Mitochondrial Transmembrane Potential Changes Support the Concept of Mitochondrial Heterogeneity During Apoptosis

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SUMMARY Dissipation of mitochondrial membrane potential (ΔΨm) and release of cytochrome c from mitochondria appear to be key events during apoptosis. The precise relationship (cause or consequence) between both is currently unclear. We previously showed in a model of serum-free cultured granulosa explants that cytochrome c is retained in a subset of respiring mitochondria until late in the apoptotic process. In this study we further investigated the issue of heterogeneity by using the ΔΨm-sensitive probe CM-H₂TMRos in combination with a DNA fluorochrome. Changes of ΔΨm were assessed qualitatively by epifluorescence microscopy and were quantified using digital imaging microscopy. This approach yielded the following results: (a) CM-H₂TMRos staining is a reliable and specific procedure to detect ΔΨm changes in granulosa cells explants; (b) dissipation of transmembrane potential is an early event during apoptosis preceding nuclear changes but is confined to a subpopulation of mitochondria within an individual cell; (c) in frankly apoptotic cells a few polarized mitochondria can be detected. These findings support the hypothesis that ATP needed for completion of the apoptotic cascade can be generated during apoptosis in a subset of respiring mitochondria and is not necessarily derived from anaerobic glycolysis. (J Histochem Cytochem 49:1277–1284, 2001)

KEY WORDS mitochondrial membrane potential apoptosis granulosa cells CM-H₂TMRos cytochrome c

Since mitochondria were discovered in 1840, they have provided fertile ground for scientific inquiry. Given the importance of mitochondria for cell life, it comes now as no surprise that mitochondrial dysfunction and failure lead to apoptotic and necrotic cell death. Apoptosis and necrosis are two forms of cell death with clearly distinguishing morphological and biochemical features (Wyllie et al. 1980). Recent advances suggest, however, that apoptosis and necrosis have some common steps. Bcl-2 and caspase inhibitors had been believed to specifically inhibit apoptosis, but preventive effects of Bcl-2 on necrosis were also proved by the demonstration that Bcl-2 and its relative, Bcl-xL, inhibit necrotic cell death induced by oxygen depletion, respiratory chain inhibitors such as KCN and antimycin A, or by glutathione depletion (Tsujimoto 1997). Necrotic cell death was also retarded by caspase inhibitors, including tetrapeptide inhibitors and a serpin, CrmA, derived from cowpox virus (Tsujimoto 1997). Moreover, the mitochondrial permeability transition (MPT) represents a pathway that is shared both in apoptosis and necrosis (Lemasters et al. 1999).

There is general agreement that apoptosis, in contrast to necrosis, is an active, energy-requiring process. Richter et al. (1996) were the first to propose that the cellular ATP level is an important determining factor for cell death, either by apoptosis or necrosis. They hypothesized that a cell stays alive as long as a certain ATP level is maintained. When ATP falls below this level apoptosis ensues, provided that enough ATP is still available for energy-requiring apoptotic processes such as enzymatic hydrolysis of macromolecules, nuclear condensation, and bleb formation. Only low ATP concentrations can switch an apoptotic death towards a necrotic fate (Richter et al. 1996). This hypothesis has been confirmed by later studies of Leist et al. (1997). These authors demonstrated that demise of
human T-cells caused by two classic apoptotic triggers (staurosporine and CD95 stimulation) changed from apoptosis to necrosis when cells were depleted of ATP. There seems to be an apparent contradiction between the obligatory induction of the MPT associated with a cessation of mitochondrial ATP synthesis, on the one hand, and a need for the maintenance of intracellular ATP levels during the development of apoptosis, on the other. This controversy could be resolved if the apoptosis-inducing changes were restricted to a subset of mitochondria or if the energy level can be maintained by anaerobic glycolysis. In a previous study we have characterized by ultrastructural localization of cytochrome c the persistence of a subset of respiring mitochondria until late in the apoptotic process in a model of granulosa explants cultured under serum-free conditions, which elicits apoptosis by gonadotropin withdrawal (D’Herde et al. 2000). The aim of the present study was to investigate whether apoptosis is accompanied by an early drop of \( \Delta \Psi m \) and whether the detected heterogeneity of the mitochondrial population with respect to cytochrome c loss is paralleled by a similar heterogeneity at the level of the mitochondrial membrane potential. To measure \( \Delta \Psi m \) we used a chloromethyl rosamine-derived probe, CM-H\(_2\)TMRos, which becomes fluorescent only when it is oxidized in the cell. This dye has an alkyllating chloromethyl moiety attached. Owing to their membrane potential, functional mitochondria take up the dye. Once the probe accumulates in the mitochondria, the chloromethyl group can react with accessible thiol groups of peptides and proteins to form an aldehyde-fixable conjugate (Macho et al. 1996; Poot et al. 1996).

