Protease Activation and Cleavage of Poly(ADP-ribose) Polymerase: An Integral Part of Apoptosis in Response to Photodynamic Treatment

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ABSTRACT

Apoptosis induced by numerous cancer chemotherapeutic and other toxic agents has been shown to proceed through a cascade of proteases, now termed caspases, culminating in cleavage of a set of proteins. The ability of photodynamic treatment (PDT) with the phthalocyanine Pc 4 to activate cellular caspases has been assessed during the rapid apoptosis in murine lymphoma L5178Y-R cells. Cells were exposed to combinations of Pc 4 and activating red light that result in ≥90% cell death, as judged by a clonogenic assay. The rate of entry of cells into apoptosis was dose dependent. For 0.5 μM Pc 4 and either 2.1 or 3 kJ/m², which kill 90% of the cells, oligonucleosomal fragmentation was visible on agarose gels as early as 60 or 30 min after PDT, respectively. To assess caspase activation, cells were harvested at various times after PDT, and cell proteins were subjected to electrophoresis and Western blot analysis, using an antibody to poly(ADP-ribose) polymerase (PARP). The cleavage of the normally M₆, 116,000 PARP into fragments of M₆, ~90,000 was observed at approximately the same time as the earliest DNA fragmentation. An antibody to the polymer, poly(ADP-ribose), did not recognize the M₆, ~90,000 PARP cleavage products, in contrast to the parent enzyme. This analysis also revealed that levels of a poly(ADP-ribosylated) U, reaction, as measured in cell lysates with the fluorogenic substrate DEVD-AMC, was elevated almost immediately after PDT. The cell-permeable, irreversible caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(OMethyl)-fluoro-methylketone, inhibited PDT-induced apoptosis and PARP cleavage, whereas the inactive peptide analogue, benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone, was without effect. The results indicate that PDT-induced apoptosis is mediated by activation of caspase-3 and/or other similar caspases.

INTRODUCTION

Photodynamic therapy, a newly approved cancer treatment, uses a photosensitizer and visible light to produce singlet oxygen and other reactive oxygen species (1, 2), leading to lipid peroxidation and damage to membranes, DNA, cytoskeleton, and other sites (3), and eventual cell death and tumor ablation (4). PDT of mammalian cells in culture induces programmed cell death or apoptosis in some cell lines (5–8). In mouse L5178Y-R lymphoma cells, PDT induces rapid cell death by apoptosis that appears to be dependent upon the activation of phospholipases C and A₂ but not upon new gene expression (9). Evidence for the importance of protein serine/threonine phosphatases and protein kinase C in the progress of apoptosis in HL-60 cells has been obtained with the aid of the inhibitors calyculin and staurosporine, respectively (10).

Apoptosis is a programmed event characterized by chromatin condensation, cell membrane blebbing, and DNA cleavage (11–13), which are the most commonly used indicators of apoptosis. The endonucleolytic cleavage of DNA proceeds in two phases, first generating large (50–300 kb) chromosomal fragments and subsequently, in many but not all situations, oligonucleosome-sized (multiples of 180 bp) fragments.

