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Studies with cytotoxic agents suggest that apoptosis is not a major determinant of clonogenic death in neuroblastoma cells

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Abstract

We have previously reported the selection of a radioresistant human neuroblastoma cell line, Clone F, from IMR32 cells. We have shown that clonogenic radioresistance in these cells is accompanied by a reduced level of radiation-induced apoptosis [Cancer Res 55 (1995) 4915]. Here, we measured the response of these lines to several cytotoxic agents, in terms of clonogenicity and apoptosis. In the clonogenic assay, the radioresistant line was also resistant to cisplatin, melphalan and doxorubicin, but not to perillyl alcohol. However, all these agents produced less apoptosis in the Clone F cells, except cisplatin, which failed to induce any apoptosis in either cell line. Reduced apoptosis cannot be the cause of the Clone F cells' resistance to cisplatin. By extension, the Clone F cells' resistance to radiation and other agents cannot be due to diminished apoptosis either. Based on these results, apoptosis may not be a useful surrogate for clonogenic outcome.

Keywords: Neuroblastoma; Apoptosis; Cisplatin; Doxorubicin; Perillyl alcohol; Tamoxifen; Melphalan; Radiation

1. Introduction

The role of apoptosis in the response of cancers to therapy has been a major source of interest to researchers, stemming largely from the excitement around p53's role as the 'Guardian of the Genome' [1]. Most transformed cells do not undergo apoptosis in response to ionising radiation or other cytotoxic agents. Those that do are usually non-epithelial in origin, e.g. lymphomas and neuroblastomas. Such cells are often unusually sensitive to clonogenic death following ionising radiation, and the tumours tend to be responsive to radiation and chemotherapy [2]. Thus, it is natural to assume that apoptosis sensitises cells by making them commit suicide in response to levels of damage that would not otherwise be lethal. However, transfection of *bcl-2* can suppress radiation or chemotherapy-induced apoptosis, without any corresponding decrease in their radiation sensitivity, as measured by clonogenic survival [3–5]. This suggests that, in these cases at least, apoptosis simply represents an alternative mode of death for cells that were already doomed.

We have previously reported the selection of a radioresistant human neuroblastoma cell line, which was obtained by exposing the parental cells (IMR32) to repeated doses of 2 Gy [6]. The resistant cell line, which was named Clone F, required approximately twice as much radiation as the parental cells to produce equivalent levels of clonogenic death. However, they showed no enhanced capacity for DNA double-strand break repair, and both lines were equally susceptible to radiation-induced chromosome aberrations. The resistant cells displayed markedly less apoptosis following radiation, leading us to conclude that the difference between the cell lines was confined to the apoptotic pathway, and that the reduced levels of apoptosis found in Clone F cells caused the increased clonogenic survival. If this reasoning was correct, then in IMR32 cells, apoptosis was contributing to radiosensitivity.

We report here how these two cell lines respond to various cytotoxic agents. The drugs used were cisplatin, doxorubicin, and melphalan, and perillyl alcohol. Melphalan, doxorubicin and cisplatin are all used in the treatment of neuroblastoma [7,8]. Perillyl alcohol is a

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novel compound currently being investigated in phase I clinical trials [9]. It has been shown to depress transforming growth factor β2 (TGFβ2) receptor expression [10] and to interfere with ras farnesylation [11]. We expected, on the basis of published results with other cell systems, that perillyl alcohol would induce apoptosis in the neuroblastoma lines [12]. It was our aim to investigate whether the radioresistant cells had acquired resistance to other forms of DNA damage, and also whether the defect in their apoptotic pathway would extend to apoptosis induced by non-DNA damaging events.

