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Changes of Plasma Membrane Properties in a Human Pre-T Cell Line Undergoing Apoptosis

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Abstract. A variety of cellular functions are modulated by the physical properties of the cell membrane, and the modification of intracellular transfer, resulting from loss of membrane integrity, may contribute toward setting the cell onto the pathway of apoptosis. Apoptosis in lymphoid cells can be induced in different ways and biochemical modifications occur at an early phase of cell death, while the morphological features of apoptosis are evident later. We previously reported that DMSO is an efficient apoptosis-inducing factor in the human RPMI-8402 pre-T cell line. The aim of the present study was to verify the effect of DMSO on the plasma membrane fluidity, the intracellular calcium concentration and the phosphodiesterase activity in DMSO-induced apoptosis. Our results show a modification of membrane fluidity associated with an increase of intracellular Ca^{2+} concentration. Moreover, we demonstrate that these modifications are related to a decrease in the phosphodiesterase (PDE) activity. The correlation between the proceedings of added DMSO and the induction of apoptosis will provide significant information regarding the first part of the apoptotic process.

Key words: Membrane fluidity — Intracellular calcium — PDE — DMSO — Bcl-2 — Apoptosis

Introduction

Apoptosis, also called programmed cell death (PCD), is a gene-controlled process in multicellular organisms undergoing regression or remodelling. It represents the most common mechanism of eukaryotic cell death and occurs in embryogenesis, metamorphosis, tissue atrophy, and tumor regression (Arends & Wyllie, 1991; Choen & Duke, 1992; Ashwell et al., 1994). Typical morphological and biochemical changes are observed in cells undergoing apoptosis. These include DNA fragmentation, changes in membrane symmetry, activation of caspases, and alteration in the cell death-related protein levels (Earnshaw, 1995; Fadeel, Zhivotovsky & Orrenius, 1999). Apoptosis can be induced by various extracellular factors including UV, γ-irradiation, cytokines and activation of death receptors expressed on the cell surface (Sellins & Cohen, 1987; Riccardi et al., 2000; Strasser, O'Connor & Dixit, 2000). In any case, following a signal, which may be either intrinsic or extrinsic to the cell, the cell reaches a committed phase of apoptotic program that ends in a final degradation phase (Kroemer et al., 1995). Thus, it is important to investigate the intracellular mechanism of a drug that induces apoptosis. The experimental study and manipulation of PCD have been greatly supported by the identification of genetic, chemotherapeutic and biochemical agents that can modulate cell lethality (Wertz & Hanley, 1996), one of these last is the dimethyl sulfoxide (DMSO) (Trubiani et al., 1996). As a powerful solvent DMSO is widely used in biological and biochemical studies but its role as apoptotic factor has not yet been understood. Previous studies suggest that a number of causes may contribute to the DMSO-induced apoptosis in lymphoid cells but the specific pathway is still unclear (Trubiani et al., 1998, 1999; Liu et al., 2001). It has been demonstrated that mitochondrial function is disturbed by DMSO treatment; this disturbance has been shown by depolarization of the membrane, release of cytochrome *c* and decrease of Bcl-2 protein levels (Liu et al., 2001). Moreover, previous studies reported the ability of DMSO to decrease membrane viscosity and thereby increase membrane fluidity (Dai & Sheetz, 1995; Hochmuth et al., 1996; Hui & Nayak,

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2001). It is well known that various cellular functions are modulated by the physical properties of the cell membrane (Kinnunen, 1991), thus the perturbation of the lipid bilayer and the alteration of its fluidity may contribute toward setting the cell onto the pathway of apoptosis. In fact, several studies suggest that a number of drugs or lipid compounds promote apoptosis in many cell types, enhancing the membrane fluidity at a very early stage of apoptosis (Fujimoto et al., 1999). The loss of membrane integrity may result in an alteration of the selective transport of ions and solutes across the plasma membrane, thus inducing apoptosis. For example, the disruption of Ca^{2+} homeostasis has been shown to induce Ca^{2+}-dependent endonuclease activation, perturbation of mitochondrial function and activation of other degradative enzyme cascades (Götz et al., 1994; Martin, Green & Cotter, 1994).