Materials and Methods

Isolation and Culture of Granulosa Cell Sheets

Granulosa cell (GC) sheets were prepared from ovarian follicles of adult regularly laying Japanese quail (Coturnix japonica). The animals were reared under continuous artificial illumination, with food (fresh lettuce and complete breeding food; Biofor AVEVE, Belgium) and water ad libitum. Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives 86/6091 EEC. The monolayered granulosa epithelium of the largest preovulatory follicle (F1) was isolated from the surrounding thecal covering as previously described (Gilbert et al. 1977). This method provides large sheets of vital GCs sandwiched between their basement membrane and vitelline membrane. In the avian granulosa, three regions were defined by us (Farioli–Vecchioli et al. 2000). The transition region (animal-vegetative pole) and the animal pole region are both in close contact with the germinal disc that contains several cell proliferatory factors, which could influence survival of GCs (Tischkau and Bahr 1996). Therefore these regions were selectively discarded from the rest of the GC sheet. The remaining vegetative pole region of \( \sim 5.8 \) cm\(^2\) was divided into smaller squares of \( \sim 4 \) mm\(^2\), followed by culture in 35-mm petri dishes under serum-free conditions for up to 72 hr in humidified room air at 38°C. The culture medium was M199 (Sigma; Bornem, Belgium) phenol red-free supplemented with 0.1% w/v bovine serum albumin fraction V (Sigma), 6.0 g/liter HEPES (Acros; Geel, Belgium), 1% v/v penicillin-streptomycin (Gibco BRL, Paisley, UK) at pH 7.4. To inhibit the apoptotic process, the culture medium of controls was supplemented with luteinizing hormone (LH, 100 ng/ml; Sigma) and insulin-like growth factor-I (IGF-I, 10 ng/ml; Sigma) (Onagbesan and Peddie 1995).

Mitochondrial Staining by CM-H\(_2\)TMRos

CM-H\(_2\)TMRos (Molecular Probes; Leiden, The Netherlands) was stored desiccated at \(-20^\circ\)C (following the instructions from the manufacturer) and dissolved in dimethylsulfoxide (DMSO; UCB, Leuven, Belgium) to give a 1 mM stock solution before use. Staining media were prepared immediately before use by adding the dye stock solution to culture medium to obtain the desired final dye concentration of 200 nM. Living GC sheets were incubated with the dye in phenol red-free medium in serum-free conditions for 15, 20, 30, and 60 min at 37°C shaking in the dark. Specific mitochondrial staining was obtained after a minimum of 30 min of incubation and was totally absent at shorter incubation times; at 60 min of incubation the signal-to-noise ratio further improved. Therefore, all images shown, as well as quantitative measurements of CM-H\(_2\)TMRos, are derived from experiments with a 60-min incubation period. After the incubation the GC sheets were gently rinsed in PBS at 37°C before fixation in freshly prepared 4% paraformaldehyde in PBS at 37°C for 15 min (following the manufacturer’s instructions). Thereafter, GC sheets were rinsed in PBS and permeabilized by incubation in ice-cold acetone at \(-20^\circ\)C for 10 min (following the manufacturer’s instructions). This acetone permeabilization step appeared to improve signal retention (data not shown). Finally, GC sheets were rinsed in PBS, mounted in a drop of bidest water, and allowed to air-dry. Samples were examined on a Leica DM IRB/E inverted microscope equipped with epifluorescence optics, suitable filters for FITC and TRITC detection, and an MPS-60 camera.

