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The Phosphatase Inhibitor Calyculin Antagonizes the Rapid Initiation of Apoptosis by Photodynamic Therapy

Yu Luo and David Kessel[1]

Department of Pharmacology, Wayne State University School of Medicine, 540 E. Canfield Street, Detroit, Michigan 48201

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DNA fragmentation and internucleosomal cleavage were rapidly initiated after lysosomal photodamage to murine leukemia cells, with apoptotic chromatin and DNA 'ladders' detected within 30 min after irradiation. Apoptosis was inhibited by concurrent exposure of cells to a 10 nM concentration of the serine-threonine phosphatase inhibitor calyculin A and promoted by a serine/threonine kinase inhibitor. These results indicate that a late stage in apoptosis requires serine/threonine dephosphorylation.

The term 'photodynamic therapy' (PDT) refers to the production of cytotoxic photodamage to cells which have exposed to photosensitizing drugs (1,2). PDT can result in a very rapid initiation of apoptosis (3–6), although this effect is dependent, in part, on the cell line and photosensitizing agent (5,6). Whether DNA fragmentation leads to only 50 kb particles or to nucleosomal multimers, yielding DNA 'ladders', depends on the site of subcellular photodamage (6). Two photosensitizing agents, the tin etiopurpurin SnET2 (7) and a porphycene dimer (8), localize in lysosomes of murine leukemia L1210 and P388 cells and catalyze lysosomal photodamage upon irradiation (9,10). The resulting photodamage leads to the rapid initiation of apoptosis, with DNA 'ladder formation detected within 30 min (6).

There have been several reports indicating that the level of protein-tyrosine (11–14) or -serine/threonine phosphorylation (15–17) can affect initiation and progression of apoptosis. In this report, we describe the inhibition of PDT-induced apoptosis by calyculin A, a selective inhibitor of serine/threonine phosphatases 1 and 2A (18). Effects of the protein kinase C inhibitor staurosporin were also assessed. We also compared the effects of PDT with those of didemnin B, a peptolide agent which has also been reported to cause a rapid initiation of apoptosis (19).

MATERIALS AND METHODS

Cells and cell culture. Murine leukemia P388 and L1210 cells were maintained in Fischer's medium (Gibco-BRL) supplemented with gentamicin, glutamine, and 10% horse serum. Cells were used during the exponential period of growth, and were suspended in growth medium with HEPES (pH 7.2) replacing $NaHCO_3$ to increase the buffering capacity.

Chemicals. Calyculin A and staurosporin were purchased from Sigma Chemical Co., St. Louis MO., didemnin B was provided by Prof. Peter Twogood, Department of Chemistry, University of Michigan. The tin etiopurpurin SnET2 was supplied by Dr. Allan Morgan, PDT Pharmaceuticals, Hollister, CA; the porphycene dimer by Dr. C.K. Chang, Department of Chemistry, Michigan State University.

Incubation and irradiation. Cells (density = 5 mg/ml wet weight) were incubated with photosensitizing agents (2 μM) for 15 min at 37°C, then collected by centrifugation and resuspended in fresh HEPES-buffered growth medium. Irradiation was carried out at 10°C to minimize temperature-dependent repair systems. The light dose was 1.5 $J.cm^{-2}$, using a bandpass of 600–700 nm. A further description of this procedure is provided in Refs. 6 and 20. After irradiation, cells were resuspended in HEPES-buffered Fischer's medium for 60 or 120 min 37°, at a density of 3×10^5 cells/ml, then collected for gel electrophoresis and fluorescence microscopic examination. Where specified, 10 or 50 nM Calyculin A, or 1 μM staurosporin was present during these incubations. Additional experiments were carried out with cells exposed to 1 μM didemnin B for 120 min at 37°C. No irradiation was involved in the latter protocol.

[1] Corresponding author. FAX:313 577 6739.

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In studies reported here, L1210 cells were used, but identical effects could be produced with the P388 cell line. Similarly, either SnET2 or the porphycene dimer yielded similar results; data for SnET2 are shown.

Viability studies. Cell viability was assessed by an MTT assay; this yields results which are well-correlated with clonogenic assay procedures (21).