In recent years, it has become apparent that a cascade of proteases with properties resembling those of ICE is also activated during apoptosis (14–17). Some of the earliest indications of the regulation of cell death by proteases were described for CTLs and natural killer cells, which induce apoptosis upon contact of their cytotoxic granules with target cells. The proteins that are released from the granules and are responsible for the induction of cell death are perforin as well as a series of serine proteases, including granzyme B/fragmentin-2, which cleaves its substrates at Asp residues (18). Further evidence of the involvement of proteases in the induction of programmed cell death came from studies of the nematode Caenorhabditis elegans, in which the cell death protein Ced-3 was recognized to be a protease with cysteine at its active site; Ced-3 is a homologue of ICE (19), having 29% overall sequence identity, 43% in the most highly conserved region, and complete identity in the pentapeptide QACRG that contains the active site cysteine (20). A family of ICE-like cysteine proteases has been described in mammalian cells based on the varying degrees of sequence homology among the family members and on their preferred cleavage sites (16, 17). All members of this family of proteases have in common an essential cysteine residue at the active site and an absolute requirement for aspartic acid at the substrate cleavage site. A systematic nomenclature, as proposed by Alnemri et al. (21), established the term caspases for cysteine proteases acting on aspartic acid, and the properties of the caspases and their known substrates have been reviewed recently (17). Among the caspases are: Net12-Flc-1/caspase-2 (22, 23); CPP32/YAMA/Apopain/caspase 3 (24, 25); TX/Ich-2/caspase-4 (26, 27); and Mch-2/caspase-6 (28), Mch3/ICE-LAP3/caspase-7 (29, 30), Mch4/caspase-10 (31), Mch5/FLICE/caspase-8 (31, 32), and Mch6/ICE-LAP6/caspase-9 (33, 34). Overexpression of the caspases in cultured cells has been shown to induce apoptosis, and it is inferred that physiological levels participate in apoptosis after appropriate stimuli. Among the most commonly observed final substrates that become cleaved during apoptosis are a set of nuclear proteins, including lamin B1 (35, 36), topoisomerases I and II and histone H1 (37, 38), PARP (14, 39), and others (17). The cleavage of one or more of these proteins is an important hallmark of apoptosis.

During apoptosis, PARP most often undergoes proteolytic cleavage between Asp 216 and Gly 217. This reaction cleaves the M₆, 116,000 native enzyme into a M₆, ~90,000 fragment, which contains the COOH-terminal catalytic domain, and a M₆, ~26,000 fragment, which contains a truncated NH₂-terminal DNA-binding domain (14, 39). Asp 216 is the preferred cleavage site for caspase-3 and other closely related proteases (14, 17, 20).

When apoptosis is induced by PDT, hallmarks such as chromatin

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1 These abbreviations used are: PDT, photodynamic treatment; PARP, poly(ADP-ribose) polymerase; ICE, interleukin-1β converting enzyme; TdT, terminal deoxynucleotidyl transferase; AMC, 7-amino-4-methyl-coumarin; caspase, cysteine-dependent aspartate-directed protease; zFA-fmk, benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMethyl)-fluoromethylketone.
condensation (5), DNA fragmentation (5–8), and inhibition by Bcl-2 (40) have been shown to be components of the process. Until recently, caspase activation was not characterized during the rapid apoptosis produced by PDT. After submission of this report, caspase activation and PARP cleavage were reported in HL60 cells treated with PDT sensitized by benzoporphyrin derivative (41), and PARP cleavage was briefly demonstrated in PDT-treated L1210 and P388 cells with aluminum phthalocyanine (42). The purpose of the present study was to determine the ability of PDT to activate caspases, as assessed by PARP cleavage, and to better define the relationship to the extent and timing of PDT-induced cell death.

**MATERIALS AND METHODS**

**Photosensitizer, Protease Inhibitors, and Substrate.** The phthalocyanine photosensitizer Pc 4 (43) was provided by Drs. Ying-syi Li and Malcolm E. Kenney, Department of Chemistry, Case Western Reserve University, Cleveland, OH. Stock solutions (0.5 mM in dimethyl formamide) were stored at 0–4°C. zVAD-fmk and zFA-fmk were purchased from Enzyme Systems Products (Dublin, CA). Stock solutions (50 mM in DMSO) were stored at −70°C. DEVD-AMC was from BIOMOL Research Laboratory, Inc. (Plymouth Meeting, PA). A stock solution (10 mM in DMSO) was stored at −20°C.

**Cell Culture.** Mouse lymphoma L5178Y-R (LY-R) cells are designated R (45). LY-R cells were grown in suspension culture in Fischer’s medium containing 1.5% agarose gel. After electrophoresis, the gel was stained with 0.5 µg/ml ethidium bromide and photographed under UV light.

