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Changes in intramitochondrial cardiolipin distribution in apoptosis-resistant HCW-2 cells, derived from the human promyelocytic leukemia HL-60

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Abstract Using a cytofluorimetric approach, we studied intramitochondrial cardiolipin (CL) distribution in HCW-2 cells, an apoptosis-resistant clone of human HL-60 cells. In HL-60, about 50% of total CL is distributed in the outer leaflet of mitochondrial inner membrane, while in HCW-2 a significantly higher amount of CL (about 65%) is in that site. In basal conditions, HSW-2 cells also show a reduced mitochondrial membrane potential even if they are able to proliferate as the parental line. Taking into account the complex functions that CL plays in the regulation of mitochondrial activity, it is likely that HCW-2 could produce ATP utilizing more glycolytic pathways rather than mitochondrial respiratory chain.

Key words: Mitochondrion; Cardiolipin; Mitochondrial membrane potential; Apoptosis; Flow cytometry

1. Introduction

Mitochondria are involved in many types of programmed cell death/apoptosis. In some experimental models, they provide the energy required for the process to occur [1–3]. In others, they can trigger cell death in a number of ways: by disrupting electron transport and energy metabolism, by releasing/activating proteins that mediate apoptosis, and by altering cellular redox potential [4–6]. As an abnormal resistance to apoptosis correlates with malformations, autoimmune diseases or cancer due to the persistence of self-specific immunocytes or mutated cells, the role of such organelles is crucial for the maintenance of the body homeostasis [7,8].

Recent studies have suggested that cardiolipin (CL), a diacidic phospholipid that was isolated from beef heart and has an unusually high content of linoleic acid ester residues [9], could be involved in apoptotic cell death [10,11]. CL is present throughout the eukaryotes including animals, plants and fungi. In mammalian tissues and in yeast, CL is found exclusively in mitochondria [12,13], a site in which it is specific and essential for the normal function of mitochondrial inner membrane system, and in particular for: (i) the activity of cytochrome bc_1 and cytochrome *c* oxidase, key components of the electron transport chain; (ii) the attachment of cytochrome *c* to the inner mitochondrial membrane; (iii) the activity of mitochondrial transporters, including the ADP/ATP translocase, the mono-, di-, tri-carboxylate carriers, the α-ketoglutarate, aspartate/glutamate and palmitoylcarnitine carriers, and the (acyl)carnitine translocase system; (iv) the functionality of F_0F_1-ATP synthase [14].

In order to further investigate the role of such a molecule, we analyzed the apoptosis-resistant clone called HCW-2, derived from the human promyelocytic cell line HL-60. This clone has been selected from parental cells by treatment with a cytotoxic dose of 8-Cl-cAMP, and shows resistance to cycloheximide- and staurosporine-induced cell death [15].

In the present paper, using a cytofluorimetric approach that allows the study of intact cells, we describe a direct assay for analyzing intramitochondrial CL transverse distribution [16]. The assay is based upon the following peculiar properties of the fluorescent dye 10-*N*-nonyl-3,6-bis(dimethylamino)acridine (nonyl acridine orange, NAO), i.e.: (i) two molecules of NAO can bind with a high affinity one single CL molecule, forming NAO dimers; (ii) the dye is unable to bind zwitterionic phospholipids and has a low affinity for other anionic phospholipids; (iii) NAO is capable of changing its emission due to its spectral properties, as after dimer formation the fluorescence can shift from green (monomeric form) to red (dimeric form) [16,17]. Using such an assay, we could show that the distribution of CL was markedly different in HCW-2 cells when compared with the parental line HL-60.

2. Materials and methods

2.1. Chemicals

Fetal calf serum and RPMI 1640 were from Life Technologies Ltd. (Oxford, UK). NAO and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were from Molecular Probes (Eugene, OR, USA). Valinomycin (Val) and other common chemicals were from Sigma (St. Louis, MO, USA) and were of analytical grade.

