Photodynamic Therapy with the Phthalocyanine Photosensitizer Pc 4 of SW480 Human Colon Cancer Xenografts in Athymic Mice

Cecilia M. Whitacre, Denise K. Feyes, Taroh Satoh, Johannes Grossmann, John W. Mulvihill, Hasan Mukhtar, and Nancy L. Oleinick

Departments of Medicine [C. M. W., T. S., J. G.], Radiation Oncology [J. W. M., N. L. O.], and Dermatology [D. K. F., H. M.], Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4937

ABSTRACT

Photodynamic therapy (PDT) using the silicon phthalocyanine photosensitizer Pc 4 [HOSiPcOsi(CH$_2$)$_3$(CH$_3$)$_2$N-(CH$_2$)$_3$] is an oxidative stress associated with induction of apoptosis in various cell types. We assessed the effectiveness of Pc 4-PDT on SW480 colon cancer xenografts grown in athymic nude mice. Animals bearing xenografts were treated with 1 mg/kg body weight Pc 4 and 48 h later were irradiated with 150 J/cm$^2$ 672-nm light from a diode laser delivered at 150 mW/cm$^2$. Biochemical studies were performed in xenografts resected at various time points up to 26 h after Pc 4-PDT treatment, whereas tumor size was evaluated over a 4-week period in parallel experiments. In the tumors resected for biochemical studies, apoptosis was visualized by activation of caspase-9 and caspase-3 and a gradual increase in the cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) from its NH$_2$-terminal DNA binding domain of PARP to its COOH-terminal catalytic domain (16, 22), generating two fragments of $M_r$ ~90,000 and 26,000. It has been previously shown that tumor growth resumed after a delay of 9–15 days. Our results suggest that: (a) Pc 4-PDT is effective in the treatment of SW480 human colon cancer xenografts independent of p53 status; (b) PARP cleavage may be mediated by caspase-9 and caspase-3 activation in the Pc 4-PDT-treated tumors; and (c) p38 phosphorylation may be a trigger of apoptosis in response to PDT in vivo in this tumor model.

INTRODUCTION

PDT, recently approved by the U.S. Food and Drug Administration for the treatment of esophageal and lung cancer, is undergoing clinical trials for the treatment of a variety of solid cancers as well as numerous noncancerous conditions (1). PDT is a novel mode of cancer treatment in which visible light of an appropriate wavelength activates a tumor-associated photosensitizer to produce reactive oxygen (2–5). Although the primary mechanism of tumor regression by PDT is still not completely understood (6, 7), it is known to induce damage to the tumor vasculature and elicits an immune and inflammatory response in the treated tumors (1). PDT causes lipid peroxidation and damage to multiple cellular sites, including membranes, DNA, and cytoskeleton (8, 9), followed by apoptosis in some cell lines (8, 10–14) and apoptosis and rapid regression of solid tumors (1, 5, 15). Apoptosis is characterized by a pattern of PARP cleavage (10, 16–18) distinctive from the pattern observed during necrosis (19). PARP is one of many regulatory and structural proteins that are cleaved during apoptosis after activation of a cascade of caspasases (20, 21). This proteolytic cleavage by caspasases divides the NH$_2$-terminal DNA binding domain of PARP from its COOH-terminal catalytic domain (16, 22), generating two fragments of $M_r$ ~90,000 and 26,000. It has been previously shown that PDT induces cleavage of PARP in murine lymphoma cells (10) and in other cell lines (23, 24).

In cell culture, the rapid oxidative stress induced by PDT results in a complex series of events, including activation of phospholipases and sphingomyelinase and release of the lipid second messengers inositol-1,4,5-trisphosphate and ceramide (25), induction of p21(WAF1/Cip1) (26), activation of nitric oxide synthase and release of nitric oxide (27), activation of stress kinases (8, 28–30) and nuclear factor kB (31), and increased expression of early response genes such as c-fos, c-jun, c-myc, and egr-1 and cellular stress proteins such as GRP-78 and heme oxygenase (4, 32, 33). p38 MAP kinase, also named...
Pc 4-PDT of Colon Cancer Xenografts

Therefore, the goals of this study were: (a) induction of apoptosis by Pc 4-PDT is not known. Several tumor models (15, 41, 42), the mechanism for the damage occurs after light activation (40). Although Pc 4 [HOSiPcOSi(CH₃)₂(CH₂)₃N(CH₃)₂] seems to preferentially localize into the tumor parenchyma where the network that regulates cellular responses to cytokines and stress triggered by osmotic shock, inflammatory cytokines, lipopolysaccharides, UV light, and growth factors (34–38). In cell culture, PDT strongly activates this stress kinase (28, 30, 39).

