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Intermediate Filament Proteins of Lamin A/C and Cytokeratin 18 are involved in Apoptotic Induction by Photodynamic Therapy with Hexaminolevulinate in Human Colon Carcinoma Cells

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Abstract

Hexaminolevulinate (HAL), a hexylester of 5-aminolevulinic acid, is a precursor of the photosensitizer protoporphyrin IX (PpIX) and clinically used for photodynamic therapy (PDT) of cancer, an established modality with a light-activated drug. However, the mechanism involved in the killing cancer cells is still not fully understood. Our previous report (Cancer Lett., 2013; 339: 25-32) has shown a crucial role of caspase-6-mediated cleavage of lamin A/C, a member of type-V intermediate filaments (IFs), in the HAL-PDT-induced apoptosis in human B-cell lymphoma cells. Lamin A/C is present in all cell types; while cytokeratin 18, a major component of type-I IFs, is expressed only in the epithelial cells and carcinoma. This study has focused upon the roles of the two IF proteins, lamin A/C and cytokeratin 18 in the HAL-PDT-mediated apoptotic induction in the human colon carcinoma COLO 205 and HCC2998 cell lines. HAL-PDT-induced apoptosis was confirmed by fluorescence microscopy, electron microscopy and M30 CytoDeath™ ELISA in both cell lines.

Fluorescence microscopy, immunoblots and immunocytochemistry showed that both lamin A/C and cytokeratin 18 were involved in the apoptotic induction and the specific caspase-6 inhibitor stopped not only the cleavages of the two IF proteins, but also the apoptotic induction. Knockdown of both lamin A/C and cytokeratin 18 by siRNAs induced the cells to be apoptotic, further supporting the hypothesis that the disruption of both lamin A/C and cytokeratin 18 is required in the apoptotic induction by HAL-PDT in the human carcinoma cells.

Keywords: Photodynamic therapy; Hexaminolevulinate; Colon carcinoma; Apoptosis; Lamin A/C; Cytokeratin 18; Caspase-6

Abbreviations: ALA: 5-Aminolevulinic Acid; HAL: Hexaminolevulinate; PDT: Photodynamic Therapy; PpIX: Protoporphyrin IX

Introduction

Photodynamic therapy (PDT) is an established modality based on a combination of a photosensitizer with light exposure to generate reactive oxygen species including singlet oxygen in a lesion. PDT with protoporphyrin IX (PpIX) precursors has shown a promising non-invasive treatment for several oncological and non-oncological disorders [1-4]. PDT can cause cell death via necrosis and apoptosis [5], depending largely upon the parameters affecting the PDT protocol and intrinsic biological properties of cells [6,7]. However, the exact mechanism involved in the PDT-mediated killing effect is still not fully understood.

Apoptosis is characterized by several morphological changes including cytoskeleton disruption, chromatin condensation and margination, and nuclear fragmentation into membrane-enclosed apoptotic bodies. The activation of the caspase cascade responsible for the stepwise cleavage of critical cellular proteins is a key feature of the apoptotic process such as activation of pro-caspases and reorganization of cyto/nucleo-skeletons [8,9].

The cytoskeleton of eukaryotic cells is a complex network of three major classes of filamentous biopolymers: microfilaments, microtubules and intermediate filaments (IFs). Based on the chemical and structural homologies IFs are divided into six different types. Lamin A/C is a type-V IF and expressed in all cell types; while the type-I cytokeratin

18 is only present in the epithelial cells [9]. A large number of studies have concluded that many IF proteins contain a caspase consensus site in their conserved L-2 linker region of the rod domain that can be targeted by activated caspases, the caspase-6 in particular [9-11].

Hexaminolevulinate (HAL) is a PpIX precursor used for both clinical PDT and photodetection [12,13]. Our previous study has shown that the cleavage of lamin A/C by caspase-6 activation is responsible for the apoptotic induction by HAL-PDT in human B-cell lymphoma cells [14]. In this report we have studied the roles of both lamin A/C and cytokeratin 18 in the apoptotic induction by HAL-PDT in two human colon carcinoma COLO 205 and HCC2998 cell lines.