2.2. Cell culture

Mycoplasma-free HL-60 human promyelocytic leukemic cells and its apoptosis-resistant clone HCW-2 (kindly provided by Drs. J.H. Wyche and Z. Han, Brown University, Providence, RI, USA) were grown in suspension in complete culture medium (RPMI 1640 sup-

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Abbreviations: CL, cardiolipin; NAO, nonyl acridine orange; $\Delta\Psi$, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; Val, valinomycin

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plemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin) and kept at 37°C in a humidified atmosphere (5% CO_2 in air). Cells were collected during the log phase of growth, washed in phosphate-buffered saline (PBS), counted and adjusted at a density of 1×10^6 cells/ml.

2.3. Analysis of CL distribution

In order to analyze CL intramitochondrial distribution, we took advantage from the spectral characteristics of NAO [16,17]. In particular, we used its capacity to form dimers when it interacts with diacidic phospholipid, and the fact that its fluorescence emission shifts from 525 nM (monomeric form of the dye) to about 640 nm (under dimeric conditions, in the presence of a stoichiometric ratio of 2:1 with CL). Cells were fixed in 1% formaldehyde (in PBS) for 15 min at room temperature, then washed twice in cold PBS and adjusted at the concentration of 0.5×10^6 cell/ml in PBS. Increasing amounts of NAO (0.1–35 μM) were added, and cells were kept at room temperature for 15 min, washed twice with PBS and resuspended in a total volume of 400 μl of PBS and analyzed.

The red and green fluorescence emission intensity was plotted as function of the amount of NAO present in the incubation mixture; however, only the red fluorescence was used for the analysis of CL distribution.

2.4. Analysis of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was measured by lipophilic cationic probe JC-1 [18], a dye we are using for this purpose since several years, and that reversibly changes its color from green to orange/red as $\Delta\Psi$ increases (over values of about 80–100 mV). This property is due to the reversible formation of JC-1 aggregates upon membrane polarization that causes shifts in emitted light from 530 nm (i.e. emission of monomeric form) to 590 nm (i.e. emission of J-aggregates) when excited at 490 nm [19,20]. Cells were stained with 2.5 μg/ml JC-1 and kept at room temperature for 20 min, washed twice with PBS, resuspended in a total volume of 400 μl PBS and analyzed as described [21,22]. For a complete depletion of $\Delta\Psi$, the potassium ionophore Val (used at the concentration of 100 nM) was used as a positive control [18].

2.5. Flow cytometry and data analysis

Cytofluorimetric analyses were performed using a FACScan cytometer (Becton Dickinson, San José, CA, USA), equipped with an argon ion laser tuned at 488 nm. Green fluorescence was detected through the standard band-pass filter centered at 520 ± 10 nm, and red fluorescence through the long pass filter (615 ± 15 nm). A standard cytogram based on the measurement of right angle scatter versus forward angle scatter was defined to eliminate cellular debris and aggregates.

Samples stained with scalar amounts of NAO were acquired in linear scale, and the median fluorescence values from the resulting histograms were then used to calculate the intensity of the fluorescence mission. Median values were then plotted and analyzed by using Grafit 3.0 software.

Cells stained with JC-1 were acquired and analyzed in logarithmic scale; the FL1/FL2 ratio was calculated after linearization of data, as described [23]. In all cases, a minimum of 10^4 events per sample were acquired in list mode and analyzed with WinMDI 2.8 software (by Dr. Trotter, Scripps Institute, La Jolla, CA, USA). Statistical analysis was performed by a two-tailed Student's *t* test.

