Niemann–Pick Human Lymphoblasts Are Resistant to Phthalocyanine 4-Photodynamic Therapy-Induced Apoptosis

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Stress-induced activation of sphingomyelinase (SMase) leading to generation of ceramide, a lipid mediator, has been associated with apoptosis in several malignant and nonmalignant cell lines. Photodynamic therapy (PDT), with the phthalocyanine photosensitizer Pc 4 [HOSiPcOSi(CH₃)₂(CH₂)₃N(CH₃)₂], is an oxidative stress associated with increased ceramide generation and subsequent induction of apoptosis in various cell types. We assessed the role of SMase in photocytotoxicity. Normal human lymphoblasts accumulated ceramide and underwent apoptosis after Pc 4-PDT. In contrast, Niemann–Pick disease (NPD) lymphoblasts, which are deficient in acid sphingomyelinase (ASMase) activity, failed to respond to Pc 4-PDT with ceramide accumulation and apoptosis, suggesting that ASMase may be a Pc 4-PDT target. NPD lymphoblasts were exposed to exogenous bacterial sphingomyelinase (bSMase) to test whether these defects are reversible. Treatment of NPD cells with bSMase itself led to elevated ceramide formation, which did not translate into induction of apoptosis. However, a combination of Pc 4-PDT + bSMase induced a significant apoptotic response. Thus, the combined treatment of Pc 4-PDT + bSMase, rather than bSMase alone, was required to restore apoptosis in NPD cells. These data support the hypothesis that SMase is a proapoptotic factor determining responsiveness of cells to Pc 4-PDT.© 1999 Academic Press

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Photodynamic therapy is a cancer treatment that uses a photosensitizer, light and oxygen to generate singlet oxygen, as well as other reactive oxygen species (ROS). The formation of ROS in cellular targets results in cell death and tumor ablation (1). PDT, as an oxidative stress, initiates apoptosis in vitro (2) and in vivo (3). Stress inducers, such as tumor necrosis factor-α, ionizing or UV radiation, heat shock, and H₂O₂, initiate apoptosis via ceramide generation (4–6). We have demonstrated that Pc 4-photodynamic treatment of LY-R mouse lymphoma, U937 human leukemia, or CHO Chinese hamster ovary cells leads to elevated intracellular ceramide levels and subsequent induction of apoptosis. Furthermore, exogenous cell-permeable C2-ceramide mimics the effect of Pc 4-PDT, as evidenced by DNA fragmentation in these cells (7, 8).

Sphingomyelinase (SMase), the enzyme that catalyzes the initial step in the sphingomyelin pathway, can be activated in response to pro-apoptotic stimuli, resulting in the generation of ceramide (9). Several isoforms of SMase have been identified, two of which have been cloned (10, 11). The enzymes are distinguished based upon their pH optima, cellular localization and cation dependence (12). Acid sphingomyelinase (ASMase) requires zinc for its activity and can be found in lysosomes or be secreted, depending on post-translational processing (13, 14). ASMase, as well as...
neutral SMase (NSMase), have been associated with apoptosis in a variety of cells involving various receptors. However, in human lymphoblasts ASMsase alone appears to be involved in apoptosis induced by ionizing radiation and Fas (15, 16). To test the role of SMase in Pc 4-PDT-induced apoptosis, cultured Niemann-Pick disease (NPD) human lymphoblasts were used. NPD is an autosomal recessive disorder caused by loss-of-function mutations within the ASMsase gene (10). We demonstrate that ASMsase deficiency correlates with suppressed ceramide generation and apoptosis after Pc 4-PDT. The defect in apoptotic response is reversed by the combined treatment of Pc 4-PDT and bacterial sphingomyelinase (bSMase).

MATERIALS AND METHODS

Materials. The phthalocyanine photosensitizer Pc4, HOSIPDSI-(CH3)3(CH3)2N(CH3)2 (17), was supplied by Drs. Ying-syi Li and Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). E. coli sn-1,2-DAG kinase were from Calbiochem (La Jolla, CA), while sphingomyelinase (S. aureus) was from Sigma (St. Louis, MO). Fas ligand was from Chemicon International (Temecula, CA). The horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). TLC plates (aluminum sheets of silica gel 60) were from EM Industries (Gibbstown, NJ).

