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CALYCULIN A, A POTENT INHIBITOR OF PHOSPHATASES-1 AND -2A, PREVENTS APOPTOSIS

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Summary: Exposure of the Burkitt's lymphoma cell line BM13674 cells to γ-radiation or heat results in extensive DNA fragmentation and morphological changes characteristic of apoptosis. When cells were γ-irradiated in the presence of calyculin A, a potent inhibitor of the catalytic subunit of phosphatases 1 and 2A, apoptosis was prevented. This was shown to be concentration dependent with maximal inhibition occuring at 20nM. Both DNA fragmentation and the morphological features characteristic of apoptosis were prevented by incubation with calyculin A. The concentration required to inhibit apoptosis (20nM) was considerably less than that reported previously for okadaic acid (500nM), also an inhibitor of phosphatase activity as well as apoptosis. In addition, while okadaic acid caused a marked condensation of nuclear material in both control and irradiated cells, while preventing apoptosis, no such changes in chromatin were evident in the presence of calyculin A. It is clear from these data and other results with protein synthesis inhibitors that the complete machinery for apoptosis, induced under certain conditions, preexists in the cell, and phosphatase activity plays a key role in this process.

Protein phosphorylation is a major regulatory mechanism for the control of cellular metabolism, growth, and differentiation (1,2). Although the level of phosphorylation of a particular protein depends on the relative activities of protein kinases and protein phosphatases, the latter enzymes have received less attention. In eukaryotic cells,

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serine/threonine phosphatases have been grouped in two classes based on their sensitivity to inhibitors and on their *in vitro* substrate specificity (3,4). Type 1 phosphatases are sensitive to the thermostable Inhibitors-1 and -2 and selectively dephosphorylate the β-subunit of phosphorylase kinase while the phosphatase 2 group are resistant to these inhibitors and preferentially dephosphorylate the α-subunit of phosphorylase kinase (4). The type 2 phosphatases comprise three different enzymes: type 2A, like type1 shows no requirment for bivalent cations while type 2B and type 2C are Ca^{2+}/Calmodulin- and Mg^{2+}-dependent, respectively (5)

Although their precise functions are not yet known, several biological roles of protein phosphatases have been revealed in a number of recent studies (6-8). Type1 from higher eukaryotes has been extensively characterized *in vitro*, and is involved in regulating major metabolic pathways, including glycogen metabolism, muscle contraction, and protein synthesis (9, 10). It has been reported that protein phosphatases play an important role in meiosis, mitosis, and in other events coupled to the cell cycle(11-13). Anaphase separation of daughter nuclei is inhibited by antibodies against type 1 enzyme (14) and phosphatases have distinct, essential roles in the control of the fission yeast cell division cycle (7).There is evidence that phosphatases negatively regulate B-cell and T-cell activation (15-16). Phosphatase type 1 appears to be a major regulator of cAMP-responsive element binding protein (CREB) (17), and it has been suggested that these enzymes may regulate the activity of multiple transcription factors (18).

Recent results from this laboratory have provided evidence that dephosphorylation of proteins may play an important role in apoptosis (19). Furthermore, okadaic acid, a potent inhibitor of phosphatases 1 and 2A prevented apoptsis induced by heat and ionizing radiation in human tumour cell lines (19). This compound also prevented apoptosis induced by a number of cytotoxic compounds (20). Cycloheximide, an inhibitor of protein synthesis, failed to inhibit apoptosis in these cells (21). These

results suggest that modification of existing proteins may be as important as *de novo* protein synthesis during the process of apoptosis. In order to investigate this possibility further we have used calyculin A, a more effective inhibitor of phosphatase activity.

Materials and Methods

Cells and cell culture: A spontaneous Epstein-Barr virus negative Burkitt's lymphoma cell line, BM13674 was used in this study. BM13674 cells were kindly provided by Dr Brian Harmon, School of Life Science, Queensland University of Technology. Cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum at 37°C under 5% CO_2 in a humidified atmosphere. Exponentially growing cells were used for all experiments at a plating density of $1X10^6$ cells/ml. Calyculin A was added at concentrations varing from 0 - 20nM prior to exposure to 20Gy of Gamma-rays from a ^{137}Cs source (4Gy/min). Calyculin A was obtained from Calbiochem and dissolved in ethanol (4μM).

Cell viability assay: Cell viability was determined using the trypan blue dye exclusion assay (22) at a cell concentration of $1X10^6$ cells/ml.

