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ROS PRODUCTION BY ENDOGENOUSLY GENERATED PROTOPORPHYRIN IX IN MURINE LEUKEMIA CELLS

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Abstract – Endogenous production of Protoporphyrin IX (PpIX) is successfully exploited for photodynamic therapy (PDT) on malignant cells, following 5-aminolevulinic acid (ALA) administration and light irradiation. This treatment kills cancer cells by damaging organelles and impairing metabolic pathways via cellular reactive oxygen species (ROS) generation. We studied the efficiency of PpIX synthetized from ALA on ROS generation, in the Vincristine resistant (LBR-V160), Doxorubicin resistant (LBR-D160) and sensitive (LBR-) murine leukemia cell lines. Cells were incubated 4 hr with 1 mM ALA and then irradiated during different times with fluorescent light. One hour later, production of ROS was analyzed by flow cytometry using different fluorescent probes: Hydroethidine (HE) for superoxide anion, 2',7' Dichlorodihydrofluorescein diacetate (DCFH-DA) for hydrogen peroxide; mitochondrial damage was examined with 3,3' Dihexyloxacarbocyanine iodide (DiOC6). We found that superoxide anion production in the three cell lines increased with irradiation time whereas no peroxide hydrogen was detected. Mitochondrial damage also increased in an irradiation time dependent manner, being higher in the Vincristine resistant line. Previous studies have demonstrated that apoptotic cell death increased with irradiation time, which is consistent with these results, indicating that ROS are critical in ALA-PDT efficiency to kill malignant cells.

Key words: 5-aminolevulinic acid, porphyrins, photodynamic therapy

INTRODUCTION

Porphyrins are compounds with photodynamic properties, which are exploited in the cancer treatment known as Photodynamic Therapy (PDT). It involves the administration of a given photosensitizing agent (PS), the preferential accumulation of the PS in malignant tissue and the subsequent activation by visible light; the interaction between the excited PS and molecular oxygen produces singlet oxygen ($^1O_2$) as well as other reactive oxygen species (ROS) selectively destroying the target cells (13).

Abbreviations: **ABMT**, autologous bone marrow transplantation; **ALA**, 5-aminolevulinic acid; **DCFH-DA**, 2', 7' Dichlorodihydrofluorescein diacetate; **DiOC6**, 3, 3' Dihexyloxacarbocyanine iodide; **HE**, Hydroethidine; **MDR**, multidrug resistance; **MMP**, mitochondrial membrane potential; **PpIX**, Protoporphyrin IX; **PDT**, photodynamic therapy; **ROS**: reactive oxygen species

A clear demonstration that porphyrins are unique and powerful endogenous PSs are the human disorders known as porphyrias, in which porphyrin intermediates are accumulated because of the deficiency of some enzymes of the heme biosynthetic pathway (2). Abnormal quantities of circulating porphyrins result in skin photosensitivity and development of cutaneous lesions; in patients with Erythropoietic Protoporphyria (EPP), Protoporphyrin IX (PpIX) is the predominant elevated porphyrin (8).

Endogenously generated porphyrins after 5-aminolevulinic acid (ALA) administration has become one of the most promising fields in cancer research, it has been successfully used to visualize and destroy malignant cells (6). ALA-based PDT was reported to inactivate leukemic cells (7) and it can be used for purging residual tumor cells in autologous transplants (10)

Autologous bone marrow transplantation (ABMT) is a common treatment for

hematopoietic disorders; however this therapy is limited by a high relapse rate which may be attributed to the presence of residual tumor cells. Moreover, the presence of drug resistant cells contributes to the ineffectiveness of the ABMT (15). Among the methods capable of eliminating occult leukemic cells from bone marrow remission grafts, apart from pharmacological and immunological procedures commonly employed, the potential use of PSs based purging methods are being examined (14).

In the present study initiated to explore if ALA-based PDT could overcome multidrug resistance when it is applied to eradicate minimal residual disease in patient with leukemia, ROS generation after ALA exposure and subsecuent light irradiation was examined in the vincristine resistant (LBR-V160), doxorubicin resistant (LBR-D160) and sensitive (LBR-) murine leukemia cell lines.