Previous studies have demonstrated that certain subsets of lymphoid cells undergo apoptosis after treatment with agents that elevate intracellular 3′, 5′ cyclic adenosine monophosphate (cAMP) (Lerner, Kim & Lee, 2000). Calcium and cAMP have many interactions in several cell types (Rasmussen et al., 1992; Choi et al., 1993). The cAMP molecule is a ubiquitous second messenger that induces the death of mature T lymphocytes and thymocytes through internucleosomal DNA cleavage (Kizaki et al., 1990; Mentz et al., 1995). The levels of cAMP are controlled, balancing the cAMP synthesis and hydrolysis through, respectively, activity of adenylate cyclase and phosphodiesterases (PDEs). The presence of membrane fluidity variations could modulate these enzymes (Kitagawa, Kotani & Kametani, 1990).

It has been previously shown that specific inhibitors of phosphodiesterases (PDEs) induce cAMP-dependent apoptosis in B cells from B-chronic lymphocytic leukaemia patients (Mentz et al., 1996; Siegmund et al., 2001); in addition, the inhibition of PDE activity could modulate T-cell responses either by inhibiting cytokine production or by inducing apoptosis (Lerner, Kim & Lee, 2000; Ogawa et al., 2002). Several new PDEs have been discovered in the past ten years, although their specific functions are often still unclear due in part to the lack of availability of selective inhibitors. Within each family there may be several subtypes of enzyme (Arp et al., 2003).

Since we found that dimethyl sulfoxide (DMSO) provokes cell death by apoptosis in the human pre-T cell line RPMI-8402 (Trubiani et al., 1996), the aim of the present study was to investigate a possible mechanism by which T-cell death is regulated. In particular we focused our attention on earliest biochemical and morphological modifications of cell membrane, examining the plasma membrane fluidity, the intracellular calcium concentration $[Ca^{2+}]_i$, and the phosphodiesterase activity in DMSO-induced apoptosis throughout apoptotis progression.

Materials and Methods

Cell Cultures

The human TdT-positive pre-T cell line (RPMI-8402), described by Huang et al. (1974), was maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-Glutamine, 100 mM Na-pyruvate and 25 mM HEPES. Cells were grown at 2.5×10^5/ml, with more than 98% viability as determined by trypan blue exclusion test. During the log growth phase, the cells were treated with 1.5% (v/v) of DMSO (gas chromatography grade) for a time ranging between 0 to 72 h.

To investigate the reversibility of DMSO effects, treated cells were washed twice with RPMI-1640 medium, seeded at 3×10^5/ml, and grown again in the DMSO-free medium for 72 h. Treated and untreated samples were immediately processed for morphological and biochemical analyses.

Cell Cycle Analysis

To evaluate the effect of DMSO treatment on the cell cycle, BrdU incorporation was analyzed by flow cytometry as described (Trubiani et al., 1996). Cell cycle analysis was performed by FACStar ^{Plus} flow cytometer (Becton Dickinson, USA) equipped with an argon ion laser tuned at 488 nm, 200 mW output, for excitation of FITC and PI. Data were analyzed as the percentage of cells at different phases of the cell cycle (i.e., G_0/G_1-S-G_2/M) and as the mean channel of FITC fluorescence of BrdU-positive cells. Both anti-BrdU and PI fluorescences were measured on a linear scale.

Apoptosis Estimation

Apoptosis was detected by morphological examinations performed by mixing 10 μl of 1×10^6 cells/ml with 10 μl of ethidium bromide (EB) (100 μg/ml) and acridine orange (AO) (100 μg/ml) dye solution and examined by fluorescence microscopy. Cells were scored as either viable or non-viable and either normal or apoptotic. Cells with bright green chromatin were scored as viable; those with bright orange chromatin were scored as non-viable. Normal nuclei are those with chromatin in organized structures, while apoptotic nuclei are those with highly condensed or fragmented nuclei. Two hundred cells were counted for each treatment.

