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Lamin A/C cleavage by caspase-6 activation is crucial for apoptotic induction by photodynamic therapy with hexaminolevulinate in human B-cell lymphoma cells

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ABSTRACT

Photodynamic therapy (PDT) with a light-activated drug is an approved modality for cancer treatment. Hexaminolevulinate (HAL), a hexylester of 5-aminolevulinic acid as the photosensitising protoporphyrin IX (PpIX) precursor, is clinically used for both PDT and photodetection. Our previous studies have shown that HAL–PDT can effectively induce apoptosis in several human blood malignant cell lines. However, the mechanisms involved in the apoptotic induction are still not fully elucidated. In this study we have focused on the role of cellular lamin A/C in the apoptotic induction. HAL–PDT-mediated apoptosis was confirmed by various techniques including fluorescence microscopy and electron microscopy in both human B-cell lymphoma Ramos and Daudi cell lines. The lamin A/C, together with caspases-6 and -3, was cleaved during the apoptosis. Western blots, immunocytochemistry, fluorescence microscopy and electron microscopy demonstrated that the specific caspase-6inhibitor abrogated the HAL-PDT-mediated cleavages of both caspase-6 and lamin A/C and subsequent apoptosis in these two cell lines, suggesting that the cleavage of lamin A/C by the caspase-6 activation is crucial for such apoptotic induction.

1. Introduction

Photodynamic therapy (PDT), a modality using a light-activated drug, is based on the energy transfer from the photosensitiser to oxygen to generate cytotoxic singlet oxygen. PDT with protoporphyrin IX (PpIX), endogenously induced from 5-aminolevulinic acid (ALA) or its esters via heme biosynthetic pathway, has shown to be an effective and safe treatment for several premalignant and malignant diseases [1–3]. However, the mechanisms of action on cell-killing are still not fully elucidated.

Apoptosis is well defined with morphological and biochemical changes of the cell nucleus including chromatin condensation (pyknosis), nucleosomal-sized DNA fragmentation (DNA laddering) and nuclear fragmentation (karyorrhexis) [4]. Active cytosolic aspartate-specific (cysteine) proteases, named caspases, are primarily responsible for such characteristic features of the nucleus.

Although more than 14 caspases have been identified [5], due to their structural homology only caspases-3, -6 and -7 are classified as executioner caspases responsible for cleaving downstream target polypeptides causing apoptotic cell death [6–8]. The role of caspase-3 in apoptotic execution has widely been investigated, involving the cleavage of poly(ADP-ribose) polymerase [9] as well as the release of the active DNA fragmentation factor [10]. However, the specific role of caspase-6 is poorly understood with conflicting information. In some reports the caspase-6 activation depends upon the caspase-3 activation [11,12]; while in others does not [13] or takes place before the caspase-3 activation [14]. The caspase-6 has been reported to exclusively cleave the lamin A/C responsible for typical apoptotic nuclear morphology [15].

Nuclear lamina is a dense network of lamin filaments and lamin-associated proteins, both of which are physically associated with the inner nuclear membrane and peripheral chromatin. Besides regulating important cellular events such as DNA replication, the nuclear lamina also provides mechanical support and determines chromatin organization [16]. Lamins are classified as types

Abbreviations: ALA, 5-aminolevulinic acid; HAL, hexaminolevulinate; PDT, photodynamic therapy; PpIX, protoporphyrin IX; TdT, terminal deoxynucleotid transferase.

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A/C and B. The two types differ in their biochemical properties [17]. The type A/C has been shown to form functional association with polynucleosomes, histones and DNA [18].

In the present report we have investigated the effect of caspase-6 activation on the lamin A/C cleavage during apoptosis in the human Ramos and Daudi B-cell lymphoma cell lines after PDT with hexaminolevulinate (hexylester of ALA), a PpIX precursor used for both clinical PDT and photodetection [19,20].

2. Materials and methods

2.1. Chemicals

Hexaminolevulinate (HAL) was kindly provided by Photocure ASA (Oslo, Norway). Stock solution of 12 mM was freshly prepared for each experiment. The pan-caspase inhibitor (InSolution™ Q-VD-OPh, Non-O-methylated) and caspase-6 inhibitor-I were from Calbiochem® (USA). The anti-cytochrome C antibody was from Santa Cruze Biotechnology (Santa Cruz, CA) and the secondary antibody conjugated with DyLight®549 from AbD seroTec (Oxford, UK). All other antibodies used in this work were from Cell Signaling (Beverly, MA).

