# Involvement of Fas (APO-1/CD-95) during Photodynamic-Therapy-Mediated Apoptosis in Human Epidermoid Carcinoma A431 Cells

Nihal Ahmad, Sanjay Gupta, Denise K. Feyes, and Hasan Mukhtar*

Department of Dermatology, Case Western Reserve University, Cleveland, Ohio, U.S.A.

**Photodynamic therapy is a promising treatment modality for a variety of cutaneous neoplasms and other skin disorders. Studies suggest an involvement of multiple pathways during photodynamic-therapy-mediated cell death. A complete knowledge of the mechanisms involved in photodynamic therapy may lead to an improvement in its therapeutic efficacy. *In vitro* as well as *in vivo* studies have shown the involvement of apoptosis during photodynamic-therapy-mediated cell death. The pathways by which photodynamic therapy causes this are not fully understood. In this study, employing human epidermoid carcinoma (A431) cells and silicon phthalocyanine 4 photodynamic therapy, we show that the cell surface death receptor Fas (also known as APO-1 or CD-95) pathway is an important contributor to photodynamic-therapy-mediated apoptosis. Employing flow cytometric analysis and confocal microscopy we first established that silicon phthalocyanine 4 photodynamic therapy results in a significant induction of apoptosis in A431 cells. Immunoblot analysis revealed a significant time-dependent increase in the protein expression of Fas at 5, 15, 30, and 60 min post-photodynamic therapy followed by a decrease at later time-points (2 and 3 h post-photodynamic therapy). A Fas enzyme-linked immunosorbent assay demonstrated an increase in this protein in cell culture medium starting at 1 h post-photodynamic therapy and showing a time-dependent response up to 3 h following therapy, suggesting a diffusion of soluble Fas from cells into the medium from 1 h after photodynamic therapy. Silicon phthalocyanine 4 photodynamic therapy also resulted in a time-dependent increase in (i) the multimerization of Fas protein, (ii) the protein expression of Fas ligand, (iii) FADD, an adapter molecule for Fas, and (iv) the binding of FADD with Fas. Silicon phthalocyanine 4 photodynamic therapy also caused a significant activation of FLICE, as evident from the appearance of cleaved products of pro-caspase 8. Further, a pretreatment of cells with rhFas:Fc fusion protein or general caspase inhibitor Z-VAD-FMK followed by silicon phthalocyanine 4 photodynamic therapy resulted in a significantly enhanced cell survival. Taken together, our data, for the first time, delineate an involvement of the Fas pathway as an important contributor to photodynamic-therapy-mediated apoptosis of cancer cells. These observations may be important for improving the efficacy of photodynamic therapy for the treatment of skin cancer and possibly other skin disorders.**

***Key words: FADD/Fas/FasL/FLICE/PDT. J Invest Dermatol 115:1041–1046, 2000***

Photodynamic therapy (PDT) is a relatively new therapeutic procedure used for the management of a variety of solid tumors including skin cancer and is showing promise for the management of many nonmalignant diseases including dermatologic disorders (Colussi *et al*, 1998; Dougherty *et al*, 1998; Fritsch *et al*, 1998; Oleinick and Evans, 1998; Kalka *et al*, 2000). The United States Food and Drug Administration has approved the use of photodynamic therapy for endobronchial and esophageal tumors (Dougherty *et al*, 1998; Oleinick and Evans, 1998). More recently, PDT has also been approved for treating actinic keratoses. The approval of PDT for management of skin cancers and metastatic cutaneous and subcutaneous breast cancers is expected, based on investigator-initiated and other clinical trials. Investigator-initiated clinical trials for psoriasis, alopecia areata, atherosclerosis, vascular restenosis, age-related macular degeneration, rheumatoid arthritis, and wound healing are currently ongoing (Colussi *et al*, 1998; Dougherty *et al*, 1998; Fritsch *et al*, 1998; Oleinick and Evans, 1998; Kalka *et al*, 2000). PDT relies on a bimodal protocol with two components: (i) chemical photosensitization, and (ii) light irradiation. Both of these components, individually, are nontoxic but they are tumoricidal in combination (Colussi *et al*, 1998; Dougherty *et al*, 1998; Fritsch *et al*, 1998; Oleinick and Evans, 1998). PDT involves a selective uptake of a porphyrin-based photosensitizing chemical in the tumor relative to the surrounding normal tissue followed by light irradiation in the visible or near infrared region that is typically derived through a laser. This causes a photoactivation leading to oxidative damage to a variety of cellular targets that subsequently results in the death of cancerous cells and thereby ablation of the tumor. The molecular mechanism(s) of PDT-mediated cell killing and the subsequent tumor ablation is not well understood. PDT-mediated

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Reprint requests to: Dr. Hasan Mukhtar, Department of Dermatology, Case Western Reserve University, 11100 Euclid Avenue, Cleveland, OH 44106. Email: hxm4@po.cwru.edu

Abbreviations: Pc4, silicon phthalocyanine 4; PDT, photodynamic therapy.