Ultrastructural Analyses

Treated and untreated samples were fixed with 1.25% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.6 for 1 h at 4°C. After rinsing in the same buffer the cells were postfixed with 1% OsO_4 in cacodylate buffer for 1 h in a dark cold room. The cells were then washed with buffer and double-distilled water and stained overnight using a saturated uranyl acetate aqueous solution. The cell suspensions were then washed, dehydrated in a series of ethanol and embedded in Spurr medium. Thin sections were placed on copper grids and stained with lead citrate. Sections were then analyzed using a Philips CM10 electron microscope operating at 60 kV.

Fluorescence Measurements

To measure the fluidity of the hydrophilic region (outer membrane) of the cell membrane, the fluorescent probe 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), a hydrophilic derivative of DPH, was used. Cell incubation with the probe was performed as described by Sheridan and Block (1988). Briefly, 3 μl of

TMA-DPH (10^{-3} M) was incubated for 5 min at room temperature with 2 ml of freshly prepared cells in 50 mM Tris-HCI buffer solution, pH 7.4. Fluorescence intensities (100 readings each) of the vertical and horizontal components of the emitted light were measured by a Perkin-Elmer LS 50 B spectrofluorometer equipped with two glass prism polarizers (excitation wavelength 365 nm, emission wavelength 430 nm). Membrane fluidity is expressed as the inverse of fluorescence polarization and fluorescence polarization of TMA-DPH was calculated from the intensity measurements by $P = (I_v - I_h)(G) / (I_v + I_h) (G)$ where I_v is the intensity measured when the polarizer and analyzer prisms are in the vertical position and I_h is the intensity when the analyzer prism is in the horizontal position. G is the correction given by the ratio of the vertical to the horizontal components when the excitation light is polarized in the horizontal direction (Kuhry et al., 1985).

Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured in intact cells using the fluorescent probe FURA 2-AM as previously described (Grynkiewicz, Poenie & Tsien, 1985). Determinations were performed using a Perkin-Elmer LS 50 B spectrofluorometer at 37 °C according to the method of Rao (1988). Fluorescence intensity was evaluated at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm). At the end of any experiment, maximum and minimum fluorescence values, at each excitation wavelength, were obtained by first lysing the cells with 0.1% Triton X-100 (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340/380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a Fura–2 Ca^{2+} binding constant (135 nM) and the formula described by Grynkiewicz et al. (1985).

PDE Assay

The PDE enzymatic analysis was carried out using the method of Spoto et al. (1991) with minor modifications. Briefly, the reaction was started by the addition of 44 μM of cAMP, in a final volume of 500 μl. The time course of reaction was 60 min. The reaction was terminated by transferring the tubes with the reaction mixture to a boiling water bath for 3 min after acidification with HCl 2 M. The samples were then centrifuged and filtered through a nylon-66 filter, 0.2 μm (Rainin Corporation). The clear filtrate obtained was used directly for HPLC assay, performed as previously described (Spoto et al., 1994), or stored at −80°C. Control experiments were performed using a traded preparation (SIGMA) in which the enzyme concentration was 0.4 μM. Protein content was determined using a bicinchoninic acid protein determination kit from Sigma with bovine serum albumin as a standard.

Fluorescence-activated Cell Sorter (FACS) Bcl-2 Analysis

Untreated and DMSO-treated cells were washed once with PBS and suspended in RPMI-1640 medium containing 10% FBS to a density of 2×10^6 cells /ml. A 0.1 ml aliquot of the cell suspension was placed in a plastic tube and treated with human lgG at a final concentration of 15 μg/ml at 4°C for 15 min to reduce the unspecific binding of antibody. The cells were then incubated, at 4°C for 30 min, with an appropriate amount of FITC-conjugated mAb anti-Bcl-2. Stained cells were analyzed with a FACStar^{Plus} flow cytometer (Becton Dickinson, USA).