Gel electrophoresis. Cell pellets were suspended in 0.5 ml of buffer containing 150 mM NaCl, 15 mM sodium citrate, 10 mM EDTA and 25 μg of Sarkosyl, mixed with 50 μl of proteinase K (1 mg/ml) and incubated at 50°C for 2 h. The mixture was cooled to 0°C and 1 ml of ethanol at −20°C added to precipitate DNA which was collected by centrifugation (12,000 × g, 20 min), dried in air and dissolved in 50 μl of 10 mM Tris 1 mM EDTA at pH 8 (TE buffer). DNA levels were quantitated by observing optical density at 280/260 nm; in all studies the ratio was > 1.5. Each DNA preparation (30 μl = approx. 10 μg) was mixed with 1 μl of RNase (10 mg/ml) and 6 μl of loading buffer (30% glycerol, 0.1% bromphenol blue) and incubated for 15 min at 37°C. Samples were then loaded onto 1.5% agarose gels and electrophoresed for approx. 3.5 h (2V/cm gradient). A series of markers (154–2165 bp) from Boehringer/Mannheim (Mol.-Wt. marker kit VI) were initially used to calibrate the electrophoresis system (6,9). Gels were stained with ethidium bromide and the DNA bands were visualized under 312 nm light.

Fluorescence microscopy. A Nikon Labophot system was used to assess the fluorescence of treated vs. control cells after staining with 5 μM HO342 5 min at 37°C (6). The excitation wavelength was 330–380 nm with emission monitored at 420–450 nm. Numbers of apoptotic nuclei per field of 100 cells were scored. Images were acquired using a Dage 68 SIT camera fitted with a digital signal processor (10).

RESULTS

Fig. 1 shows the typical DNA 'ladder' pattern 60 min after irradiation of cells previously incubated with sensitizers and given a light dose sufficient to reduce viability by 50% (lane 2). This pattern could be detected as early as 30 min after PDT. Apoptosis was abolished when a 10 nM concentration of the serine-threonine phosphatase inhibitor calyculin A was present during irradiation and the subsequent 60 min incubation (lane 4). A 60 min exposure to calyculin A alone (lane 3) did not induce endonucleosome cleavage, nor was cell viability affected.

Fluorescence microscopy of HO342-stained cells showed blue fluorescent nuclei in control cells (Fig. 2, top left). One hour after an LD_{50} PDT dose, there were approx. 40% apoptotic cells (Fig. 2, top right). Treatment with calyculin A alone yielded much more a granular chromatin (Fig. 2, bottom left). Irradiation of photosensitized cells in the presence of calyculin A resulted in many cells exhibiting a featureless chromatin staining pattern (Fig. 2, bottom right), but (see Fig. 1) no

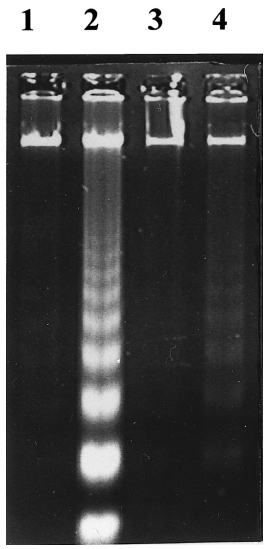

FIG. 1. Gel electrophoresis analysis of DNA from cells 60 min after specified procedures: lane 1 = control, 2 = PDT, 3 = calyculin A alone, 4 = PDT + calyculin A.