Double Staining (CM-H\(_2\)TMRos and DAPI)

GC sheets were stained with CM-H\(_2\)TMRos according to the standard procedure (see above), fixed with paraformaldehyde, permeabilized with acetone, and rehydrated with PBS. Then GC sheets were stained with 2',6'-diamidino-2-fenyldinole (DAPI; Sigma) at a concentration of 1 mg/ml in PBS at room temperature for 10 min. Then the GC sheets were mounted on slides and samples were observed under an epifluorescence microscope Leica (see above). Apoptotic cells were identified as cells showing condensed chromatin masses and/or fragmented nuclei. Small groups of apoptotic bodies were counted as remnants of one apoptotic cell. Apoptosis was expressed as the number of apoptotic nuclei per total number of nuclei counted in the same microscopic field and expressed as the percent apoptotic nuclei. This apoptotic index (AI) was averaged for 10 fields, giving a total...
number of about 1500 cells counted for each independent experiment.

Cytochrome c Staining

Living GC sheets were stained at 37°C in culture medium, pH 7.4, for 40–90 min with 2 mg/ml 3′-diaminobenzidine-tetrahydrochloride dihydrate (DAB). For light microscopic evaluation, whole mounts of the sheets were counterstained with methyl green. The DAB technique used in the present study to localize cytochrome c in individual mitochondria was described previously by us (D’Herde et al. 2000). Because it depends on cytochrome oxidase activity, it also gives evidence for an active respiration.

Pretreatment with Mitochondrial Poisons

Valinomycin (Sigma) was dissolved in 95% ethanol to obtain a 10 mM stock solution. The stock solution was stored in small aliquots at −20°C and diluted in culture medium immediately before adding to the cells to obtain a final concentration of 1 mM. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; Sigma) was dissolved in ethanol. The stock solution was stored in small aliquots at −20°C and diluted in culture medium immediately before adding to the cells to obtain a final concentration of 50 μM.

To investigate whether mitochondrial staining with CM-H$_{2}$TMRos is dependent on the ΔΨm, uncultured GC sheets and GC explants cultured for 72 hr supplemented with IGF-I and LH were analyzed in which the ΔΨm was dissipated with valinomycin (5 min at 37°C) and with FCCP (15 min at 37°C).

Quantitative Measurements of CM-H$_{2}$TMRos Fluorescence

Quantitative measurements of CM-H$_{2}$TMRos fluorescence were performed by digital imaging epifluorescence microscopy. GCs were viewed with an inverted Nikon Eclipse TE 300 epifluorescence microscope using a ×40 oil-immersion lens. CM-H$_{2}$TMRos fluorescence images were obtained by excitation at 546 nm, reflection off a dichroic mirror with a cut-off wavelength at 564 nm, and longpass emission filtering at 590 nm. Images were captured with an intensified CCD camera (Extended Isis camera; Photonic Science, East Sussex, UK) and were stored in a PC equipped with an image acquisition and processing board (Data Translation, type DT3155; Marlboro, MA).

To study the effect of mitochondrial poisons on CM-H$_{2}$TMRos fluorescence, images were acquired in the two experimental groups: uncultured GC sheets and GC explants cultured for 72 hr supplemented with IGF-I and LH.

To investigate ΔΨm changes during apoptosis induction by gonadotropin withdrawal, fluorescence intensity generated by CM-H$_{2}$TMRos was quantified in 72-hr cultures of GCs compared to control cultures supplemented with IGF-I and LH. All images were corrected for background fluorescence (culture medium without sheets).

Statistical Analysis

Data are expressed as mean ± SEM with n denoting the number of experiments on different animals. Statistical significance was tested using a Student’s t-test for unpaired observations. p values less than 0.05 were considered as statistically significant.