Western Blotting

8402-RPMI cells were resuspended in cold hypotonic lysis buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF, 10 μM leupeptin and 10 μg/ml soybean trypsin inhibitor). Protein concentration was determined by the Bio-Rad protein assay and detected spectrophotometrically. Proteins (60 μg/sample) were separated using SDS-12% polyacrylamide gel, and then electrophoretically transferred to a 0.2 μm PVDF membrane for 1 h. The transfer buffer contained 25 mM Tris-HCI, 192 mM glycine, 0.037% (w/v) SDS and 20% (v/v) methanol. The membranes were blocked by TBS (20 mM Tris-HCI, 150 mM NaCI, pH 7.5) containing 5% (w/v) skimmed milk and 0.1% Tween-20 for 1 h. The membrane was then incubated overnight at 4°C with the appropriate dilutions of mouse monoclonal anti-Bcl-2 as primary antibody. This was followed by an incubation with a goat horseradish peroxidase-conjugated anti-mouse lgG as secondary antibody at 1:1000 dilution for 1 h at room temperature. DAB reaction was used to visualize immune complex. Pre-stained standard size markers and β-actin control (*data not shown*) were used.

Source of Materials

Antibodies, reagent grade materials and culture reagents were from Sigma Chemical (St. Louis, MO). Polyvinylidene difluoride membrane was from Millipore, (Bedford, MA). Reagents for electron microscopy were from Polyscience (Warrington, VA), while products for electrophoresis were from Bio-Rad Laboratories (Richmond, VA).

Statistical Analysis

The *t*-test of Student was used to compare the results. P values less than 0.05 were considered statistically significant.

Results

DMSO induces several biological modifications in the RPMI-8402 human cell line, such as growth arrest, phenotypic changes and progression of programmed cell death, including DNA fragmentation (Trubiani et al., 1999). In resting cells these effects are completely reversed after removal of DMSO (Trubiani et al., 1996). Indeed, when DMSO-synchronized cells were transferred to DMSO-free medium, all the morphological and biochemical parameters of apoptosis tended to return at the basal values.

Flow cytometry analysis was carried out to assess the effect of DMSO on cell growth. The results reported in Table 1 demonstrate that DMSO provokes a reduction of cell growth and this effect is due to a decrease in the number of cells entering the S-phase of the cycle. This action is particularly evident after 24 h of treatment. Beside the decrease in the S phase, an accumulation of the cells in the G1 phase is consistent. After culturing in DMSO-free medium the treated cells return to the basal state.

Other than the evidence of RPMI-8402 cell cycle modification, a gradual change in cell structure was assessed by the analysis of forward scatter parame-

Table 1. Cell cycle phases as assessed by flow cytometry

	Incubation time (h)									
Phase	0[a,b]	12[a]	12[b]	24[a]	24[b]	48[a]	48[b]	72[a]	72[b]	*R*
$G_0 + G_1$	40 ± 0.01%	44 ± 0.03%	51 ± 0.01%	45 ± 0.02%	64 ± 0.01%	46 ± 0.02%	80 ± 0.01%	58 ± 0.01%	87 ± 0.02%	41 ± 0.02%
S	42 ± 0.02%	40 ± 0.01%	36 ± 0.02%	39 ± 0.02%	22 ± 0.03%	38 ± 0.02%	12 ± 0.03%	27 ± 0.02%	7 ± 0.03%	41 ± 0.01%
$G_2 + M$	18 ± 0.05%	16 ± 0.04%	13 ± 0.04%	17 ± 0.05%	14 ± 0.03%	16 ± 0.04%	8 ± 0.03%	15 ± 0.04%	6 ± 0.05%	18 ± 0.03%

The data report the means of six separate experiments ± SD.
R: treated cells re-cultured in DMSO-free medium for 72 h.
[a]: Control cells; [b]: DMSO-treated cells.

ters, showing the presence of a hypodiploid peak starting at 48 h of treatment (*data not shown*). This peak indicates the apoptotic cell population, which was quantified by double staining with AO and EB in fluorescence analysis. The results, obtained from five different experiments, show that the means of apoptotic cells in untreated sample was less than 8 ± 3 % at 72 h, while in DMSO-treated cells at 0, 24, 48 and 72 h they were, respectively, 3 ± 2% , 6 ± 3%, 20 ± 5 % and 36 ± 8%.

