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5-Aminolevulinic Acid-Mediated Photodynamic Therapy on Hep-2 and MCF-7c3 Cells

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The cytotoxic effect of 5-aminolevulinic acid (ALA) induced protoporphyrin IX (PPIX) on two human carcinoma cell lines, MCF-7c3 cells and Hep 2 cells, was studied. In both cell lines, PPIX content depends on the ALA concentration and incubation time. The maximal PPIX content was higher in the MCF-7c3 cells, reaching a value of 8 $\mu g/10^6$ cells, compared to the Hep-2 cells, which accumulated 3.2 $\mu g/10^6$ cells. Treatment of cells with the iron chelator desferrioxamine prior to ALA exposure enhances the amount of PPIX, consequently diminishing enzymatic activity of ferroquelatase. Photosensitization of the cells was in correlation with the PPIX content; therefore, conditions leading to 80% cell death in the MCF-7c3 cells provoke a 50% cell death in the Hep 2 cells. Using fluorescence microscopy, cell morphology was analyzed after incubation with 1 mM ALA during 5 hr and irradiation with 54 Jcm^{-2}; 24 hr post-PDT, MCF-7c3 cells revealed the typical morphological changes of necrosis. Under the same conditions, Hep-2 cells produced chromatine fragmentation characteristic of apoptosis. PPIX accumulation was observed to occur in a perinuclear region in the MCF-7c3 cells; while in Hep-2 cells, it was localized in lysosomes. Different mechanisms of cell death were observed in both cell lines, depending on the different intracellular localization of PPIX.

KEY WORDS: 5-aminolevulinic acid, photodynamic therapy, photosensitisation, apoptosis, necrosis

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Introduction

All living cells synthesize heme from the natural precursor 5-aminolevulinic acid (ALA). On exogenous administration, ALA is enzymatically converted into the potent photosensitizer (PS) protoporphyrin IX (PPIX); by this procedure, the feedback control of heme biosynthesis is bypassed and accumulation of PPIX is consequently induced.[1]

It has been observed that higher amounts of PPIX are accumulated in malignant cells compared to normal cells, which is attributed to differences in the metabolizing ability of the heme pathway, an increased activity of PBG deaminase, and a decrease in ferrochelatase (FC) activity.[2] On exposure to light of appropriate wavelength, the energy of the activated PPIX is transferred to molecular oxygen, producing singlet oxygen and other oxygen molecular species, triggering cytotoxic events that ultimately conduce to cell death. This is the basis of the photodynamic therapy (PDT) of tumors employing ALA, which has been receiving increased attention in recent years.[3] ALA-induced PDT has been successfully applied in various medical fields, such as dermatology, urology, and gastroenterology, also including photodiagnosis of tumors by means of PPIX fluorescence detection.[4]

In many types of cells and tumors, PDT induces apoptosis, explaining the rapid ablation of tumors that occurs after the treatment.[5] However, the mode of cell death can be apoptosis or necrosis, depending on the cell type, the photosensitiser and its subcellular localization, and the light dose.[6]

In the present work, we studied the cytotoxic effect of ALA-induced PPIX on two human carcinoma cell lines. Photosensitization kinetics were analyzed by means of chemical porphyrin extraction, cell morphological changes after irradiation with visible light, and PPIX subcellular localization.

Materials and Methods

Chemicals

5-aminolevulinic acid (ALA) and desferrioxamine (DFX) were purchased from Sigma Chem Co.

ALA was dissolved in Dulbecco's phosphate buffered saline (PBS), adjusting the pH with sodium hydroxide. All other chemicals used were of the highest purity commercially available.

Cell Culture

MCF-7c3 cells derived from the human breast cancer MCF-7 (WS8) cell line transfected with the pBabepuro retroviral vector encoding human procaspase-3 cDNA was provided by Dr. C.J. Froelich (Northwestern University, Evanston, IL). The cell line was cultured in RPMI 1640 medium containing 10% fetal bovine serum and 2 μg/ml puromycin. Culture doubling times were approximately 24 hr. The cultures were maintained at 37°C in the humidified atmosphere of 5% CO_2. The Hep-2 human larynx-carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacional de Enfermedades Virales Humanas, Pergamino, Argentina) was kept frozen in liquid nitrogen. The cells were grown as a monolayer employing Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 50 μg/ml gentamycin as antibiotic. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and the medium was changed daily. Both cell lines were routinely checked for the absence of mycoplasma contamination.