2. Materials and methods

2.1. Cell culture and reagents

IMR32 and Clone F cells were cultured in MEM with 10% fetal bovine serum (Sigma, St Louis, MO, USA), L-glutamine, (2 mM) and penicillin/streptomycin (100 U/ml). Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. Perillyl alcohol was purchased from Aldrich (St Louis, MO, USA). Hoechst 33258, doxorubicin, tamoxifen, cisplatin and melphalan were obtained from Sigma. Doxorubicin was dissolved in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml; cisplatin was dissolved in dimethyl sulphoxide immediately prior to use; melphalan was dissolved in 50% ethanol, 0.05 N HCl, to give a stock solution of 16 mM. Perillyl alcohol solutions were prepared by adding drug to medium at 37 °C for 1 h prior to use. Anti-Bcl2 was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA); anti-proliferating cell nuclear antigen (PCNA) from Calbiochem (San Diego, CA, USA) and anti-mouse-horseradish peroxidase conjugate from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Clonogenic assay

For clonogenic assays, exponentially growing cells were seeded into T25 flasks at numbers ranging from 10^3 to 10^5/flask. After 6 h, when the cells had attached, the medium was removed and replaced with fresh drug-containing medium. At this stage, lethally-irradiated (50 Gy) IMR32 feeder cells were added, to give a total of 5×10^5 cells per flask. After 24 h, the medium was removed, the flasks were rinsed in PBS, and fresh medium added. The rinse was added to the drug-containing medium, which was spun down to collect the detached cells. The pellet was rinsed again, resuspended in medium and added back to the appropriate flasks. This procedure was employed because previous experiments with perillyl alcohol had shown that cells that had detached during treatment were capable of re-attaching to flasks and forming colonies. Cells were allowed 10–14 days to form colonies, after which they were stained in crystal violet (10%). The colonies were counted by microscope, using the standard definition of a colony, as containing at least 50 cells. For radiation exposures, exponentially growing cells were seeded into T25 flasks along with feeder cells and irradiated immediately. Radiation exposures were performed using a Gammacell ^{137}Cs source, (Nordion, Ottawa, ON, Canada), at a dose rate of approximately 0.9 Gy/min.

2.3. Assays for apoptosis and caspase activation

Cells were fixed in ice-cold methanol–acetic acid (3:1) and spread on slides. The slides were stained with Hoechst 33258 and apoptotic cells counted. A total of 500 cells were counted per point. Apoptotic cells were recognised on the basis of chromatin condensation and the frequent fragmentation of the nucleus into multiple bodies. Slides were coded before counting; the codes were broken after all the slides had been counted.

Caspase activation was measured using a carboxyfluorescein labelled pan-caspase inhibitor FAM-VAD-FMK, (Intergen, Purchase, NY, USA) according to manufacturer's instructions, and analysed by fluorescent cell sorting analysis (FACS). A total of 10^4 cells were counted.

2.4. Western blotting

Protein extracts were prepared by lysing cells on ice in lysis buffer (Tris, 50 mM, pH 8.0; NaCl, 150 mM; Triton-X 100, 1%; protease inhibitor cocktail, 1%; phenylmethyl sulphonyl fluoride (PMSF) 50 μM). After 30 min on ice, the lysate was centrifuged for 10 min at 10 000*g* and the supernatant collected. Proteins were denatured by boiling for 5 min in gel loading buffer (Tris, 62.5 mM, pH 6.8; glycerol, 5%; sodium dodecyl sulphate (SDS), 2%; β-mercaptoethanol, 2%; bromophenol blue, 0.01%). Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 12.5% polyacrylamide gels, and transferred to nitrocellulose membranes. Blocking and antibody exposure were in 5% non-fat-milk in PBS with 0.05% Tween-20, for 1 h at room temperature. Anti-Bcl-2, (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used at a dilution of 1:100; anti-PCNA and the secondary antibody were used at 1:1000. After the membrane had been probed for Bcl-2, it was stripped in Tris, 62.5 mM pH 6.8; SDS, 2%; β-mercaptoethanol, 0.8%, for 20 min at 60 °C, and re-probed for PCNA.

2.5. Statistics

To test for differences between the apoptotic fractions, we used the Fisher's exact test, available on the web at http://www.pauldickman.com/teaching/excel.html.