3. Results

3.1. Measurement of the distribution of CL between the leaflets of mitochondrial inner membrane: the theoretical approach

Cells were labelled with different concentrations of NAO to take advantage of the capacity of this fluorescent dye to gradually bind CL with high affinity [16]. At low doses, NAO binds CL on the outer leaflet of mitochondrial inner membrane in a monomeric form; this phenomenon gives a green fluorescence emission after excitation at 490 nm. Increasing NAO concentrations result in a qualitative change in fluorescence emission because of the capacity of NAO to form dimers (i.e. two NAO molecules bind one CL molecule) [16,17], and cells emit in the red channel. Simultaneously, while increasing the red fluorescence, they loose the capacity to emit in the green channel (as the number of NAO monomers decreases).

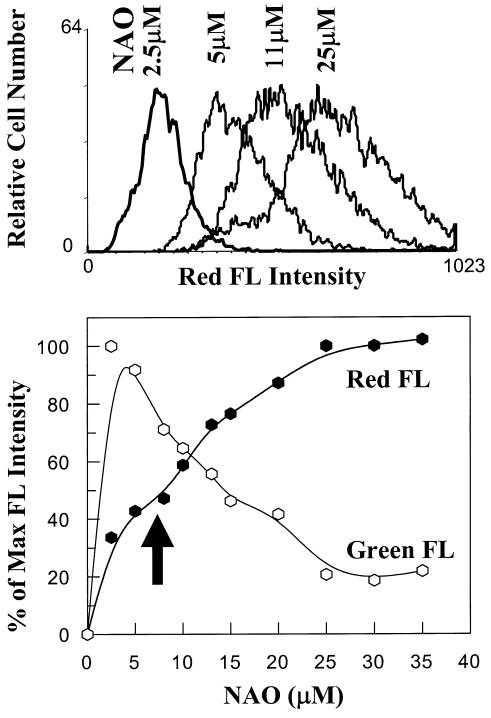

Fig. 1. Analysis of intramitochondrial CL distribution in HL-60 cells. Upper panel: fluorescence histograms after staining with different doses of NAO (only the red fluorescence is shown). Lower panel: analysis of green and red fluorescence intensity after staining with increased doses of NAO. Data are expressed as percentages of the maximum fluorescence intensity (i.e. that obtained with the highest concentration of NAO), and were calculated on the median value of each histogram. Arrow indicates the first plateau, i.e. the point of saturation of the CL residues present on the outer leaflet of the inner mitochondrial membrane. One experiment representative of four is shown.

The curve of red emission shows a peculiar phenomenon related to the dose of dye and its distribution into the mitochondrial inner membrane [17]. Indeed, when NAO dimers occupy all the possible residues of CL that are present on the outer leaflet of mitochondrial inner membrane, a plateau in fluorescence emission is reached. Then, the interactions between NAO and CL induce modifications of the inner membrane permeability, so that the dye may cross the membrane and may have free access to the CL present in the matrix side, with the subsequent formation of other dimers, and an increase in red fluorescence. Finally, when also the inner leaflet is saturated, i.e. when all the phosphate residues of CL have bound NAO, a new and final plateau is reached. The first plateau indicates the saturation of the CL residues exposed on the outer leaflet, the second the saturation of all the intramitochondrial CL, i.e. the maximal fluorescence. The ratio between the fluorescence intensity of the first plateau and that of the second corresponds to the percentage of CL present on the cytoplasmic (outer) face of mitochondrial inner membrane.

Finally, it is noteworthy that, apart a low affinity, the stoichiometry of NAO binding to other phospholipids such as