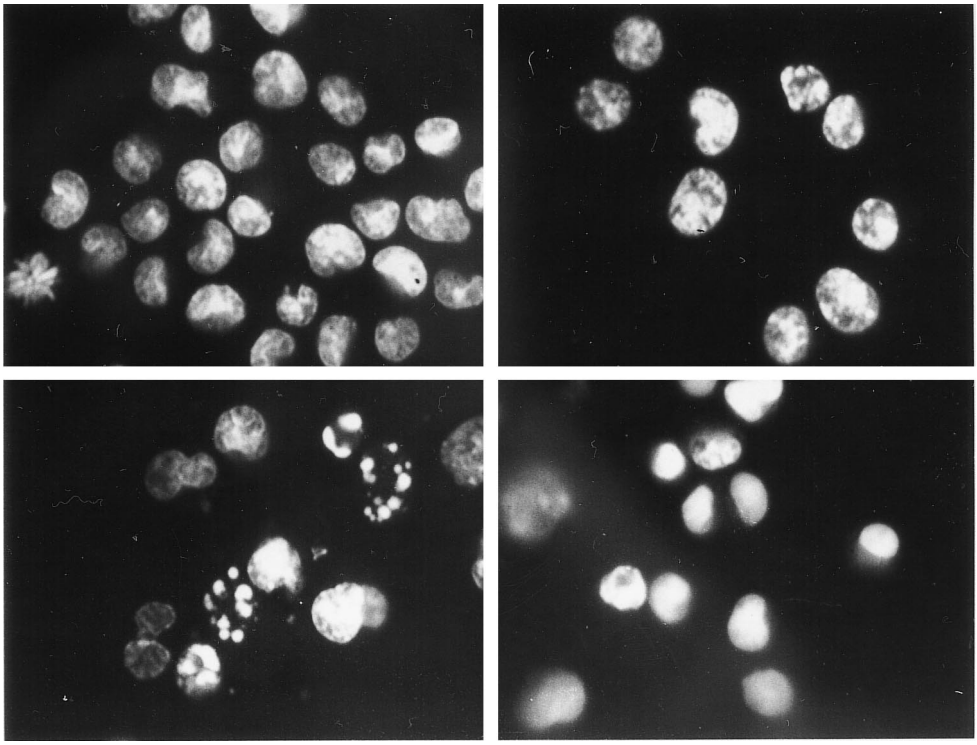

FIG. 2. Fluorescence microscopy of HO342-stained L1210 cells 60 min after specified treatment: top left = control; top right = calyculin A alone (10 nM); bottom left = PDT; bottom right = PDT + calyculin A.

DNA 'ladders'. No evidence of apoptotic chromatin fragmentation was observed over the next 24 hr (not shown).

At a 50 nM Calyculin concentration, further changes in chromatin structure were observed (Fig. 3, top left), cell viability was reduced to 50–60% of control values. Presence of the protein kinase C inhibitor staurosporin during the subsequent 60 min incubation after PDT markedly promoted PDT-induced apoptosis (Fig. 3, bottom left). In the absence of PDT, this drug concentration did not affect cell viability or initiate apoptosis if incubations did not exceed 60 min.

Didemnin B has also been reported to induce the rapid initiation of apoptosis in HL-60 cells within 140 min (19). With L1210 cells, a 2 hr exposure to 1 μM Didemnin B had only a minor effect (Fig. 3, top right). In contrast, an LD_{99} PDT dose of SnET2 led to 100% apoptotic chromatin fragmentation after 60 min (Fig. 3, bottom right).

DISCUSSION

The initiation of apoptosis by photooxidative processes is not unexpected, since other intracellular oxidative processes can have the same effect (22). We interpret the rapid appearance of apoptosis after PDT to indicate that a late stage in the process has been initiated. The common resistance of neoplastic cells to chemotherapy appears to be associated with the failure of drug therapy to initiate apoptosis (23,24). The broad spectrum of responses to PDT (1,2) may result from the ability of the latter procedure to circumvent early steps in the apoptotic process which are absent or strongly inhibited in many neoplastic cell types.

The results shown here indicate that apoptosis induced by lysosomal photodamage requires a cellular capacity for protein- serine hreonine dephosphorylation. PDT-induced apoptosis was further promoted by the action of staurosporin, an inhibitor of PKC activity. The latter finding is also