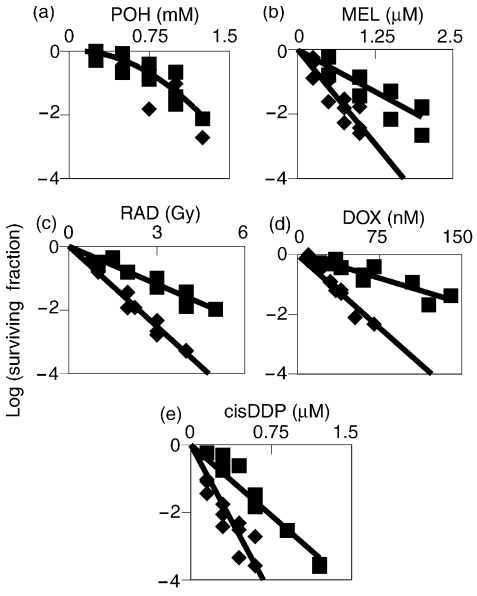

Fig. 1. Survival of IMR32 (◆) and Clone F cells (■) after exposure to: perillyl alcohol (a); melphalan (b); radiation (c); doxorubicin (d); and cisplatin (e).

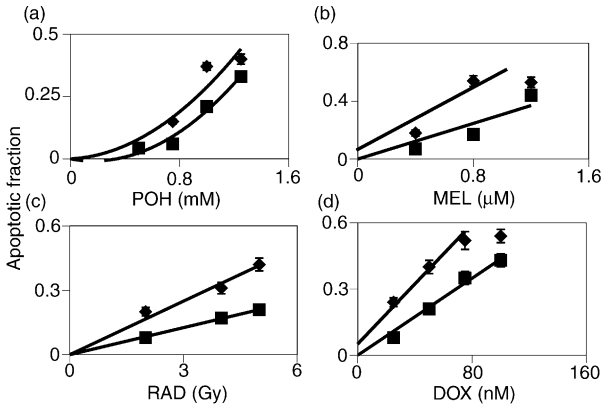

Fig. 2. Apoptosis in IMR32 (◆) and Clone F cells (■) after exposure to: perillyl alcohol (a); melphalan (b); radiation (c); and doxorubicin (d).

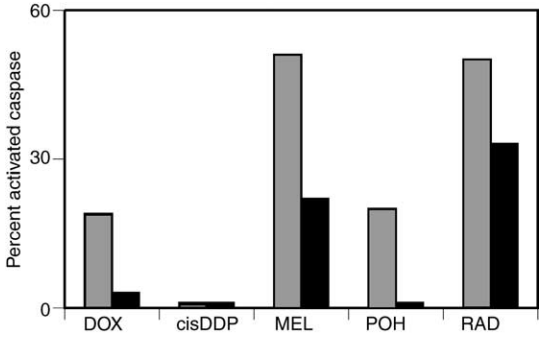

Fig. 3. Caspase activation in IMR32 (hatched) and Clone F cells (solid). The caspase-positive fraction in the untreated controls was approximately 12% for both lines, and this has been subtracted from the treated values.

3. Results

Fig. 1 shows the survival curves of IMR32 and Clone F cells after exposure to perillyl alcohol (a), melphalan (b), radiation (c), doxorubicin (d) and cisplatin (e).The response of both cell lines to the DNA damaging agents is monotonic; for perillyl alcohol there is a threshold dose below which there is little or no killing. The ID_{90} values are shown in Table 1. As before [6], the Clone F cells were significantly more resistant to radiation than the parental cells. Similarly, the Clone F cells were significantly more resistant to cisplatin, melphalan and doxorubicin. For radiation, the Clone F cells required approximately twice the dose to achieve the same level of cell kill. For cisplatin and melphalan, the Clone F cells showed a 2.2-fold gain in resistance, and for doxorubicin, a 3.1-fold increase. The cell lines were equally sensitive to clonogenic killing by perillyl alcohol.