Materials and Methods

Chemicals

Hexaminolevulinate (HAL) was kindly provided by Photocure ASA

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(Oslo, Norway). Stock solution of 12 mM was freshly prepared for each experiment. The pan-caspase inhibitor (InSolution™ Q-VD-OPh, Non-O-Methylated), caspase-6 inhibitor-I and caspase-3 inhibitor were from Calbiochem® (Madison, WI, USA). The monoclonal mouse anti-human lamin A/C antibody was from Cell Signaling (Beverly, MA); while the monoclonal mouse anti-human cytokeratin 18 antibody (Clone DC 10) was from DakoCytomation, Denmark A/S. Alexa Fluor® 568 anti-mouse donkey secondary antibody was from Molecular Probes. USA. The donkey serum was from AbD SeroTec (Oxford, UK).

Cell culture

Human colon carcinoma cell lines COLO 205 and HCC2998 were sub-cultured in RPMI-1640 medium (PAA Laboratories GmbH, Fisher Scientific, Norway) supplemented with 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% CO₂ humidified incubator. In all experiments 70-80% of confluent cells were used.

PpIX production induced by HAL

The cells in 3 ml growth medium were seeded in 6-well (10 cm² in each) plastic tissue-culture plates (Nunc, Denmark) at a cell density of 5×10⁴ cells/cm² and allowed to attach the substratum at 37°C for 15 h before further treatment. The wells were washed once with RPMI medium without serum and filled with 4 ml of serum-free medium containing HAL at different concentrations (0-100 µM). Serum-free medium was used to avoid PpIX extraction from the cells. After incubation for 4 h at 37°C the HAL-containing medium was removed and the cells were washed three times with ice-cold PBS. Finally, the cells were brought into a solution of 1 N HClO₄ in 50% methanol by scraping off the cells from the substratum using a cell scraper (Coster, Cambridge, MA). This solution has been found to monomerize PpIX, so that its concentration can be reliably determined by fluorescent measurements. A Perkin-Elmer LS50B spectrofluorimeter (Norwalk, CT) was used to measure the PpIX content in the cells. The excitation wavelength was set at 405 nm and the emission was measured at 605 nm using a long-pass cut-off filter (530 nm) on the emission side. The fluorescence in each sample was compared with that of a PpIX standard of known concentrations and calculated relative to the protein content in control cells as measured by the Bradford method using the BCA protein assay kit (Pierce Inc., Rockford, IL).

PDT treatment with HAL

COLO 205 and HCC2998 cells (5×10⁵ cells) were seeded in 6-well tissue plates and allowed to attach the substratum for 15 h. The cells were then washed once with serum-free medium before being incubated for 4 h in the dark with serum-free RPMI 1640 medium containing 20 and 5 µM of HAL for the COLO 205 and HCC2998 cell lines, respectively. The cells were then exposed to the light from a bank of four fluorescent tubes (model 3026, Applied Photophysics, London, UK) emitting light mainly around 450 nm. The fluence rate of the light reaching the cells was 12 mW/cm². In the experiments on apoptotic induction the light doses were adjusted to reach a rate of 60-70% cell killing. After irradiation the medium was immediately replaced with HAL-free medium containing 10% FBS. In some experiments the inhibitors (20 µM) of pan-caspase, caspase-3 or caspase-6 were added to the samples after irradiation.

Cell survival

Cell survival was measured by the MTS assay. Immediately after

HAL-PDT 1 ml of single-cell suspension was made with a scraper and 100 µl of such cell suspension were added to each well of the 96-well plastic micro plates to incubate for 23 h at 37°C, followed by the addition of 20 µl of the CellTiter 96® Aqueous One Solution Reagent (Promega Corp., Madison, WI) into each well for an additional 1-h incubation. The absorbance at 490 nm was recorded using a 96-well plate reader (Multiskan Ex, LabSystems, Helsinki, Finland).

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.

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% OsO₄ 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 formvar/carbon coated copper grids, stained with 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.

Western blot

Control and PDT-treated cells (5×10⁵) at various times after HAL-PDT were collected by a scraper. After being centrifuged and washed once with cold PBS, the pellets were resuspended in 100 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. The whole-cell extracts 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) 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 were 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 and probed overnight at 4°C with mouse primary antibodies. The membranes were then washed three times and incubated for 1 h at room temperature with a secondary anti-mouse donkey 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: 5000/10000 dilutions) allowed re-confirmation of the total amount of proteins loaded on the gels.