Cell culture and treatments. Both normal and NPD human lymphoblasts (MS1418) were Epstein–Barr virus (EBV)-transformed and were kindly provided by Drs. Edward H. Schuchman and Adriana Haimovitz-Friedman. The cells were cultured in RPMI 1640 supplemented with 5 mM L-glutamine and 15% heat-inactivated fetal bovine serum (FBS). The cells were grown in T-25 flasks maintained upright at 37°C under 5% CO2 and were subcultured twice weekly at a density of 2 × 10^6/mL. The lymphoblasts were not maintained in continuous culture for more than two months. For experiments, an aliquot of a stock solution of Pc 4 (0.5 mM in DMF) was added to the cells (5 × 10^6) in T-25 culture flasks containing 10 mL of a low serum medium (1% FBS + RPM) to give the desired concentration of 200 nM. After overnight incubation, the cells were irradiated using an LED array (EFOS, Mississauga, Ontario, Canada; λmax = 670-675 nm) and then incubated at 37°C for desired periods of time before harvest. In experiments in which bSMase was used, the enzyme was added to the cells in 0.1% FBS + RPM medium. Membrane integrity of either of the two cell lines was not significantly affected by the treatments, as assessed by trypan blue dye exclusion.

TUNEL assay (APO-DIRECT) and flow cytometry. The procedures for cell fixation and staining with fluorescein isothiocyanate (FITC)/dUTP, using terminal deoxynucleotidyl transferase (TdT), and propidium iodide (PI), were performed according to the manufacturer's instructions (Phoenix Flow Systems, San Diego, CA). Cells were analyzed by an EPICS ESP flow cytometer (Coulter Corp., Hialeah, FL) in the Flow Cytometry Facility of the Case Western Reserve University/Ireland Comprehensive Cancer Center. Fluorescence measurements were made using the following parameters: 488 nm (excitation), 520 nm (Fluorescein emission) and 623 nm (PI emission). Data analysis was performed using version 4.1 of the instrument software.

Measurement of ceramide level. Ceramide mass was determined by the DAG kinase assay as described previously (8). Following Pc 4-PDT treatment and incubations, cells were extracted with 2 mL chloroform/methanol/1 M HCl (500/500/5, v/v/v). Lipids in the lower phase were dried under nitrogen and subjected to alkaline hydrolysis (0.1 M methanolic KOH for 1 h at 37°C) to remove glycerophospholipids. Samples were reextracted, and lipids from the chloroform-phase extract were quantified by the DAG kinase reaction. Ceramide was resolved by TLC using chloroform/methanol/acetonitr/glacial acetic acid (50/15/20/10, v/v/v), and quantified using a PhosphorImager 445 Si (Molecular Dynamics, Sunnyvale, CA) and by comparison to a concomitantly run standard curve comprised of known amounts of ceramide.

Western immunoblot analyses. Whole cell extracts were prepared by direct lysis of PBS-washed cells in lysis buffer [6 M urea, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8)], sonicated with a fifteen second burst using a Fisher 550 Sonic dismembrator. Equal amounts of protein were heated at 65°C for 10 min and separated by 10% SDS-PAGE. Separated proteins were transferred to Immobilon-P (Millipore, Bedford, MA), while fetal bovine serum was from Intergen (Purchase, NY). The filters were allowed to dry and were placed in scintillation vials. The radioactivity was counted in a L3801 Beckman Liquid Scintillation Counter (Fullerton, CA).