Fig. 2 shows the apoptotic response of IMR32 and Clone F cells to perillyl alcohol (a), melphalan (b), radiation (c), and doxorubicin (d). For each agent, the Clone F cells underwent significantly less apoptosis than the IMR32 cells. The error bars represent the standard error of the mean (SEM); where error bars are absent, it is because they are smaller than the symbol. For doxorubicin, the difference between IMR32 and clone F cells is much reduced at higher doses (0.54 against 0.43, at 100 nM), and a similar pattern is seen with melphalan. However, these differences were statistically significant ($P<0.05$); the only treatment not to show a statistically significant difference between the cell lines was the lowest dose of perillyl alcohol. No apoptosis was seen in either cell line after exposure to 0.75 μM cisplatin (a dose which produced approximately two decades of clonogenic killing in IMR32 cells), even up to 72 h after treatment.

These results were mirrored in the fraction of cells showing caspase activation (Fig. 3). Except for cisplatin, all the agents tested produced a significant increase in

Table 1
ID_{90} values for IMR32 and Clone F cells

	PA (SEM) (Mm)	Cisplatin (SEM) (μM)	ADR (SEM) (μM)	MEL (SEM) (μM)	RAD (SEM) (Gy)
IMR32	0.81 (0.05)	0.34 (0.02)	0.030 (0.03)	0.43 (0.03)	1.19 (0.03)
Clone F	0.89 (0.04)	0.72 (0.04)	0.094 (0.01)	0.95 (0.08)	2.50 (0.13)

SEM, standard error of the mean; RAD, radiation; ADR, doxorubicin; MEL, melphalan; PA, perillyl alcohol; ID_{90}, concentration resulting in 90% cell death.

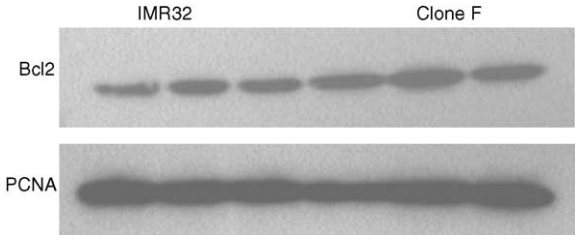

Fig. 4. Bcl2 expression in IMR32 and Clone F cells. Left three lanes: independent protein samples of IMR32 cells; right three lanes: independent protein samples of Clone F cells.

caspase activation in the IMR32 cells, and this was observed to a lesser extent in the Clone F cells.

Because other workers have reported that a high ectopic expression of Bcl-2 can reduce radiation-induced apoptosis, without affecting clonogenic survival, we examined Bcl-2 expression in these cell lines. As Fig. 4 shows, there is no evidence to suggest that IMR32 cells express more Bcl-2 than the clone F cells.

4. Discussion

Clone F cells were obtained from the parental IMR32 population after exposure to 15 fractions of 2 Gy. They were found to be less sensitive to radiation in the clonogenic assay, and less prone to radiation-induced apoptosis. We considered two interpretations of these results. The clone F cells may be less susceptible to radiation damage, perhaps through enhanced DNA repair. The decreased levels of apoptosis would simply reflect this change. Alternatively, the reduced clonogenic sensitivity of the Clone F cells may be a direct consequence of them being less prone to enter apoptosis after radiation.

Since the Clone F cells did not show greater repair of DNA double-strand breaks, and since both cell lines showed equal levels of micronucleus formation after radiation, we concluded that reduced levels of apoptosis in these cells was not a consequence of their enhanced radioresistance. Instead, we felt that our results pointed to a diminished apoptotic response as the cause of the Clone F cells' clonogenic radiation resistance. Logically, it followed that the extreme radiosensitivity of the IMR32 cells was a consequence of their susceptibility to radiation-induced apoptosis. More generally, our results suggested that radiation-induced apoptosis might explain the extreme intrinsic radiosensitivity often found in neuroblastoma cells. This work is described in Ref. [6].