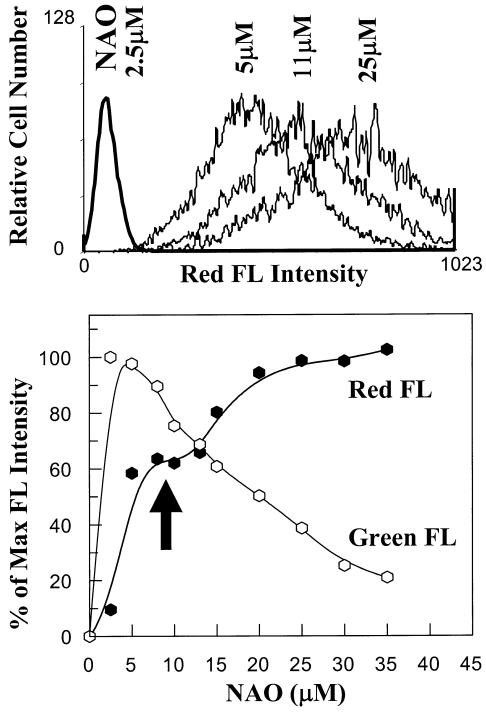

Fig. 2. Analysis of intramitochondrial CL distribution in HCW-2 cells. Panels as in the legend to Fig. 1.

phosphatidylserine and phosphatidylinositol (1 mol dye/1 mol phospholipid) prevents the dimerization of the dye, and the subsequent appearance of red fluorescence [24].

3.2. Distribution of CL into mitochondrial inner membrane

NAO is a dye that has high affinity for CL, but it is also a cationic probe which is sensitive to $\Delta\Psi$. Thus, cells were pre-treated with formaldehyde to collapse $\Delta\Psi$ and prevent the incorporation of NAO due to $\Delta\Psi$ rather than specific binding to CL [25]. Such a treatment also ensures a better preservation of cellular integrity, since high concentrations of NAO can induce cell damages and disruptions [26].

The binding curves obtained from the red fluorescence analysis in both cell types (HL-60 and HCW-2) were biphasic, with two plateaus (arrows in the lower part of Figs. 1 and 2, respectively). As described above, the first saturation level corresponds to the amount of CL located in the outer leaflet of mitochondrial internal membrane [17,27]. The second saturation level reflects penetration of NAO into mitochondria and titration of CL in both halves of inner membrane. As expected, increasing the NAO dose resulted either in an increase in red fluorescence or a decrease in the green fluorescence (lower part of Figs. 1 and 2).

The distribution of CL on the cytoplasmic face (outer leaflet) of the inner membrane in HL-60 was $50.2 \pm 2.8\%$ of the total mitochondrial CL content (mean of four independent experiments). Such a value is similar to that reported in other studies on eukaryotic or mammalian cells [16,17,24,27,28]. The apoptosis-resistant clone HCW-2 shows a significant higher percentage of CL present in the outer leaflet, i.e. $64.8 \pm 2.4\%$. The analysis of four separate experiments showed only negligible variations, and the statistical analysis revealed a level of significance < 0.01.

3.3. HCW-2 mitochondria respond normally to depolarizing stimuli

We then studied the mitochondrial membrane potential in HCW-2 stained with JC-1, and found that, in basal conditions, such cells had a slight reduction in orange fluorescence (FL2) and a parallel increase in green fluorescence (FL1), with respect to the parental cell line HL-60. Consequently, after linearization of the median values obtained from the fluorescence histograms, the FL1/FL2 ratio was higher in HCW-2 compared to HL-60 cells (0.83 ± 0.05 vs. 0.67 ± 0.04, $P < 0.05$). Adding Val resulted in the same marked decrease in FL2 with a concomitant increase in FL1, which reveals a similar $\Delta\Psi$ drop in both cell types (Fig. 3).

4. Discussion

CL appears to be involved, either directly or indirectly, in the modulation of a variety of mitochondrial processes including the activation of mitochondrial enzymes, and hence production of energy by oxidative phosphorylation [29]. CL is specific and essential for the normal function of mitochondrial inner membrane system, even if its precise role is not completely understood [14]. CL–protein interactions orient membrane proteins, matrix proteins, and, on the outer face of inner mitochondrial membrane, receptors, enzymes and some leader peptides for import. Moreover, they activate enzymes or keep them inactive unless the inner membrane is disrupted and modulate formation of non-bilayer HII-phases [30].