Mechanisms of PDT may be markedly different when assessed in vitro and in vivo (1) and, further, may differ in rodent tumor models compared with human tumors. Some photosensitizers that are porphyrins or porphyrin derivatives tend to accumulate in cells of the tumor vasculature and upon photoillumination cause various types of damage including stasis, vessel collapse, and vessel leakage (1, 5) in the tumor. In contrast, Pc 4 [HOSiPcOSi(CH₃)₂(CH₂)₃N(CH₃)₂] seems to preferentially localize into the tumor parenchyma where the damage occurs after light activation (40). Although Pc 4-PDT has been shown to be a strong inducer of apoptosis in several tumor models (15, 41, 42), the mechanism for the in vivo induction of apoptosis by Pc 4-PDT is not known. Therefore, the goals of this study were: (a) to evaluate the effectiveness of PDT in a human colon cancer model in vivo; (b) to further evaluate in vivo the participation of signal transduction pathways shown to operate during Pc 4-PDT-induced apoptosis in vitro; and (c) to test the utility of PARP cleavage and other previously observed responses as potential indicators of apoptosis in human tumors treated with Pc 4-PDT. We chose the human SW480 colon cancer model for this study because its growth as a xenograft in athymic nude mice has been well characterized, and it is known to undergo apoptosis in response to topoisomerase inhibitors (43, 44). Furthermore, SW480 colon cancer cells are known to undergo apoptosis in response to PDT with merocyanine-540 and chemotherapeutic agents (44, 45).

**MATERIALS AND METHODS**

**Cell Lines.** SW480 colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing 10% fetal bovine serum at 37°C in an atmosphere of 95% air and 5% CO₂ in a humidified incubator.

**Pc 4 Formulation.** The compound used in this study was obtained from the National Cancer Institute (NSC 676418). The silicon Pc 4 stock solution (1 mg/ml) was prepared as described previously (15, 46) by dissolving Pc 4 in 50% Cremophor EL and 50% absolute ethanol (National Cancer Institute diluent 12), followed by addition of 9 volumes of normal saline while vortexing at low speed. The solution was passed through a 0.22-µm syringe filter, and the precise concentration was determined by absorption spectrophotometry and then aliquoted for storage at −20°C. For injection, this solution was further diluted with an equal volume of 5% Cremophor EL, 5% ethanol, and 90% saline solution.

**Xenograft Generation and Animal Treatment.** Four-week-old female athymic mice (athymic nu/nu) were injected s.c. on the flank with 5 × 10⁶ SW480 colon cancer cells in 0.2 ml of serum-free MEM, as described previously (43, 44). Animals were maintained under pathogen-free conditions. After xenografts reached ~50–100 mm³ in size, the animals were randomized into four groups of four animals (four xenografts) per group to determine tumor growth rate after the following treatments: (a) control, no treatment; (b) 1 mg/kg bw Pc 4 alone; (c) light alone; and (d) PDT, 1 mg/kg bw Pc 4 and light exposure. Pc 4 was delivered via tail vein injection, and 48 h later a 1-cm-diameter area encompassing the tumor was irradiated (power density, 150 mW/cm²; λ, 670 ± 1 nm; fluence, 150 J/cm²) with light from a 250-mW diode laser (Applied Optonics Corp., South Plainfield, NJ) coupled to a 400-µm quartz fiber-optic cable terminating in a micro lens to distribute light uniformly throughout the treatment field. Growth curves representing tumor regrowth for the control and treated groups were estimated by measuring tumors in three dimensions using a caliper. Tumor volume (V) was determined by the following equation: 

\[ V = (L \times W \times H) \times 0.5236 \]

where L is length, W is the width, and H is the height of the xenograft (44).

For biochemical studies, animals were sacrificed at the following times after treatment: 0, 0.08, 0.25, 0.5, 1, 3, 6, 10, 18, and 26 h. Xenografts were resected and frozen instantly in liquid nitrogen and stored at −80°C for further biochemical studies.

