

Photodynamic Therapy Induces Rapid Cell Death by Apoptosis in L5178Y Mouse Lymphoma Cells¹

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Abstract

The mode of cell death of two strains of mouse lymphoma L5178Y cells was studied following photodynamic therapy (PDT) sensitized by chloroaluminum phthalocyanine. Strains LY-R and LY-S differ in their relative sensitivities to UVC radiation, X-radiation, and PDT; both responded to PDT by undergoing apoptosis. The DNA was degraded into fragments with lengths which are multiples of approximately 180–190 base pairs (*i.e.*, oligonucleosome size), a biochemical marker of apoptosis. The DNA fragmentation was dose and time dependent which indicates this response to be an enzymic process related to cell killing. Cycloheximide, a protein synthesis inhibitor, and actinomycin D, an RNA synthesis inhibitor, enhanced the endonucleolytic DNA fragmentation. Transmission electron microscopy revealed chromatin condensation around the periphery of the nucleus, which is also characteristic of apoptosis. The induction of apoptosis in L5178Y cells by PDT was rapid, with marked degradation of DNA occurring in as little as 30 min. The rapidity of the response to PDT suggests that cellular damage produced by PDT can directly activate endonucleolysis and chromatin condensation, thereby by-passing many of the early steps in the signal transduction program which are acted upon by other agents causing apoptosis.

Introduction

PDT³ is an experimental cancer treatment modality in which systemic administration of a tumor-localizing photosensitizer is followed by irradiation of the tumor with visible light (1, 2). The photochemical interaction of the sensitizer, light, and molecular oxygen produces singlet oxygen (¹O₂) and other forms of active oxygen, such as peroxide, hydroxyl radical, and/or superoxide ion (3, 4). The resultant damage to organelles within the malignant cells, as well as to stromal elements of the tumor, leads to tumor ablation (1, 2, 5). Membranous organelles, including mitochondria, plasma membrane, and lysosomes, have been suggested to be major sites of PDT damage (6). However, in no case is the mechanism of PDT-induced cell death known.

The two closely related strains of mouse lymphoma L5178Y cells differ in their relative sensitivities to a variety of toxic treatments. Strain LY-S is more sensitive to the lethal effects of ionizing radiation than is strain LY-R and is deficient in the rejoining of X-ray-induced DNA double-strand breaks, whereas strain LY-R is the more sensitive of the two strains to UVC radiation and is deficient in the repair of UVC-induced pyrimidine dimers (*e.g.*, 7–9). Strain LY-R is also more sensitive to

PDT sensitized either by Photofrin, a porphyrin mixture used in clinical trials of PDT, or AlPcCl, one of a class of potential second generation photosensitizers (9, 10). Previous studies in this laboratory revealed that the differential response of strains LY-R and LY-S to PDT was associated with a greater formation of DNA-protein cross-links in the case of strain LY-R and a more rapid and extensive degradation of DNA in strain LY-R (11).

Here we report that cells of both strains LY-R and LY-S respond to PDT by inducing apoptosis (12): the chromatin becomes condensed around the periphery of the nucleus and the DNA is degraded into nucleosome-size fragments apparently by activation of a constitutive endonuclease. In contrast to many other agents and treatments (*e.g.*, glucocorticoids and antibodies to CD3/T-cell receptor complex) which induce apoptosis over a period of several hours or days (13–15), the induction by PDT in L5178Y cells is rapid, which suggests that PDT is affecting a late step in the pathway of apoptosis.

Materials and Methods

Cell Culture. Mouse lymphoma L5178Y cells, strains LY-R and LY-S, were isolated and named based on their relative sensitivity to X-radiation and were subsequently shown to be inversely cross-sensitive to UVC radiation and to PDT (7, 9, 11). The cells were grown in suspension culture in Fischer's medium containing 0.1% Pluronic F68 (BASF Wyandotte, Parsippany, NJ), 2 mM sodium pyruvate, and 10% heat-inactivated horse serum.

Photodynamic Treatment. AlPcCl was purchased from Eastman Kodak and recrystallized from α -chloronaphthalene in the laboratory of M. E. Kenney, Case Western Reserve University, Cleveland, OH. Cells were treated with 1 μ M AlPcCl by addition of aliquots of a 1 mM stock solution in dimethylformamide to the culture medium 15 h prior to exposure of the cultures to the indicated fluences of red light. The light source was a 500-W tungsten-halogen lamp located approximately 29 inches below the surface of a glass exposure tray. The light was filtered (Lee primary red filter No. 106; Vincent Lighting, Cleveland, OH) to remove light with wavelengths below 600 nm.