**Materials and Methods**

**Flow Cytometry.** Cell fixation and staining were performed based on the APO-DIRECT kit (Phoenix Flow Systems, Inc.; Ref. 48). In brief, cells were fixed in 1% paraformaldehyde for 15 min and stained in 70% ethanol at −20°C overnight. Fixed cells were then washed with PBS, resuspended in 50 µl of TdT reaction buffer containing: 10 µl of 5X concentrated buffer solution (1 M cacodylic acid, 125 mM Tris-HCl (pH 6.6), and 1.26 mg/ml BSA), 2.5 mM cobalt chloride, 6 units TdT, and 0.25 mM FITC-dUTP and incubated at 37°C for 1 h. The cells were rinsed twice and incubated in 1 ml of a solution containing 2.5 µg/ml propidium iodide and 0.1% DNase-free RNase A in the dark at room temperature for 30 min. Analysis was carried out in the Flow Cytometry Facility of the Case Western Reserve University/Ireland Cancer Center. Fluorescence measurements were made on an EPICS ESP flow cytometer (Coulter Corp.); fluorescence was activated at 488 nm, and fluorescence emission was monitored at 520 nm (fluorescein) and 623 nm (propidium iodide).

**Fluorescence Microscopy.** The cell pellets from control or PDT-treated cultures were resuspended at 1×10^7 cells/ml in fresh medium, and a 25-µl aliquot was mixed with 1 µl of a solution of acridine orange and ethidium bromide (100 µg/ml). The mixture was immediately placed on the stage of an Olympus fluorescence microscope. The samples were excited at 330–380 nm, and the image was observed and photographed under a combination of a 400-nm dichroic mirror and the 420-nm high-pass filter. Live, apoptotic, and necrotic cells were scored under the microscope. Cells with a green, condensed and the image was observed and photographed under a combination of a 400-nm long-pass filter. The fluence rate was 75 mW/m². Unless otherwise indicated, irradiations were performed at room temperature. The temperature during irradiation did not exceed 34°C.

**Gel Electrophoresis of Proteins and Western Blot Analysis.** Following PDT, cultures were returned to the 37°C incubator until harvest. Cells were lysed and sonicated in a solution comprised of 0.5% sodium deoxycholate, 0.2% SDS, 1% Triton X-100, 5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride in PBS. An equal volume of SDS sample buffer [50 mM Tris (pH 6.8), 1% SDS, 1% mercaptoethanol, 0.1 mM DTT, and 5% sucrrose] was added to the cell lysate. Equivalent amounts of protein were loaded onto an 8% polyacrylamide gel, subjected to electrophoresis, transferred to a polyvinylidene difluoride membrane, and incubated with either antibody 4C10-5 (45), anti-poly(ADP-ribose)-oic, or anti-topoisomerase I antibody overnight at 4°C. The immune complexes were detected by the ECL system (Amersham). The images of the Western blots were scanned, and the intensity of each band was determined using SigmaGel (Jandel Scientific, San Rafael, CA). In most cases, gels were also probed with anti-actin to correct for unequal loading of protein. Because the 4C10-5 antibody recognizes the M, 116,000 native PARP band and the M, ~90,000 PARP cleavage fragments, the percentage of the total PARP in each lane that is present as fragments was calculated as follows:

\[
\text{% PARP cleaved} = \frac{\text{Intensity of M, 90,000 PARP bands}}{\text{Intensity of M, 116,000 PARP bands}} \times 100
\]

The intensity of the bands for the native and cleaved PARP deviated from a linear dependence on amount of protein loaded. However, in test samples, the % PARP cleaved was independent of protein loading for control (0% cleaved) and fully cleaved (100%) samples and fluctuated <15% about the average value over a 10-fold range of protein loaded for a partially cleaved sample. Therefore, no further correction was made for nonlinear band intensity.

**RESULTS**

**Fig. 1a** presents a Western blot analysis of the time course of PARP cleavage after each of three doses of PDT. Cells not treated with PDT revealed only a single protein band reactive with the anti-PARP antibody at M, 116,000, the expected size of unmodified, intact PARP. When cells were treated with 0.5 µM Pc 4 and 2.1 kJ/m² of red light, a PDT dose resulting in 90% loss of colony-forming ability (LD90).
of PDT dose to LY-R cells. LY-R cells were exposed to 0.5 μM Pc 4 and either 2.1, 3, or 10 kJ/m² of red light. At the indicated times thereafter, cells were collected, subjected to SDS-PAGE, transferred, and reacted with the 4C10-5 antibody. A representative immunoblot analysis of PARP cleavage. C, untreated control cells. *h*, quantitation of PARP cleavage. The relative intensity of each band in experiments such as a was measured using Sigma Gel (Jandel Scientific), and the percentage of PARP cleaved was calculated as described in “Materials and Methods.” Data represent the means from at least three independent experiments, except for data from cells treated with 10 kJ/m², which were from a single experiment; bars, SE.