Results

Sensitivity of CM-H$_{2}$TMRos to Mitochondrial Poisons

To confirm that the mitochondrial CM-H$_{2}$TMRos dye accumulation was dependent on mitochondrial transmembrane potential, uncultured (i.e., freshly isolated) GC sheets and 72-hr IGF-I- and LH-supplemented cultures were treated with the mitochondrial uncoupling agents FCCP and valinomycin. Pretreatment of GC sheets with FCCP or valinomycin before probe loading resulted in absence of punctiform mitochondrial staining and showed only diffuse cytoplasmatic staining on visual inspection (Figures 1A, 1E, and 1F). Quantification of the CM-H$_{2}$TMRos signal under these conditions showed significantly reduced fluorescence intensity with FCCP or valinomycin compared to control (Figures 2A and 2B). Both mitochondrial poisons had no effect on the CM-H$_{2}$TMRos fluorescence when added after loading of the mitochondrial probe.

Subcellular Distribution of Mitochondria During Apoptosis

In uncultured GC sheets, staining of polarized mitochondria was intense and the pattern was uniform throughout the sheet. In images of GC sheets stained with CM-H$_{2}$TMRos in combination with DAPI, mitochondria appeared as threadlike and granular structures homogeneously distributed throughout the GCs. The mitochondrial staining revealed by CM-H$_{2}$TMRos was similar to the appearance of respiring mitochondria identified by cytochrome c staining (Figures 1A and 1B).

Double staining (CM-H$_{2}$TMRos–DAPI) of 72-hr IGF-I- and LH-supplemented cultures, i.e., the control condition in which the apoptotic process is inhibited (AI of circa 0.64 ± 0.27% SEM; n=4), showed a homogeneous pattern in contrast to the serum-free cultures. All normal cells showed a similar number of polarized mitochondria localized in compact masses at one side of the nucleus (Figure 1C).

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Seventy-two-hour cultured GC explants cultured in the absence of IGF-I and LH (AI of circa 29 ± 8.03% SEM; n=4) showed a non-uniform pattern of CM-H$_{2}$TMRos staining throughout the GC explant cell layer (Figure 1D). Some cells with normal nuclei were surrounded by very few stained mitochondria, whereas other adjacent cells displayed a sizable number of polarized mitochondria localized around nuclei with normal morphology. Apoptotic cells were observed with condensed and fragmented nuclei but still containing polarized mitochondria (Figure 1G). In the
Figure 1  (A–G) Epifluorescence microscopy of paraformaldehyde-fixed GC sheets double stained with CM-H₂TMRos and DAPI. Polarized mitochondria are stained as separate red dots. (A) Large numbers of polarized mitochondria in all cells of freshly isolated GC sheet. (B) Brightfield light microscopy; cytochrome c localization (brown) in freshly isolated living GC sheet, methyl green counterstaining of nuclei. Large numbers of reactive mitochondria; image similar to A. (C) Polarized mitochondria are concentrated in compact masses at one side of the nucleus in GC sheet cultured for 72 hr and supplemented with IGF-I and LH. (D) Few polarized mitochondria when GC sheet is cultured for 72 hr in serum-free conditions; in comparison to A and C, a smaller area of the cytoplasm is taken in by punctiform CM-H₂TMRos staining, while the largest part displays only background fluorescence. (E) Mitochondria depolarized by treatment with FCCP before loading with CM-H₂TMRos do not take up the probe; there are no granules. GCs cultured for 72 hr with IGF-I and LH. (F) Polarized mitochondria stained with CM-H₂TMRos before treatment with FCCP in freshly isolated GC sheet. Note necrotic cells with absence of polarized mitochondria. (G) Mitochondrial heterogeneity in GC sheet cultured for 72 hr in serum-free conditions. Remark persistence of polarized mitochondria surrounding apoptotic chromatin bodies (arrowheads). Other apoptotic nuclei show no stained granules (arrow). Bars: A,B,E,F = 10 μm; C,D,G = 5 μm.
CM-H₂TMRos fluorescence was expressed as the whole field fluorescence. GCs were either pretreated with FCCP (FCCP+CM-H₂TMRos), pretreated with valinomycin (Valin+CM-H₂TMRos), or treated with FCCP after probe loading (CM-H₂TMRos+FCCP), treated with valinomycin after probe loading (CM-H₂TMRos+Valin). Intensity of CM-H₂TMRos fluorescence was expressed as the whole field fluorescence. *p<0.001 compared to control; #, changes were not significant compared to control. (A) Uncultured GC sheets. The data represent the mean ± SEM of four independent experiments. In each experiment, fluorescence intensity of CM-H₂TMRos was measured in 10 fields. (B) GC explants cultured for up to 72 hr supplemented with IGF-I and LH. Data represent mean ± SEM of five independent experiments. In each experiment, fluorescence intensity of CM-H₂TMRos was measured in five fields.