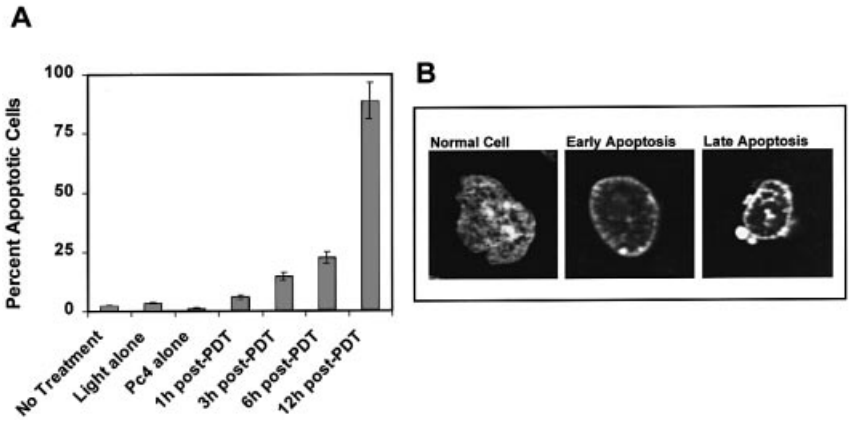


**Figure 1. Induction of apoptosis by Pc4-PDT.** (*A*) Flow cytometric analysis. The A431 cells were subjected to Pc4-PDT, labeled with deoxyuridine triphosphate using terminal deoxynucleotide transferase and propidium iodide, and analyzed by flow cytometry as described in *Materials and Methods*. The data are displayed as percentage apoptotic cells and represent the mean ± SEM from three experiments. (*B*) Confocal microscopy analysis. The A431 cells were subjected to Pc4-PDT and analyzed for morphologic changes as described in *Materials and Methods*. *Panel A* represents normal nonapoptotic cells; *panel B* shows an early apoptotic cell (data shown here represent a picture of a cell taken 1 h post-PDT); *panel C* shows a late apoptotic cell (data shown here represent a picture of a cell taken 12 h post-PDT). The data shown here are from a representative experiment repeated twice with similar results.

oxidative stress has been shown to increase the expression of early response genes (*c-fos*, *c-jun*, *c-myc*, *egr-1*), as well as glucose-regulated protein and heme oxygenase (Luna *et al*, 1994; Oleinick and Evans, 1998). The involvement of heat shock protein HSP-70 (Gomer *et al*, 1996), nuclear factor κB (Ryter and Gomer, 1993), and the mitogen-activated protein kinase (MAPK) pathway (Tao *et al*, 1996; Klotz *et al*, 1998; Assefa *et al*, 1999) has also been documented in PDT response.

PDT is known to exert oxidative stress and induce apoptosis both *in vitro* and *in vivo* (Colussi *et al*, 1998; Dougherty *et al*, 1998; Fritsch *et al*, 1998; Oleinick and Evans, 1998). The involvement of apoptosis has been shown to be an early response of PDT, both *in vitro* and *in vivo* (Agarwal *et al*, 1993, 1996; Zaidi *et al*, 1993; Ahmad *et al*, 1998; Colussi *et al*, 1998; Colussi *et al*, 1999). Based on many studies from this laboratory and elsewhere, it is becoming clear that multiple pathways are involved during PDT-mediated cell death. Therefore, a hypothesis is put forward that a synergistic activation of apoptosis-inducing pathways via a combination therapy may enhance the therapeutic efficacy of PDT. This warrants concerted detailed efforts to decipher the mechanism(s) involved in PDT-mediated apoptosis.

