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Caspase-independent apoptosis in Friend's erythroleukemia cells: role of mitochondrial ATP synthesis impairment in relocation of apoptosis-inducing factor and endonuclease G

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Abstract Mitochondria have emerged as the central components of both caspase-dependent and independent apoptosis signalling pathways through release of different apoptogenic proteins. We previously documented that parental and differentiated Friend's erythroleukemia cells were induced to apoptosis by oligomycin and H_2O_2 exposure, showing that the energy impairment occurring in both cases as a consequence of a severe mitochondrial F_0F_1ATPsynthase inactivation was a common early feature. Here we provide evidence for AIF and Endo G mitochondrio-nuclear relocation in both cases, as a component of caspase-independent apoptosis pathways. No detectable change in mitochondrial transmembrane potential and no variation in mitochondrial levels of Bcl-2 and Bax are observed. These results point to the osmotic rupture of the mitochondrial outer membrane as occurring in response to cell exposure to the two energy-impairing treatments under conditions preserving the mitochondrial inner membrane. A critical role of the mitochondrial F_0F_1ATP synthase inhibition in this process is also suggested.

Keywords Mitochondrial F_0F_1ATPsynthase · Friend's erythroleukemia cells · Apoptosis · Apoptosis inducing factor · Endonuclease G · H_2O_2 · Oligomycin · Energy impairment · Mitochondrial outer membrane permeabilization

Abbreviations

AIF	apoptosis inducing factor
BSA	bovine serum albumin
CS	citrate synthase
CsA	cyclosporine A
D-FELC	differentiated FELC
$\Delta\psi_m$	mitochondrial transmembrane potential
Endo G	endonuclease G
FELC	Friend's erythroleukemia cells
HMBA	N,N′-hexamethylene bisacetamide
IAPs	inhibitor of apoptosis proteins
IMP	intermembrane space
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide
LDH	lactate dehydrogenase
MIM	mitochondrial inner membrane
MOM	mitochondrial outer membrane
P-FELC	parental FELC
PBS	phosphate-buffered saline
PMSF	phenylmethanesulfonyl fluoride
PTP	permeability transition pore
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
z-VAD-fmk	Z-Val-Ala-DL-Asp(Ome)-fluoromethylketone

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Introduction

Apoptosis is an active process of programmed cell death in which cells die in a coordinated and stereotyped manner, ultimately exhibiting characteristic structural features such as reduction in cell volume, membrane blebbing, phosphatidyl serine exposure on the surface of the plasma membrane, chromatin condensation and nuclear fragmentation (Kroemer et al. 2007). Today, it is well recognized that cell death, in both its physiological and pathological occurrence, is closely linked to mitochondrial structure and (dys)function. Specifically, most cell death in vertebrates proceeds via the intrinsic or mitochondria-dependent pathway of apoptosis. which generally originates from permeabilization of the mitochondrial outer membrane (MOM), resulting in different sequences of biochemical events leading to cellular demise (Kroemer et al. 2007; Ravagnan et al. 2002; Green and Kroemer 2004). The death-associated MOM permeabilization is a tightly regulated phenomenon. Apoptogenic proteins, which usually are retained within the mitochondrial intermembrane space (IMS), when released into the cytoplasm exert pleiotropic effects (Kroemer et al. 2007; Ravagnan et al. 2002; Green and Kroemer 2004; Wang 2001; Kroemer and Matin 2005). Thus, in addition to the vital functions of mitochondria in the maximization of cellular energy production by oxidative phosphorylation, these organelles also play key roles in programmed cell death by acting as reservoirs for apoptogenic proteins and deciding the mode of cell death through energy supply over the bioenergetic threshold required for apoptosis to occur (Richter et al. 1996). Mitochondria-dependent apoptosis is mediated by two main different pathways, the most studied of which culminates in the cytosolic activation of the proteolytic enzymes caspases from proenzyme forms. This pathway is triggered by the mitochondrial intermembrane protein cytochrome c that, when released to the cytosol, initiates a cascade of caspase-activating events thereby promoting the formation of the so-called “apoptosome” (Li et al. 1997). Caspases are further activated by Smac/Diablo, which binds the inhibitor of apoptosis proteins (IAPs) and blocks their caspase inhibitory activity (Chai et al. 2000).

The second apoptosis signalling pathway, which was more recently identified, results in fragmentation of nuclear DNA independently of caspase recruitment and is initiated by MOM permeabilization-dependent release into the cytosol and translocation to the nucleus of some other intermembrane mitochondrial proteins, such as endonuclease G (Endo G) and apoptosis-inducing factor (AIF). Endo G, which normally functions in the maturation of mitochondrial DNA, is compartmentalized in substantial amount in the IMS in healthy cells. Once released into the cytoplasm after induction of apoptosis, Endo G translocates to the nucleus and causes oligonucleosomal DNA fragmentation (Schafer et al. 2004; van Loo et al. 2001). AIF, a redox-active flavoenzyme exhibiting pro-apoptotic properties, is embedded in the mitochondrial inner membrane (MIM) where it exerts a vital function in bioenergetic and redox metabolism (Churbanova and Sevrioukova 2008). The C-terminal domain of AIF is oriented towards the IMS and released into the cytosol after proteolytic maturation to a soluble form, in response to specific death signals (Otera et al. 2005). According to some authors, a mature AIF pool is soluble in the IMS and co-exists with the MIM-associated isoform (Modjtahedi et al. 2006). Once released, the AIF soluble form translocates to the nucleus, where it mediates chromatin condensation and DNA cleavage in large fragments through a process that may involve a direct binding to DNA (Joza et al. 2001; Susin et al 1999). Caspase requirement in Endo G and AIF pathways is still controversial (Kroemer et al. 2007). In fact, the nuclear effects of these two proteins are mostly caspase-independent, but there is evidence that caspases are required upstream of AIF and Endo G release in some cases, e.g. in cells treated with Bax/Bak-dependent pro-apoptotic drugs (Lorenzo and Susin 2004; Arnoult et al. 2003).