Ref. 49), a new immunoreactive protein band migrating at M₉ ~90,000 was visible by 1 h after PDT, and the cleavage of PARP was nearly complete by 4 h. Treatment of cells with 0.5 μM Pc 4 and 3 kJ/m² of red light, a PDT dose resulting in 99.9% loss of colony-forming ability, revealed a faster time course of PARP cleavage, with the cleavage fragments visible by 30 min and all PARP appearing in the cleaved form by 1 h. After an even higher dose, 0.5 μM Pc 4 and 10 kJ/m² of red light, which results in more than a four-log loss of colony-forming ability, PARP cleavage was essentially complete by 10 min after PDT. The marked reduction in anti-PARP-reactive material in the last two lanes of Fig. 1a is due to underloading of the lanes, because cell destruction was extensive and protein recovery was poor after the highly supralethal dose.

The percentage of PARP in the combined cleaved form has been estimated by densitometry, and the data from several experiments are combined in Fig. 1b. Because the density of the bands was not linearly related to the amount of protein throughout the entire range, the data are most useful for determining the time period over which PARP cleavage occurs. On average, approximately 50% cleavage of PARP required 80, 30, or <10 min after PDT with 2.1, 3, or 10 kJ/m² red light, respectively. A comparison of the time course of PARP cleavage to that of other hallmarks of apoptosis was made after a single PDT dose (0.5 μM Pc 4 and 3 kJ/m²; Fig. 2).

In LY-R cells, PDT induces apoptosis that is monitored by oligonucleosomal DNA fragmentation, observable as DNA “ladders” upon static-field agarose gel electrophoresis (Ref. 5; Fig. 2a). Quantification of the number of cells in apoptosis was accomplished by flow cytometry after marking cells with FITC-labeled dUTP catalyzed by TdT. To compare the kinetics of PARP cleavage (Fig. 2b), oligonucleosomal DNA fragmentation (Fig. 2a), and the appearance of FITC-positive apoptotic cells (Fig. 2c), cultures were treated with PDT, and aliquots were taken at various times after treatment were analyzed for all three features. In the examples shown in Fig. 2, the earliest evidence of increases in each of the three measures was at 20 min after PDT. By 45 min after PDT, PARP cleavage was complete, whereas only 30% of the cells appeared to be in apoptosis, as estimated by flow cytometry, and much of the cellular DNA remained too large to enter the gel. Although the progress of apoptosis in PDT-treated LY-R cells is rapid, an increase in caspase activity appears to occur prior to endonuclease activity.

To investigate the role of the caspase(s) responsible for the cleavage of PARP during PDT-induced apoptosis, we first directly assayed for caspase-3 activity by incubating cell lysates with DEVD-AMC and measuring the release of AMC. As shown in Fig. 3, peptide cleavage activity was increased above control levels as early as 10 min and reached a maximum level by 45 min after PDT. We then exposed the cells to PDT in the presence of zVAD-fmk, a cell-permeable peptide inhibitor of ICE-like proteases, or zFA-fmk, an inactive peptide analogue (50). These experiments were carried out in 96-well plates (Fig. 4a). PARP cleavage started at ~30 min and was complete at 60 min. No cleavage of PARP was found at any time up to 4 h after PDT when 200 μM zVAD-fmk was present, whereas the inhibitor did not affect the size of PARP in the absence of PDT. As shown in Fig. 4b, zVAD-fmk was less effective in inhibiting PARP cleavage at concentrations of 50 or 100 μM, and zFA-fmk was ineffective at all concentrations between 50 and 200 μM. Following a lower dose of PDT (0.5 μM Pc 4 and 2.1 kJ/m² of red light), PARP cleavage was partial by 2 h and nearly complete by 4 h; in this case as well, 200 μM zVAD-fmk provided complete inhibition of PARP cleavage (data not shown).