2.2. Cell culture

Human B-cell lymphoma cell lines, Ramos and Daudi, were subcultured every second day in RPMI-1640 medium (PAA Laboratories GmbH, Fisher Scientific, Norway) containing 10% fetal bovine serum (FBS) (Saveen & Werner, Oslo, Norway), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine (Gibco, Invitrogen, Norway) at 37 °C in a 5% CO_2 humidified incubator. All experiments were carried out at a cell density of 8×10^5/ml.

2.3. PDT with HAL

Ramos and Daudi cells were incubated for 4 h in the dark in serum-free RPMI 1640 medium containing 5 or 20 μM of HAL. The cells were then exposed to the light from a bank of four fluorescent tubes (model 3026, applied Photophysics, London, UK) emitting light mainly in the region of 410–500 nm with a maximum around 440 nm. The fluence rate of the light reaching the cells was 12 mW/cm^2. The light doses were 0.18 J/cm^2 for Ramos cells and 0.78 J/cm^2 for Daudi cells, respectively. After irradiation the medium was immediately replaced with HAL-free medium containing 10% FBS. In some experiments the inhibitor of pan-caspase or of caspase-6 (20 μM) was added to the samples before and after irradiation.

2.4. Assessment of apoptotic cells

Typical nuclear morphology of apoptotic cells such as chromatin condensation, chromatin margination and nuclear fragmentation was assessed by fluorescence microscopy after staining cells with 4 μg/ml Hoechst 33342 (H342) (Sigma, USA) at 37 °C for 10 min. The percentage of apoptotic cells was calculated by counting at least 200 cells in each sample and each experiment was always done in triplicate. Such counting followed the apoptotic verification by electron microscopy as described below. Furthermore, DNA fragmentation was determined in control and treated samples by using the Apoptotic DNA Ladder Kit (Life technologies, USA) according to the manufacture's instructions. An equivalent amount of DNA from samples at various times after HAL–PDT were resolved on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide in Tris–borate ethylenediaminetetraacetic acid (EDTA) (1xTBS). The bands had been visualised under an UV Tran-illuminator before the photographs were taken.

2.5. Electron microscopy

Control and PDT-treated cells were washed with phosphate buffered saline (PBS), fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 2% OsO_4 and 1.5% KFeCN, followed by the staining with 1% uranyl acetate. The cells were then dehydrated through a graded ethanol series and embedded in Epon/Araldite mixture. Semithin sections were cut with glass knives, mounted on glass slides and stained with toluidine blue; and observed under a light microscope for the orientation. Ultrathin sections were cut with diamond knives, floated onto a 100-mesh copper grid, stained with uranyl acetate and lead citrate, and finally examined by transmission electron microscopy (TEM) (JEOL-JEM 1230 at 80 kV). The TEM images were recorded on a digital camera (Morada, Olympus, Tokyo, Japan) and further processed using the Adobe Photoshop software.

2.6. Caspase-3 activity

The activity of caspase-3 was measured with a colorimetric assay kit (Chemicon, Temecula, CA). Subsequent to PDT (4 h for Ramos and 10 h for Daudi; respectively) the cells were collected and suspended in lysis buffer at 0 °C for 10 min. Lysates were centrifuged to precipitate cellular debris. The amount of protein in the lysates was determined. 150 μg of the lysate proteins from each sample were incubated at 37 °C for 3 h in a reaction buffer containing caspase-3 substrate (N-acetyl-Asp-Glu-val-Asp(DEVD)-p-nitroanilide) according to the manufacture's instruction. Absorbance at the 405 nm was read on a microtiter plate reader (Multiskan Ex, Labsystems, Finland).