**Western Blotting.** Xenografts were pulverized using a mortar and pestle on dry ice and then lysed by sonication in a 1% Triton X-100, 1% NP40, 5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride in ice-cold PBS (all reagents were from Sigma Chemical Co., St. Louis, MO). Samples (35 µg of protein determined by DC Bio-Rad protein assay from Bio-Rad, Hercules, CA) were separated by PAGE consisting of a 5% (w/v) acrylamide stacking gel and a 12.5% (w/v) acrylamide separating gel containing 0.1% SDS (47). The running buffer comprised 0.1% SDS, 25 mM Tris, and 250 mM glycine (pH 8.3). Electrophoretic fractionation was carried out at a constant current of 15 mA until...
bromphenol blue migrated ~10 cm. Proteins were electrotransferred onto an Immobilon p15 membrane (Millipore, Bedford, MA). The filters were blocked with 5% nonfat dry milk in 0.1% Tween 20 in PBS and then incubated overnight at 4°C with 1 μg/ml primary antibody (directed to PARP, caspase-9, caspase-3, p38, phospho-p38, p21/WAF1/Cip1, or actin). The secondary antibody was horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (1:1000 in blocking solution). Bands were visualized with enhanced chemiluminescence reagent and subsequent exposure to Hyperfilm-enhanced solution). Bands were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). The intensity of the bands was quantified by densitometric scanning (SCiScan 5000 USB densitometer; United States Biochemicals, Cleveland, OH) and normalized with respect to actin. The percentage of PARP cleavage was estimated as described previously (44, 45): percentage of PARP cleavage was estimated as described previously (44, 45): percentage of PARP cleaved = intensity of M90,000 PARP band × 100/(intensity of M90,000 PARP band + intensity of M116,000 PARP band).

**Origin of Antibodies.** The monoclonal antibody to purified human PARP (4C10–5) is an IgG1 previously (44, 45): percentage of PARP cleaved = intensity of M90,000 PARP band × 100/(intensity of M90,000 PARP band + intensity of M116,000 PARP band).

The monoclonal antibody to purified human PARP (4C10–5) is an IgG1 that shows specificity for the NAD binding domain of PARP and reacts with an M90,000 degradation product in mitogen-stimulated human lymphocytes (PharMingen, San Diego, CA). The polyclonal antibody to phospho-p38 (Thr-180 and Tyr-182) MAP kinase was from New England Biolabs (Beverly, MA); monoclonal anti-human p38 MAP kinase, mouse anti-human p21 monoclonal, and anti-human caspase-9 monoclonal antibodies were from PharMingen. Monoclonal anti-human caspase-3 antibody was from Transduction Laboratories (Lexington, KY). Peroxidase-linked anti-rabbit and anti-mouse immunoglobulin were from Amersham.

**RESULTS**

**Growth Inhibition of SW480 Xenografts by Pc 4-PDT.** Treatments were performed as described in “Materials and Methods.” Untreated tumors or those receiving Pc 4 alone or light alone grew with a doubling time of ~6 days. Fig. 1 shows the growth rate of untreated xenografts, xenografts treated with Pc 4 alone or light alone, and xenografts exposed to Pc 4-PDT. Treatment with Pc 4-PDT resulted in almost complete tumor ablation in all four xenografts within 26 h after treatment. The curves revealed a growth delay of ~9–15 days in tumor growth in the Pc 4-PDT-treated tumors. Treatment with Pc 4 alone or light alone did not exert any detectable effect on tumor growth.

**Apoptosis in Pc 4-PDT-treated SW480 Xenografts.** To determine whether evidence of apoptosis could be observed in the SW480 xenografts after Pc 4-PDT, animals were sacrificed at various time points after treatment. The xenografts were excised, and lysates were prepared as described in “Materials and Methods.” The time course of the cleavage of PARP, as measured by Western blot analysis, is shown in Fig. 2. Trace amounts of the M90,000 PARP cleavage product were detected in the control untreated samples and those treated with light alone or photosensitizer (Pc 4) alone. In contrast, a gradual increase in levels of PARP cleavage was observed in the first 6 h after PDT with a significant increase of ~60% PARP cleavage at 26 h after treatment. Interestingly, Pc 4-PDT-induced PARP cleavage appeared to be mediated by caspase 9 and caspase-3, which were evaluated in the same blots. Activation of caspase-9 and caspase-3 was shown by proteolytic cleavage of the respective proenzymes after Pc 4-PDT (Fig. 3A). The intensity of the bands corrected to actin as determined by densitometric scanning is shown in Fig. 3B. There was continued processing of both proenzymes over the first 26 h after PDT.