Although the permeabilization of MOM and the release of apoptogenic proteins from mitochondria is well established, the mechanism for this permeabilization/release is not yet defined. According to some authors, intermembrane proteins spill out to the cytosol after physical disruption of MOM due to transient osmotic swelling of the matrix (Vander Heiden et al. 1997; Petit et al. 1998; Desagher and Martinou 2000). According to others, intermembrane proteins enter the cytosol by passing through non-specific large pores or channels that involve proteins of the Bcl-2 family and specifically Bax/Bak oligomers assembled within or at the surface of MOM (Chipuk et al 2006; Wang 2001; Desagher and Martinou 2000; Shimizu et al. 1999). It is nonetheless clear that within the Bcl-2 family there are antiapoptotic members, such as Bcl-2, that stabilize MOM (Yang et al. 1997; Kluck et al. 1997; Marzo et al. 1998) and proapoptotic members, such as Bax, that permeabilize the membrane (Desagher and Martinou 2000; Gross et al. 1998). Moreover, both Bax and Bcl-2 may exert their function independently or in coordination with the permeability transition pore (PTP) complex, whose opening, whatever its mechanism is, leads to a long-lasting or permanent dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) across MIM (Chipuk et al 2006; Marzo et al. 1998). $\Delta\psi_m$ is a key event in many but not all paradigms of apoptosis (Zamzami et al. 2005). Actually, MOM permeabilization, with or without the contribution of MIM and consequently with or without $\Delta\psi_m$ loss, is considered as the real “point of no return” in the cascade of

events leading to apoptosis (Samraj et al. 2007; Ricci et al. 2004; Bouchier-Hayes et al. 2005).

We previously documented that differentiated Friend's erythroleukemia cells (D-FELC), but not parental FELC (P-FELC), undergo cell death with apoptotic features upon exposure to mild acute treatment with H_2O_2 (Comelli et al. 2003). Apoptosis in D-FELC was attributed to a reduction of energy charge, due to an impairment of mitochondrial ATP synthesis which was more marked to that observed in P-FELC (Comelli et al. 1998, 2003). In accordance, apoptosis was triggered in P-FELC by mimicking the cell energy charge and mitochondrial ATP synthesis impairment observed in D-FELC through cell exposure to oligomycin, the F_0F_1ATP synthase specific inhibitor (Comelli et al. 2003). Thus, the results of the present study refer to the two different triggers inducing energy impairment-related apoptosis: i.e. exposure to oligomycin for P-FELC and to H_2O_2 for D-FELC. The aim of this study was to evaluate which one of the two main apoptosis signalling pathways was involved and if the pathway was the same in the two sets of apoptotic cells. Specifically, the experiments were aimed at defining if the same apoptogenic proteins were released from MOM in both cases and if $\Delta\psi_m$ loss and MIM permeabilization occurred. The results, while documenting an additional paradigm of cell death involving Endo G- and AIF-related pathways independent of caspases, provide evidence for an essential role of the impairment of mitochondrial ATP synthesis in MOM rupture under conditions preserving $\Delta\psi_m$.

Materials and methods

Chemicals and reagents Trypan blue, glutamine, penicillin and streptomycin were purchased from Biochrom (Berlin, Germany) and H_2O_2 was obtained from Merck (Darmstad, Germany). All other materials were purchased from Sigma (St. Louis, MO, USA), unless specifically indicated.

Cell culture Friend's erythroleukemia cells (FELC), clone 3CL8, were routinely cultured in complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μU/ml penicillin and 100 μg/ml streptomycin) and induced to erythroid differentiation with N, N′-hexamethylene bisacetamide (HMBA) as previously described (Comelli et al. 1994). Experiments were performed both with undifferentiated parental cells in exponential phase of growth (P-FELC) and with differentiated cells 5 days after induction (D-FELC).

Apoptosis induction and detection P-FELC and D-FELC were pelleted and resuspended at 37°C in phosphate-buffered saline (PBS) plus 5 mM glucose to a concentration of 2.0×10^6 cells/ml. In some experiments, we added Z-Val-Ala-DL-Asp(Ome)-fluoromethylketone (z-VAD-fmk), a broad spectrum, cell-permeable caspase-inhibitor (Hsu et al. 1997), to a final concentration of 100 μM and the cells were preincubated at 37°C in a CO_2 cell culture incubator for 1 h. In other experiments, cells were preincubated for 1 h with 0.5 μM cyclosporin A (CsA), a specific inhibitor of the PTP complex (Zoratti and Szabò 1995).

Apoptosis *via* caspase-dependent mitochondrial pathways was induced by incubating both P-FELC and D-FELC for 30 min with 1 μM camptothecin, a well-known DNA-damaging agent, inducing apoptosis accompanied by loss of cytochrome c and $\Delta\psi_m$ (Stefanis et al. 1999).

Two different treatments were performed to induce apoptosis by mitochondrial energy impairment: i.e. 0.25 mM H_2O_2 in D-FELC and 10 μM oligomycin in P-FELC; such treatments were responsible for a reduction of about 50% of ATP cell content (Comelli et al. 2003). Conditions were set up to separate in the two sets of experiments the effects of the selective inhibition of F_0F_1ATP synthase from those of oxidative stress associated to the erythroid differentiation program *per se* (Comelli and Mavelli 2001). In fact, it was difficult in our hands to control the exposure of D-FELC to oligomycin and to avoid cytolysis in this case (not shown), likely as a consequence of cumulative effects resulting from the inhibition of F_0F_1ATP synthase in a pro-oxidant cell/mitochondrial environment.

After incubating the cells for 30 min with H_2O_2, or oligomycin or camptothecin in the CO_2 incubator, they were pelleted, resuspended in an equal volume of pre-warmed complete medium without additions, and returned to the CO_2 incubator for 2 h prior to assay. These conditions guaranteed a substantial amount of DNA degradation without cytolysis (Comelli et al. 2003), as monitored by assaying cell viability with the trypan blue dye exclusion test (Cook and Mitchell 1989). Values were expressed as percentage of viable cells with respect to the total number of cells (viable, excluding trypan blue, plus damaged, taking up trypan blue, cells).