The above results indicate that lethal doses of PDT activate proteases capable of the specific cleavage of PARP in LY-R cells. Because zVAD-fmk inhibits the cleavage, we next asked whether zVAD-fmk prevented PDT-induced apoptosis. The extent of apoptosis was estimated by fluorescence microscopy of acridine orange- and ethidium bromide-stained cells. Representative fields are shown in Fig. 5a, and quantification of live, apoptotic, and necrotic cells is recorded in Fig. 5b. No apoptotic cells were found in any of the cultures that were not exposed to PDT, although in cultures treated with zFA-fmk alone, about 18% of the cells appeared to be necrotic (ethidium bromide positive but lacking condensed chromatin). After PDT, ~20% of the cells were scored as necrotic, and this percentage was unaffected by either zVAD-fmk or zFA-fmk. The majority of the PDT-treated cells (65%) were morphologically apoptotic, and the presence of 200 μM zVAD-fmk, but not 200 μM zFA-fmk, prevented the appearance of apoptotic cells.
PARP CLEAVAGE IN RESPONSE TO PC 4-PDT

Fig. 2. Kinetics of apoptotic events and PARP cleavage in PDT-treated LY-R cells. Cells were treated with 0.5 μM Pc 4 and 3 kJ/m² red light and collected at various times as indicated. a, cell lysates were processed for agarose electrophoresis of DNA. b, immunoblot analysis, upper panel, PARP; middle panel, actin; lower panel, quantitation of PARP cleavage. c, TUNEL assay and flow cytometry to determine the percentage of apoptotic cells. Similar results were obtained in three independent experiments.

During the course of these studies, the fragmented PARP occasionally appeared as a double band migrating at Mr ~85,000 and Mr ~90,000 (e.g., Fig. 1a, Lanes 5 and 6; Fig. 2b, Lane 6; Fig. 4a, Lane 3). Because PARP contains only one DEVD ↓ G site, we considered the possibility that the two apparent PARP fragments may differ with respect to the extent of poly(ADP-ribosylation). The presence of poly(ADP-ribose) on various proteins was assessed with an anti-poly(ADP-ribose) antibody. As shown in the top panel of Fig. 6, the intact form of PARP was poly(ADP-ribosylated) in the control and in treated samples collected up to 30 min after PDT. The antibody did not recognize the Mr 116,000 PARP from samples taken 45 min or later after PDT, when intact PARP was completely cleaved (Figs. 1a and 2b). No PARP fragments were detected with this antibody at any time during the 2-h post-PDT period. However, a strong poly(ADP-ribosylated) protein band was observed at Mr ~100,000. Western blotting indicated that topoisomerase I is one of the proteins migrating at this position (Fig. 6, middle panel). PDT does not appear to modify either the poly(ADP-ribosylation) of the Mr 100,000 protein or the amount of topoisomerase I protein until 45–120 min after PDT.

DISCUSSION

The present study provides direct evidence for the involvement of caspases in PDT-induced apoptosis. Three lines of evidence support this conclusion for apoptosis in LY-R cells:

(a) The nuclear protein PARP is cleaved in response to PDT (Fig. 1–2, 4, 6). Although all of the specific proteases that are activated by PDT have not been identified, the data of Fig. 3 suggest that one or more caspases that cleave at the sequence of DEVD ↓ AMC, which is also the cleavage site for PARP (DEVD ↓ G) (14), are activated after PDT. In HL-60 cells exposed to PDT sensitized by benzoporphyrin derivative, evidence was provided for the activation of caspase-3, but caspase-1 activation was not detected (41). Caspase-3 and caspase-7 preferentially cleave PARP at that sequence into a Mr 90,000 fragment (51). Caspase-6, although cutting at the same sequence, has a much lower activity toward PARP but readily cleaves lamin A (51). More-
PARP CLEAVAGE IN RESPONSE TO PC 4-PDT

Fig. 5. Morphological evidence for the effects of zVAD-fmk and zFA-fmk on PDT-induced apoptosis in LY-R cells. Cells were grown in 96-well plates and treated as described in Fig. 4. Cells were collected 2 h after PDT, double-stained with acridine orange and ethidium bromide, and viewed and photographed in a fluorescence microscope. The results are shown as representative fluorescence images (a) and quantification of apoptotic cells and necrotic cells (b). Similar data were obtained from two additional experiments.