**PDT Induces Phosphorylation of p38 in SW480 Xenografts.** Several laboratories have reported that treatment of cultured cells with PDT promotes the phosphorylation and activation of SAPK/JNK and p38/HOG (28, 30, 39). We have demonstrated that the p38 inhibitor SB202190 can interfere with PDT-induced apoptosis, suggesting that the activation of p38 is necessary for apoptosis in PDT-treated cells in culture (30). Therefore, we have evaluated the possible involvement of p38 in apoptosis by Pc 4-PDT in the SW480 human colon cancer xenografts. Treatment of the xenografts with Pc 4-PDT induced rapid phosphorylation of p38 at Thr-180 and Tyr-182, which could be observed by Western blot analysis in samples taken within 5 min after Pc 4-PDT. Levels of phospho-p38 remained elevated for ~1 h and then gradually decreased to baseline levels within the next 25 h. Control untreated samples or samples treated with light alone or Pc 4 alone showed little or no p38 phosphorylation. Levels of p38 protein remained constant in all samples analyzed (Fig. 4).

**Effect of PDT on p21/WAF1/Cip1 Levels.** Activation of p21 has been observed in human A431 skin carcinoma in
vitro (26) and in human OVCAR-3 xenografts after Pc 4-PDT treatment (15). Therefore, levels of p21 were evaluated by immunoblotting of the same membranes used to detect p38 and phospho-p38. PDT appeared not to affect levels of p21 in SW480 xenografts. Fig. 5 shows that control and treated samples express similar levels of p21.

**DISCUSSION**

The mechanisms by which PDT induces cell death have been extensively evaluated with a variety of photosensitizers in tissue culture and to a lesser extent in animal models (1, 8). Interestingly, apoptosis has been observed in all animal tumors in which it has been sought, arguing that this is an important process contributing to the rapid responses of the tumors to PDT. For Pc 4-PDT, apoptosis has been demonstrated in the early post-treatment hours in a murine transplantable (RIF-1) tumor in C3H mice (41), in an induced skin tumor in SENCAR mice (42), and in a human ovarian tumor xenograft (OVCAR-3) in athymic nude mice (15). In a search for molecular markers of human tumor response to Pc 4-PDT, both PARP cleavage and induction of p21/WAF1/CIP1 were found to be correlated with response to Pc 4-PDT treatment in the OVCAR-3 cancer model, a tumor that is wild type for p53 (15). Among the response markers that have been studied in vitro, both p38 phosphorylation (30) and release of ceramide (25) have been strongly correlated with PDT-induced cell death. In the present report, we have examined the response of the SW480 human colon cancer xenograft, a tumor that expresses mutant p53 (48). The possible role of p53 in PDT response has been examined by Fisher et al. (49) in three cell systems. With either Photofrin or SnET2 as photosensitizer, PDT-induced cell killing was greater for HL60 cells that expressed a transfected wild-type p53 than in HL60 cells with either deleted or mutated p53; however apoptosis was prominent in all three cell lines (49). Comparison of