In this study, we have attempted to investigate our hypothesis that the cell surface death receptor Fas pathway is an important contributor to PDT-mediated apoptosis. The rationale for this hypothesis is based on three major observations. First, it is becoming clear that multiple pathways are involved during PDT-mediated apoptosis. This is based on our recent studies showing that silicon phthalocyanine 4 (Pc4)-PDT-mediated cell cycle deregulation and/or apoptosis involves (i) an increased production of nitric oxide (Gupta *et al*, 1998), (ii) a WAF1/p21-mediated inhibition of the cyclin-cyclin dependent kinase network (Ahmad *et al*, 1998), and (iii) a deregulation of pRb/E2F-DP machinery (Ahmad *et al*, 1999). Several other laboratories have shown the involvement of $bcl_2$/bax/bcl-x(l) (He *et al*, 1996; Granville *et al*, 1998; Kim *et al*, 1999), phospholipases A2 and C (Agarwal *et al*, 1993), intracellular $Ca^{2+}$ (Agarwal *et al*, 1993; Tajiri *et al*, 1998), ceramide (Separovic *et al*, 1997; 1998), caspases (Granville *et al*, 1998; Varnes *et al*, 1999), c-Jun N-terminal kinase (JNK)/p38 MAPK (Klotz *et al*, 1998; Assefa *et al*, 1999), and cytochrome C release (Granville *et al*, 1998; Varnes *et al*, 1999) during PDT-mediated apoptotic cell death. Thus, it is becoming clear that multiple pathways are involved during Pc4-PDT-mediated cell death. The involvement of multiple pathways is also evident from our earlier study where we showed that PDT results in G0-G1-phase arrest of A431 cells mediated by WAF1/p21 cyclins (E and D1)–cyclin dependent kinases (2 and 6) that ultimately leads to their apoptotic death (Ahmad *et al*, 1998). In this study, we observed that at 12 h following PDT only 69% cells were arrested in the G0-G1 phase whereas 89% cells were found to be apoptotic at this time. This observation indicated that in addition to the cell-cycle-mediated apoptosis, other mechanism(s) of apoptosis may also be operative. Second, the Fas pathway is intimately associated with sphingomyeline/ceramide, caspase activation and JNK/p38 MAPK regulation (Brenner *et al*, 1997; 1998; Juo *et al*, 1998). Finally, PDT is known to exert oxidative stress and Fas has been shown to be a redox responsive signaling molecule (Peter and Krammer, 1998; Schulze-Osthoff *et al*, 1998).

In this study, we demonstrate the involvement of the Fas pathway during Pc4-PDT-mediated apoptosis.

## MATERIALS AND METHODS

**Cells** The human epidermoid carcinoma (A431) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained in an atmosphere of 95% air/5% $CO_2$ in a 37°C humidified incubator under standard conditions.

**Photodynamic therapy** The photosensitizer Pc4, synthesized as described earlier (Oleinick *et al*, 1993), was kindly provided by Dr. Malcolm E. Kenney of the Department of Chemistry. The cells (80% confluent) were incubated overnight with Pc4 (0.5 μM in dimethylsulfoxide) in DMEM in a 100 × 20 mm style Falcon disposable cell culture dish. Cells were then washed with Hank's balanced salt solution (HBSS) (with $Ca^{2+}$ and $Mg^{2+}$) and irradiated (in HBSS with $Ca^{2+}$ and $Mg^{2+}$) with 15 kJ per $m^2$ of light, as measured with a digital photometer (Tektronix, Beaverton, OR), using a 300 W halogen lamp. The light delivered was filtered through a Lee primary red filter #106 (Vincent Lighting, Cleveland, OH) to remove light with wavelength less than 600 nm. After irradiation, the cells were washed with phosphate-buffered saline (PBS) and incubated, in DMEM, in the dark for selected times in a humidified incubator at 37°C. Appropriate controls, as specified at appropriate places, were also included. After the specified times, the medium was aspirated and the cells were washed with cold PBS (10 mM, pH 7.4) and processed as desired.

**Detection of apoptosis by flow cytometry** The cells were treated with Pc4 and light as described above and harvested at 1, 3, 6, and 12 h following PDT. The cells were trypsinized and collected in a 50 ml Falcon tube. In this experiment, the floating cells were also collected and included in the assay. The cells were washed with PBS twice and the extent of apoptosis was quantified by using APO-DIRECT™ flow cytometry kit (Phoenix Flow System, San Diego, CA) as per the manufacturer's protocol.