RESULTS AND DISCUSSION

The apoptotic response of the two lymphoid cell lines to Pc4-PDT was assessed using the TUNEL assay and flow cytometry. Pc4-PDT induced a dose- and time-dependent increase in apoptosis in normal lymphoblasts (Fig. 1). Apoptosis was not detected before 2 h at the lowest PDT dose (200 nM Pc 4 + 45 mJ/cm^2; data not shown) and was maximal by 6 h at the highest PDT dose (200 nM Pc 4 + 90 mJ/cm^2; data not shown). In contrast, apoptosis was significantly suppressed in the NPD cells after Pc 4-PDT. An increase in the NPD apoptotic cell population was only detected at the highest PDT dose (200 nM Pc 4 + 90 mJ/cm^2) 6 h after treatment. Curiously, the apoptotic response of the normal human lymphoblasts to ionizing radiation (15) and Pc 4-PDT was different, since (i) the peak effect induced by radiation and Pc 4-PDT in these cells was approximately 40 and 90%, respectively, and (ii) the onset of the process was detected by 8 and 2 h, respectively. The data indicate that Pc 4-PDT is a more effective inducer of apoptosis than ionizing radiation. These differences suggest potential underlying mechanistic differences between radiation- and Pc 4-PDT-induced apoptosis.
To assess whether the deficiency in apoptosis in NPD cells correlated with alterations in ceramide formation, the kinetics of ceramide generation in response to Pc 4-PDT were measured in both cell lines. Following exposure of normal lymphoblasts to PDT (200 nM Pc 4 + 67 mJ/cm²), ceramide was elevated above control levels by 66, 67, 39 and 24% at 5, 10, 15, and 30 min, respectively (Fig. 2). However, ceramide accumulation was significantly suppressed in NPD cells after Pc 4-PDT. Since NPD lymphoblasts have a normal level of NSMase activity (15) and express only 2% or less residual ASMase activity (15), our data indicate that abnormalities in apoptosis and ceramide generation in these cells post-Pc 4-PDT correlate with the loss of ASMase function. These results agree with the similar defects observed in NPD cells in response to ionizing radiation (15). By contrast, Boesen-deCock et al. (19) showed that ASMase is not required in anti-Fas-induced ceramide formation and apoptosis in NPD cells. Perhaps the difference in the responses to two different stimuli, radiation and anti-Fas, is due to phenotypic changes that can occur upon continuous in vitro culture of NPD cells (19).

Treatment of cells with bSMase results in generation of ceramide with a subsequent restoration of the lost ceramide responses, such as apoptosis (20). To test if bSMase can restore apoptosis in NPD lymphoblasts after Pc 4-PDT, the cells were treated with the exogenous enzyme. While bSMase alone (50 mU/mL) did not induce apoptosis, the combined treatment, bSMase + Pc 4-PDT, initiated the process in a PDT dose-dependent fashion in NPD cells (Fig. 3A). Similar results were also obtained in the presence of higher doses of bSMase (300 or 500 mU/mL) ± Pc 4-PDT (data not shown). In normal cells, in contrast to Pc 4-PDT, bSMase itself (50 mU/mL), did not induce apoptosis. In the presence of both treatments the extent of apoptosis was not further increased. The combined treatment was more effective in generating an apoptotic cell population in normal lymphoblasts than in NPD cells. Perhaps ASMase is a critical Pc 4-PDT target for the induction of apoptosis, and the absence of a functional ASMase in NPD cells may account for the observed difference.

We then examined whether the synergistic apoptotic response to the combined treatment of Pc 4-PDT + bSMase would translate into altered ceramide formation in NPD cells. bSMase alone induced a 7.7-fold increase in ceramide levels (Fig. 3B), without subsequent apoptosis (Fig. 3A). Following combined Pc 4-PDT and bSMase treatments, ceramide levels were elevated to the same extent as with bSMase alone and apoptosis was induced. In normal lymphoblasts bSMase alone increased ceramide levels 7.4-fold above baseline (Fig. 3B). However, exogenous bSMase did not induce apoptosis (Fig. 3A), suggesting that the ceramide effectors and the primary components of the apoptotic machinery are out of reach. The combined treatment with Pc 4-PDT + bSMase did not further increase ceramide accumulation beyond that observed.
in the presence of bSMase alone. Curiously, a smaller ceramide increase, ranging from 35% to 82%, correlated with a significant and a dose-dependent induction of apoptosis in normal cells post-Pc 4-PDT. Perhaps ceramide must reach a threshold concentration at a critical Pc 4-PDT target, e.g. the ASMase pool, to trigger apoptosis.

To confirm the observed apoptotic changes following treatment, another apoptotic marker was used. Cysteine-aspartate specific proteases (caspases) are central mediators of the late stages of apoptosis (21). PDT (22), as well as ceramide (23), can activate these enzymes. In particular, caspase-3, the protease that cleaves poly(ADP-ribose) polymerase (PARP) into 89 and 24 kDa fragments from the 113 kDa full-length peptide, is activated post-PDT (22). Here we show that after treatment of normal lymphoblasts with Pc 4-PDT ± bSMase, PARP cleavage was induced (Fig. 4). In contrast, only the highest PDT dose (200 nM Pc 4 + 90 mJ/cm²) induced partial PARP cleavage in NPD cells. In addition, a low level of cleavage product appeared following treatment of NPD cells with Pc 4-PDT + bSMase. However, bSMase itself (50 mU/mL) did not induce PARP cleavage in either of the two cell lines. These results are in agreement with previous observations that treatment with exogenous bSMase did not cause apoptosis when administered without Pc 4-PDT.