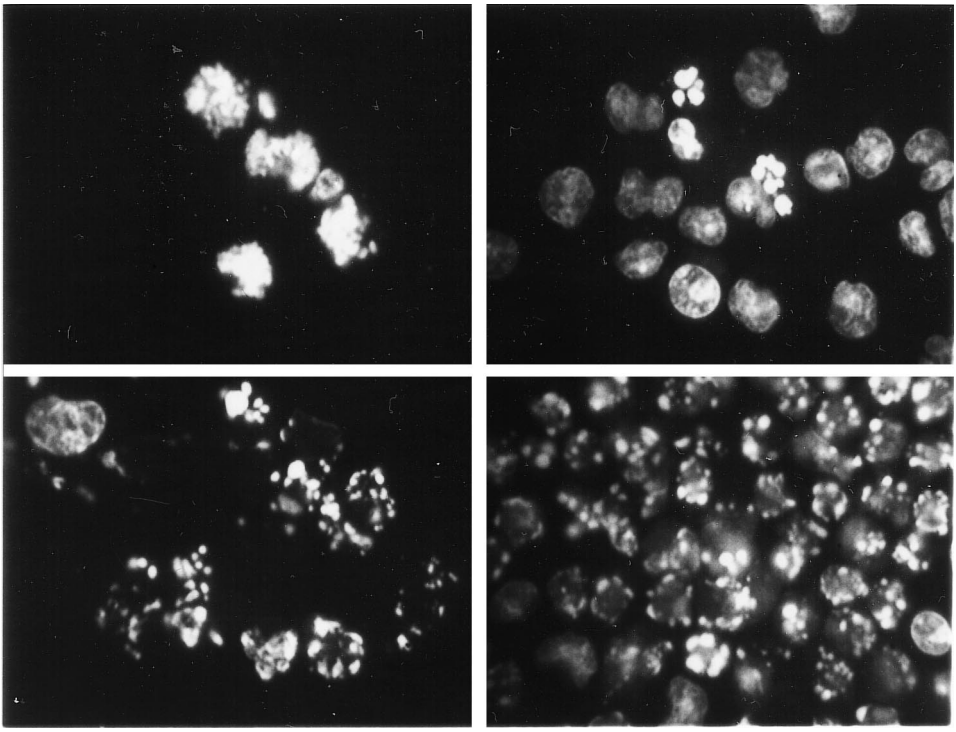

FIG. 3. Fluorescence microscopy of HO342-stained L1210 cells 60 or 120 min after specified treatment: top left = 50 nM calyculin A (60 min); top right = didemnin B (120 min); bottom left = PDT + staurosporin (60 min); bottom right = PDT (LD_{99} conditions).

consistent with the hypothesis that inhibition of serine hreonine phosphorylation promotes PDT-induced apoptosis after lysosomal photodamage. Whether this proposal will hold true with regard to other sites of photodamage remains to be established.

The requirement of serine-threonine dephosphorylation for the initiation of apoptosis initiation is not unique to the PDT-induced effect (14–18). Calyculin A was also shown to inhibit apoptosis induced by y-irradiation and heat shock (18). In order to place these observations in the proper perspective, we consider it important to recall suggestions that cell survival may depend on maintenance of an optimal level of protein phosphorylation, and that excess kinase or phosphatase activity could initiate apoptosis (13,25).

The mechanism of modulation of late stage apoptosis by protein phosphorylation remains to be established. While a 60 min exposure of cells to 10 nM calyculin did not initiate apoptosis (Fig. 1), we did observe a more granular nuclear staining pattern (Fig. 2), suggesting chromatin condensation at matrix attachment regions. At higher calyculin levels, this pattern became more distinct (Fig. 3). It is possible that the condensed chromatin could limit the access of PDT-induced endonuclease activity to internucleosomal linkers. Such a phenomenon was suggested by effects of the phosphatase inhibitor okadaic acid in another system (26). In a Burkitt's lymphoma cell line, no changes in chromatin structure were observed upon exposure to calyculin A (18), but the degree of resolution did not permit a more definitive conclusion. While exposure to staurosporin resulted in the initiation of apoptosis in many other cell lines (27), the exposure time in our experiments was too short for this to occur.

The rate of apoptosis after PDT (Figs. 1 and 2) clearly exceeds the rate reported for didemnin B (19). The latter studies were carried out with HL-60 cells which may be more responsive to didemnin B than L1210 or P388 cells. Additional studies will be required to assess the role of

protein-tyrosine phosphorylation reactions in the initiation of the late-stages of apoptosis by PDT and other procedures.

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