The cellular modifications induced by DMSO were detailed by ultrastructural analyses (Fig. 1). Section *A* reports the ultrastructural features of untreated cells recovered at the starting time of the experiment. In response to DMSO, cell changes both in shape and organization can be detected. After 8 h (section *B*), treated cells show a remodelling of the plasma membrane. Membrane ruffling and several short microvilli-like protrusions can be seen. Subsequently these projections are substituted by finger-like protrusions of plasma membrane know as filipodia, mostly at 12 h (section *C*), and then by sheet-like protrusions known as ruffles or lamellipodia. The latter are particularly evident after 24 h of treatment, as seen in sections *D* and *E*. Moreover, at 24 h (section *D*), changes other than in cell surface arrangement are also evident: modification of cell-size and, at the nuclear level, initiation of the clumping of the chromatin, representing nuclear condensation. At 48 h (section *E*), we can observe the condensation of nucleoplasm and the preliminary step of the nuclear fragmentation. These cells, after 72 h (section *F*) show the typical apoptotic features with the chromatin aggregates forming half-moon images, presence of micronuclei, and apoptotic vesicle formation representing the final step of the apoptotic process. At this last phase the plasma membrane appears completely flat. The results obtained by morphological investigation suggest that the addition of DMSO induces detailed modification of the plasma membrane, noticeable at an early phase of the treatment. These morphological changes are completely abolished in re-cultured cells (*data not shown*).

In order to confirm this hypothesis we analyzed the membrane fluidity, the intracellular calcium level and the phosphodiesterase activity within the first 48 h of the DMSO treatment. As reported in Fig. 2, the plasma membrane fluidity of RPMI-8402 cells shows a significant decrease of TMA-DPH P values (i.e., increased fluidity of the superficial part of the membrane) after 8, 12, 24 and 48 h of incubation with DMSO. As concerns the intracellular calcium concentration, it is significantly increased after 8, 12, 24 and 48 h of incubation (Fig. 3). Regarding the PDE activity determination (Fig. 4), treated cells show a slight increase of the enzymatic activity along the first 12 h of incubation with DMSO, followed by a downregulation that reaches the lowest value at 48 h of treatment. In the first 12 h the upregulation of PDE enzymatic activity represents the immediate cellular answer to the action of DMSO, while the down-regulation of the enzymatic activity, starting from 24 h of incubation, indicates the apoptotic phenomenon progression, morphologically noticeable at 48 h of incubation. In DMSO-free condition the treated cells return to the basal state, as observed in untreated cells.

Finally, to establish a gene regulation of DMSO-dependent cellular injury and apoptosis, Bcl-2 determination has been performed. By flow cytometry analyses, data reported in Fig. 5, and Western blotting, data reported in Fig. 6, we evaluated, respectively, the percentage of Bcl-2-positive cells and their protein levels. The data display the downregulation of the Bcl-2 expression, in terms of positive cells and protein content, following the addition of DMSO. In DMSO-free medium the cells re-establish the basal level of Bcl-2 expression.

Discussion

Programmed cell death culminating in apoptosis is essential for normal tissue homeostasis and has increasingly been implicated in mediating pathological cell loss. Although apoptosis of cells is accompanied by characteristic morphological changes, including cell volume loss and thus changes in membrane permeability to water and ions, it was not clear whether intracellular changes of ion homeostasis are a passive secondary feature of the cell death or

