the ability of 3LL cells to adhere to endothelium. Diethylaminoethyl (DEAE)-sepharose chromatography of cell extracts revealed that incubation of 3LL cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) caused a rapid translocation of protein kinase c activity from cytosol to the membrane fraction. Membrane PKC activity induced by TPA was reduced by 60% after treatment with temozolomide. Coincident with these changes, TPA induced phosphorylation of α-6 integrin, whereas temozolomide or calphostin c abolished the appearance of this phosphoprotein. These results suggest that temozolomide reduced metastatic potential by interfering with α-6 phosphorylation induced by PKC activation.

Key words: PKC, temozolomide, metastasis, calphostin c, integrins, cell adhesion, phosphorylation
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INTRODUCTION

THE CELLULAR signalling systems have recently come to the fore as potential targets for the development of new anticancer drugs. Because of its pivotal importance for signal transduction, protein kinase c (PKC) is undoubtedly a logical target for drug intervention. PKC, a family of structurally related isoforms, may play a critical role in the regulation of tumour cell invasion and metastasis. In fact, tumour cells showing high PKC activity have enhanced ability to invade and metastasise. This association has been found for mouse B16 melanoma [1], mouse Lewis lung carcinoma [2], mouse mammary adenocarcinoma [3] and murine fibrosarcoma [4]. 3LL cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) display an enhancement of adhesion to endothelial cells. This event is associated with increased phosphorylation of cell proteins [2], as well as PKC membrane translocation. Tumour cell adhesion is mediated by a large array of cell surface adhesion molecules, including P- and E-selectins [5, 6], cadherins [7, 8], integrins [9, 10], intercellular adhesion molecules and vascular adhesion molecules [11]. In particular, α-6 subunit integrin is known to be present in higher amounts in 3LL cells which possess higher capacity to metastasise to the lung [12]. However, it is not clear how cell adhesion is regulated during tumour progression to metastatic stage. Recent data suggest that adhesion molecules, in addition to their function in mediating cell–cell adhesion, play a crucial role in signal transduction [11, 13]. PKC activators, such as TPA, have been shown to activate adhesion receptors by promoting phosphorylation of integrins [14, 15], which can affect binding characteristics for the ligands [16]. Studies using various PKC inhibitors such as H-7 and calphostin c, developed against PKC at its catalytic and regulatory domain [17, 18], also provide evidence that PKC is associated with metastasis. Specificity seems more likely to be associated with agents which inhibit predominantly at the regulatory site [18]. Moreover, anticancer drugs in clinical use, such as tamoxifen [19] and doxorubicin [20–21], have been described as possessing PKC inhibitory properties.

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