2.7. Western blot

Control and PDT-treated cells (8×10^8) at various times after HAL-PDT were collected by centrifugation. After being washed once with cold PBS, the pellets were resuspended in 200 μl lysis buffer (Sigma, St. Louis, MO), followed by incubation on ice for 30 min and sonication for 15 s to obtain whole-cell extracts. For the cytosolic fraction the cell pellets were resuspended in 200 ml HEPES medium containing digitonin (20 mg/ml) (Sigma, St. Louis, MO), incubated for 5 min at room temperature and then 30 min on ice to permeabilize the cells to leak their cytosolic proteins into the medium. The cells were finally centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was collected as the cytosolic fraction. The whole-cell extracts and cytosolic fractions were kept at −70 °C before use. Total proteins were quantified by the Bradford method using the bovine serum albumin (BSA) protein assay kit (Pierce, Inc., Rockford, IL) according to the protocol. An equal amount of proteins (20 μg per lane) from the whole-cell lysates and cytosolic fractions was electrophoresed on SDS–polyacrylamide gels (10–15%) and the gel-separated proteins were transferred to polyvinylidene difluoride membranes by a semi-wet transfer apparatus (Bio-Rad, CA). The membranes had been washed once (5 min) with 0.1% TBS-Tween, blocked with 5% non-fatty milk in the TBS-Tween for 1 h at room temperature before they were probed overnight at 4 °C with primary antibodies. The membranes were then washed three times and incubated for 1 h at room temperature with an anti-mouse or anti-rabbit secondary antibody. Finally, they were visualized using a chemiluminescence detection kit, ECL-PLUS (Amersham Biosciences, Piscataway, NJ). The recognition of β-actin with its antibody (1:5000 dilution) allowed reconfirmation of the total amount of proteins loaded on the gels.

2.8. siRNA and transfection

Two siRNA sequences targeting lamin A/C, (NM_005572) (5′-CAGGCAGTCTGCTGAGAGGAA-3′ and 5′-CCCACCAAAGTTCACCCTGAA-3′) and one irrelevant siRNA (control) sequence (5′-UUGAUGUGUUUAGUCGCUA-3′) were purchased from Qiagen (USA). Transfections of both cell lines with siRNAs were performed using electroporation technique as described previously [21]. Briefly, 2.5×10^6 cells were suspended in 0.5 ml serum-free RPMI-1640 medium containing 2 μg siRNA using the BTX electroporation apparatus in a 4-mm BTX cuvette and placed on ice for 5 min. The cells were then pulsed at 500 V for 2 ms. After transfection the cells were diluted in 2.5 ml of pre-warmed medium and incubated at 37° in 5% CO^2 for 48 h. After that the protein extracts were prepared and separated as described at the Section 2.7; and then probed with the anti-lamin A/C monoclonal antibody. After densitometric analyses of the signals and normalisation with β-actin signals the percentages of the lamin A/C inhibition by siRNAs were calculated.

2.9. Cell Death Detection ELISA^{PLUS}

The kit of Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Mannheim, Germany) was used to determine the cytosolic histone-associated DNA fragments (mono- and oligonucleosomes) in the lamin A/C-targetting siRNA transfected and untransfected cells according to the manufacture's instructions. The ELISA signals representing the cytoplasmic histone-associated DNA fragments released from the nuclei after apoptotic cell death were quantified by measuring the absorbance at 405 nm using an ASYS UVM340 96-well plate reader at 48 h after transfection.

2.10. Immunocytochemistry of lamin A/C

Controlled and PDT-treated cells (5×10^4) were washed once with PBS containing 1% FBS before being cytospun on specially coated slides and then air-dried overnight. The cells were permeabilized with 0.1% Saponin for 5 min, blocked with 10% sheep serum in PBS for 30 min, incubated with the primary antibody for 90 min at room temperature; and subsequently with the secondary sheep anti-rabbit IgG antibody conjugated with DyLight®549 for 45 min. The fluorescence images of the lamin A/C were obtained by a fluorescence microscope (Nikon Eclipse E800, Nikon) equipped with a highly light-sensitive thermo-electrically cooled charge-coupled device camera ($ORCA_{11}$-ER, Hamamatsu, Japan).

2.11. Isolation of cell nuclei

To address if there was a direct effect of HAL-PDT on the cleavage of the lamin A/C, the Nuclei EZ Prep Kit (Sigma, St. Louis, MO) was used to isolate the nuclei of the Ramos and Daudi cell lines according to the manufacture's instructions. The nuclei of the cells were immediately incubated with PpIX at a concentration of 0.1 or