Fig. 6. By Western blot analysis with anti-poly(ADP-ribose), the Mr 116,000 PARP appears to be poly(ADP-ribosylated). The antibody, which recognizes all size classes of the polymer, did not react with the PARP cleavage fragments, arguing that removal of poly(ADP-ribose) from PARP precedes or accompanies cleavage, or that only free, inactive PARP is cleaved. However, modifications of PARP that would not be detected by this antibody and that could remain in the cleavage products include phosphorylation and mono(ADP-ribosylation).

(b) PARP cleavage has a PDT dose and time dependence (Figs. 1, 2, and 4) that is approximately the same as that for the appearance of other markers of apoptosis, i.e., DNA fragmentation and Tdt responsiveness, as measured by flow cytometric identification of cells that have incorporated fluorescently labeled nucleotides (Fig. 2). Based on the induction of apoptosis that follows the overexpression of proteases in cultured cells and the demonstration that exogenous proteases can produce morphological apoptosis in isolated nuclei, protease action has been considered a precursor to endonuclease action and chromatin condensation. After treatment of a series of tumor cell lines with the chemotherapeutic agent topotecan, an excellent correlation was observed between the extent of PARP cleavage and the appearance of apoptotic cells, as detected by staining with acridine orange. In the case of PDT-treated LY-R cells, the very rapid induction and progress of apoptosis has precluded a clear demonstration of temporal order of the measured events. In fact, in each experiment where comparison of the kinetics was sought, the initiation of PARP cleavage and of DNA fragmentation was nearly simultaneous, although PARP cleavage seemed to be completed earlier than the entry of the final cells into apoptosis, when measured by flow cytometry. However, activation of the cascade of proteases that are ultimately responsible for cleavage of PARP and other proteins must have preceded the appearance of PARP fragments. As estimated by the cleavage of the fluorogenic peptide substrate DEVD-AMC (Fig. 3), caspase-3-like activity began to increase almost immediately after PDT and was 2–3-fold elevated in 10 min and 100-fold elevated in 30 min. Because
it is not known what level of activity increase is needed to initiate the next steps in apoptosis, the small early elevation may be sufficient.

It is interesting that very rapid and complete PARP cleavage was observed for the highest PDT dose tested (Fig. 1), a dose that kills LY-R cells without producing oligonucleosomal DNA fragmentation (58). Perhaps the excessive oxidative damage produced by a markedly supraphotodynamic PDT dose allows apoptosis to be initiated but interferes with the late steps of the process. A similar proposal has been made by Dellinger (59), based on studies of the morphology of cells undergoing apoptosis in response to PDT sensitized by Photofrin. However, Luo and Kessel (42) found that PARP cleavage and oligonucleosomal DNA fragmentation were blocked in P388 cells treated with a high dose of PDT sensitized by aluminum phthalocyanine.

(c) An inhibitor of ICE-like cysteine proteases, zVAD-fmk, blocks both PARP cleavage and the appearance of morphologically apoptotic cells, whereas an inactive peptide analogue, zFA-fmk, blocked neither event (Figs. 4 and 5). zVAD-fmk has broad specificity for cysteine proteases (50), and its ability to block PDT-induced apoptosis is strong evidence that one or more of such proteases are critical for apoptosis after PDT. However, whether the cleavage of PARP per se is important cannot be discerned from these results. PARP is one of a set of nuclear proteins that has been demonstrated to be proteolytically cleaved during apoptosis. Which, if any, of these proteins is critical for initiating the later stages of apoptosis is not known.

The demonstration that proteases are important in PDT-induced apoptosis has implications for the treatment of tumors by this modality. PDT produces rapid tumor ablation in experimental animals, with complete loss of visible tumor often occurring within 1–2 days (4). Apoptosis is frequently observed at early times during tumor ablation (60–62). If protease action and/or the cleavage of PARP is important in tumor response, PARP cleavage may be a useful marker of tumor cell death and overall response of PDT-treated tumors.

REFERENCES


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