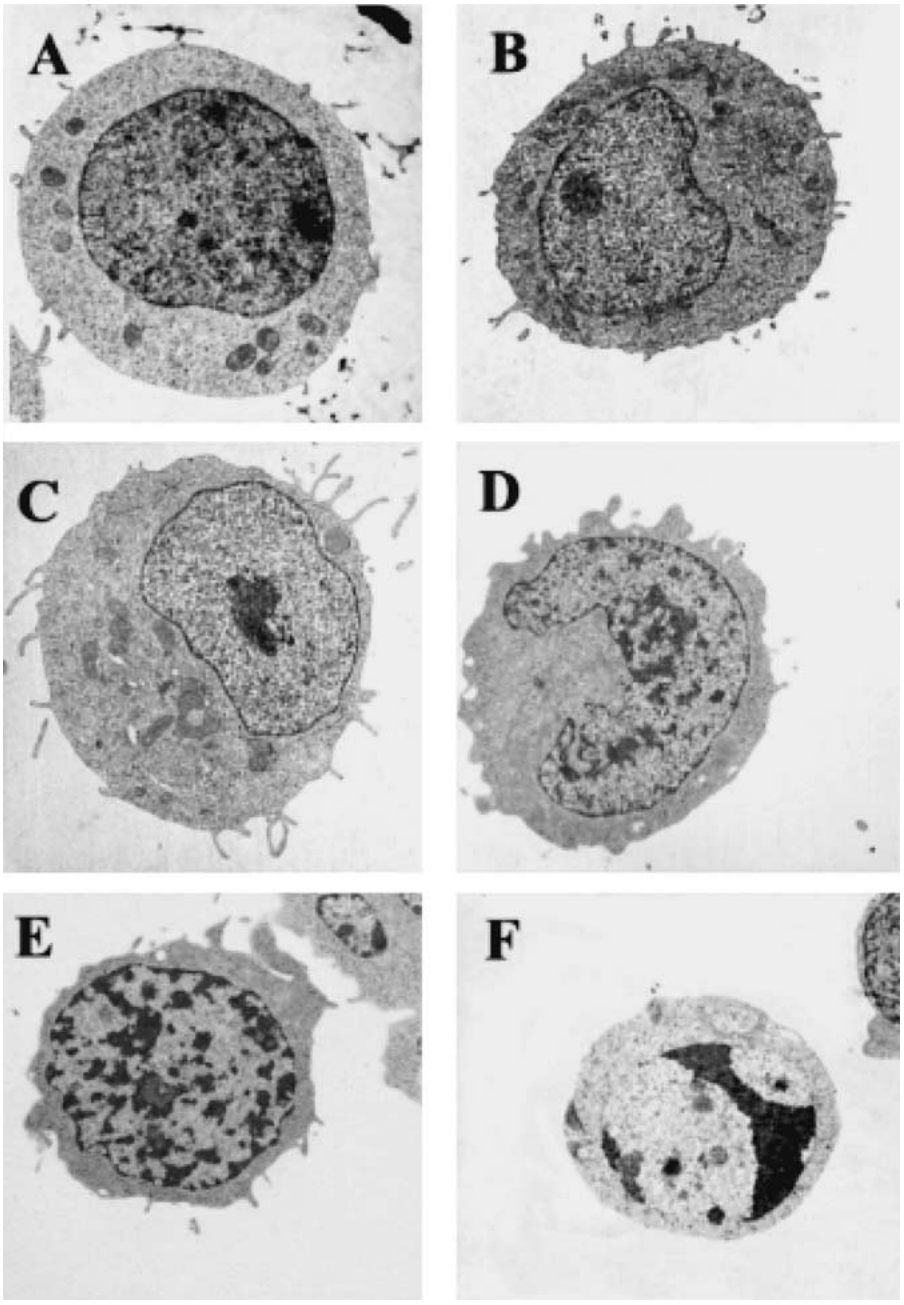

Fig. 1. Ultrastructural analyses of DMSO-treated cells. Untreated cells (*A*), 8 h (*B*), 12 h (*C*), 24 h (*D*), 48 h (*E*) and 72 h (*F*) DMSO-treated cells. The pictures show cellular modifications due to apoptotic program progression. Depending on treatment time, the cells show differences in the plasma membrane organization. After 8 h the cell surface shows protrusions and ruffles and the presence of many microvilli, while at 12 h filipodia are present and distributed over the whole cell surface. These particular membrane structures .are substituted then at 24 h and mostly at 48 h by blebs and lamellipodia that characterize the cell surface of treated cells. At 72 h a complete flattening of the plasma membrane is evident. Moreover, at 24 h (*D*) changes other than in the membrane arrangement are evident: shrinkage of cell size and at the nuclear level, initiation of clumping of the chromatin, representative of nuclear condensation. These cells, after 72 h, show the typical apoptotic features with the presence of micronuclei or the chromatin being aggregated, forming half-moon images. 1 cm = 1 μm.

a driver of the process (Yu, Canzoniero & Choi, 2001).