CL is greatly asymmetrical without involving proteins, has the only phospholipid headgroup that can collect and donate pumped protons to transmembrane porters at physiological pH levels, and mediates an adaptively responsive proton-selective leak that regulates non-phosphorylating oxidations [31]. The regulatory properties which govern CL biosynthesis, its remodelling and trafficking, are beginning to emerge [29]. However, a consensus has not yet been established on the role and meaning of the submitochondrial localization of CL. This question is important for understanding its biological functions, and to unravel the topographic relationships in the mitochondrial structural framework [13]. Interestingly, recent data indicate that, in yeast, the transmembrane asymmetry of CL changes during the switch from fermentative to gluconeogenic metabolism, and influences the transmembrane redox potential [27]. This could indicate that a different distribution of CL in the inner membrane could be related to a higher non-oxidative metabolism in apoptosis-resistant HCW-2 cells, compared to HL-60 cells.

We have investigated the distribution of CL in the mitochondrial inner membrane in cells that were pre-treated with formaldehyde to prevent the incorporation of the dye due to $\Delta\Psi$ [25,32]. The higher amount of CL we found in the outer leaflet of apoptosis-resistant HCW-2 cells could suggest that, in comparison with the parental line HL-60, the glycolytic pathway gives a more relevant contribution to the establishment of the intracellular cellular ATP pool [33]. This is corroborated by preliminary data on the respiration of such cells, indicating that their metabolism is skewed towards anaerobic pathways (V. Bobyleva, personal communication).

To ascertain whether a relationship exists between CL topography and membrane redox potential [27], we then evaluated $\Delta\Psi$ in both cell lines by using the specific probe JC-1.