DNA Isolation and Gel Electrophoresis. Immediately after irradiation, the cells were collected by centrifugation and resuspended in fresh medium. After incubation for various periods at 37°C, total DNA was isolated from 5×10^6 cells for each treatment. The cells were washed in phosphate-buffered saline, and the cell pellet was resuspended in 1 ml 0.15 M sodium chloride-0.015 M sodium citrate, pH 7.0/10 mM EDTA containing 1% (w/v) sodium lauryl sarkosinate and 0.5 mg/ml proteinase K. Proteolytic digestion was allowed to proceed at 50°C for 2 h. The DNA was precipitated with 2 volumes of absolute ethanol, resuspended in 10 mM Tris-Cl-1 mM EDTA buffer (pH 8.0), heated to 70°C, and loaded onto a 1.5% agarose gel containing 0.1 mg/ml ethidium bromide. Electrophoresis was carried out in 40 mM Tris-acetate-1 mM EDTA, pH 8.0, until the marker dye had migrated 4–5 cm. DNA was visualized under UV light.

Electron Microscopy. LY-R and LY-S cells were exposed to LD₉₀ doses of PDT (1 μ M AlPcCl plus 10 and 15 kJ/m² of red light, respectively) or not treated, as described above. One h after light exposure, PDT-treated cells and controls were recovered from the

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³ The abbreviations used are: PDT, photodynamic therapy; AlPcCl, chloroaluminum phthalocyanine; LD₉₀, dose lethal to 90% of cells.

suspension cultures and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde. The fixed cells were embedded in Polybed 812, sectioned, stained with uranyl acetate and lead citrate, and observed in a JEOL 100CX transmission electron microscope.

Results and Discussion

In order to investigate the mechanism of the molecular response of L5178Y cells to PDT, cultures of strains LY-R and LY-S were loaded with 1 μM AIPcCl and then exposed to graded fluences of red light to produce 10, 50, 90, or 95% cell killing, as determined by a clonogenic assay. Two h posttreatment, the DNA was extracted and subjected to electrophoresis in 1.5% agarose gels. As shown in Fig. 1, two h after LD₉₀ and LD₉₅ doses of PDT (10 and 15 kJ/m² for strain LY-R and 15 and 18 kJ/m² for strain LY-S), there was extensive DNA fragmentation. The degraded DNA was found in the gel as a ladder of fragments with lengths which are multiples of approximately 180–190 base pairs, *i.e.*, oligonucleosome size. Similar results were obtained for PDT sensitized by Photofrin (data not shown). Such patterns of fragmented DNA are diagnostic of apoptosis (12, 13).

The chromatin condensation characteristic of apoptotic cells was clearly shown by transmission electron microscopy (Fig. 2). Untreated cells of both strains had a similar appearance, with ruffled plasma membranes and normal appearing mitochondria, Golgi, nuclei, and cytoplasmic vacuoles. One h after a LD₉₀ dose to each strain, the majority of cells displayed relaxation of the plasma membrane, which resulted in a rounding of the cells, and extensive swelling and fragmentation of cytoplasmic organelles, in particular, mitochondria. Many of the cells had lost all definition of structure in most or all of the cytoplasm. While the nuclear membrane appeared intact, there

Strain	LY-S						LY-R					
AIPcCl	-	+	+	+	+	+	-	+	+	+	+	+
kJ/m ²	0	0	6	10	15	18	0	0	3	6	10	15

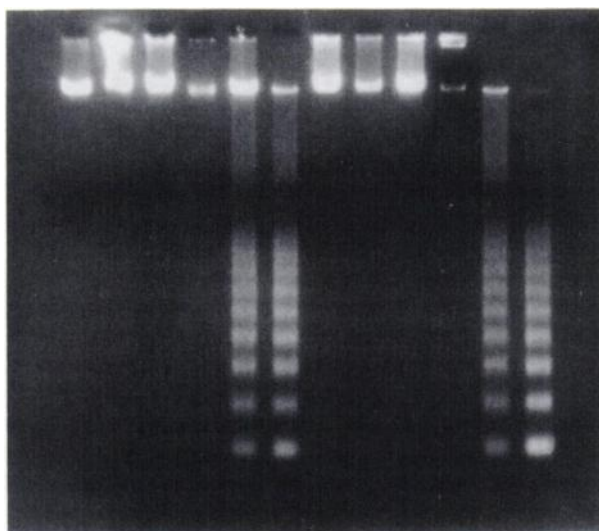


Fig. 1. PDT dose response for the nucleosomal DNA fragmentation in two cell strains of mouse leukemic lymphoblasts, L5178Y-S and L5178Y-R. Cultures were given 1 μM AIPcCl 15 h prior to exposure to various fluences of red light. Two h after PDT, total DNA was isolated and analyzed electrophoretically on a 1.5% agarose gel. DNA from PDT-treated cells shows a clear ladder of oligonucleosome size DNA bands. This pattern of DNA fragmentation is characteristic of apoptosis.