Detection of apoptosis: Cell morphology was evaluated using fluorescence microscopy. At the end of each incubation, cells were pelleted at 1000 rpm for 5 min and resuspended in fresh RPMI-1640 medium containing 10% fetal calf serum. The cells were stained with acridine orange (5μg/ml) and observed using a Zeiss Axioskop-20 model fluorescence microscope. Extent of apoptosis was determined by the number of cells undergoing micronuclear fragmentation and condensation.

DNA extraction and electrophoresis: DNA was extracted from cells using a modified form of the procedure described by Miller et al., (23). Briefly, cells were pelleted by centrifugation at 1000 rpm for 5 min at room temperature. Cell pellets were resuspended at 2 $X10^6$ cells/ml in cell lysis buffer (10mM NaCl, 1mM EDTA, 1% SDS, 10mM Tris-HCl, pH 7.8) containing 0.5 mg/ml proteinase K. The cell lysates were digested overnight at 37°C. After completion of digestion, saturated NaCl (approximately 6M) was added and the cell lysates were shaken vigorously. Each tube was then centrifuged at 3000 rpm on a benchtop centrifuge for 15 min. The supernatant containing DNA was transferred to another polypropylene tube. An equal volume of absolute ethanol at room temperature was added and the tubes inverted several times until the DNA precipitated. The DNA precipitates were then transferred to1.5ml microcentrifuge tubes, washed with 70% ethanol three times, air-dried for 2 h at room temperature and resuspended in TE buffer (10mM Tris-HCl, 0.2mM EDTA, pH 8.0). The DNA was allowed to dissolve for 2 h at 37°C and treated with RNase A at a concentration of 0.2 mg/ml before quantitation. Approximately 10μg of DNA was loaded into each well and

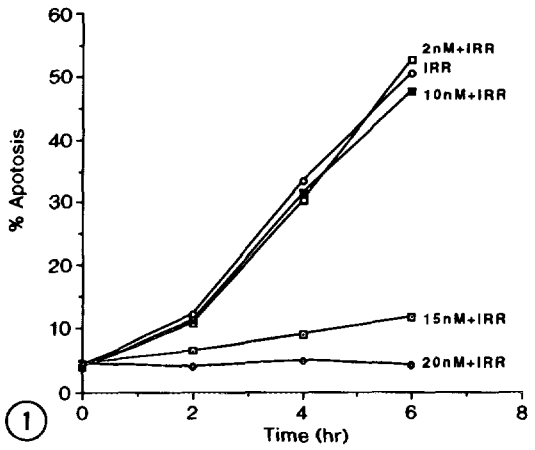
electrophoresis was carried out at 10 mA in 1.3% agarose gels with TBE buffer (89mM Tris-HCl, 89mM Boric Acid, 3mM EDTA, pH 8.0) . After electrophoresis, each gel was stained in H_2O containing 2μg/ml ethidium bromide for 10 min and destained in H_2O for 10 min. The DNA was visualized by UV illumination.

Results

Ionizing radiation has previously been shown to induce apoptosis in the Burkitt's lymphoma cell line BM13674 within a few hours of exposure (19). Calyculin A was used to investigate the possible role of phosphatase activity in this process. Over the concentration range 0 - 10nM this compound did not interfere with the appearance of apoptosis induced by ionizing radiation over a period of 6h (Fig.1). A rather dramatic inhibition of apoptosis was evident when calyculin A was increased to 15nM, and inhibition was maximal at 20nM (Fig1.) When cells were incubated with 20nM calyculin A for 1h followed by extensive washing of the cells in medium no protection against radiation-induced apoptosis was observed up to 6hr post-irradiation (results not shown).

Gel electrophoresis of DNA extracted from BM13674 cells exposed to 20 Gy of X-rays revealed a typical fragmentation ladder at 4 h post-irradiation (Fig. 2, lane 3). Incubation of cells in the presence of calyculin A (20nM) completely prevented the DNA fragmentation (lane 4). Incubation with calyculin A alone did not alter the migration pattern of the DNA (lane 2).

These results agree with those obtained previously with okadaic acid but the effective concentration of calyculin A was 25-fold less. Since we observed premature chromatin condensation in cells exposed to okadaic acid plus ionizing radiation (20), it was possible that the inhibitory effect of okadaic acid was indirect, causing the chromatin condensation and thus preventing access to an endonuclease capable of fragmenting DNA, a prerequisite for apoptosis in those cells. In order to investigate this cells were exposed to calyculin A and radiation prior to morphological examination by fluorescence microscopy. Peripheral