Advanced DNA fragmentation typical of apoptosis was assayed by an immunoenzymatic assay based upon mono- and oligonucleosome identification (Cell Death Detection ELISA kit, cat. number 1544 675; Roche Diagnostics). Values were expressed as percentage of cells exhibiting DNA fragmentation.

Preparation of mitochondrial, cytosolic and nuclear fractions and purity assessment by detection of subcellular markers Subcellular fractions of FELC were prepared according to a modification of the method described by Abou-Khalil (Abou-Khalil et al. 1985). Briefly, after experimentation, cells were washed twice with ice-cold

PBS and resuspended at a concentration of 7.5×10^7 cells/ml in extraction solution (250 mM sucrose, 2 mM EDTA, 1 mg/ml bovine serum albumin (BSA), 50 µg/ml phenylmethanesulfonyl fluoride (PMSF) and 2 µg/ml apoprotinin, adjusted to pH 7.4 with KOH). Cell suspensions were sonicated on ice three times for 10 s at 30-s intervals, in order to disrupt 90%-95% of cells (Abou-Khalil et al. 1985). The homogenates were centrifuged at 1,000×g at 4°C for 10 min, and the pellets were used as the nuclear fraction after washing twice with PBS. The supernatants were centrifuged at 14,000×g at 4°C for 20 min to separate the cytosolic fraction (supernatant liquid) from the crude mitochondrial fraction (pellet, after washing twice with PBS).

Protein concentrations of the subcellular fractions were determined by the method of Lowry (Lowry et al. 1951) using BSA as standard. Correct subcellular fractionation was assessed by measuring lactate dehydrogenase (LDH) as a cytosolic marker, citrate synthase (CS) as a mitochondrial marker, and DNA content as a nuclear marker. LDH activity was determined according to Zhang et al. (Zhang et al. 2004), CS activity according to Morgunov and Srere (Morgunov and Srere 1998), and DNA content as described by Sambrook et al. (Sambrook et al. 1989).

Western blotting Cytochrome c, Endo G and AIF were immunochemically assessed in the cytosolic fraction (100 µg protein), mitochondrial fraction (10–20 µg) and nuclear fraction (20 µg). Levels of Bax and Bcl-2 were assessed in the mitochondrial fraction (50 µg).

Western blotting was performed as described by Green and colleagues (Bossy-Wetzel et al. 1998). Briefly, samples were electrophoresed in 15% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose membrane. Membranes were incubated separately with: mouse monoclonal antibody against cytochrome c (1:1,000; Pharmingen, San Diego, CA, USA), rabbit polyclonal antibody against Endo G (1:2,000; Chemicon, Temecula, CA, USA), rabbit polyclonal antibody against AIF (1:2,000; Chemicon), rabbit polyclonal antibody specific for Bcl-2 (1:1,000; Oncogene Research Products, Cambridge, MA, USA) or mouse monoclonal antibody specific for Bax (1:1,000; Oncogene Research Products). Bound primary antibodies were revealed by incubation with peroxidase-conjugated anti-mouse (1:10,000) or anti-rabbit (1:10,000) antiserum (Amersham, Little Chalfont, United Kingdom) and detected by enhanced chemiluminescence (Super Signal West Chemiluminescent Substrates; Pierce, Rockford, IL, USA).

Cytofluorimetric analysis of mitochondrial transmembrane potential Mitochondrial transmembrane potential ($\Delta\psi_m$) was measured in P-FELC and D-FELC, in control conditions (30 min incubation in PBS-glucose solution) and immediately after the 30-min induction of energy impairment-related apoptosis in the two sets of experiments: i.e. with oligomycin in P-FELC and with H_2O_2 in D-FELC. $\Delta\psi_m$ was also measured in the mid-point (1 h) and at the end of the 2-h incubation in complete medium after apoptosis induction. $\Delta\psi_m$ was measured using two mitochondrial dyes: i.e. the lipophilic cationic probes 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA) and tetramethyl rhodamine methyl ester (TMRM; Molecular Probes, Eugene, OR, USA). JC-1 normally exists in solution as a monomer emitting a green fluorescence, but depending on $\Delta\psi_m$, JC-1 accumulates in mitochondria and forms aggregates that are associated with a large shift in emission (red fluorescence) (Reers et al. 1991). TMRM monovalent cation reversibly accumulates into mitochondria according to $\Delta\psi_m$ with a Nernstian distribution (Ehrenberg et al. 1988).

Suspensions of control and treated cells were incubated in the dark, in complete medium containing 10 µg/ml JC-1 for 10 min at 37°C or 20 nM TMRM for 5 min at room temperature. Thereafter, cells were washed twice in PBS at 4°C and analyzed with a FACScan flow cytometer (Becton Dickinson, New York, USA) equipped with a single 488 nm argon laser. The excitation wavelength was 488 nm for both probes and emission was monitored at 530 nm (FL1) for JC-1 and 582 (FL2) for JC-1 and TMRM. Data were acquired in list mode and analyzed with CellQuest software (Becton Dickinson, New York, USA).

Statistical analysis Differences between samples were assessed using the unpaired two-tailed *t* test. A value of $p<0.05$ was considered to be significant.

Results

Pan-caspase inhibitor z-VAD-fmk did not prevent mitochondrial ATP synthesis impairment-related apoptosis in FELC

To investigate if caspases play a role in the apoptosis that follows mitochondrial ATP synthesis impairment, apoptosis induction (treatment of D-FELC with H_2O_2 or P-FELC with oligomycin for 30 min, followed by incubation for 2 h in complete medium) was performed in the absence and presence of the pan-caspase-inhibitor z-VAD-fmk. The percentage of cells exhibiting characteristic advanced DNA fragmentation is reported in Fig. 1. Treatment with H_2O_2 significantly increased the percentage of D-FELC with DNA fragmentation, although the cells remained intact

excluding trypan blue (>90%). Preincubation with the pan-caspase-inhibitor z-VAD-fmk did not prevent H_2O_2-induced apoptosis in D-FELC, suggesting that the caspase-dependent apoptotic pathway was not involved. As already documented (Comelli et al. 2003), in parental cells H_2O_2 treatment had no apoptotic effect, while oligomycin treatment did. Interestingly, the oligomycin-induced apoptosis was also caspase-independent (no effect of z-VAD-fmk). Control P-FELC and D-FELC exposed to z-VAD-fmk alone did not show any difference from untreated control cells (data not shown). In addition, in both D-FELC and P-FELC, z-VAD-fmk fully blocked caspase-dependent apoptosis due to camptothecin treatment, performed as a positive control.