majority of the apoptotic cells, however, CM-H₂TMRos fluorescence was no longer detected (Figure 1G). In necrotic cells, often present at the edge of the explants and recognized by their small pyknotic nuclei, polarized mitochondria were never observed (Figure 1F).

Quantification of the CM-H₂TMRos signal in the 72-hr cultured GC explants revealed that the fluorescence intensity was significantly lower (Figure 3) when IGF-I and LH were absent in the culture medium compared to the control condition (with both survival factors).

Discussion

Cationic lipophilic fluorochromes such as 3,3′-dihexyloxocarbocyanine iodide (DiOC₆), rhodamine 123 (R123), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) have been widely used to assess the functionality of mitochondria in diverse biological scenarios, including differentiation (Mancini et al. 1997), aging (Hagen et al. 1997), and apoptosis (Zamzami et al. 1995a,b). Comparing to DiOC₆ and R123, JC-1 is a reliable and sensitive probe, as shown in several studies. Its loading is impaired by several drugs able to collapse ΔΨm, which is not the case for DiOC₆ and R123 owing to a high sensitivity to changes in plasma membrane potential for DiOC₆ and energy-independent binding sites for R123 (Salvioli et al. 1997). Moreover, this dichromatic probe permitted detection of the coexistence of mitochondria in different energetic conditions within an individual cell. A disadvantage of JC-1 is that it cannot be combined with other fluorescent probes in the FITC/TRITC emission spectra. In addition, the use of DiOC₆, R123, and JC-1 is incompatible with chemical fixation.

Macho et al. (1996) described the main characteristics of a class of fixable fluorescent probes: the MitoTracker probes, focusing on MitoTracker Red (both the oxidized and the reduced form CMXRs and H₂-TMRos respectively). In the present study we used MitoTracker Orange (in its reduced form: CM-H₂TMRos) to study mitochondrial transmembrane potential changes during apoptosis in relation to nuclear morphology as revealed by DAPI staining. CM-H₂TMRos enters the mitochondria due to the negative mitochondrial membrane potential and only after that, in a second step when it is oxidized, binds to SH-groups, which makes this probe aldehyde-fixable. Uncoupling treatments were ineffective after probe loading (Figures 1F, 2A, and 2B), indicating that binding of the probe to SH-groups occurs only after entering of CM-H₂TMRos in mitochondria due to their membrane potential. The first step of our work was to demonstrate the usefulness of CM-H₂TMRos in measuring ΔΨm. We analyzed fluorescence intensity after impairing mitochondrial function with commonly used, mechanism-specific mitochondrial poisons (FCCP, a proton translocator, and valinomycin, a potassium ionophore) in uncultured GC sheets and in 72-hr cultures supplemented with IGF-I and LH. Pretreatment with FCCP and valinomycin before probe loading gave no punctiform mitochondrial staining (Figure 1E) and significantly reduced the intensity of fluorescence (Figures 2A and 2B). A possible explana-
tion for the presence of the diffuse cytoplasmic staining could be that cells still oxidized the probe in their cytoplasm but, as mitochondrial respiration is already uncoupled, the dye is not sequestered in the mitochondria but is distributed in the cytoplasm.