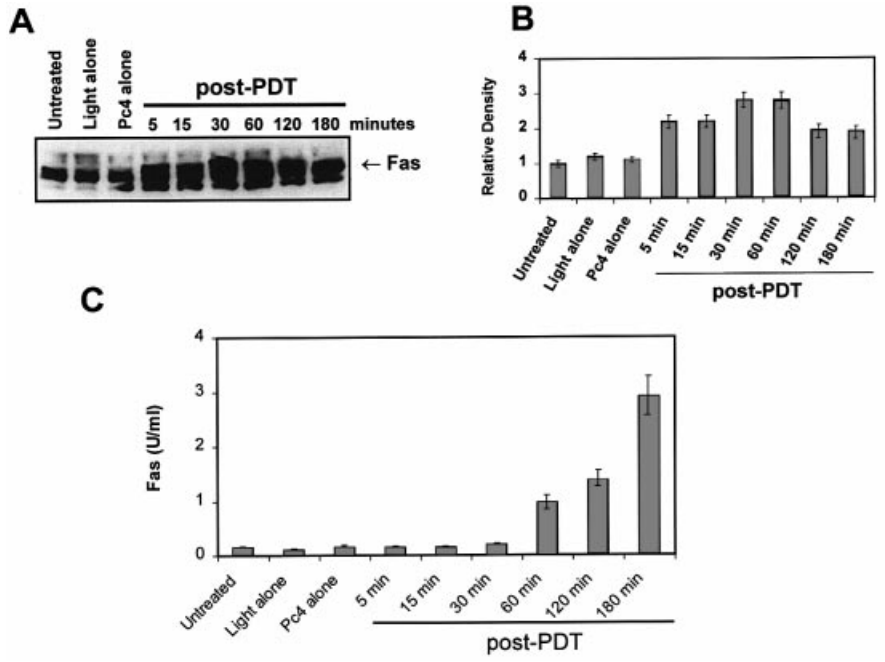


**Figure 2. Effect of Pc4-PDT on protein expression of Fas in A431 cells.** (*A*) Immunoblot analysis. The cells were subjected to PDT and harvested at 5, 15, 30, 60, 120, and 180 min following the light treatment. Total cell lysates were prepared and 50 μg protein was subjected to SDS-PAGE followed by immunoblot analysis using anti-Fas antibody. The proteins were detected by chemiluminescence. The details are described in *Materials and Methods*. Equal loading was confirmed by stripping the membrane and re-probing it with β-actin (data not shown). The data shown here are from a representative experiment repeated three times with similar results. (*B*) Quantification of the protein bands. The protein bands were quantified using Scion Image Software. The density of the band corresponding to the untreated control was taken as unity. The data represent relative density normalized to β-actin and are expressed as mean ± SEM of three experiments. (*C*) Effect of Pc4-PDT on Fas protein in culture medium by ELISA. Following PDT the cells were harvested at specified times and the amount of Fas protein in the culture medium was detected by Fas/APO-1 ELISA kit obtained from Oncogene Research Products according to the manufacturer's protocol. The details are described in *Materials and Methods*. The data represent the mean ± SEM from three experiments.

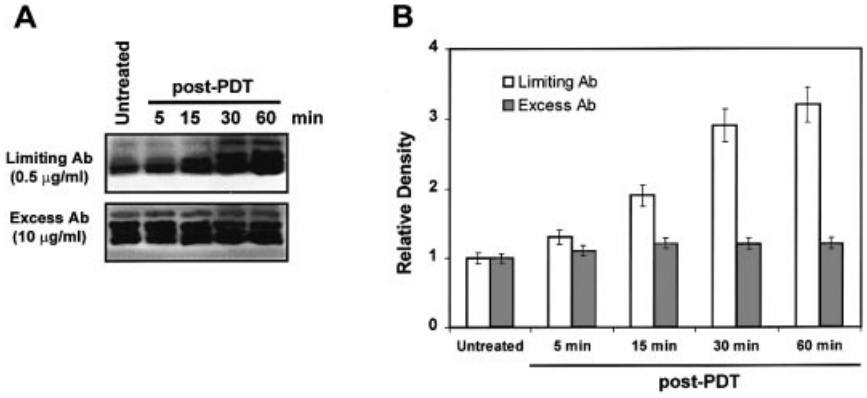


**Figure 3. Multimerization of Fas by Pc4-PDT in A431 cells.** (*A*) Immunoblot analysis. The cells were subjected to Pc4-PDT and harvested at 5, 15, 30, and 60 min following the light treatment. Total cell lysates were prepared and Fas protein (50 μg) was immunoprecipitated using anti-Fas antibody (Apo-1, Kamiya Biomedical Company) at 0.5 μg per ml (limiting antibody) or 10 μg per ml (excess antibody). The immunocomplex was subjected to SDS-PAGE followed by immunoblot analysis using anti-Fas antibody. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and re-probing it with β-actin (data not shown). The details are described in *Materials and Methods*. Data from a typical experiment repeated twice with similar results are shown. (*B*) Quantification of the protein bands. The protein bands were quantified using Scion Image Software. The density of the band corresponding to the untreated control was taken as unity. The data represent relative density normalized to β-actin and are expressed as mean ± SEM of three experiments.

**Detection of apoptosis by confocal microscopy** The cells were cultured on round glass coverslips in a 60 mm culture dish to about 70% confluency and subjected to Pc4-PDT for selected times as described above. The cells were then labeled with 10 μM SYTO 13 (Molecular Probes, Eugene, OR) by incubating with the dye for 20 min in complete medium at 37°C. The coverslips were washed with PBS and mounted. The SYTO 13 fluorescence was excited by an argon-krypton laser with 488 nm wavelength and imaged through a 460 nm dichroic reflector and 514–540 nm emission filter with a Zeiss 410 laser scanning confocal microscope. Apoptosis was characterized by morphologic changes, namely chromatin condensation, nuclear condensation, and formation of apoptotic bodies.