These results indicate that in FELC the two different treatments leading to mitochondrial ATP synthesis impairment trigger apoptosis through pathways not involving caspase activation.

Nuclear translocation of AIF and Endo G in apoptotic FELC

To determine if mitochondrial ATP synthesis impairment-related apoptosis was associated with the release of apoptogenic factors from mitochondria, immunodetection of mitochondrial intermembrane proteins was performed in three main subcellular fractions, in control and apoptotic conditions. We first assessed the purity of the subcellular fractions (i.e. minimal cross-contamination) on the basis of cellular markers for mitochondria, cytosol and nucleus (Table 1). In both P-FELC and D-FELC, the mitochondrial marker CS was found predominantly in the mitochondrial fraction (>93% of the total cellular amount), with undetectable amounts in the cytosolic fraction and <8% in the nuclear preparation. The cytosolic marker LDH was found prevalently in the cytosol (>96%), with ≤0.5% in the mitochondrial and nuclear fractions. Finally, 92%–96% of total DNA content was associated with the nuclear fraction, with <5% in the other fractions. These distribution patterns did not change after the induction of apoptosis (data not shown). These results indicate good overall fractionation, with a low level of cross-contamination; in particular, the cytosolic and nuclear fractions had a low enough level of mitochondrial contamination to permit subsequent experiments.

Figure 2a–c shows the results of AIF, Endo G and cytochrome c immunodetection after the two different apoptosis-inducing treatments in P-FELC and D-FELC. In control samples, AIF was present exclusively in the mitochondrial fraction, as it was also in P-FELC treated with H_2O_2 (which has no apoptotic effect) (Fig. 2a). In contrast, in cells induced to undergo apoptosis (H_2O_2-treated D-FELC and oligomycin-treated P-FELC), AIF was most abundant in the nuclear fraction, with a small amount

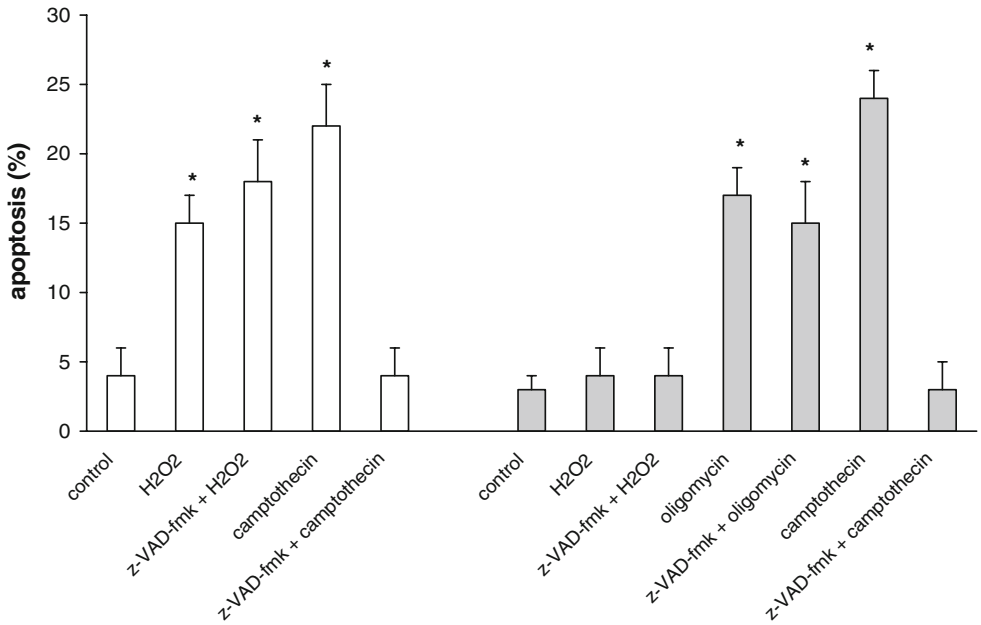

Fig. 1 z-VAD-fmk does not prevent H_2O_2-induced apoptosis in differentiated FELC or oligomycin-induced apoptosis in parental FELC. D-FELC (open columns) and P-FELC (gray columns) were preincubated for 1 h in the absence or presence of 100 μM z-VAD-fmk before oligomycin or H_2O_2 exposure. As positive control, cells were treated with 1 μM camptothecin for 30 min to induce a caspase-dependent apoptosis. After treatments, cells were returned to fresh complete medium for 2 h and then tested for apoptosis, expressed as percentage of cells exhibiting DNA fragmentation. Control P-FELC and D-FELC exposed to z-VAD-fmk alone did not show any difference from untreated control cells (data not shown). Values are means and SD of four independent experiments. *$p<0.001$ vs. controls

Table 1 Distribution of subcellular markers in fractions from P-FELC and D-FELC and percentage of total cellular values demonstrate negligible levels of cross-contamination

Subcellular fraction	*Citrate synthase*		*Lactate dehydrogenase*		*DNA*	
	mU	*%*	*mU*	*%*	*μg*	*%*
P-FELC						
Mitochondrial	1.45 (0.22)	93.3	1.1 (0.2)	0.3	20.6 (2.9)	4.4
Cytosolic	<0.1	-	378.1 (4.0)	97.2	8.4 (2.1)	1.8
Nuclear	0.12 (0.09)	7.6	1.9 (0.2)	0.5	425.0 (25.4)	92
Whole cells	1.58 (0.24)	100	389.2 (4.5)	100	462.5 (26.8)	100
D-FELC						
Mitochondrial	1.53 (0.28)	93.7	1.3 (0.2)	0.3	16.6 (2.2)	4.0
Cytosolic	<0.1	-	377.2 (43.4)	96.3	4.5 (1.8)	1.0
Nuclear	0.11 (0.08)	6.7	1.6 (0.1)	0.4	425.5 (32.6)	96
Whole cells	1.63 (0.35)	100	391.6 (5.2)	100	450.5 (35.4)	100