Assays

The cells (7×10^4 cells/ml) were seeded in triplicate in 30 mm diameter dishes and incubated 48 hr at 37°C. Afterward, the cells were incubated in a serum-free medium containing specific concentrations of ALA during different time periods. Irradiations were performed 3 hr after ALA incubations. After irradiation, the medium was replaced by an ALA-free medium plus FBS, and the cells were incubated for another 19 hr to let photodamage occur and then tested for viability. When testing DFX, the chelating agent was added 3 hr previous to ALA addition.

Measurement of Porphyrin Synthesis

Total porphyrins accumulated within the cells were extracted twice with 5% HCl after removing the media, leaving the cells for a half-hour in the presence of the acid at 37°C. The media were acidified and measured directly in a Shimadzu RF-510 spectrofluorophotometer. The excitation and emission wavelengths producing the highest fluorescence were 406 and 604 nm, respectively.

MTT Viability Assay

Cell viability was assessed by the MTT assay.[7] Following appropriate treatments, MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) in DMEM solution was added to each well at a final concentration of 50 µg/ml and plates were incubated at 37°C for 3 hr. The resulting formazan crystals were dissolved by the addition of dimethyl sulfoxide and absorbance was read at 540 nm. Cell viability was expressed as a percentage of non-treated control cells.

Cell Number

The number of cells seeded per well and the cell number employed for the calculations of porphyrins per cell were determined by counting viable cells with the Trypan blue exclusion method.

Irradiation

The light source used was a Kodak slide projector equipped with a 150 W lamp and a wavelength range of 350–800 nm was selected using optical filters. The light was filtered through a 3 cm water layer to absorb heat. The light intensity at the treatment site was 60 mW/cm2.[8]

Morphology and Cell Counting

Changes in cell morphology were analyzed using fluorescence microscopy. After fixation with methanol at −20°C for 10 min, cells were stained with Hoechst-33258 (H-33258 10 µg/ml in distilled water for 5 min) to visualize the DNA chromatin. After washing and air drying, preparations were mounted in a mixture of distyrene, plasticizer, and xylene (DPX, Serva, Heidelberg, Germany) and observed under UV excitation.

Statistical Treatment

The values in the figures are expressed as means ± standard error of the mean, and they are the average of three independent experiments run in triplicate. A paired two-tailed Student's t-test was used to determine the statistical significance between means. The p values < 0.05 were considered significant.

Results

PPIX Accumulation as a Function of ALA Concentration and Incubation Time

Figures 1A and 1B show the dependence of porphyrin synthesis on ALA concentration in MCF-7c3 and Hep-2 cells, respectively. PPIX is rapidly accumulated in the cells and tends to a saturation value between 5 and 24 hr in both cell lines. The intracellular accumulation of PPIX increased with ALA concentration, reaching a plateau after 5 hr incubation in all the concentration ranges tested. The amount of porphyrins accumulated was markedly lower in the Hep-2 cell line. When incubated with 1 mM ALA, the maximal porphyrin synthesis was approximately 8 $\mu g/10^6$ cells for MCF-7c3 cells and 3.2 $\mu g/10^6$ cells in the Hep-2 cells. Desferrioxamine (DFX) is an iron chelator agent that sequesters iron and thus inhibits the ferroquelatase activity. As expected, treatment with 0.1 µM DFX increased the amount of PPIX accumulated in the cells from 8 $\mu g/10^6$ to 15.3 $\mu g/10^6$cells in the MCF-7c3 cells and from 3.2 $\mu g/10^6$ to 9.5 $\mu g/10^6$ cells in the Hep-2 cells (Figs. 1A and 1B).