**Preparation of cell lysates and immunoblot analysis** The cells were treated with Pc4 and light as described above and incubated for the specified time. The cells were then washed with cold PBS (10 mM, pH 7.4); ice-cold lysis buffer (50 mM Tris-HCl, 150 nM NaCl, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 mM ethylenediamine tetraacetic acid, 20 mM NaF, 100 mM $Na_3VO_4$, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg per ml aprotinin, 10 μg per ml leupeptin; pH 7.4) was added and the cells were left over ice for 30 min. The cells were scraped, and the lysate was collected in a microfuge tube and passed through a $21^{1/2}$ G needle to break up the cell aggregates. The lysate was cleared by centrifugation at

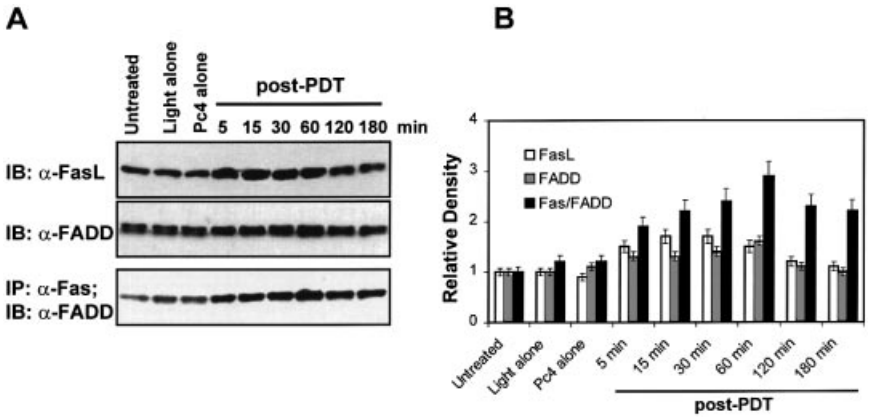


**Figure 4. Effect of Pc4-PDT on the protein expression of FasL, FADD, and binding of FADD with Fas in A431 cells.** (*A*) Immunoblot analysis. For the effect of Pc4-PDT on FasL and FADD, the cells were harvested at designated times following PDT, total cell lysates were prepared, and 50 μg protein was subjected to SDS-PAGE followed by immunoblot analysis using appropriate primary antibodies and secondary horseradish peroxidase conjugates. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and re-probing it with β-actin (data not shown). For the effect of Pc4-PDT on the binding of FADD with Fas, the Fas protein (50 μg) was immunoprecipitated using excess of anti-Fas antibody (Apo-1, Kamiya Biomedical Company). The immunocomplex was subjected to SDS-PAGE followed by immunoblot analysis using anti-FADD antibody. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and re-probing it with β-actin (data not shown). The details are described in *Materials and Methods*. Data from a typical experiment repeated three times with similar results are shown. (*B*) Quantification of the protein bands. The protein bands were quantified using Scion Image Software. The density of the band corresponding to the untreated control was taken as unity. The data represent relative density normalized to β-actin and are expressed as mean ± SEM of three experiments.

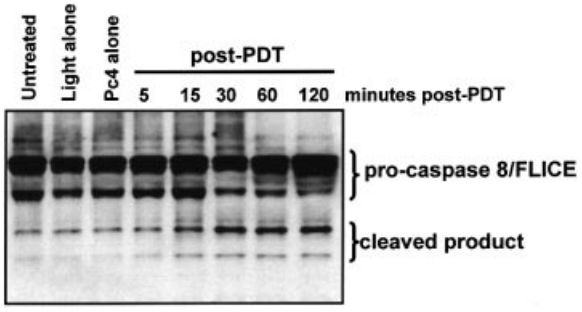


**Figure 5. Effect of Pc4-PDT on FLICE in A431 cells.** The cells were subjected to PDT and harvested at 1, 5, 15, 30, and 60 min following the light treatment. Total cell lysates were prepared and 50 μg protein was subjected to SDS-PAGE followed by immunoblot analysis using anti-FLICE antibody. The proteins were detected by chemiluminescence. Equal loading was confirmed by stripping the membrane and re-probing it with β-actin (data not shown). The details are described in *Materials and Methods*. The data shown here are from a representative experiment repeated three times with similar results.