With the present study we demonstrate that biochemical modifications at the plasma membrane level occur at an early phase of the apoptotic program and that the evidence of nuclear alterations corresponds to the final step of cell death progression. Consistent with previous studies demonstrating that DMSO enhances membrane fluidity (Hui & Nayak, 2001), the incubation of RPMI-8402 cells with DMSO is associated with a significant increase in the fluidity of the more superficial part of the plasma membrane, as demonstrated by the lower TMA-DPH *P* values. These data are in agreement with previous results suggesting that apoptotic lymphocytes are characterized by a membrane fluidization that mainly occurs on the cell membrane surface (Benderitter et al., 2000). Our results strongly suggest that changes in membrane structure may be relevant to initial aspects of the apoptotic process. A secondary effect of the plasma membrane modifications might be changes in cytosolic Ca^{2+} concentration caused by altered transmembrane calcium fluxes. Our data show, in fact, a significant increase of the intracellular calcium concentration after 8, 12, 24 and 48 h of incubation with DMSO and this increase was parallel to the

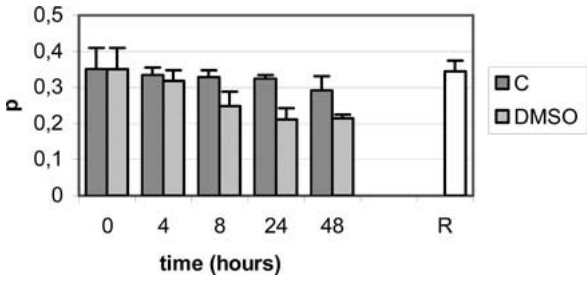

Fig. 2. Effect of DMSO treatment on polarization (*P*) values of TMA-DPH in RPMI-8402 cells at time 0 and after 8, 12, 24 and 48 h of incubation. *C*: control samples; *DMSO*: treated samples. *R*: treated cells re-cultured in DMSO-free medium for 72 h. The means ± SD of four different experiments are reported. Significance: 8 h: DMSO vs. *C*, $p < 0.05$; 12 h: DMSO vs. *C*, $p < 0.05$; 24 h: DMSO vs. *C*, $p < 0.001$; 48 h: DMSO vs. *C*, $p < 0.01$.

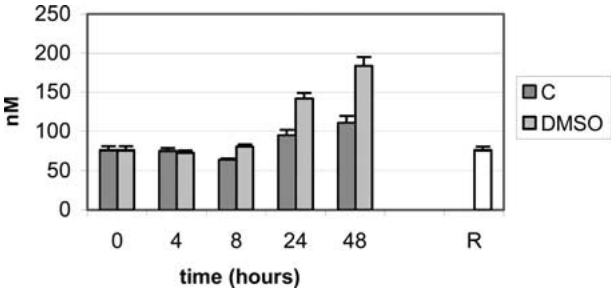

Fig. 3. Effect of DMSO treatment on $[Ca^{2+}]_i$ in RPMI-8402 cells at time 0 and after 8, 12, 24 and 48 h of incubation. *C*: control samples; *DMSO*: treated samples. *R*: treated cells re-cultured in DMSO-free medium for 72 h. The means ± SD of four different experiments are reported. Significance: 8, 12, 24 and 48 h: DMSO vs. *C*, $p < 0.001$

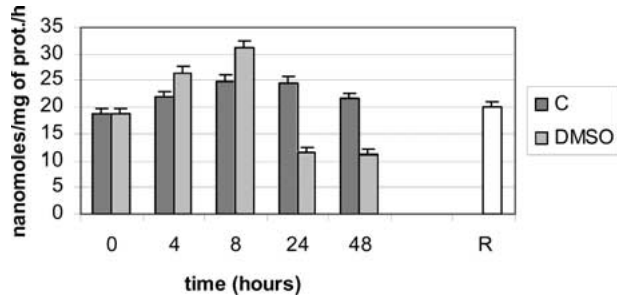

Fig. 4. Effect of DMSO treatment on phosphodiesterase activity in RPMI-8402 cells at time 0 and after 8, 12, 24 and 48 h of incubation. *C*: control samples; *DMSO*: treated samples. *R*: treated cells re-cultured in DMSO-free medium for 72 h. The means ± SD of four different experiments are reported. Significance: 8, 12, 24 and 48 h: DMSO vs. *C*, $p < 0.001$