Figures 2A and 2B show the ALA-PDT-induced damage as a function of the increasing light

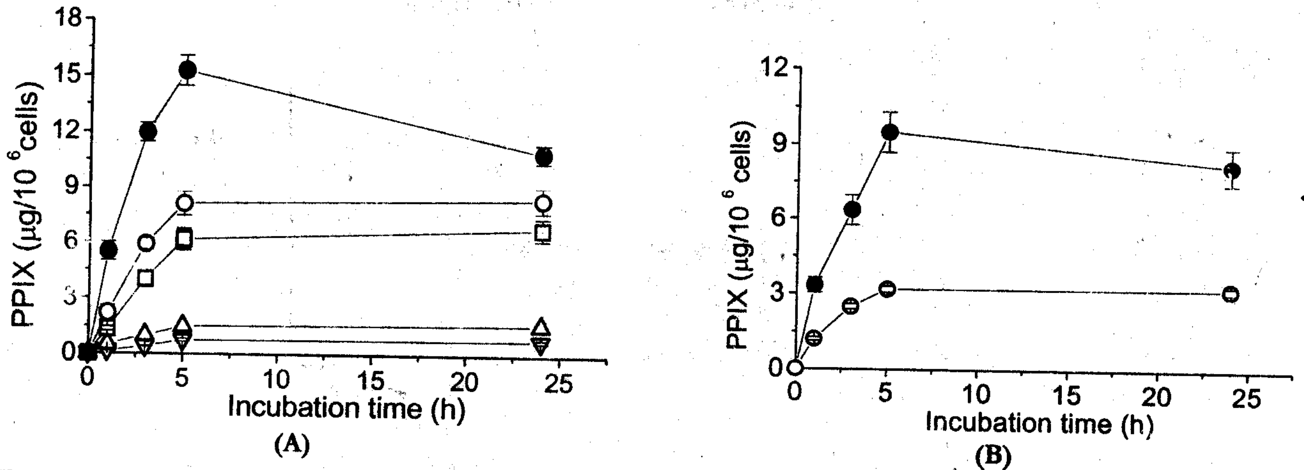

FIGURE 1. Time course of porphyrin synthesis **(A)** in MCF-7/c3 cells and **(B)** Hep-2 cells. Cells were incubated with 0.1 (▽), 0.3 (Δ), 0.8 (□), and 1.0 (O) mM 5-ALA, or 1.0 mM 5-ALA plus 0.1 μM DFX (●). At different time periods intracellular porphyrins were extracted, determined fluorometrically, and normalized to 10^6 cells. Values represent mean ± standard deviation of three separated experiments.

dose in MCF-7c3 and Hep-2 cells, respectively. Cell survival by means of MTT assay indicates that in the absence of light, ALA does not affect cell viability up to 1mM concentration in both cell lines. In contrast, the combination of ALA with visible light induces a decrease in cell survival that depends on the drug concentration and light dose. With 15 min irradiation, which corresponds to 54 J/cm2, 80% of cell death was observed in the MCF-7c3 cells, and 50 % in the Hep-2 cells, values that are in accordance with the different levels of PPIX content. Similar results were obtained with the Trypan blue exclusion method. When DFX was added 3 hr prior to ALA incubation, the photokilling activity of ALA-induced PPIX was enhanced in good correlation with the greater accumulation of PPIX accumulated (Figs. 2A and 2B).

Morphological analysis of MCF-7c3 cells performed with Hoechst-33258, after incubation with 1 mM ALA during 5 hr and irradiation with 54 Jcm^{-2} light dose, revealed the typical morphological changes of necrosis 24 hr post-PDT, although the apoptosis pattern was also observed (Fig. 3A). Similar results were obtained with toluidine blue staining. Apoptotic cell death was identified not only by the occurrence of cell rounding, shrinkage, and defor-