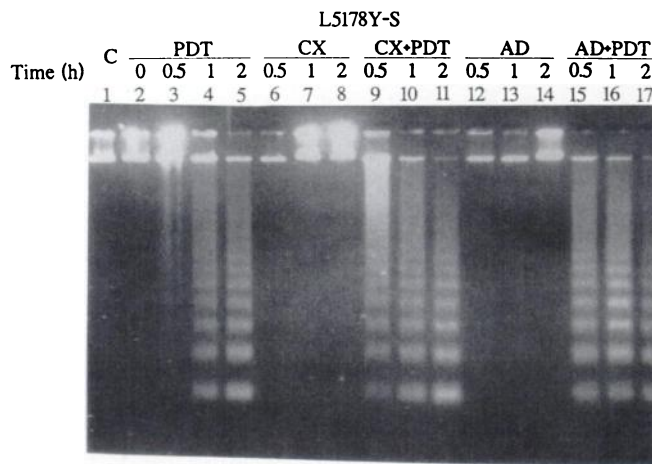


Fig. 3. Kinetics of the PDT-induced fragmentation of DNA and the effect of cycloheximide and actinomycin D on the DNA fragmentation. LY-S cultures were treated with a LD₉₀ dose of PDT (1 μM AIPcCl plus 15 kJ/m²), with 5 $\mu\text{g}/\text{ml}$ cycloheximide (CX), with 10 $\mu\text{g}/\text{ml}$ actinomycin D (AD), or with combinations of these agents as indicated. When present in combination with PDT, the antibiotics were given 30 min before red light irradiation and were left in the culture until the cells were harvested. A similar response was observed for LY-R cells exposed to 1 μM AIPcCl + 10 kJ/m². C, control untreated culture.

were regions of swelling of the space between the inner and outer nuclear membranes, and masses of condensed chromatin were prominent features around the nuclear periphery.

The data of Figs. 1 and 2 indicate that the signs of apoptosis appear early after PDT. A partial time course for DNA fragmentation in strain LY-S is shown in Fig. 3 (Lanes 2–5). DNA fragmentation was visible in as little as 30 min after a LD₉₀ dose, and there was increased fragmentation of DNA at 1 and 2 h post-PDT. The increase in fragmentation with time indicates that this is an enzymic process and not a direct consequence of photochemical damage to DNA. The morphological changes in the cells are also time dependent (data not shown). Further evidence for the involvement of an endonuclease in the DNA fragmentation is provided by the observation that the process is inhibited by post-irradiation incubation at 0–4°C (data not shown).

The activation of apoptosis in other systems, *e.g.*, lymphocytes treated with γ -radiation, is blocked in the presence of inhibitors of protein or RNA synthesis (16), providing evidence for the induction of the endonuclease or other proteins needed for activation of the endonuclease. In L5178Y cells, the protein synthesis inhibitor cycloheximide alone caused a low level of DNA fragmentation which is barely detectable in Fig. 3 (Lanes 6–8); more extensive fragmentation was observed after treatment of the cells with 5 $\mu\text{g}/\text{ml}$ cycloheximide for 2–4 h (data not shown). In the presence of cycloheximide, the response to PDT was accelerated (Fig. 3, Lanes 9–11). The RNA synthesis inhibitor actinomycin D also accelerated the PDT-induced fragmentation of DNA (Lanes 15–17), although no independent effect of the inhibitor was apparent within the time course shown (Lanes 12–14).

From the rapid appearance of DNA fragments, it is likely that PDT is affecting a late step in the sequence of events leading to activation of an endogenous endonuclease and scission of the DNA in the internucleosomal linker region. PDT is known to produce ion imbalances and membrane depolarization (17), and the endonuclease responsible for DNA fragmentation in apoptosis is Ca²⁺ dependent in some cell systems (18, 19). PDT induces elevation of intracellular free Ca²⁺ in Chinese