14,000 × *g* for 15 min at 4°C and the supernatant (total cell lysate) was either used immediately or stored at –70°C. The protein concentration was determined by DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

For immunoblot analysis, 25–50 μg protein was resolved over 12% polyacrylamide sodium dodecyl sulfate gels and transferred onto a nitrocellulose membrane. The membrane was incubated in blocking buffer (5% nonfat dry milk, 1% Tween 20, in Tris-buffered saline, pH 7.6) for 1 h at room temperature and incubated with appropriate human reactive primary antibody (anti-Fas from Santa Cruz Biotechnology, Santa Cruz, CA; anti-FasL and anti-FADD from Transduction Laboratories, Lexington, KY; anti-FLICE from Upstate Biotechnology, Lake Placid, NY) in blocking buffer for 1 h to overnight at 4°C. The membrane was then incubated with antimouse or antirabbit secondary antibody horseradish peroxidase conjugate (Amersham Life Sciences, Arlington Heights, IL) followed by detection using an enhanced chemiluminescence kit (Amersham Life Sciences).

The density of the bands was quantified using Scion Image Software (Scion Corporation, Frederck, MD), which is based on NIH Image for Macintosh and modified for Windows by Scion Corporation.

**Immunoprecipitation** For immunoprecipitation, the cell lysates containing about 50 μg of protein (in a total volume of 1.0 ml) were precleared by incubating with 1 μg of normal mouse IgG and 20 μl of Protein-A-Sepharose 4B fast flow (Pharmacia Biotech, Piscataway, NJ) for 1 h at 4°C. The supernatant was collected and incubated with 1.0 μg of anti-Fas antibody (Apo-1, Kamiya Biomedical Company, Seattle, WA) for 2 h. Then, 20 μl of Protein-A-Sepharose was added and it was incubated overnight at 4°C. The beads were collected by centrifugation at 2500 rpm at 4°C and washed with RIPA buffer three times following the centrifugation steps. The immunocomplexes were resolved over a 12% polyacrylamide sodium dodecyl sulfate gel, followed by immunoblot analysis and chemiluminescent detection as described above.

**Fas enzyme-linked immunosorbent assay (ELISA)** To assess the effect of Pc4-PDT on the levels of soluble Fas protein, we performed Fas/APO-1 quantitative ELISA using a kit obtained from Oncogene Research Products (Cambridge, MA). The cells were incubated overnight with Pc4 and then irradiated with light as described above. The cells were then incubated in the dark for 5, 15, 30, 60, 120, and 180 min following light treatment in a humidified incubator at 37°C. The medium was collected and Fas protein was analyzed by ELISA performed according to the manufacturer's protocol.

**Treatment of cells with Fas:Fc fusion protein and Z-VAD-FMK** To assess the effect of blockade of the Fas pathway on Pc4-PDT-mediated cell survival, we employed Fas:Fc fusion protein kit (Alexis, San Diego, CA) as per the manufacturer's protocol. Briefly, A431 cells (50,000 cells in 100 μl complete DMEM) were incubated simultaneously with 100 μg per ml of rhFas:Fc fusion protein and 1 μg per ml enhancer protein (for 24 h) and Pc4 (0.5 μM, overnight) in a 96 well plate at 37°C, and this was followed by light treatment as described above. The cells were washed with PBS and incubated in the dark (in DMEM) for 6 or 12 h. The viability of cells was measured by 3-(4,5-dimethylthiazol)-2-yl (MTT)-based colorimetric assay employing a kit obtained from Roche Molecular Biochemicals (Indianapolis, IN) exactly as the manufacturer's protocol.

To assess the effect of general caspase inhibitor Z-VAD-FMK on Pc4-PDT-mediated cell survival, we treated the A431 cells (50,000 cells in 100 μl complete DMEM) simultaneously with 100 μM Z-VAD-FMK (for 24 h) and Pc4 (0.5 μM, overnight) in a 96 well plate at 37°C, followed by light treatment as described above. The cells were washed with PBS and incubated in the dark (in DMEM) for 6 or 12 h. The viability of the cells was measured by MTT assay as described above.

## RESULTS AND DISCUSSION

This study was designed to define the involvement of the cell surface death receptor Fas pathway as an important contributor of

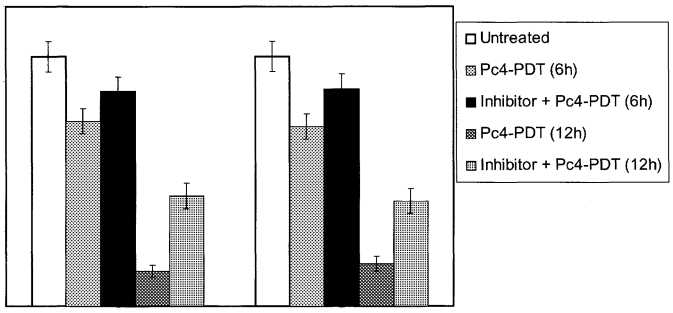


**Figure 6. Effect of pretreatment of cells with Fas:Fc fusion protein and caspase inhibitor Z-VAD-FMK on Pc4-PDT-mediated cell survival in A431 cells.** Cells were incubated simultaneously with 100 μg per ml of rhFas:Fc fusion protein and 1 μg per ml enhancer protein (for 24 h) or 100 μM Z-VAD-FMK and Pc4 (0.5 μM, overnight) in a 96 well plate at 37°C, and subjected to light treatment. Six hours or 12 h following light treatment, the viability of the cells was assessed by MTT assay. The details are described in *Materials and Methods*. The data are the mean ± SEM from three experiments.