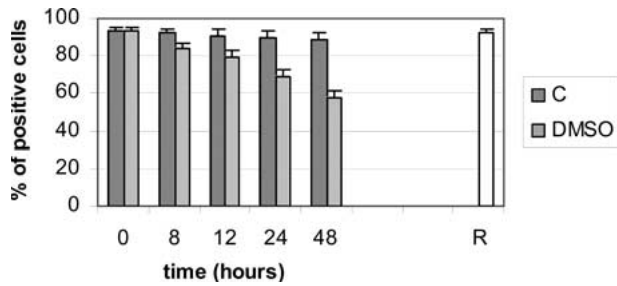

Fig. 5. Immunocytochemical analysis of DMSO-treated cells by flow cytometry. The decrease of Bcl-2 positive cells, expressed as percentage, is detectable starting at the time of treatment. *R*: treated cells re-cultured in DMSO-free medium for 72 h. The means ± SD obtained from six different experiments are reported. Significance: 8, 12, 24 and 48 h: DMSO vs. *C*, $p < 0.001$

membrane fluidity enhancement. Calcium is a crucial regulator of many physiological processes and in most cell lines apoptosis is induced by increased intracellular Ca^{2+} concentration associated with enhanced cell membrane permeability (Pozzan et al., 1994; Yu et al., 2001). In particular, studies on immature thymocytes have documented a critical role of a transient increase in cytosolic calcium in apoptosis signalling (Lalli & Sassone-Corsi, 1994). Concerning the PDE, our results show a significant reduction of PDE activity after 24 and 48 h of incubation with DMSO. A consequence of such reduction is the increase of cAMP levels; the enhancement of this intracellular second messenger, in turn, is responsible for the induction of apoptosis.

Previous studies demonstrated that certain subsets of lymphoid cells undergo apoptosis after treatment with PDE inhibitors (e.g., methylxanthines, rolipram, theophylline) (Mentz et al., 1996; Lerner et al., 2000; Siegmund et al., 2001). Finally, the incubation with DMSO provokes a downregulation of the Bcl-2 protein levels, in agreement with previous studies demonstrating a decrease of Bcl-2 protein

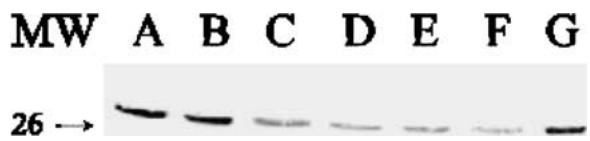

Fig. 6. Western blotting, performed as previously described, shows the downregulation of Bcl-2 protein during the DMSO treatment. (*A*) Untreated cells. (*B*), (*C*), (*D*), (*E*) and (*F*) indicate, respectively, 8, 12, 24, 48 and 72 h of treatment, while (*G*) represents the level of Bcl-2 appearing in untreated cells cultured for 72 h in DMSO-free medium. The decrease of Bcl-2 starts after 12 h of treatment. Untreated cells analyzed at 0 (*A*) and at 72 (*G*) h show the same levels of Bcl-2. *MW*: molecular weight.

levels in DMSO-induced apoptosis of EL-4 murine T lymphoma cells (Liu et al., 2001). Bcl-2 participates in the composition of the permeability transition pore (PT pore) complex; so, downregulation of the Bcl-2 level following DMSO treatment may cause the release of pro-apoptotic factors from the mitochondrial inter-membrane space and may be essential for induction of apoptosis.

In summary, our investigation indicates that DMSO-induced apoptosis is a gene-driven process

accompanied by characteristic morphological and biochemical apoptotic changes that initiate with early alterations of cytoplasmic membrane homeostasis. In DMSO-free condition the treated cells return to the basal state.

To completely clarify the effect of DMSO, further investigations are still required. The study of DMSO will not only uncover the effects of this molecule on cell functions but also be a benefit for better understanding the apoptotic signal transduction.

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