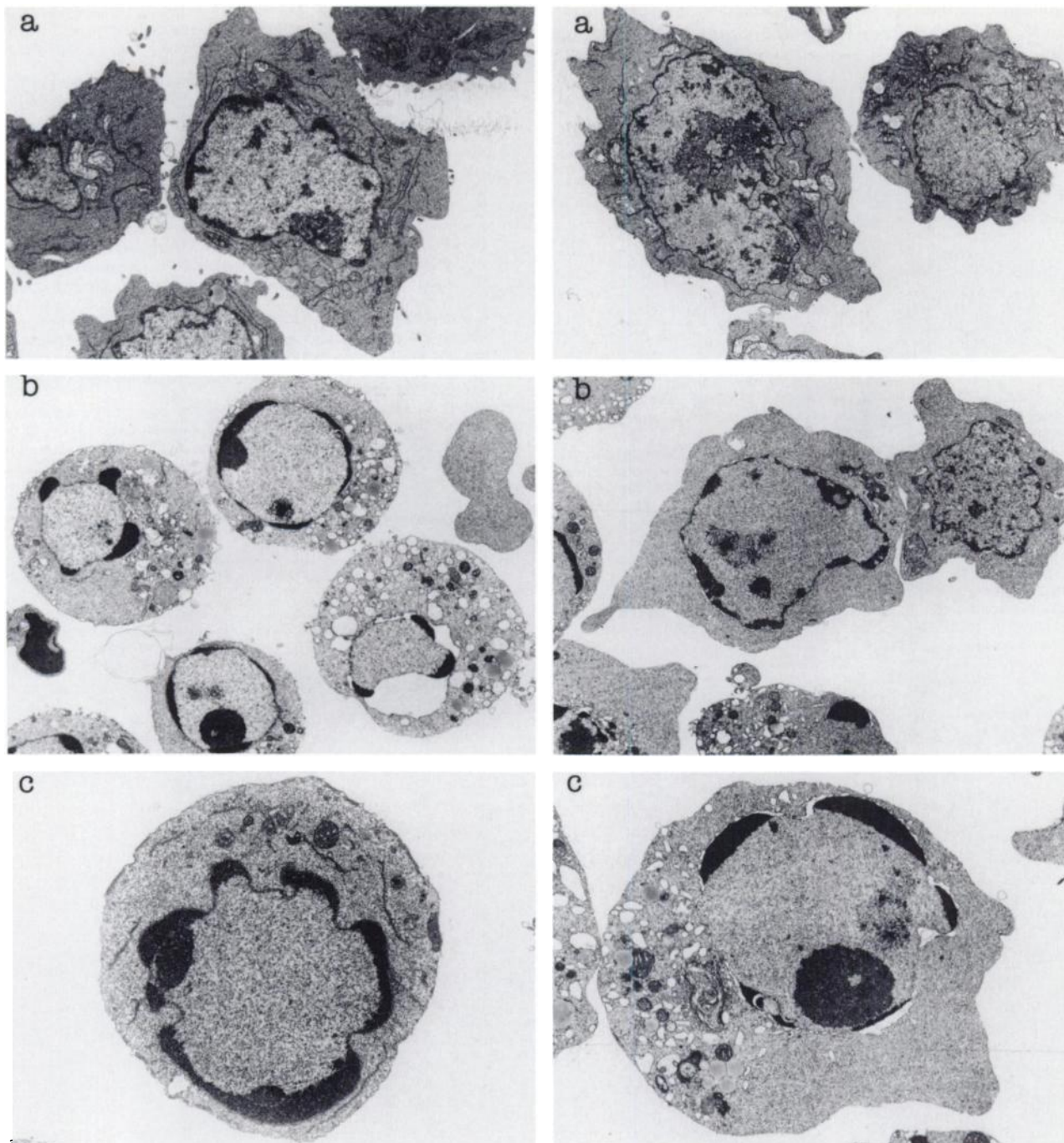


Fig. 2. Morphological changes in PDT-treated L5178Y cells. *Left*, LY-R cells; *right*, LY-S cells. *a*, control untreated cells, $\times 9000$; *b*, PDT-treated cells, $\times 9000$; *c*, PDT-treated cells, $\times 30,000$. Chromatin condensation at the nuclear periphery is prominent in the apoptotic cells. Extensive damage to cytoplasmic structures has also occurred.

hamster cells (20). However, we did not observe endonucleolytic fragmentation of DNA when L5178Y cells were treated with the Ca^{2+} ionophore A23187 (data not shown), which suggests that simple elevation of intracellular Ca^{2+} is insufficient to explain the action of PDT. Additional factors may result from the extensive damage to cytoplasmic organelles, especially mitochondria. Recently, Hockenbery *et al.* proposed that the *bcl-2* gene product, a protein of the inner membrane of the mitochondrion, can suppress apoptosis in B-lymphocytes (21). If this or an analogous protein is expressed in L5178Y cells, which are derived from lymphoblasts of the T lineage, perhaps

PDT-induced damage to mitochondria serves to limit the ability of such a suppressor to block apoptosis. The independent activation of endonucleolysis by cycloheximide and the acceleration of the PDT-induced DNA fragmentation in the presence of cycloheximide or actinomycin D suggest the possibility that control over apoptosis in L5178Y cells is exerted by one or more protein(s) with short half-lives. The identification of these putative regulators should aid in the elucidation of the mechanism of apoptosis.

The results reported herein describe the induction of apoptosis by direct PDT damage to the killed cells. Since the tumor

vasculature is thought to be an important target of PDT *in vivo* (1, 2, 5), it will be necessary to ascertain whether vascular damage can also lead to apoptosis. It may be that arachidonic acid metabolites (22) or other products of PDT damage to host cells can trigger apoptosis in the malignant cells.

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