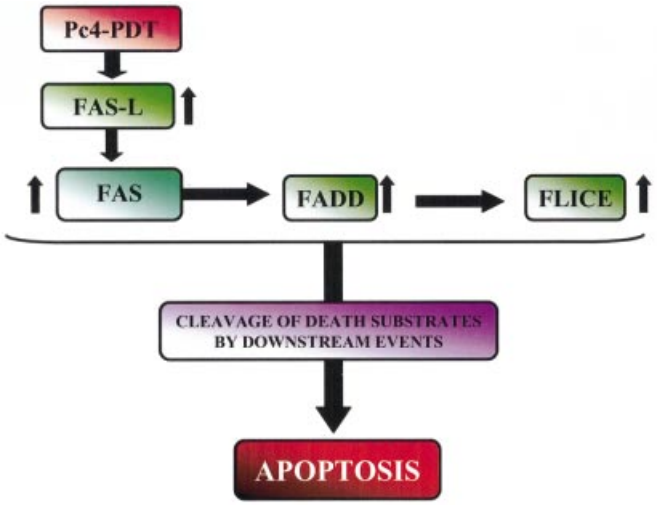


**Figure 7. Proposed scheme showing the involvement of the Fas pathway as a mechanism of PDT-mediated apoptosis.** Up arrows (↑) demonstrate an activation/upregulation of the specified molecule.

PDT-mediated apoptosis. In our experiments, we employed (i) A431 cells, a keratinocyte-derived cell line, as the model cell line, and (ii) silicon phthalocyanine, termed "Pc4", as the model photosensitizer. Employing flow cytometric analysis of the cells labeled with F-dUTP and propidium iodide, we first established that Pc4-PDT causes apoptosis of A431 cells. As shown by data in **Fig 1**(***A***), PDT was found to result in a significant induction of apoptosis in A431 cells at 3, 6, and 12 h post-PDT (**Fig 1*A***). The induction of apoptosis by Pc4-PDT was also evaluated by confocal microscopy after labeling the cells with SYTO 13, as this method can identify the early apoptotic cells. As shown by the data in **Fig 1**(***B***), early apoptotic morphology was evident even at 1 h following PDT. As the Fas pathway is known to be activated much before the fragmentation of DNA, we chose 5, 15, 30, 45, 60, 120, and 180 min time-lag following PDT (unless otherwise stated) for the rest of the study.

As Fas and Fas ligand (FasL) are the complementary receptor-ligand proteins eliciting apoptosis via caspase activation, and studies have shown that PDT results in activation of caspases (Granville *et al*, 1998; Varnes *et al*, 1999), employing immunoblot analysis, we studied the effect of Pc4-PDT on the protein expression of Fas in A431. As shown by the data in **Fig 2**(***A***, ***B***), a significant time-dependent increase in the protein expression of Fas was observed at 5, 15, 30, and 60 min post-PDT in A431 cells. In immunoblot analysis Fas protein was observed as a doublet, a strong upper band and a lower band. This is consistent with earlier reports and may be due to the insoluble and soluble forms of Fas proteins, respectively (Catlett and Bishop, 1999). The observed rapid increase in the Fas protein is an interesting observation, as 5 min does not appear to be a sufficient time for *de novo* protein synthesis. The reason for this rapid increase is not clear at present. It appears that it may be due, however, to extremely fast protein synthesis that might have started during the treatment of cells with light, because the time to deliver 15 kJ per $m^2$ light was about 7.5 min in our experimental setup, which, in fact, increases the time-lag for 5 min post-PDT samples to 12.5 min. Another interesting observation of this experiment was that at 2 h and 3 h post-PDT the level of Fas protein was found to decrease. This decrease in Fas is probably due to the diffusion of this soluble protein from the cells into the cell culture medium. To further investigate this possibility, we performed ELISA using Fas quantitative assay kit (Oncogene Research Products). We evaluated the effect of Pc4-PDT on the levels of Fas protein released in the culture medium. As shown by the data in **Fig 2**(***C***), we found an increase in Fas protein in cell culture medium starting at 1 h post-PDT and showing a time-dependent response up to 3 h following PDT. These data further support our assumption of diffusion of the soluble Fas protein from cells into the medium after 1 h following PDT.