Values are means (SD) and percentage of three determinations made on control (not treated) cells (5×10^6 cells). Results are equivalent to treated cells.

also detected in the cytosol and only a residual amount in the mitochondria. Similar behavior was observed for the subcellular compartmentalization of Endo G (Fig. 2b). In contrast, no effect on the subcellular localization of cytochrome c was evidenced, as no detectable extra-mitochondrial amount and no noticeable variation in the mitochondrial content were observed in apoptotic (H_2O_2-treated D-FELC and oligomycin-treated P-FELC) with respect to not treated cells (Fig. 2c). Together, these results suggest that the apoptotic effects (i.e. DNA fragmentation) of the two treatments leading to mitochondrial ATP synthesis impairment depend on the nuclear translocation of mitochondrial proteins AIF and Endo G, and do not involve cytochrome c release into cytosol, in line with the activation of caspase-independent processes in both cases.

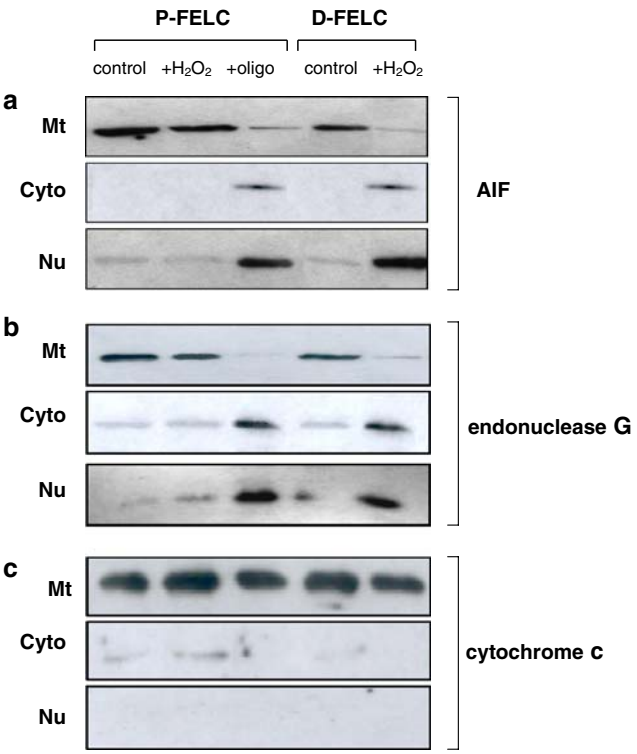

Fig. 2 Subcellular distributions of AIF, Endo G and cytochrome c in control and apoptotic FELC. Cells suspended in PBS-glucose solution were treated with oligomycin (P-FELC), H_2O_2 (P- and D-FELC) or vehicle for 30 min and returned to fresh medium for 2 h prior to preparation of mitochondrial (Mt), cytosolic (Cyto) and nuclear (Nu) fractions for immunochemical detection of mitochondrial proteins. Blots are representative of three separate experiments

Mitochondrial transmembrane potential was not modified during mitochondrial ATP synthesis impairment-related apoptosis

We next investigated whether mitochondrial ATP synthesis impairment-related apoptosis involved a change in $\Delta\psi_m$, as revealed by FACScan analyses after staining with the mitochondrial dye JC-1 (Fig. 3a). In control parental and differentiated cells, 96% and 94% of cells, respectively, exhibited double green-red fluorescence. At the end of the treatments for induction of apoptosis with oligomycin (P-FELC) and H_2O_2 (D-FELC), the percentage of cells exhibiting double fluorescence remained high (95% and 94%, respectively), indicating no apparent change in $\Delta\psi_m$. The same finding was also observed at the mid-point and at the end of the 2-h incubation in complete medium (data not shown). To validate these data with a non-toxic $\Delta\psi_m$ probe, TMRM was also used under the same conditions, namely at the end of the induction of apoptosis (Fig. 3b), at the mid-point and after 2 h (data not shown). Oligomycin and H_2O_2 treatments never shifted significantly the fluorescence intensity peak of their controls (P-FELC and D-FELC respectively). Therefore, we can safely state that the two treatments