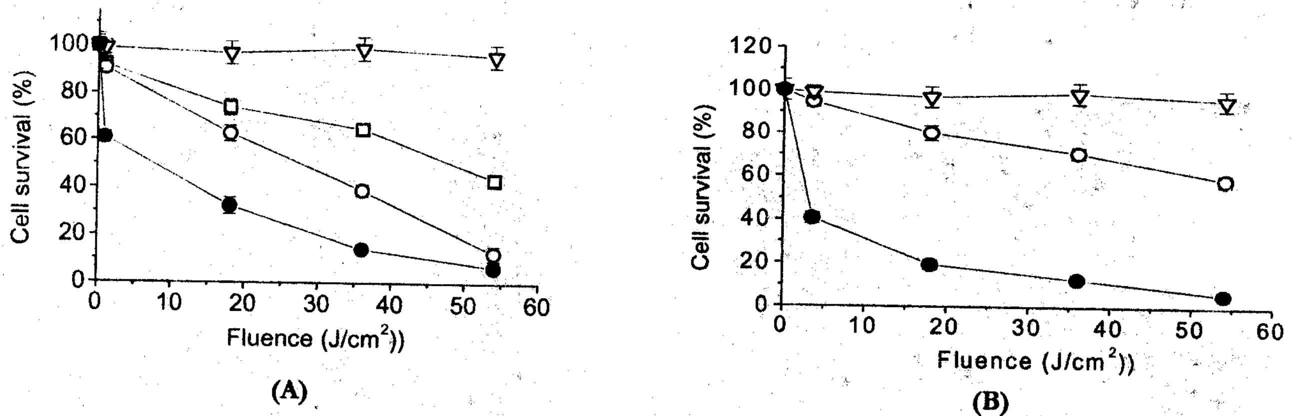

FIGURE 2. Survival of **(A)** MCF-7/c3 cells and **(B)** Hep-2 cells after 5-ALA-PDT with different light doses. Cells were incubated 5 hr in SFM with 0.8 (□) and 1.0 (O) mM 5-ALA or 1.0 mM 5-ALA plus 0.1 μM DFX (●), and then irradiated with different light doses (360–800 nm, 60 mW/cm²). After incubation in medium containing FBS for further 24 hr, MTT assay was performed. Cell survival is expressed as percentage of the nonirradiated control (▽) incubated in the presence of 5-ALA. Values represent mean ± standard deviation of three separated experiments.

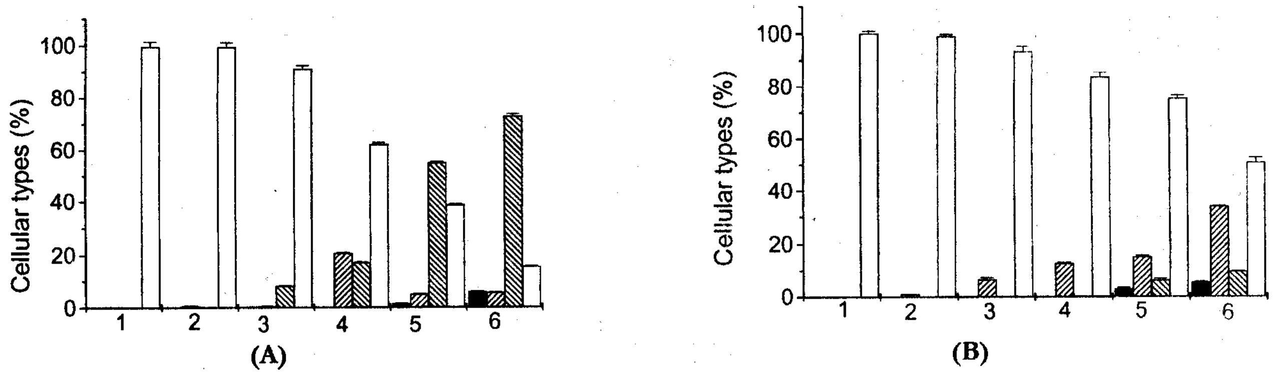

FIGURE 3. Effects of 5-ALA-PDT. **(A)** MCF-7c3 cells and **(B)** Hep-2 cells were incubated with 1 mM 5-ALA for 5 hr, followed by different irradiation times; light (1) and dark (2) controls. Light doses: 3.6 J/cm² (3); 18 J/cm² (4); 36 J/cm² (5); 56 J/cm² (6). (□), normal cells; □ with /, apoptotic cells; □ with \, necrotic cells; ■, ghost.

mation, but also by the typical nucleosomal DNA fragmentation, and formation of apoptotic bodies. The same conditions applied to the Hep-2 cells produced chromatine fragmentation characteristic of apoptosis (Fig. 3B).