MATERIALS AND METHODS

Chemicals

ALA, hydroethidine (HE), dichlorohydrofluorescein diacetate (H2DCF-DA), diehxyloxacarbocyanine iodide (DiOC6), menadione and trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) were obtained from Sigma Chem. Co.

Cell lines

Vincristine resistant (LBR-V160), doxorubicine resistant (LBR-D160) and senstitive (LBR-) murine leukemic cell lines (IDEHU, Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, UBA), were cultured in RPMI 1640 medium (GIBCO, Gran Island, NY. USA), supplemented with 10% FCS, gentamycin and l-glutamine, at 37º C in a 5% CO_2 humidified atmosphere.

Treatments

Cells were incubated in 15 ml Falcon tubes during 4 hr with 1mM ALA in RPMI 1640 complete medium. At the end of incubation cells were pelleted and mixed with 5% chlorhydric acid to extract PpIX. Fluorescence spectrum was recorded in a Perkin Elmer spectrofluorophotometer (emission wavelength at 604 nm, excitation wavelength at 406 nm), using PpIX as standard reference.

Photodynamic experiments: after incubation with ALA, the cells were resuspended in ALA free medium and then irradiated with light from a bank of two fluorescent lamps (Osram L 18W/765); the spectrum of light was between 400 and 700 nm with the highest radiant power at 600 nm. The tubes were located at 21 cm from the light source. Viable cells were determined by the MTT method (11).

Oxidative stress

One hour after PDT, cells were centrifuged, resuspended in PBS and loaded with the fluorescent probes for ROS, superoxide anion and mitochondrial damage detection. Stained cells were examined with a Partec PAS III flow cytometer.

For ROS detection, especially hydrogen peroxide, cells were incubated with 1 uM H_2DCF-DA (λ excitation: 485 nm; λ emission: 530 nm) during 30 minutes at 37ºC. Positive control was performed incubating a cell suspension aliquot with 0,1 mM H_2O_2 30 minutes prior to the probe addition.

Superoxide anion production was detected by incubating the cells with 2 uM HE (λ excitation: 510 nm; λ emission: 590 nm) during 30 minutes at 37º C. To perform a positive control a cell suspension aliquot was incubated with 25 uM menadione during 1 hr prior to the probe addition.

Mitochondrial damage was detected incubating the cells with 30 nM DiOC6 (λ excitation: 484 nm; λ emission: 511 nm) during 30 minutes at 37º C. For the positive control cells were incubated with 250 nM FCCP, 10 minutes prior to the probe addition (12).

Statistics

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.

RESULTS

PpIX accumulated after incubation with 1 mM ALA during 4 hr was 22.5 pmol PpIX/ 10^6 cells in the LBR- cell line, 51.6 pmol PpIX/ 10^6 cells in the LBR-V160 cell line and 27.5 pmol PpIX/ 10^6 cells in the LBR-D160 cells; these amounts produced 31%, 49.5% and 50.8% of cell death respectively, 1 hr after 10 min irradiation (data not shown).

Confocal fluorescence microscopy using specific organelle markers, showed the typical red fluorescence of PpIX after 4 hr incubation of the cells with 1 mM ALA, confined in the mitochondria. As the incubation time increases the fluorescence pattern diffuses to all the cytoplasm, including lysosomes. These results were similar in the three cell lines (data not shown).

Superoxide anion production was measured with the fluorescent probe HE. The red fluorescence intensity of HE was used to detect O_2^{2-}, as it is increased when it is oxidized. The main responsible for this oxidation is oxygen free radical, with minimal oxidation by H_2O_2. Fig 1 shows typical histograms obtained by flow cytometry after ALA-based PDT in the LBR- cell line.

Superoxide anion production increased with the irradiation time in the three cell lines. The two resistant lines showed a similar behaviour, while in the sensitive cells the generation of superoxide anion was higher and the increment too (Fig 2). Under our experimental conditions, hydrogen peroxide production was not detected.