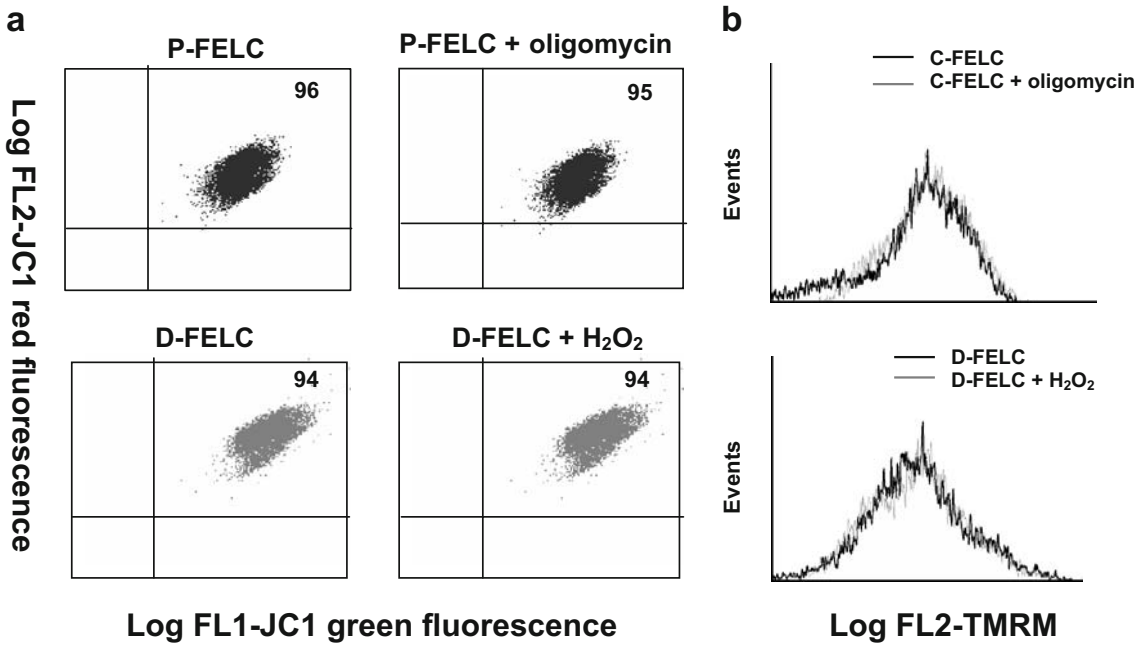

Fig. 3 Mitochondrial membrane potential is not modified during the energy impairment-related apoptosis in control and apoptotic FELC. After induction of apoptosis with oligomycin (P-FELC) or H_2O_2 (D-FELC), cells were incubated with JC-1 or TMRM as described in Materials and Methods and analyzed by flow cytometry. **a** Dot plot analyses of control and apoptotic FELC with JC-1. **b** Histogram plots of control and apoptotic FELC with TMRM. Data were acquired in list mode and analyzed with CellQuest software (Becton Dickinson, New York, USA). The data are representative of three separate experiments

triggering mitochondrial ATP synthesis impairment-related apoptosis in FELC do not involve change in $\Delta\psi_m$.

In accordance, opening of mitochondrial PTP complex was ruled out in both sets of apoptotic cells on the basis of the absence of prevention of apoptosis by CsA, a specific inhibitor of PTP. As positive control, the induction of mitochondrial caspase-dependent apoptosis was obtained by incubation of P-FELC and D-FELC with camptothecin, a well-known DNA-damaging agent, inducing apoptosis accompanied by loss of cytochrome c and $\Delta\psi_m$. As expected, camptothecin gave rise to $\Delta\psi_m$ impairment, while pretreatment with CsA blocked such a loss (Fig. 4).

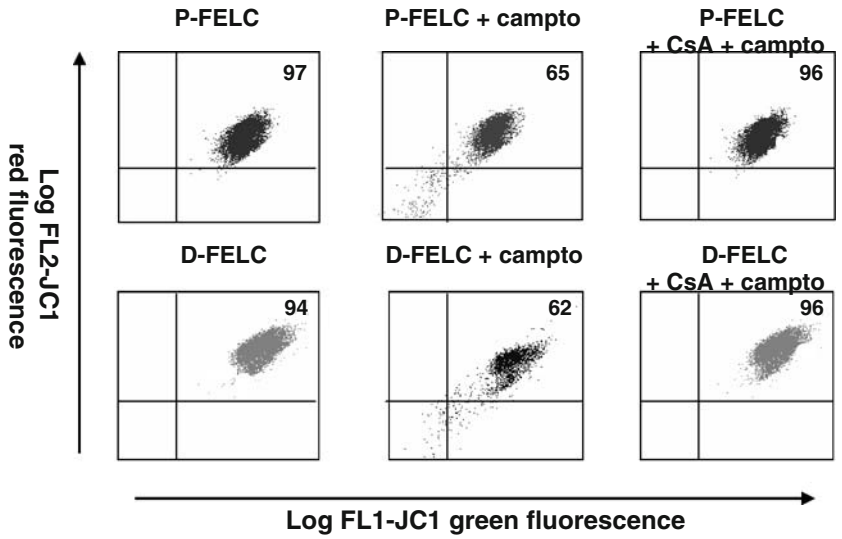

Fig. 4 Cyclosporine A prevents $\Delta\psi_m$ loss induced by camptothecin in FELC. P-FELC and D-FELC were treated for 30 min with 1 μM camptothecin (campto) after 1 h preincubation in the presence or in the absence of 0.5 μM CsA. Cells were then incubated for 10 min with 10 μg/ml JC-1 and analyzed by flow cytometry Data were acquired in list mode and analyzed with CellQuest software (Becton Dickinson, New York, USA). The data are representative of three separate experiments