**Fig. 3** Pc 4-PDT induces caspase-9 and caspase-3 activation. *A*, a time-dependent decrease in the levels of pro-caspase-9 and pro-caspase-3 was detected by Western blotting after Pc 4-PDT treatment. *B*, densitometric scanning of the Hyperfilm ECL indicates gradual decrease of pro-caspase-9 and pro-caspase-3 over the initial 26 h after PDT. Values were corrected to actin as internal standard for protein loading.
two human colon carcinoma cell lines with wild-type (LS513) or mutant (LS1034) p53 for their sensitivity to PDT revealed greater sensitivity of the LS513 line; however, differences between the two lines other than their p53 status may have explained the differential photosensitivities (50). In a recent study (51), abrogation of p53 function by transfection of the HPV16 E6 oncoprotein into the wild-type p53-expressing LS513 colon carcinoma cell line or MCF-7 human breast cancer cell line had little effect on the photosensitivity of the cells. Thus, PDT response appears to be independent of p53 status. Because we have shown tumor regression in the p53 mutant SW480 colon cancer xenografts (Fig. 1) as well as the p53 wild-type OVCAR-3 ovarian cancer xenografts (15), the in vivo tumor response appears to be independent of tumor origin and p53 status as well. Furthermore, Pc 4-PDT increased p21 levels in the OVCAR-3 xenografts (15) but not in the SW480 xenografts (Fig. 5), suggesting that induction of cell death in these tumors may be triggered by a p53- and p21-independent mechanism. However, because we have not compared SW480 xenografts that differ only in their p53 expression, it is possible that introduction of wild-type p53 to SW480 cells might confer additional sensitivity to PDT with respect to induction of apoptosis or overall tumor response. Our results further indicate that Pc 4-PDT caused tumor cells to die by apoptosis, because generation of the M, 90,000 PARP cleavage fragment and caspase-9 and caspase-3 activation were observed in association with tumor regression (Figs. 1–3). Although we have not specifically addressed the contribution of necrosis in the present study, PDT-treated tumors often contain regions of necrosis in addition to apoptosis. Some of the necrosis may represent late stages of apoptosis. However, within the time course of the present study, there was little evidence of PARP fragments at M, 35,000–50,000 that are characteristic of necrosis (19, 44). In addition, despite the fact that the majority of cells constituting a tumor are the transformed cells, the presence of host cells, such as endothelial cells, stromal fibroblasts, and peripheral blood cells, and the responses of those cells are always a concern in measuring apoptosis, because any or all of these cell types may undergo apoptosis. In the present case, although we cannot be certain that only the SW480 cells are undergoing apoptosis, the high percentage of PARP cleavage (>60%) and the marked reduction in tumor size over 26 h after treatment suggest that the bulk of the PARP cleavage derives from the malignant cells.

Although the principal mechanism of tumor regression by Pc 4-PDT is not completely understood, certain mechanisms are being elucidated in cells in vitro. Pc 4 localizes in mitochondria, as well as in other intracellular organelles, of cells in culture (52), and mitochondrial damage appears to play a central role in cell killing by Pc 4-PDT (8, 53), as it does for PDT with other mitochondria-localizing photosensitizers (24). Immediately upon photodamage,
cytochrome c is released from mitochondria into the cytosol of treated cells (53), and the caspase-9 and -3-dependent pathway of apoptosis is activated, leading to PARP cleavage and DNA fragmentation. Using a specific inhibitor of p38/HOG, we have been able to show that inhibition of this stress kinase inhibits apoptosis, providing evidence for a proapoptotic role for p38 (30). Consistent with the in vitro data, we show for the first time in an in vivo model that Pc 4-PDT induces activation of p38/HOG without altering the levels of expression of p38 protein (Fig. 4). Furthermore, as discussed above, this effect must be independent of p53 or p21 activity. The enhanced phosphorylation of p38 in response to Pc 4-PDT suggests a possible role in promoting apoptosis within the colon tumor xenografts. p38/HOG is a member of the SAPK family that also includes JNKs. Both p38 and JNK are strongly activated by PDT (28, 30, 39). Recently, it was shown that in response to genotoxic stresses, SAPK/JNK is translocated from the cytosol to the mitochondria, where it phosphorylates the antiapoptotic protein Bcl-XL (54). Both Bcl-XL and Bcl-2 inhibit PDT-induced apoptosis (55–58), and we showed earlier that overexpression of Bcl-2 confers partial resistance to loss of clonogenicity as well as to induction of apoptosis (55). Both Bcl-XL and Bcl-2 inhibit the release of cytochrome c from mitochondria (59–61). Therefore, one mechanism whereby p38 may promote PDT-induced apoptosis is through a similar translocation of activated p38 to mitochondria, where it may phosphorylate Bcl-2 and/or Bcl-XL and relieve their antiapoptotic effects, thereby facilitating the efflux of cytochrome c from mitochondria. Evidence for such a mechanism is currently being sought in in vitro studies. The results shown in this report are encouraging toward future clinical applications of Pc 4-PDT for colon cancer and/or colon cancer metastases.

ACKNOWLEDGMENTS

We thank Elizabeth Zborowska and Karl J. Mann for technical assistance.

REFERENCES


Photodynamic Therapy with the Phthalocyanine Photosensitizer Pc 4 of SW480 Human Colon Cancer Xenografts in Athymic Mice

Cecilia M. Whitacre, Denise K. Feyes, Taroh Satoh, et al.