The effect of ALA-based PDT on mitochondrial membrane potential (MMP) is dependent on the irradiation dose. As shown in

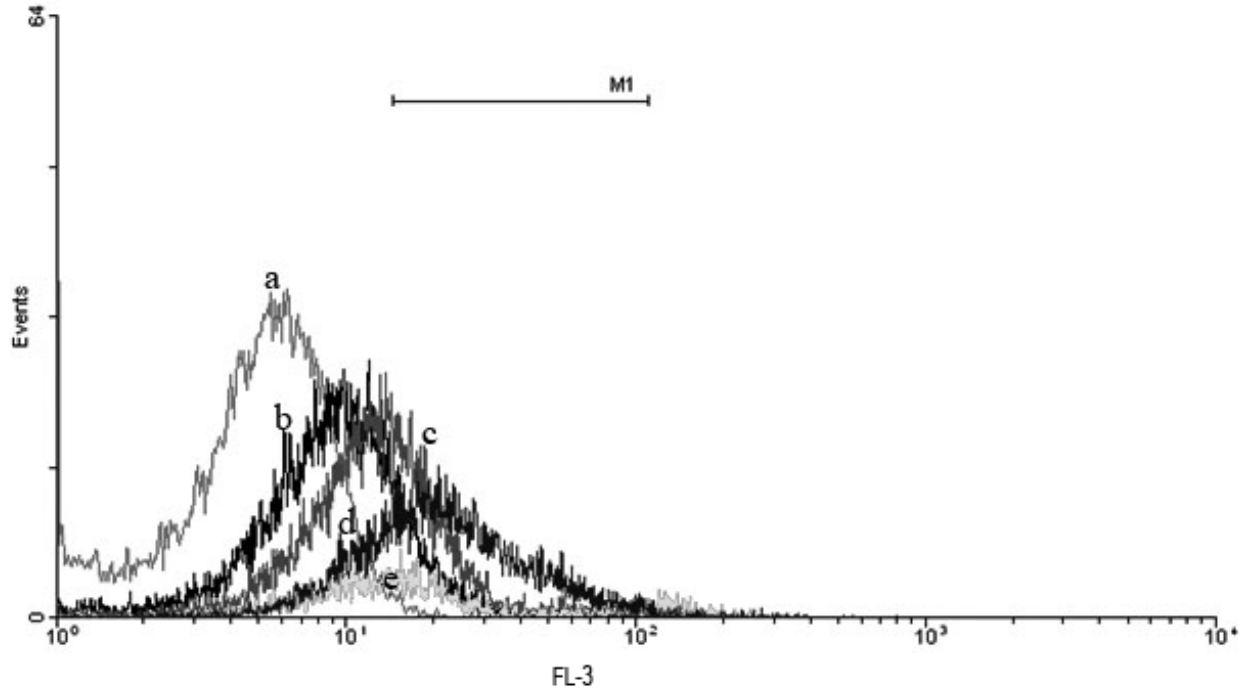

Figure 1. Flow cytometric histograms obtained by incubating LBR- cell line with HE one hr after ALA-PDT as described in Materials and Methods. a) Negative control, b) 5 minutes of irradiation, c) 10 minutes of irradiation, d) 20 minutes of irradiation and e) positive control.

Fig 3, MMP decreased to 26.3% in the LBR- cells, 28.7 % in the LBR-V160 cells, and 36.9% in the LBR-D160 cells, when cells were irradiated during 5 min. In the sensitive line no changes in the depolarization level were observed when irradiation times increased up to 20 min, while in the resistant cell lines, an increment of depolarized cells was detected. When cells were irradiated for 20 min, LBR-V160 cells showed the highest depolarization level.

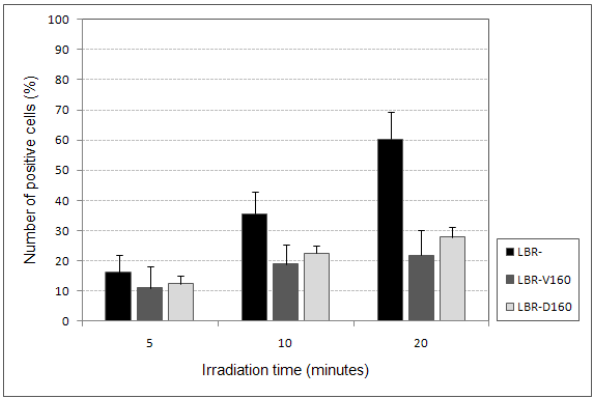

Figure 2. Superoxide anion production. It was assesed by HE staining 1 hr after ALA-PDT as described in Materials and Methods. Results are expressed as a % of stained cells relative to negative control after different irradiation times.