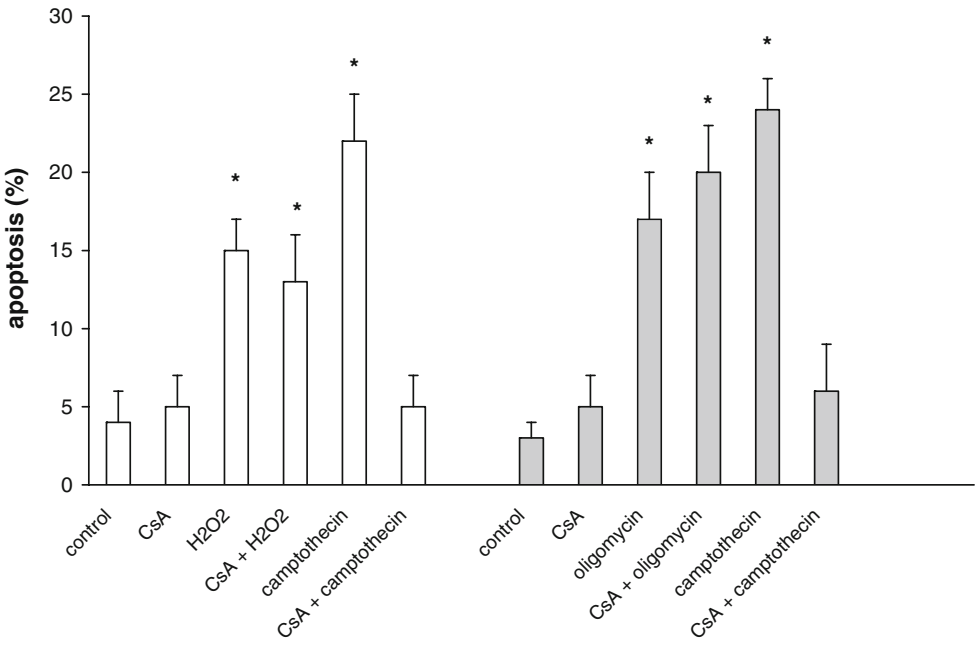

Fig. 5 Cyclosporin A does not prevent apoptosis in differentiated FELC (open columns) exposed to H_2O_2 nor in parental FELC (gray columns) exposed to oligomycin. Cells were preincubated for 1 h in the absence or presence of 0.5 μM CsA before oligomycin or H_2O_2 exposure for 30 min in PBS-glucose. As positive control, P-FELC and D-FELC after preincubation with CsA were exposed for 30 min to 1 μM camptothecin. Cells were returned to fresh complete medium for 2 h and then tested for apoptosis, expressed as percentage of cells exhibiting DNA fragmentation. Values are means and SD of 3 independent experiments, performed in duplicate

Pretreatment with CsA blocked also DNA degradation in caspase-dependent apoptosis induced with camptothecin. Conversely, CsA had no effect on the percentage of cells exhibiting DNA degradation both in the case of D-FELC induced to apoptosis by H_2O_2 and of P-FELC treated with oligomycin (Fig. 5). The conditions of CsA pretreatment had no general toxic effects on cell survival, as demonstrated by cell viability which was above 90% for all the time points and treatments.

Bcl-2 and Bax levels were not altered in mitochondrial ATP synthesis impairment-related apoptosis

We lastly monitored the mitochondrial levels of Bcl-2 and Bax, homologous proteins which exhibit opposite effects on the mitochondrial membrane permeability, thereby controlling apoptotic signals. Compared to P-FELC, D-FELC had a reduced content of mitochondrial Bcl-2 (Fig. 6). However, Bcl-2 did not undergo noticeable variations after treatment with oligomycin (P-FELC) or H_2O_2 (D-FELC). A comparable expression of Bax was found in mitochondria of control P-FELC and D-FELC and such levels did not vary in cells induced to apoptosis. These results indicate that the events leading to apoptosis associated with energy impairment in D-FELC and in P-FELC do not involve changes in mitochondrial levels of Bcl-2 and Bax, as far as immunochemically measured, suggesting that the intermembrane pro-apoptotic factors spill out to the cytosol through a mode of MOM permeabilization not mediated by Bcl-2 or Bax.

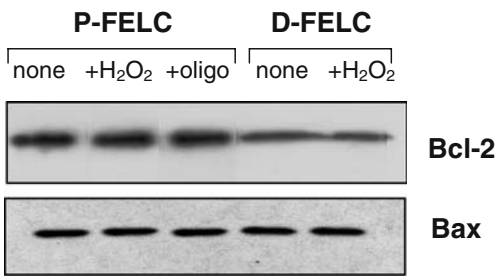

Fig. 6 H_2O_2 or oligomycin treatment does not alter mitochondrial Bcl-2 and Bax protein levels in differentiated or parental FELC. Cells suspended in PBS-glucose solution were treated with oligomycin (P-FELC), H_2O_2 (P- and D-FELC) or vehicle for 30 min and returned to fresh medium for 2 h prior to preparation of mitochondrial fraction for immunochemical detection of Bcl-2 and Bax. Blots are representative of three separate experiments

Discussion

We previously reported that D-FELC, but not P-FELC, are susceptible to mild acute oxidative stress (H_2O_2) and undergo cell death with typical apoptotic features as a result of a consequent mitochondrial energy impairment (Comelli et al. 2003), being such impairment still over the critical energy threshold required for the active events of apoptosis execution (Richter et al. 1996). Energy impairment in D-FELC was attributed to a reduction of mitochondrial F_0F_1ATP synthase which was more severe to that observed in P-FELC (Comelli et al. 1998, 2003). In accordance, both the reduction of energy charge and apoptosis was triggered in P-FELC by mimicking the F_0F_1ATP synthase impairment observed in D-FELC through cell exposure to the specific inhibitor oligomycin (Comelli et al. 2003).

Here, we report that upon the two energy-impairing apoptotic stimuli associated to inhibition of mitochondrial F_0F_1ATP synthase (e.g. oligomycin or oxidative stress resulting from mild acute H_2O_2 treatment combined with the erythroid differentiation execution program) the same signalling pathway appears to be activated. In fact, the apoptosis observed in both cases is independent of caspase activation, involves mitocondrio-nuclear relocation of AIF and Endo G without cytosolic release of cytochrome c, and occurs without any detectable change in $\Delta\psi_m$ nor PTP complex opening. The release of AIF and Endo G occurs without variations in mitochondrial levels of Bcl-2 and/or Bax. These results, while confirming our hypothesis that the impairment of mitochondrial bioenergy is crucial for triggering apoptosis (Comelli et al. 2003), also prompt us to make an hypothesis for the mechanism involved in MOM permeabilization observed in our model leading to the apoptosis execution *via* mitocondrio-nuclear translocation of AIF and Endo G. Mitochondrial release of AIF and Endo G, indicative for MOM permeabilization, appears to be selective, as cytochrome c release did not occur in the time-scale of the experiment, i.e. 2-h after apoptosis induction. Whether it does occur in later time periods could not be determined because of the confounding effects of secondary necrosis**.** In accordance with our data are results obtained by Ruchalski and collaborators, who observed a progressive accumulation of AIF in the cytosol of ATP-depleted renal epithelial cells without a concomitant cytochrome c release (Ruchalski et al. 2003) and by Zanna and collaborators, who described a caspase-independent cell death, driven by energetic failure and mediated by AIF and Endo G in Leber's hereditary optic neurophathy cybrids, thereby demonstrating that, different from cytochrome c in the case of the "classical" caspase-dependent pathway, AIF and Endo G release can account for typical apoptotic features under a rather impaired energetic status (Zanna et al. 2005). The preferential release of AIF and Endo G, observed under our conditions, with no detectable extra-mitochondrial cytochrome c, agrees with the finding that only 10–15% of cytochrome c is in the IMS, whereas the major stores of cytochrome c are sequestered within the cristae and their release requires cristae remodeling and $\Delta\psi_m$ loss (Scorrano et al. 2002). It should be emphasized in this respect that according to some authors (Modjtahedi et al. 2006) a soluble mature AIF pool co-exists in the IMS with the MIM-embedded AIF isoform and might be released into the cytosol upon MOM rupture/permeabilization. Accordingly, a recent paper of Sten Orrenius documents that in rat fibroblasts induced to apoptosis by different triggers (i.e. staurosporine, ceramide or ATP depletion) AIF translocated to the nucleus in nearly all cells in which morphological evidence of large-scale DNA fragmentation had became apparent, whereas cytochrome c translocation was observed only in a few cells, suggesting that AIF release precedes cytochrome c release (Orrenius and Zhivotowky 2005).