Intracellular localization of ALA-induced PPIX was analyzed by means of fluorescence microscopy and confirmed with colocalization with fluorescent molecular probes. In MCF-7c3 cells, red fluorescence localized in the cytoplasm and in a perinuclear region, while in the Hep-2 cells, the granular appearance of fluorescence indicates lysosomal localization (Fig. 4).

Discussion

In recent years, numerous papers have focused on the study of ALA-based PDT. We evaluated the cytotoxic effect of ALA-induced PPIX on two carcinoma cell lines, i.e., human breast adenocarcinoma (MCF-7c3) and human larynx carcinoma (Hep-2). In both cell lines, the PPIX content increased with ALA concentration and incubation time, reaching a value of 8 μg/10^6 cells in the MCF-7c3 cells and 3.2 μg/10^6 cells in the Hep-2 cells. In the range of concentrations used (0.1–1 mM), ALA does not affect survival in the dark. However, under irradiation, viability decreases according to the doses of visible light, reaching a value of 80% of cell inactivation after a dose of 54 J/cm².

It is well known that the heme biosynthetic pathway is regulated by a feedback inhibition of the initial step catalyzed by the enzyme ALA synthetase (ALA-S). Another crucial point in the heme pathway is the incorporation of iron into the PPIX molecule catalyzed by FC, an enzyme that has been

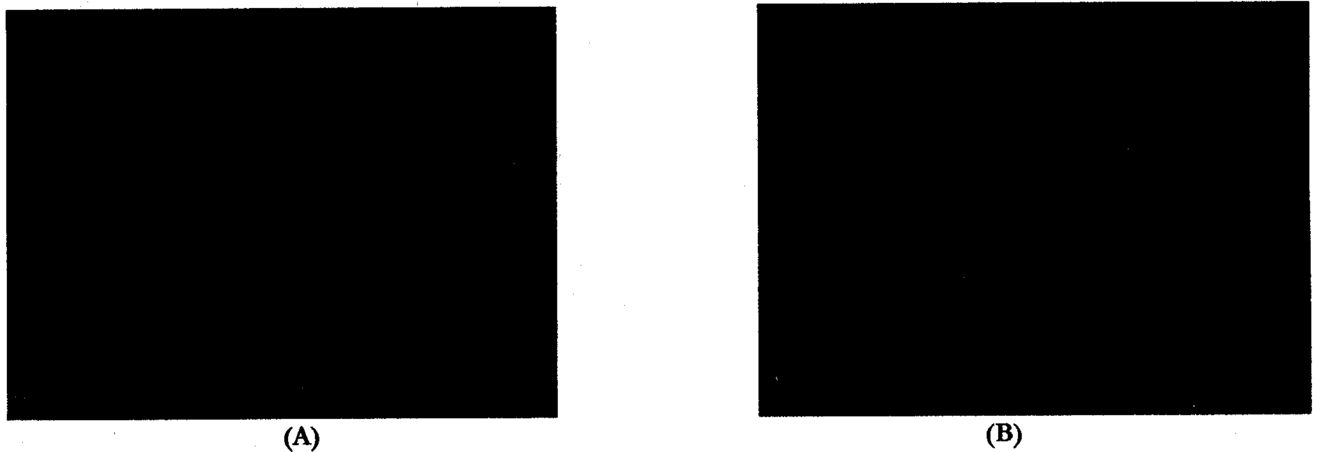

FIGURE 4. Fluorescence micrographs of living cells showing the intracellular localization of PPIX in **(A)** MCF-7c3 cells and **(B)** Hep-2 cells. Scale bars = 10 μm.

reported to be low in neoplastic cells—e.g., Schoenfeld et al. suggested that the heme biosynthetic pathway is greater in tumor cells than in normal cells, except for the insertion of iron in the last step;[9] in mouse mammary adenocarcinoma, an increase in PBGase activity was reported,[10] and in hepatoma cells, Dailey and Smith showed that the FC activity was lower compared to that in normal hepatocytes.[11] When adding exogenous ALA, PPIX is accumulated because the step catalyzed by ALA-S is by passed and FC activity is reduced. This implies that ALA-based PDT can be modulated at the level of the FC step, for instance, inhibiting the enzyme activity by iron chelators and consequently enhancing the PPIX content.