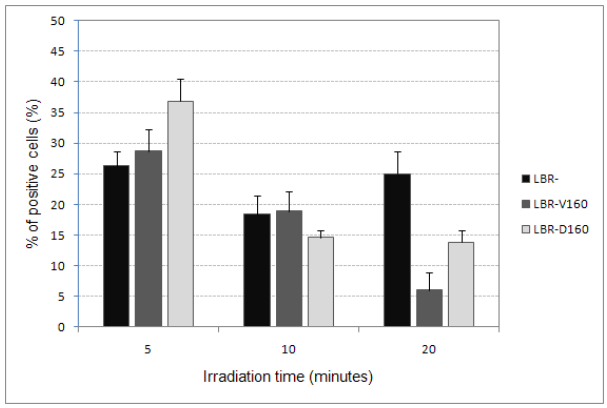

Figure 3. Evaluation of mitochondrial membrane potential. It was evaluated by DiOC6 staining 1 hr after ALA-PDT as described in Materials and Methods. Results are expressed as a % of stained cells relative to negative control after different irradiation times.

DISCUSSION

The ability of malignant cells to selectively accumulate PSs may offer the possibility of using PDT in purging of ABMT (14). This therapy is limited by a high relapse rate which may be attributed to the presence of residual tumor cells. Moreover, the presence of multidrug- resistant (MDR) cells contributes to the ineffectiveness of ABMT. In the last decade, a number of authors have studied the effect of PDT on MDR cells,

suggesting the possible application of PDT to treat MDR cancer cells (5).

Stimulation of the endogenous synthesis of the photosensitizing compound PpIX in cancer cells following the application of the metabolic precursor ALA, was investigated in many cell lines, including leukemic cells (1).

ALA-based PDT was reported to inactivate leukemic cells (7) and it can be used for purging residual tumor cells in autologous transplants (10). This treatment kills cancer cells by damaging organelles and impairing metabolic pathways via cellular reactive oxygen species (ROS) generation. PpIX is a very efficient photosensitizer having a high capacity of accumulating in the intracellular membrane organelles, like mitochondria and lysosomes. Interaction between the triplet excited state of the sensitizer and molecular oxygen leads to singlet oxygen production and other ROS, inducing deleterious effect on cells including induced apoptosis.

As PpIX is synthesized in the mitochondrial intermembrane space, alteration in MMP following ALA-PDT is expected. To assess the mitochondrial dysfunction, we used DiOC6, a lipophilic cationic probe which is commonly employed for monitoring changes of the mitochondrial and or the plasma membrane potential (3). This probe used at low concentrations (nM order) measures changes in MMP in living cells; it can be also useful to stain the endoplasmic reticulum but at higher concentrations (µM order) (12). In our case mitochondrial dysfunction progressed as the irradiation time increased from 5 min to 20 min, in the two drug-resistant lines, LBR-V160 cells showing the highest depolarization level. In the LBR- cells no variation in MMP occurred, and it is in accordance with the observation that apoptosis induction in this cell line was slower than in the resistant cell lines (data not shown).

Under our experimental conditions, hydrogen peroxide production was not detected in any of the three cell lines. The red fluorescent intensity of superoxide anion production probe HE increased with the irradiation time, this effect was more pronounced in the LBR- cell line, while the two drug-resistant lines showed similar behaviour.

These results are indicating alterations in ROS balance of the cells after ALA-PDT treatment, which may account of the serious irreversible damage conducting to cell death observed in previous experiments. It is possible that cell death mode following PDT may be differentially regulated by the type and site of ROS produced within the cells. Relocalization of PpIX within the cells observed by confocal fluorescence, may explain the different way of death found in ALA-PDT treated cells: apoptosis, necrosis and/or autophagy (4, 9). Further experiments concerning the mechanism of cell death involved are currently under investigation.

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