The second argument to support the mitochondrial specificity of the probe can be found in the similarity of the $\Delta \Psi m$ staining and cytochrome c localization in respiring mitochondria (Figures 1A and 1B). Our data are in agreement with results of Poot et al. (1996), who reported that uncoupling mitochondrial respiration with carbonyl cyanide $m$-chlorophenylhydrazone (CCCP) before staining with MitoTracker Red (H$_2$-CMXRos) significantly decreased fluorescence generated by the dye. Moreover, Poot et al. (1996) showed complete co-localization in the mitochondria of a monoclonal antibody which is specific for subunit I of human oxidase and H$_2$-CMXRos staining. By contrast, the results of Scorrano et al. (1999) indicated that staining with oxidized MitoTracker Orange (CMTM-Ros) is characterized by two different mechanisms. One is energy-independent, in that it is also observed in mitochondria de-energized by FCCP. It can be significantly prevented by N-ethylmaleimide or phenylarsine oxide (inhibitors of SH-groups). However, the data obtained with compounds such as phenylarsine oxide should be interpreted with much care because such compounds can induce mitochondrial membrane permeabilization (Costantini et al. 2000) and thereby prevent staining. The second component of CMTM-Ros staining is energy-dependent. Interpretation of this energy-dependent staining component should also be done with care because Scorrano et al. (1999) have mentioned that mitochondria sometimes spontaneously release the probe within minutes, suggesting that they became depolarized. This effect could be explained by the experiments of Minami-kawa et al. (1999), which showed that CMXRos has a strong photosensitizing action in living cells. Photoradiation of intact living cells loaded with CMXRos induces depolarization of the inner mitochondrial membrane and swelling of mitochondria, resulting in apoptosis. Based on our own findings and the above-mentioned literature data, we propose to use only the reduced chloromethylrosamine-derived probe CM-H$_2$TMRos (MitoTracker Orange) and to evaluate $\Delta \Psi m$ after fixation.

The second part of our work was to study $\Delta \Psi m$ changes in the GC explants cultured up to 72 hr under serum-free conditions, which elicits apoptosis. Both the images and the fluorescence measurements show that, in our model system, dissipation of transmembrane potential in individual mitochondria is an early event in GCs not displaying nuclear manifestations of apoptosis. Moreover, we have shown the presence of a subset of polarized mitochondria that exhibited still normal $\Delta \Psi m$ until late in the apoptotic process (Figure 1G). These data confirm our previous conclusions on mitochondrial heterogeneity during apoptosis with a subset of respiring mitochondria retaining cytochrome c function until the stage of chromatin condensation and nuclear fragmentation (D’Herde et al. 2000). The presence of polarized cytochrome c-containing and respiring mitochondria until late in the apoptotic process preserves the possibility for ATP production even in the stage of chromatin condensation and fragmentation. Bradham et al. (1998) observed by confocal microscopy in an apoptosis model consisting of TNF-α treatment in rat hepatocytes expressing an IkB super-repressor that a gradual onset of mitochondrial depolarization in a subpopulation of mitochondria occurred using the tetramethylrhodamine methyl ester (TMRM) probe, in conjunction with calcine, a probe marking MPT in individual mitochondria. The authors concluded that, for several hours during the apoptotic response, hepatocytes contain both polarized and depolarized mitochondria, the latter being the presumptive source of released cytochrome c. These results are perfectly in line with our observations. However, because these authors used a non-fixable mitochondrial-selective probe, TMRM, it was impossible to check whether vital cells with apoptotic nuclear morphology still contain polarized mitochondria as was done in the present study by using the fixable CM-H$_2$TMRos probe and DAPI staining.

Heiskanen et al. (1999) monitored mitochondrial depolarization in individual mitochondria of single cytochrome c–green fluorescent protein (GFP)-transfected PC6 cells undergoing apoptosis after staurosporine treatment. For this purpose, TMRM-loaded cells were observed at various time points after treatment by scanning laser confocal microscopy. Transfection with cytochrome c–GFP enabled the authors to study the relation between mitochondrial depolarization and cytochrome c release. In agreement with our observations, they demonstrated that mitochondrial depolarization did not occur homogenously over the entire mitochondrial population and that cytochrome c release is initially also restricted to a subpopulation yielding a pattern of mixed punctate and diffuse cytochrome c–GFP localization. Again, persistence of polarized mitochondria in cells with clearly apoptotic morphology was not documented in this study.