DFX is a highly specific membrane-permeable iron chelator with high affinity for iron.[12] Iron chelators have been applied to cells *in vitro*, and and shown to enhance photodamage as well as accumulation of PPIX.[13,14] The iron-chelator-induced PPIX accumulation is dependent on the cell line and the metal chelator employed.[15] In our case, the enhancement of PPIX content by the use of ALA and DFX was more pronounced in the laringocarcinoma cells compared to the mammary carcinoma cells—in the Hep-2 line, nearly a threefold increase from 3.2 to 9.5 μg/10^{6}cells, while in the MCF-7c3 line, enhancement was twofold from 8 μg/10^6 to 15.3 μg/10^{6}cells. Under similar conditions, Liu et al. found that addition of the nonspecific metal chelator EDTA to Hep-2 cells treated with ALA increased by 30% PPIX accumulation.[16]

It was demonstrated that reduction of FC activity is associated with ALA-induced PPIX accumulation and can contribute to the success of ALA-PDT. We found that cells became more sensitive to light when the reaction of FC was inhibited by treatment with DFX. Under the same conditions of light irradiations, the photokilling activity of ALA-induced PPIX was enhanced in good correlation with greater accumulation of PPIX (Fig. 2).

The cytotoxic response to PDT can involve apoptosis, necrosis of both,[17] and it is accepted that the intracellular localization of the PS has effects on the mechanism of cell death induced by PDT.[18,19] Kessel and Luo assessed different sites of photodamage as a determinant of cell death, utilizing agents with known patterns of subcellular localization in murine leukemia cells.[19] It was demonstrated that PDT with PS localizing in mitochondria, such as porphycenes, promote efflux of cytochrome c, initiating the pathway of apoptosis.[20] Different types of cells undergo different mechanisms of cell death. Oleinick et al. reported that aluminium porphycenesdo not induce apoptosis in V79 cells,[21] while Nood et al., employing 5-ALA, found apoptosis in the same type of cells, and necrosis in WiDr cells.[22] The mode of cell death induced by PDT seems also to be dependent on several factors such as drug concentration, incubation time, light dose, and cell density.[23] Following a similar initial insult, the balance between the two modes of cell-death mechanisms appears to be dependent on the intensity of light dose; with monocationic methoxyphenylporphyrin derivatives, the apoptotic pathway occurs mainly at low light doses, while necrotic death predominated at high light doses.[24,25]

We found that in the MCF-7c3 cells, the main mode of cell death was necrosis, while in the Hep2 cells, apoptosis was predominant. This different mechanism of cell death could not be attributed to the different porphyrin content because maximal PPIX accumulation was markedly lower in the Hep-2 cells than in the MCF-7c3 cells (3.2 μg/10^6 cells versus 8 μg/10^6 cells). Addition of the iron chelator DFX increases the amount of porphyrins, and this increase was more pronounced in the Hep2 cells. However, DFX does not change the intracellular PPIX localization. Fluorescence micrographs demonstrate that PPIX is localized in the perinuclear area in the citoplasm of the MCF cells, and is localized in the lysosomes in the Hep 2 cells. It is known that lysosomal photodamage produces the immediate release of cathepsins and other proteolitic enzymes, leading to a rapid apoptotic response. Consequently, the different modes of cell death may be explained by the different intracellular PS localization.

In conclusion, the present study shows the correlation of the intracellular content of ALA-induced PPIX with the photodamage. Furthermore, the different mechanisms of cell death can be explained by the difference in the pattern of PPIX accumulation, namely, in Hep-2 cells, fluorescence is confined into the lysosome, while in MCF-7c3 cells, fluorescence is found in the perinuclear area in the cyto-

plasm. These results also confirm that the cell response to PDT is dependent on both the light dose and the cell type.

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