M30 CytoDeath™ ELISA

Cleavage of cytokeratin 18 in the COLO 205 and HCC2998 cell lines was determined by using the M30 CytoDeath™ ELISA kit (PEVIVA

AB, Sweden) at 4 and 20 h after HAL-PDT. The M30 monoclonal antibody conjugated to horseradish peroxidase (HRP) recognizes a neo-epitope exposed after the cleavage of K18 at the aspartic acid residue 396. The signals detected at 450 nm are directly proportional to the amount of the cleaved K18Asp396 neo-epitope fragment by caspase activation only in apoptotic cells.

Immunocytochemistry

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% donkey serum in PBS for 30 min before being incubated with the primary mouse anti-human cytokeratin 18 monoclonal antibody for 90 min at room temperature; and subsequently with the secondary donkey anti-mouse antibody conjugated with Alexa fluor 568 for 45 min. The fluorescence images of the cytokeratin 18 were obtained by a fluorescence microscope (Nikon Eclipse E800, Nikon) equipped with a highly light-sensitive thermo-electrically cooled charge-coupled device camera (ORCAII-ER, Hamamatsu, Japan).

siRNA and transfection

One siRNA sequence targeting lamin A/C (NM_005572) (5'-CAGGCAGTCTGCTGAGAGGAA-3') and one siRNA sequence targeting cytokeratin 18 (5'-CCGCATCGTTCTGCAGATTGA-3') were purchased from Qiagen (USA). A control siRNA sequence targeting the *E. coli* β -galactosidase was also included. Transfections of both cell lines with siRNAs were performed using electroporation technique. Briefly, 2.5×10^6 cells were suspended in 0.5 ml serum-free RPMI-1640 medium containing 2 μ g 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 milliseconds. After transfection the cells were diluted in 2.5 ml of pre-warmed medium and incubated at 37° in 5% CO₂ for 48 h. The protein extracts for immunoblots were then prepared as described above and probed with the mouse anti-human lamin A/C and anti-human cytokeratin 18 monoclonal antibodies. After densitometric analyses of the signals and normalisation with β -actin signals the inhibitory percentages of the lamin A/C and cytokeratin 18 by siRNAs were calculated.

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 and cytokeratin 18-targeting siRNA transfected cells according to the manufacturer's instructions. The ELISA signals, representing the cytoplasmic histone-associated DNA fragments in apoptotic cells, were quantified by measuring the absorbance at 405 nm using an ASYS UVM340 96-well plate reader at 48 h after transfection.

Statistical analysis

The rank sum (Mann-Whitney) test was used to statistically analyse data and all tests were done at the 0.05 significance level

Results

HAL-induced PpIX production and its photodynamic treatment

Figure 1A shows PpIX synthesis in the COLO 205 and HCC2998 cell lines as a function of the HAL concentration. The PpIX production was increased with increasing HAL concentrations with a plateau from about 20 μ M. COLO 205 cells produced less PpIX than HCC2998 cells. Phototoxicity of the cells with HAL-PDT was studied with various light doses at the HAL concentrations of 20 μ M for the COLO 205 cells and 5 μ M for the HCC2998 cells, respectively. The PDT-mediated cell-killing effect increased with increasing light doses (Figure 1B). In addition, different light doses were screened and 120 mJ/cm² for the COLO 205 cells and 480 mJ/cm² for the HCC2998 cells were found to induce a maximal rate of apoptosis and thus used in the rest experiments.

Induction of apoptosis by HAL-PDT

Induction of apoptosis was initially studied with fluorescence microscopy using Hoechst 33342 for nuclear staining. Figure 2A shows apoptotic cells with characteristics of nuclear fragments and apoptotic bodies at 20 h after HAL-PDT in the two cell lines. Electron microscopy confirms the findings of fluorescence microscopy with typical morphological alteration of apoptosis (Figure 2B).

Figure 1: HAL-induced PpIX and its photodynamic effects in the human colon carcinoma COLO 205 and HCC2998 cell lines. (A) PpIX production as a function of the HAL concentration. (B) Cell survival after PDT with various light doses. For the PDT protocols, see the Section of materials and methods.