Goldstein et al. (2000) studied the kinetics of cytochrome c release during apoptosis, using cytochrome c tagged with GFP. With this approach they reported for HeLa cells induced to apoptosis by a variety of agents that, once initiated, the release of cytochrome c continues until all of the protein is released from all the mitochondria in an individual cell, within about 5 min. To reconcile these findings with the need for ATP production, Goldstein et al. (2000) proposed that
some cytochrome c either remains in the mitochondria or may re-enter the intermembrane space to participate in electron transport. Indeed, one has to take into account that GFP-tagged cytochrome c represents only 1% of the endogenous cytochrome c content of the mitochondria. In any case, heterogeneity in the mitochondrial population with respect to cytochrome c loss was not mentioned, although their time-lapse images (Goldstein et al. 2000; Figure 3A, upper panel) show the maintenance of some cytochrome c-containing mitochondria 10 min after release from the first mitochondria.

Recently, Salvioli et al. (2000) reported for staurosporine-treated HL60 cells that the collapse in ΔΨm is a heterogeneous phenomenon both at the single organelle level and at the cellular level, and therefore is not unequivocally related to the execution of the death process but rather is an ancillary event. Several reports described an early ΔΨm decrease before the exposure of phosphatidylserine on the cell surface or DNA fragmentation (Castedo et al. 1995; Zamzami et al. 1995a,b). In contrast, a late decrease in ΔΨm was found in several other models of apoptosis (Deckwerth and Johnson 1993; Ankarcrona et al. 1995; Cossarizza et al. 1996; Bossy–Wetzel et al. 1998). This discrepancy may be due to the use of different cell types or apoptosis-inducing stimuli.

In several experimental models, cell stress is accompanied by an early hyperpolarization of mitochondria (Banki et al. 1999; Diaz et al. 1999; Scarlett et al. 2000; Khaled et al. 2001), while subsequent mitochondrial depolarization precedes mitochondrial disruption and is dependent on the stimulus and cell-type caspase activation. Several authors reported mitochondrial clustering in various apoptosis paradigms as an early event, independent of ΔΨm changes or cytochrome c release (De Vos et al. 1998; Li et al. 1998; Diaz et al. 1999; Esposti et al. 1999). In serum-free cultured granulosa explants in which apoptosis is not synchronized (D’Herde and Leybaert 1997), we did not detect this mitochondrial clustering. Instead, a heterogeneous distribution of polarized mitochondria throughout the GC sheet could be observed (Figure 1G), with various numbers of polarized mitochondria in non-apoptotic cells and sometimes the maintenance of a few polarized mitochondria in frankly apoptotic cells. However, in control cultures supplemented with gonadotropins and IGF-I, an unexpected mitochondrial clustering in packed masses at one side of the nucleus was revealed (Figure 1C). It was previously shown by Aharoni et al. (1993) that mitochondrial clustering can be induced in GCs under the combined action of gonadotropins and IGF-I in parallel with stimulation of steroidogenesis.

In this study we have shown that (a) CM-H2TMRos staining evaluated after aldehyde fixation is a reliable and specific probe to detect ΔΨm changes in the granulosa explant system, (b) dissipation of transmembrane potential is an early event during apoptosis, preceding nuclear changes, and is again confined to a subpopulation of mitochondria within an individual cell, and (c) the majority of frankly apoptotic cells are devoid of polarized mitochondria, but in some apoptotic cells a few polarized mitochondria can be detected. Necrotic cells do not display polarized mitochondria.

The present findings support the hypothesis that ATP needed for completion of the apoptotic cascade can be generated during apoptosis in a subset of requiring mitochondria and is not necessarily derived from anaerobic glycolysis.

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