The results we describe here suggest that the above explanation for the Clone F cells' radioresistance is wrong. We compared the responses of the two cell lines to doxorubicin, melphalan, cisplatin and perillyl alcohol. We expected to find that if the Clone F cells were more resistant to any agent at the clonogenic level, they would also display less apoptosis after treatment with the agent. Alternatively, if the IMR32 and Clone F cells were equally sensitive to an agent in the clonogenic assay, they would both suffer similar amounts of apoptosis after exposure to it. Our results did not accord with these expectations.

The two cell lines' response to doxorubicin and melphalan was similar to their response to radiation: Clone F cells were more resistant to the clonogenic effects of these drugs, and less prone to undergo apoptosis after treatment. However, Clone F cells were more resistant to cisplatin than IMR32 cells, even though this drug did not induce apoptosis in either cell line. Clone F and IMR32 cells were equally sensitive to perillyl alcohol in the clonogenic assay, although this agent induced significantly more apoptosis in IMR32 cells compared with Clone F.

It could be argued that the different apoptotic responses of IMR32 and clone F cells were due to differences in the rate of appearance of apoptotic cells, and that later time points might reveal the two lines to have an equal apoptotic response. We think this is unlikely. We have reported the time course for the induction of apoptosis after radiation [6]. IMR32 cells displayed significantly more apoptosis than clone F cells at all times after irradiation, up to 42 h, which was the last point measured. We expect this to be the pattern for apoptosis in response to all other DNA damaging agents, since whatever the initial damage (radiation or doxorubicin, for example), they will initiate the same molecular events that produce apoptosis.

Clone F cells' resistance to cisplatin cannot depend on reduced apoptosis since cisplatin does not cause IMR 32 cells to enter apoptosis. This result implies that the Clone F cells are better able to survive cisplatin treatment because they are better at dealing with cytotoxic lesions. If we assume that the same mechanism produces the Clone F cells' resistance to both radiation and cisplatin, then radiation resistance cannot be due to decreased apoptosis either. The alternative to this assumption is that it is a coincidence that radiation-resistant Clone F cells are also resistant to cisplatin, and it is a further coincidence that the degree of resistance to cisplatin equals the degree of resistance to radiation.

This might seem to suggest that our first interpretation was correct: That Clone F cells display less apoptosis after cytotoxic treatment simply as a consequence of better DNA repair. However this explanation is also unsatisfactory. The Clone F cells showed markedly less apoptosis in response to perillyl alcohol, even though this agent does not damage DNA. This suggests that the Clone F cells have some general resistance to undergoing apoptosis.

We believe that these results suggest the following: Clone F cells carry some mutation or epi-mutation that

has two independent effects. It makes them less likely to enter apoptosis, and it helps them survive DNA damaging agents.

We would also observe that the absolute levels of apoptosis that we observe in IMR32 cells after radiation seem too low to account for much clonogenic killing. For example, a dose of 2 Gy reduces survival to approximately 5%, although the fraction of non-apoptotic cells is 80%. The same mis-match between levels of apoptotic and clonogenic death can also be seen after melphalan and doxorubicin treatments. Therefore, it seems likely that a tendency to enter apoptosis is not the cause of IMR32 cells' extreme radiation sensitivity.

Finally, it seems unlikely that the drug-resistant phenotype that Clone F cells display would ever compromise therapy. Alkylating agents, platinum compounds and doxorubicin are all used in combination therapy for neuroblastoma, [8] and radiation is sometimes given, particularly through the radiopharmaceutical, meta-iodobenzylguanidine [7]. The Clone F cells were originally selected using ionising radiation. Since they are also resistant to melphalan, cisplatin and doxorubicin, it may seem that these agents could also be able to select for this phenotype. A Clone F-type cell would be unlikely to emerge from drug treatment, however, since all these drugs also select for other mutations that confer a greater survival advantage. There are several reports in the literature of tumour cell sub-lines being selected on the basis of enhanced resistance to doxorubicin [13], cisplatin [14] and melphalan [15]. These lines all displayed enhanced resistance well in excess of the 2-fold increase reported here.

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