Figure 2: HAL-PDT-induced apoptosis of the COLO 205 and HCC2998 cells. (A) Fluorescence and corresponding phase-contrast images of the apoptotic COLO 205 and HCC2998 cells at 20 h after HAL-PDT. (B) Electron microscopic images of the apoptotic COLO 205 and HCC2998 cells at 20 h after HAL-PDT (Bars: 10 µm).

Involvements of caspase-6, lamin A/C and cytokeratin 18 in the apoptotic induction

Figure 3A demonstrates about 30-40% of apoptotic induction by HAL-PDT in the two cell lines. Western blots showed that the HAL-PDT cleaved both nuclear lamin A/C and cytokeratin 18 in the cell lines (Figure 3B). The HAL-PDT-mediated cleavage of cytokeratin 18 was further verified by using the M30 CytoDeath™ ELISA assay in both cell lines (Figure 3C). Furthermore, the results obtained from immunocytochemistry indicate a clear morphological change from a fine filamentous cytokeratin 18 pattern in the control cells to a granular pattern combined with a condensed and fragmented chromatin in the apoptotic HCC2998 cells (Figure 3D) as a result of the disruptive cytokeratin 18 by HAL-PDT. Similar findings were also seen in the COLO 205 cells (data not shown). Interestingly, the inhibitors of pan-caspase and caspase-6 could efficiently stop the PDT-mediated cleavages of both lamin A/C and cytokeratin 18 (Figures 3B and 3D) and thus significantly reduced the apoptotic induction ($p < 0.05$) (Figure 3A); while the specific caspase-3 inhibitor did not (Figure 3B), suggesting a crucial role of PDT-mediated caspase-6 activation in such apoptotic induction.

Confirmation of the involvements of lamin A/C and cytokeratin 18 in apoptosis by siRNA

In order to confirm if lamin A/C and cytokeratin 18 are involved in the apoptotic cell death in the COLO 205 and HCC 2998 cell lines, the cells were transfected with specific siRNAs to knockdown the lamin A/C and cytokeratin 18 in the two cell lines. The apoptotic cell death was then measured with the Cell Death Detection ELISA^{PLUS} kit. Transfection with the lamin A/C siRNA or cytokeratin 18 siRNA alone led to 93% and 75% inhibition of lamin A/C and cytokeratin 18, respectively in the HCC2998 cells (Figure 4A). However, they did not increase the amounts of cytoplasmic histone-associated DNA fragments released from the nuclei of apoptotic HCC2998 cells ($p > 0.05$, as compared to the control group with irrelevant siRNA) (Figure 4B). When the cells were treated with two siRNA sequences simultaneously targeting lamin A/C and cytokeratin 18, the expression of lamin A/C and cytokeratin 18 was still inhibited with 82% and 55%, respectively. However, the cytoplasmic histone-associated DNA fragments were released significantly more than those with the single siRNA treatment ($p < 0.05$) (Figure 4B), indicating a requirement of knocking down the two IF proteins at the same time for such apoptotic induction. Similar results were also found in the COLO 205 cell line (data not shown).

Discussion

PDT is an effective and safe treatment for several clinical indications [1,3]. A large number of reports have demonstrated that PDT can kill tumour cells via apoptosis [1,3]. Our previous studies have shown that HAL-mediated PDT induces efficiently apoptotic cell death in several human blood malignant cell lines [15-18]. The mechanisms of such apoptotic induction by PDT are largely dependent upon the intracellularly initial target sites and biological systems.

Although several pathways have been identified to be involved in apoptosis, the caspase-dependent pathway is responsible for the morphological and biochemical changes of apoptotic cells in most cases. This is because the caspase cascade activation can cleave some vital substrates [5,6]. IFs, a major class of filaments in the cytoskeleton of eukaryotic cells, are among the endogenous protein substrates of such activation. Our previous study has found that the cleavage of lamin A/C, a type-V IF, by caspase-6 activation is crucial for apoptotic induction by HAL-PDT in human B-cell lymphoma cells [14]. In this investigation we continued to focus on the roles of IFs in the apoptotic induction by HAL-PDT, but used the human colon carcinoma COLO 205 and HCC2998 cell lines as models that express not only lamin A/C, but also cytokeratin 18, a type-I of IFs.

