The inhibition of ferrochelatase enhances 5-Aminolevulinic acid-based photodynamic action for prostate cancer

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Abbreviations used are ALA: 5-aminolevulinic acid, PpIX: protoporphyrin IX, PDD: photodynamic diagnosis, PDT: photodynamic therapy, FBS: fetal bovine serum, BSA: bovine serum albumin, DFX: deferoxamine, NOC-18: 1-hydroxy-2-oxo3,3-bis(2-aminoethyl)-1-triazene, NAO: 10-nonyl acridine orange

Introduction

Protoporphyrin IX (PpIX) is excessively accumulated in tumor cells compared with normal cells. This phenomenon is related to the generally increased activity of most enzymes and decreased activity of ferrochelatase. The accumulation of PpIX in the mitochondria causes direct cell death after light irradiation. In this study, we investigated the mechanism of ALA-induced PpIX accumulation and ALA-PDT-induced cell death in PC-3 cells.

Materials and Methods

1. PpIX accumulation and PDT

PC-3 cell line derived from a poorly differentiated human adenocarcinoma. PC-3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. To induce PpIX accumulation, PC-3 cells (1 X 10^5 cells/ml) were cultured overnight. For ALA-PDT, the cells were exposed for 10 min. As a light source, a Na-Li lamp (TheraBeam, VR630, USHIO Inc.) was used. The wavelength of light was 600-700 nm, preferentially 630-670 nm. A power density of 20-65 mW/cm^2 could be attained and 29 mW/cm^2 was used in most experiments.

2. Flow cytometric analysis

The accumulated PpIX content was measured by flow cytometry with a FACScan apparatus (Becton Dickinson, San Jose, CA). Fluorescence intensity of the cells was recorded and represented the relative intracellular PpIX content. Fluorescence isothiocyanate (FITC)-annexin V/PI double staining was used in the flow cytometric detection of apoptosis and necrosis. Mitochondria were selectively stained with JC-1 or TMRE for detection of mitochondrial membrane potential.

3. Assay for caspase-like activity

Caspase-3 activity was determined in 20 mM HEPES buffer (pH7.5) containing 0.1 mM NaCl and 5 mM DTT at 37°C using 1 μM Ac-DEVD-AFC (acetyl-L-aspartyl-L -glutamyl-L-valyl-L-aspartic acid alpha-(7-amido-4-trifluoromethyl-coumarin)) as a substrate. The fluorescence of released AFC was measured using a fluorescence plate reader with excitation and emission wavelengths of 355 and 460 nm, respectively.

4. ALA-mediated PDD and PDT *in vivo*

Animal experiments were conducted in accordance with the institutional guidelines and regulations and reviewed by the animal experiment and welfare committee of Kochi medical school. Each BALB/c nu/nu mice was injected in the dorsal region subcutaneously with $2x10^6$ PC-3 cells suspended in 100 μl of DMEM.

In pathological analysis, we analyzed cell death by immunohistochemistry for single-stranded DNA (ssDNA) to detect the degenerated tissue and measured the range of degenerated lesion in the tumor. The apoptotic cells were counted in areas with high frequency under 10 high-power fields. More than 1000 tumor cells were counted to calculate the apoptosis index (AI) from these areas, and the AI values are expressed as percentage of ssDNA-positive cells.

Results

1. Increase in accumulated intracellular PpIX level by inhibition of ferrochelatase in ALA-treated PC-3 cells

Both an iron chelator, deferoxamine, and a NO generator, NOC-18, significantly increased the level of PpIX even in the presence of a low concentration of ALA, such as 0.1 or 0.25 mM.

2. Effect of PDT on the cell death of ALA-treated PC-3 cells

The results of analysis of PDT-induced PS externalization and PI stainability suggested that PDT induced cell death by both necrosis and apoptosis. After photodynamic treatment, the cells were damaged more severely by ALA-PDT plus treatment with deferoxamine or NOC-18 than ALA-PDT alone. Deferoxamine and NOC-18 significantly increased the PDT-induced cell death. The levels of mitochondrial membrane potential decreased in parallel with increased cell death in PC-3 cells after ALA-PDT in a time-dependent manner. Furthermore, the depolarization was stimulated by the inhibition of ferrochelatase activity even after incubation with 0.1 mM ALA.

3. ALA-PDT increases the activity of caspase-like enzyme

The caspase-3 activation was time-dependent and was increased by the treatment with deferoxamine and NOC-18. However, activation of the enzyme by ALA-PDT was not significantly increased. The inhibition of caspase-3 activation by z-VAD-fmk did not attenuate the ALA-PDT-induced cell death and membrane depolarization of PC-3 cells.

4. Effect of ALA-mediated PDT on prostate cancer cells *in vivo*

In the pathological analysis, the degeneration was observed within a broader range in the therapeutic group than in the control group. However, the promoting effect of ALA-PDT by deferoxamine was not significant. There was a significant difference in AI between the control group and the ALA/PDT group or the ALA/DFX/PDT group. However, there was no significant difference in AI between the ALA/PDT group and the ALA/DFX/PDT group.

Discussion

ALA is a naturally occurring metabolite that is a precursor of porphyrin in heme biosynthesis. Exogenous ALA leads to the accumulation of the potent photosensitizer PpIX in mitochondria. The mechanism of ALA-PDT-induced cell death by inhibition of ferrochelatase revealed that ALA-PDT-induced cell death led to apoptosis and necrosis. Caspase-3-like activity was not significant and a pan-caspase inhibitor, z-VAD-fmk, did not attenuate cell death and membrane depolarization. These results suggest that ALA-PDT induced not only necrosis (partially dependent on membrane depolarization) but also apoptosis that might have occurred via a caspase-independent mitochondrial pathway. We could demonstrate a weak but significant cytoreductive effect of ALA-PDT and a small promoting effect of ALA-PDT by deferoxamine in PC-3 cells *in vivo*. From the results of our present study, we conclude that ALA-mediated accumulation and distribution of PpIX are strongly regulated by heme synthesis system and hence we can achieve clinically favorable results by using ferrochelatase inhibitors.