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Original Contribution

Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage

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ABSTRACT

Lysosomal photosensitizers have been used in photodynamic therapy. The combination of such photosensitizers and light causes lysosomal photodamage, inducing cell death. Lysosomal disruption can lead to apoptosis but its signaling pathways remain to be elucidated. In this study, *N*-aspartyl chlorin e6 (NPe6), an effective photosensitizer that preferentially accumulates in lysosomes, was used to study the mechanism of apoptosis caused by lysosomal photodamage. Apoptosis in living human lung adenocarcinoma cells (ASTC-a-1) after NPe6–photodynamic treatment (NPe6–PDT) was studied using real-time single-cell analysis. Our results demonstrated that NPe6–PDT induced rapid generation of reactive oxygen species (ROS). The photodynamically produced ROS caused a rapid destruction of lysosomes, leading to release of cathepsins, and the ROS scavengers vitamin C and NAC prevent the effects. Then the following spatiotemporal sequence of cellular events was observed during cell apoptosis: Bcl-2-associated X protein (Bax) activation, cytochrome *c* release, and caspase-9/-3 activation. Importantly, the activation of Bax proved to be a crucial event in this apoptotic machinery, because suppressing the endogenous Bax using siRNA could significantly inhibit cytochrome *c* release and caspase-9/-3 activation and protect the cell from death. In conclusion, this study demonstrates that PDT with lysosomal photosensitizer induces Bax activation and subsequently initiates the mitochondrial apoptotic pathway.

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Photodynamic therapy is a minimally invasive therapy for cancer treatment, using a photosensitizer and light irradiation [1–3]. Many factors affect its efficacy, one of which being the subcellular localization of the photosensitizer. Researchers therefore have tried to improve the localization of photosensitizers in various cell organelles, including mitochondria, lysosomes, Golgi apparatus, and endoplasmic reticulum (ER) [4–6]. It is generally accepted that the location of the sensitizer coincides with the primary site of photodamage [7–10]. This is because the photogenerated singlet oxygen ($^1O_2$) has a short life and a limited diffusion path in biological systems (half-life <0.04 μs, radius of action <0.02 μm), indicating that primary molecular targets of the photodynamic process must reside within a few nanometers of the photosensitizer [11]. Recently, many lysosomal photosensitizers have been used in photodynamic therapy [1,2]. The combination of light and such photosensitizers causes lysosomal photodamage, inducing cell death. NPe6, a powerful and naturally occurring photosensitizer, preferentially accumulates in lysosomes. Recent reports have shown that irradiation of cells preloaded with NPe6 results in lysosomal disruption, dispersion of lysosomal proteases throughout the cytosol, cleavage/activation of BH3-interacting-domain death agonist (Bid), release of cytochrome *c*, and activation of downstream caspases [12–16]. The mechanism of NPe6–PDT-induced apoptosis is complicated; although activation of Bid has been shown to be involved in the induced apoptosis, other factors, such as Bax, could also play an important role.

Bax, a proapoptotic protein of the Bcl-2 family, is a key regulator of apoptosis. As reported in many apoptotic paradigms, Bax mainly resides in the cytosol of healthy cells, in an inactive state. Upon apoptotic stimulation, Bax undergoes specific conformational changes, integrates into the outer mitochondrial membrane (OMM), and oligomerizes. These oligomers are thought to induce permeabilization of the OMM, allowing the efflux of apoptogenic proteins [17–19]. Consistently, relocation of Bax from the cytosol to the mitochondria has been reported in various photodynamic therapy paradigms to occur with kinetics matching the release of cytochrome *c* [20–22]. The

Abbreviations: AIF, apoptosis-inducing factor; Bax, Bcl-2-associated X protein; Bid, BH3-interacting-domain death agonist; CFP, cyan fluorescent protein; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; H_2DCFDA, dichlorodihydrofluorescein diacetate; LD_{50}, 50% lethal dose; LD_{90}, 90% lethal dose; NAC, *N*-acetylcysteine; NPe6, *N*-aspartyl chlorin e6; OMM, outer mitochondrial membrane; PBS, phosphate-buffered saline; PDT, photodynamic treatment; RFP, red fluorescent protein; RNAi, RNA interference; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential.

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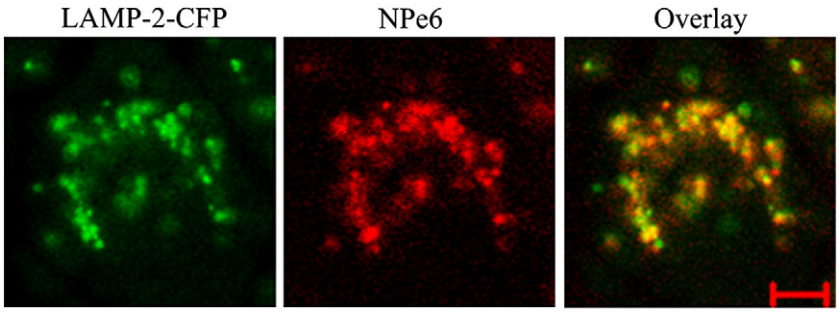