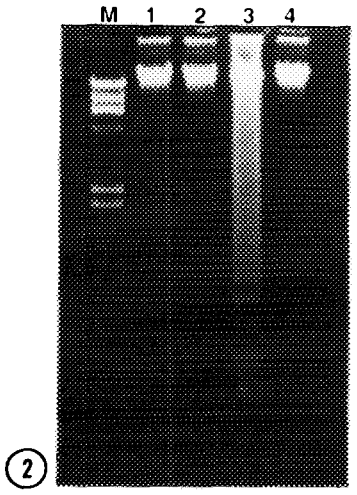


Figure 1. **Dose-response curves for inhibition of apoptosis in BM13674 cells with increasing concentration of calyculin A.** Cells were pretreated with different concentrations of calyculin A for 1h before exposure to γ-radiation. Percentage of apoptotic cells was determined by microscopic examination after 0, 2, 4 and 6h incubation. The results represent the mean ± S.D. of three separate experiments.

Figure 2. **Effect of calyculin A on γ-radiation induced DNA fragmentation in BM13674 cells.** Cells were treated with γ-radiation (20 Gy) in the presence and absence of 20nM calyculin A. DNA was extracted and electrophoresed in 1.3% agarose gels, after 4h incubation. Lane M, spp1 DNA markers; lane 1, untreated; lane 2, untreated + calyculin A; lane 3, γ-radiation; lane 4, calyculin A+ γ-radiation.

crescents of compacted chromatin, characteristic of apoptosis were observed in cells exposed to ionizing radiation (Fig 3C). None of these features were obvious when cells were incubated with calyculin A prior to irradiation (Fig. 3D). Indeed the structure of the nucleus was similar to that in untreated cells (Fig. 3A) without any evidence of chromatin condensation, as observed in the presence of okadaic acid.

Discussion

It has been established for some time that a requirement for RNA and protein synthesis exists in apoptosis (24-26). The majority of these reports have been with murine cells where only cytotoxic T cell (CTL)