Our finding that mild acute oxidative stress induces D-FELC to apoptosis in a caspase-independent manner is consistent with results obtained by other authors using different experimental models, in which ROS were involved in inactivating caspases (Fonfria et al. 2002; Carmody and Cotter 2000). Moreover, hydrogen peroxide *per se* was described as a direct inactivator of caspases (Borutaite and Brown 2001). Caspase activity in fact is optimal under reducing conditions, since their catalytic sites contain a nucleophilic cysteine prone to oxidation, thiol alkylation or thiol nitrosylation. This implies that the production of ROS can directly modulate caspase activation (Denecker et al. 2001). In addition we can hypothesize that ROS-inactivation of caspases may be involved even upon exposure of P-FELC to oligomycin. In fact up-regulation of mitochondrial ROS formation, which is inherently governed by ΔpH and $\Delta\psi_m$ through the redox state of the respiratory chain, is caused by most bioenergetic effectors by inhibiting any of the important regulatory sites for ROS generation. In particular ROS may accumulate upon increase of $\Delta\psi_m$ induced by inhibitors of mitochondrial ATP synthase like oligomycin or by acidification of the mitochondrial matrix (Brooks 2005). Then we could suggest that the caspase-independent AIF- and Endo G-mediated pathway play a fundamental role in the execution of apoptosis especially under pro-oxidant conditions possibly limiting caspase activation.

Permeabilization of MIM with loss of $\Delta\psi_m$ is considered a primary event in many paradigms of cell apoptosis (Zamzami et al. 2005). Nevertheless, in both sets of energy-impaired apoptotic cells, there was no change in $\Delta\psi_m$ just after induction of apoptosis and in the subsequent 2-h period. Accordingly, $\Delta\psi_m$ loss was reported as a not strictly required event in some cases by authors who documented the release of mitochondrial pro-apoptotic proteins, including AIF and Endo G, without a detectable decrease of $\Delta\psi_m$ (Kroemer et al. 2007; Bossy-Wetzel et al. 1998; Ruchalski et al. 2003; Krohn et al. 1999; Samraj et al. 2007; Choi et al. 2007). The determination of $\Delta\psi_m$ with fluorescent dyes is a controversial area fraught with problems (Halestrap et al. 2000), then we have validated our results by using two specific probes with different characteristics: i.e. JC-1 based on its intrinsic property to change fluorescence depending on $\Delta\psi_m$ with a large shift in emission and TMRM as a more sensitive non-toxic $\Delta\psi_m$ probe. Our results further suggest that MIM depolarization is not a universal first step in the apoptotic process. Nevetheless, as the time course of the drop in $\Delta\psi_m$ is also critical, the data can not exclude the possibility of a later reduction in $\Delta\psi_m$ or of a

transient $\Delta\psi_m$ change, missed by potentiometric probes (Bernardi et al. 2001).

With regard to MOM permeabilization the data suggest that it occurs without modifications in mitochondrial levels of Bcl-2 and/or Bax. In fact, no change was observed in the amounts of immunoreactive Bcl-2 or Bax in mitochondrial fractions isolated upon induction of ATP synthesis impairment-related apoptosis by the two treatments. Conversely, the data document a down-regulation of mitochondrial Bcl-2 upon erythroid differentiation of FELC whereas the levels of Bax were the same in mitochondria of control P-FELC and D-FELC. In accordance, down-regulation of Bcl-X_L (an antiapoptotic member of Bcl-2 family) at both mRNA and protein levels has already been observed in K562 and HEL cells after myeloid differentiation (Benito et al. 1996). Bcl-2 down-regulation together with that of other elements of the antioxidant defense system already documented in D-FELC (Comelli and Mavelli 2001), could explain *per se* the fact that the apoptotic effect of H_2O_2 was elicited selectively in these cells and not in the parental counterpart. In fact it is known that over-expression of Bcl-2 inhibits apoptosis by reducing mitochondrial generation of ROS (Hockenbery et al. 1993; Kane et al. 1993; Haddad 2004) or shifting the cellular redox potential to a more reduced state (Fleury et al. 2002; Ellerby et al. 1996).

In conclusion our data indicate that MOM permeabilization, proved by release of AIF and Endo G, occurred without $\Delta\psi_m$ loss, PTP opening or Bax recruitment to mitochondria. Then, no permeabilization of MIM or formation/activation of the Bax-dependent non specific pores or channels (Desagher and Martinou 2000) can be postulated to explain the IMS protein translocation to the cytoplasm. On this basis, we can hypothesize that the F_0F_1ATP synthase inhibition, occurring upon the two apoptotic insults, may be a causative event leading to impairment of ATP/ADP exchange and transient accumulation of protons in the IMS with MOM-disrupting osmotic swelling. A similar osmotic swelling of mitochondria sufficient to disrupt the MOM without affecting the MIM was already suggested by some authors (Desagher and Martinou 2000; Halestrap et al. 2000).

Thus, our results suggest a new mechanism responsible for the osmotic rupture of the MOM and related to inhibition of F_0F_1ATP synthase prompting us to consider such mechanism to be crucial in the execution of the apoptosis observed in our model and possibly of more general interest, considering that the mechanisms for the release of apoptogenic proteins from mitochondria are various and still a matter of debate.

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