The results obtained from this study have shown the cleavages of both lamin A/C and cytokeratin 18 by HAL-PDT in these two colon carcinoma cell lines by means of Western blots (Figure 3B). The cleavage of cytokeratin 18 was further confirmed by the M30 CytoDeath™ ELISA kit (Figure 3C). The immunocytochemistry also revealed the disruption of cytokeratin 18 as a granular pattern after HAL-PDT (Figure 3D). The use of a specific caspase-6 inhibitor can not only block the cleavages of the two IF proteins, but also the HAL-PDT-mediated apoptotic induction (Figures 3A, 3B and 3D). These findings indicate the involvements of both lamin A/C and cytokeratin 18 in the apoptotic induction by HAL-PDT in the colon carcinoma cells.

Cytokeratins constitute the largest and most complex class of IFs. Cytokeratin 18 is expressed in epithelial cells and tumors derived from such cells [19]. Caspase-6 has been reported to cleave lamin A/C. Further, the similarity and specificity of such cleavage site between lamin A/C and cytokeratin 18 [20] suggest caspase-6 to be responsible for the breakdown of cytokeratin 18 in apoptotic cells. In fact, several studies have demonstrated the cleavage of the cytokeratin 18 in apoptosis [11,21-24]. Caulin et al. [11], was the first group

who demonstrated caspase-6 as the most efficient caspase to cleave cytokeratin 18 after aspartate 238, resulting in a 23 kDa (N-terminal) and a 22 kDa (C-terminal) fragments. Cleavage at the second site nearer the C-terminal end of cytokeratin 18 exposes a neo-epitope [11] that can be identified by the antibody M30 to exclusively recognize apoptotic epithelial cells [25].

To knockdown the lamin A/C or cytokeratin 18 alone by siRNA did not increase the apoptotic induction in the two colon carcinoma cell lines. However, the reduction in the expression of both lamin A/C and cytokeratin 18 by siRNAs at the same time did significantly increase the apoptotic fraction, suggesting a requirement of such apoptotic induction with these two IF proteins to be destroyed simultaneously.

In order to understand better the roles of IFs in the apoptotic induction by HAL-PDT we have correlated the expression of lamin A/C and cytokeratin 18 with apoptotic pathways after HAL-PDT in different human blood malignant cell lines. Figure 5 shows no expression of both lamin A/C and cytokeratin 18 in the human T-cell lymphoma Jurkat cell line and non-T, non-B lymphoblastic leukemia Reh cell line; while our previous report demonstrated the expression of

only lamin A/C in the human B-cell lymphoma Ramos and Daudi cell lines [Figure 2B in the Ref.18]. In the Jurkat and Reh cell lines HAL-PDT induced the apoptosis largely via caspase-independent pathways [14,15]; whereas in the Ramos and Daudi cell lines the apoptosis was mediated through the caspase-dependent pathway with the cleavage of the lamin A/C by the caspase-6 activation [18]. In all the 4 blood malignant cell lines HAL-PDT could induce 75-90% apoptosis; while in the two colon carcinoma cell lines in the present study a maximal rate of about 40% apoptosis was induced by HAL-PDT. The different rates of apoptotic induction by the same HAL-PDT between the blood malignant cells and epithelial malignant cells may suggest that the cells with the expression of both lamin A/C and cytokeratin 18 in the epithelial origin of carcinoma cells may be more resistant to the apoptotic induction by HAL-PDT. This may be due to the fact that such apoptotic induction requires the dismantlement of both lamin A/C and cytokeratin 18 by the caspase-6 activation.

In conclusion, HAL-PDT can induce apoptosis with a rate of about 40% in the two human colon carcinoma COLO 205 and HCC2998 cell lines. Such apoptotic induction is mediated via the cleavages of both lamin A/C and cytokeratin 18 by the caspase-6 activation. Having

compared the results of our previous studies using the same HAL-PDT treatment, it appears that the carcinoma cells with the expression of both lamin A/C and cytokeratin 18 are more resistant to the HAL-PDT for such apoptotic induction than the blood malignant cells with either only expression of lamin A/C or no expression of the two IF proteins.

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