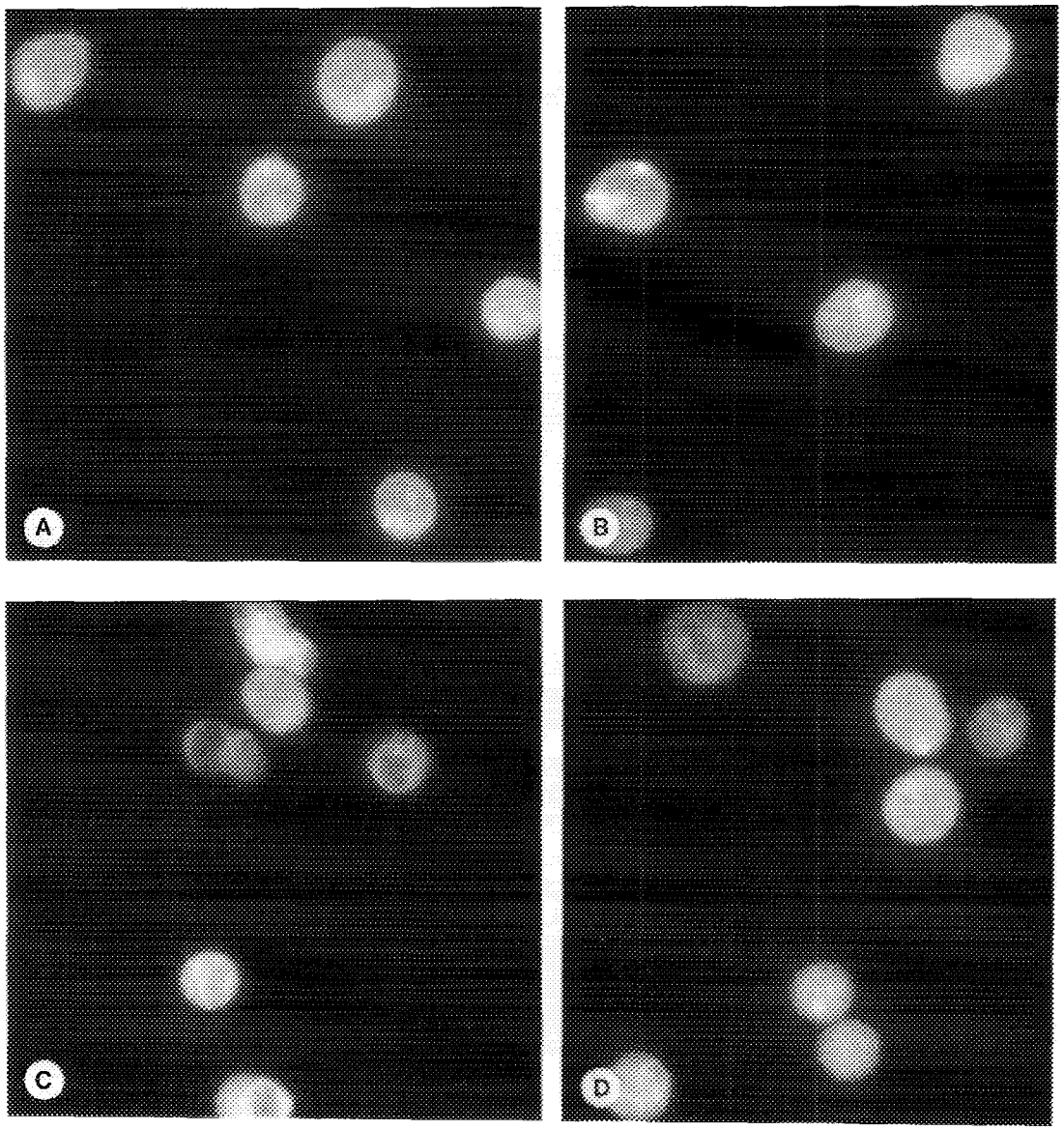


Figure 3. **Effect of calyculin A on BM13674 cells undergoing apoptosis.** Cells were pretreated with calyculin A for 1h before exposure to γ-radiation (20 Gy), stained with acridine orange (5μg/ml) after 4h incubation and observed using a Zeiss Axioskop-20 fluorescence microscope. A, untreated; B, untreated + calyculin A; C, γ-radiation; D, calyculin A + γ-radiation.

killing does not show a requirement for *de novo* synthesis (27). On the other hand in human cells there is considerable evidence that apoptosis can occur without the requirement for protein synthesis. Kelley et al

(28) showed that puromycin did not prevent DNA degradation in erythoid progenitor cells deprived of erythropoietin; diphtheria toxin-triggered DNA fragmentation and cell lysis did not require protein synthesis (29); Cycloheximide, at concentrations that inhibited protein synthesis up to 90%, did not prevent the effects of etoposide on DNA integrity (30); after deletion of all regions of the human glucocorticoid receptor except the DNA-binding domain a fragment remained capable of causing cell death (31), presumably on its ability to bind DNA only. Furthermore RNA and protein synthesis inhibitors can also cause or enhance apoptosis (21,32-34). These results suggest that different cell types under different conditions possess some or all of the factors required for apoptosis.

The results described here lend further support to the above observations. Calyculin A a specific inhibitor of phosphatases type 1 and 2A prevented apoptosis induced by ionizing radiation in the human tumour cell line BM13674. This inhibition was evident as soon as 2h post-irradiation. This result further confirms a role for phosphatase activity associated with the process of apoptosis. Dephosphorylation of a limited number of proteins has been reported in this cell type during radiation- or heat-induced apoptosis (19). Okadaic acid inhibited the dephosphorylation of 3 of these proteins and also prevented apoptosis. The high concentrations of okadaic acid (>500nM) required to prevent apoptosis caused a premature chromation condensation distinct from apoptosis (20). This condensation was also observed in human myeloid leukaemic cells after treatment with high concentrations of okadaic acid and coincided with arrest of cell proliferation (35). Failure to observe apoptosis under these conditions could be due to a physical barrier in the form of condensed chromatin, depriving an endogenous endonuclease of access to internucleosomal linkers. The results obtained here with calyculin A, also a potent phosphatase inhibitor, would seem to rule out the physical barrier explanation and point to a direct participatory role of phosphatase. Longer term exposure to these inhibitors failed to arrest the onset of apoptosis (19,20). This delayed effect could be due to a broad specificity of phosphatase activity with inhibition eventually

causing lethality. The latter explanation is supported by the rariety of phosphatase mutants for cell cycle control (36).

The higher concentration (0.5-1μM) of okadaic acid required to prevent apoptosis would suggest that phosphatase type 1 is important. Since calyculin A has similar potency for type 1 and type 2A (37), inhibition data with this compound do not discriminate between the enzymes. However, as referred to above phosphatase mutants are rare which could mean that the activities of type 1 and type 2A are functionally redundant and it may be necessary to mutate or inhibit both genes to detect a phenotype (36). Inhibition of type 1 and type 2A may be required to prevent apoptosis. Preliminary data with antisense oligonucleotides common to type 1 and 2A, support this contention (unpublished results).

In conclusion calyculin A, a potent phosphatase inhibitor, prevented the onset of radiation-induced apoptosis in BM13674 cells. These results add further support to the growing body of evidence that in some systems all of the factors necessary for apoptosis are constitutively present in the cell and phosphorylation/dephosphorylation is of central importance in this process.

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