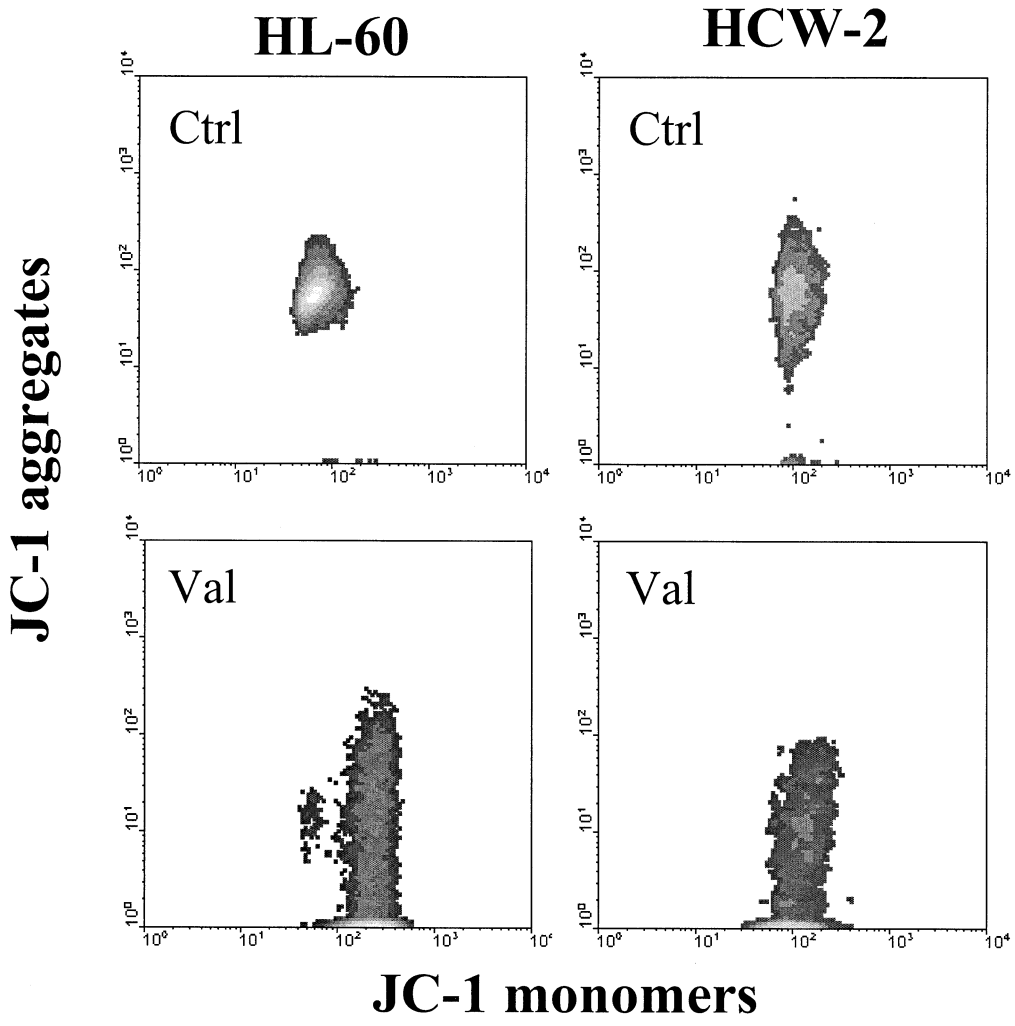

Fig. 3. Analysis of $\Delta\Psi$ in HL-60 and HCW-2 cells. Upper panels show that, in basal conditions, HCW-2 have a lower $\Delta\Psi$ than HL-60 cells. Lower quadrants evidence a similar sensitivity of both cell lines to the depolarization of mitochondrial membrane induced by Val. Ctrl: control, Val: valinomycin. One experiment representative of four is shown.

By using flow cytometry, we observed that, in comparison with HL-60, HCW-2 cells have a similar viability in culture and the same capacity to undergo cell proliferation (not shown), but displayed in basal conditions a lower $\Delta\Psi$. Moreover, when Val was added, both cell types had the same, marked drop in $\Delta\Psi$. The difference in JC-1 FL2 emission between the two cell lines could indicate first of all the presence of a lower $\Delta\Psi$ in HCW-2 cells, but, alternatively, could be explained taking into account: (i) the physico-chemical and spectral properties of JC-1; (ii) the fact that different cell types could require different staining protocols [3]. However, preliminary data obtained by electron microscopy indicate that, in comparison with HL-60, HCW-2 cells are much bigger and possess a consistently higher number of mitochondria, that display an 'unhealthy' aspect (D. Quaglino, personal communication). This suggests that the reduced fluorescence after JC-1 staining could be due to an effective decrease in $\Delta\Psi$. It remains to be established whether the lower $\Delta\Psi$ observed in HCW-2 cells could contribute to the different CL distribution in the mitochondrial internal membrane or viceversa.

In fact, the possibility exists that the different topography of CL distribution could be responsible per se for a lower $\Delta\Psi$ because of the role of CL during oxidative phosphorylation, namely because of its capacity to regulate state 4 respiration by returning ejected protons across and over mitochondrial membrane phospholipids, and to regulate state 3 respiration through the relative contributions of proteins that transport protons, electrons and/or metabolites [30,31]. CL has special properties, which include the organization into surface headgroup microdomains, asymmetry in bilayer membranes, ability to bind and donate protons at physiological pH, and propinquity to proton acceptors in mitochondrial structure. These properties are relevant for a recently proposed model in which outer surface CL microdomains act as a proton antenna that collects protons and donates them, via a water-shuttle, to transmembrane porters: transient water-molecule-chains between extended phospholipid acyls, protonophores, and uncoupling proteins [31].

In conclusion, our data show that apoptosis-resistant clone HCW-2 has a higher amount of CL in the outer leaflet of the mitochondrial inner membrane compared to the parental, apoptosis-sensitive cell line. Further studies are needed to ascertain whether this could influence the metabolism of these cells, and how this could be related with the resistance to apoptosis.

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