Studies have shown that, upon activation, Fas is trimerized to transduce the death signal to FADD/MORT1 (Fas associated through death domain) via a dynamic interaction with it through its death domain, which itself can induce cell death upon over-production (Chinnaiyan *et al*, 1995; Fraser and Evan, 1996; Peter *et al*, 1998; Schulze-Osthoff *et al*, 1998). Therefore, in order to detect Fas multimerization following Pc4-PDT, we immunoprecipitated Fas employing a protocol described by Rehemtulla *et al* (1997). When a limited anti-Fas antibody concentration is used to immunoprecipitate, more Fas molecules will be immunoprecipitated following PDT-mediated aggregation of Fas. In contrast, when an excess of antibody is used, the immunoprecipitated Fas antigen will be constant irrespective of PDT. As shown by the data in **Fig 3**, at limiting antibody concentration, a PDT time-dependent increase in Fas was observed, whereas with excess antibody no change in the amount of Fas protein was observed. This suggested that Pc4-PDT results in a multimerization of Fas, for its activation.

Employing immunoblot analysis, we next evaluated the effect of Pc4-PDT on the protein expression of FasL. As shown by the data in **Fig 4**, Pc4-PDT was found to result in a significant time-dependent increase in FasL and followed a similar pattern to that shown by Fas. We also investigated the effect of Pc4-PDT on (i) FADD, and (ii) the binding of FADD to Fas. Our data (**Fig 4**) demonstrate that Pc4-PDT of A431 cells results in a time-dependent increase, for up to 1 h post-PDT, in FADD protein levels. To investigate the effect of Pc4-PDT on the binding of FADD with Fas, we immunoprecipitated Fas-FADD complex using an antibody directed against Fas, followed by gel electrophoresis and immunoblot analysis using an antibody directed against FADD. As shown by the data in **Fig 4**, Pc4-PDT results in a time-dependent increase in the binding of FADD with Fas. As FADD is known to recruit an interleukin-1β-converting enzyme (ICE)-like protease known as FLICE (FADD-like-ICE, also known as MACH-5 or caspase 8) to initiate cystein-like protease cascade (Muzio *et al*, 1996), we also assessed the effect of Pc4-PDT on FLICE activation. Our data (**Fig 5**) show that Pc4-PDT causes an activation of FLICE in A431 cells, which is evident from the appearance of cleaved products of pro-caspase 8. Previous studies have shown the involvement of caspase 8 and cytochrome C during PDT-mediated cell death (Granville *et al*, 1998). Another recent study has also shown a caspase-8 mediated caspase 3 activation and cytochrome C release during singlet-oxygen-induced apoptosis (Zhuang *et al*, 1999). As the generation of singlet oxygen and other reactive oxygen intermediates is believed to be a prerequisite for the PDT effect, it is possible that the Fas-FADD-mediated activation of caspase 8 shown here is preceded by caspase 3 activation and subsequent release of cytochrome C from mitochondria resulting in an apoptotic death of A431 cells.

Finally, to further confirm the involvement of the Fas pathway during Pc4-PDT-mediated apoptosis, we employed an approach of blocking FasL-mediated lysis of the cells using rhFas:Fc fusion protein. As shown by the data in **Fig 6(*A*)**, a pretreatment of cells with Fas:Fc followed by Pc4-PDT resulted in a significant increase in cell viability compared with Pc4-PDT-treated cells. This observation confirms that the Fas pathway is, at least in parts, involved during Pc4-PDT-mediated apoptosis. Further, a pretreatment of cells with general caspase inhibitor Z-VAD-FMK followed by Pc4-PDT also resulted in a significant increase in cell survival (**Fig 6*B***) showing a definite involvement of caspases, which are downstream effector molecules in the Fas pathway.

Taken together, as shown in **Fig 7**, the results presented here for the first time delineate that PDT-caused apoptosis may be mediated via the Fas-FasL system, which triggers the downstream FADD-FLICE death effector pathway. These observations are important because recently it has been shown that Fas-FasL-mediated apoptosis is important for skin homeostasis and that dysregulation of the Fas-FasL system may be critical to the development of skin cancer (Hill *et al*, 1999). Therefore, our study delineating the involvement of the Fas-FasL system during PDT-mediated apoptosis may be important for improving the efficacy of PDT for the treatment of skin cancer and possibly other cancers and/or similar pathologic conditions. It is also conceivable that a synergistic activation of Fas-FasL pathways via a combination therapy may enhance the therapeutic efficacy of PDT.

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