To rule out the possibility that bSMase could have affected the uptake of Pc 4, leading to altered photocytotoxicity, both cell lines were treated with [14C] Pc 4 (200 nM ± bSMase (500 mU/mL) for 15 h, and the level of cell-associated Pc 4 was assessed. Cell uptake was similar in the two cell lines and was not altered by the presence of bSMase (data not shown).

The observations that bSMase-induced ceramide accumulation did not result in apoptosis and that in NPD cells a potentiated apoptotic response to the combined treatment did not lead to altered ceramide generation beyond that induced by bSMase itself suggest that the effect of bSMase alone is insufficient to initiate the death process. The inability of bSMase to induce apoptosis under conditions effectively generating ceramide has been shown (24, 25). Ceramide generated at the plasma membrane by bSMase did not trigger apoptosis probably because: (i) bSMase-generated ceramide is found in a plasma membrane compartment that is distinct from specialized plasma membrane domains (caveolae) where the "signaling" ceramide is formed (26); (ii) ceramide formed by bSMase in the outer leaflet of the cell membrane is unavailable to intracellular apoptotic effectors (24); or (iii) the high levels of ceramide are converted or degraded into non- or anti-apoptotic agents, such as glucosylceramide (27) or sphingosine-1-phosphate (28).

The mechanism(s) underlying the synergistic apoptotic responses in NPD cells following treatment with Pc-4-PDT + bSMase have yet to be determined. Several possibilities are suggested. First, Pc-4-PDT may affect membrane fluidity in such a way that flipping of
ceramide from the outer to the inner leaflet is facilitated, allowing the lipid interaction with the downstream targets involved in signaling and/or execution of apoptosis. Second possibility is that ceramide generation does not suffice for triggering apoptosis and that additional signals are needed (e.g., ROS) to induce the death machinery. Conversely, positive feedback loops may have to be activated, i.e. ceramide stimulates ROS production (29), and ROS in turn activates ceramide formation leading ultimately to initiation of apoptosis.

The hypothesis that ceramide signals Pc-4-PDT-induced apoptosis in normal human lymphoblasts is supported by the observation of an early rise (i.e., 5 min after PDT) in ceramide levels that preceded the ap-
pearance of apoptotic cells (2 h post-PDT). Similar data were obtained for other cells (7, 8). If ceramide mediates apoptosis in response to Pc-4-PDT, the downstream targets of ceramide need to be identified. PDT, like other stresses (15, 30), stimulates the SAPK/JNK cascade (31). Thus, the SAPK/JNK signaling pathway may play a role in Pc-4-PDT-induced ceramide-mediated apoptosis. In addition, a variety of evidence implicates mitochondrial damage and the release of cytochrome c as key events in PDT-induced apoptosis (22). Ceramide may participate by enhancing the level of oxidative damage to mitochondria (32).

Caspases are potential downstream ceramide targets, since apoptosis is blocked by caspase inhibition, while induced ceramide accumulation is not (20, 33). These findings, however, have been challenged by observations that caspase inhibitors can prevent accumulation of the lipid (34). More direct studies using caspase knockout models, are required to assess the role of ceramide in caspase activation and subsequent apoptosis.

The aim of the present study was to assess the role of SMase in Pc 4 photocytotoxicity. The data suggest that ASMase may be a Pc 4-PDT target, since ceramide formation correlates with Pc-4-PDT-induced apoptosis in normal lymphoblasts, but not in NPD cells. The attempt to reverse the abnormalities in NPD cells using bSMase led to potentiated apoptosis without augmented ceramide generation beyond that induced by bSMase alone. These novel observations are consistent with the notion that SMase is a proapoptotic factor determining responsiveness of cells to Pc4-PDT. More extensive investigations using other photosensitizers are required to test whether ASMase is a general photocytotoxic target. Further and more direct elucidation of the role of SMase in PDT cytotoxicity may suggest methods to improve the therapeutic efficacy of the treatment.

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