Fig. 1. Localization of NPe6 in ASTC-a-1 cells. ASTC-a-1 cells were transfected with LAMP-2–CFP and loaded with NPe6. The fluorescence of LAMP-2–CFP (left) and NPe6 (middle) was visualized by confocal microcopy. The overlay fluorescence image (right) of LAMP-2–CFP and NPe6 indicates that NPe6 is primarily localized in the lysosomes. Scale bar, 10 μm.

extent of Bax involvement in PDT-induced apoptosis depends on a number of factors, including the nature of the photosensitizer and the cell type that is being affected. The apoptotic response of Pc4-mediated PDT was studied in human breast cancer cells (MCF-7c3) treated with Bax antisense oligonucleotides to suppress Bax expression and in the human prostate cancer DU-145 cells, which do not express Bax. In these photosensitized cells the hallmarks of apoptosis, including cytochrome *c* release, caspase activation, and nuclear fragmentation, were inhibited, whereas restoration of Bax expression in DU-145 cells reconstituted these events and precipitated apoptosis [23]. Furthermore, reexpression of mitochondria-targeted Bax is both necessary and sufficient to fully restore caspase activation and apoptotic cell death after hypericin–PDT in apoptosis-deficient $Bax^{-/-}/Bak^{-/-}$ MEF cells [20]. Whether Bax plays an important role in apoptosis induced by lysosomal photodamage is still unknown.

Two separate pathways leading to procaspase activation have been clearly documented: the death receptor-mediated pathway (or extrinsic pathway) and the mitochondria-mediated pathway (or intrinsic pathway) [24,25]. In either pathway, the activation of initiator caspases (caspase-8 or caspase-9) leads to the activation of effector caspases (caspase-3, -6, and -7). The critical role of the mitochondrial pathway in PDT-induced apoptosis has been largely documented [1,2,10]. PDT causes release of mitochondrial apoptogenic proteins into the cytosol, followed by the caspase activation cascade, leading to an apoptotic morphotype. This is especially true for those photosensitizing agents with preferential mitochondrial localization [1,2,7]. Other recent studies have shown that mitochondria are also critical executers of lethal pathways emanating from photodamage to other subcellular sites or organelles [1,2,10,13].

The aim of this study was to investigate the molecular mechanisms of apoptosis induced by lysosomal photodamage. We focused, in particular, on the molecular involvement of Bax in this PDT protocol. By using real-time single-cell analysis, the following events in response to NPe6–PDT were investigated: generation of ROS, disruption of lysosomes, activation of Bax, release of cytochrome *c*, loss of mitochondrial membrane potential ($\Delta\Psi_m$), and activation of caspases.

Materials and methods

Cell culture and transfection

The human lung adenocarcinoma cell line ASTC-a-1 was obtained from the Department of Medicine, Jinan University (Guangzhou, China) and cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 15% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) in 5% CO_2 at 37 °C in a humidified incubator. Transfections were performed with Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The solution of Lipofectamine reagent was replaced with fresh culture medium after 5 h. Cells were examined 36 h after transfection.

Photodynamic treatment

Cells (1×10^4 per well) growing in 35-mm petri dishes were incubated in the dark at 37 °C with 50 μM NPe6 (Light Science Corp., USA) for 1 h. After the culture medium was removed, the cells were rinsed with phosphate-buffered saline (PBS) and then irradiated with a semiconductor laser at 635 nm (NL-FBA-2.0-635, Shanghai, China). The laser light was delivered using an optical fiber (core diameter 400 mm) with a custom distal microlens. The laser power was measured using a laser power meter (FieldMate; Coherent, Santa Clara, CA, USA). The light intensity was set at 2 mW/cm^2.

Time-lapse confocal fluorescence microscopy

Cellular GFP, CFP, H_2DCFDA, fluorescein isothiocyanate (FITC), rhodamine 123, and MitoTracker red were monitored confocally using a laser-scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3 NA oil DIC objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows. GFP, H_2DCFDA, FITC, and rhodamine 123 were excited at 488 nm with an

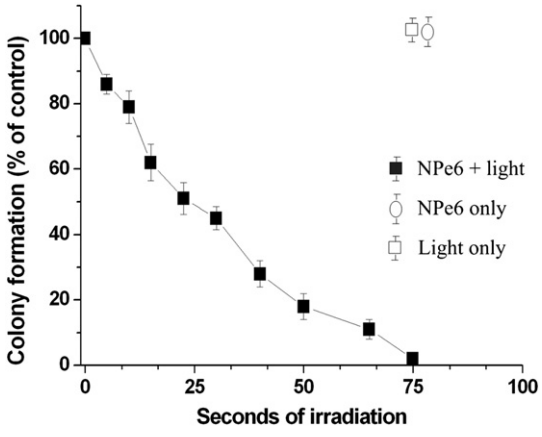

Fig. 2. NPe6 cytotoxicity as assessed in colony-forming assays. ASTC-a-1 cells were plated at densities of 500–1200 cells per 60-mm dish. Approximately 24 h later cultures were loaded with 50 μM NPe6 for 1 h before being washed and refed, and then the cells were irradiated. After the indicated times of irradiation, cultures were returned to the incubator. Colonies were counted 8–10 days after irradiation. Data represent means ± SD of three plates per treatment group.

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