Expression of P-170 glycoprotein sensitizes lymphoblastoid CEM cells to mitochondria-mediated apoptosis

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Multidrug resistance caused by P-glycoprotein (P-170) is a phenomenon by which cells exposed to a single drug acquire resistance to other structurally and functionally unrelated drugs. This is a widespread phenomenon described *in vivo* in the management of infectious as well as non-infectious diseases. Several *in vitro* models have been developed in order to evaluate physiopathological properties of P-170. Among these are P-170-expressing variants of the human T-lymphoblastoid CEM cell line called VBL100. As a general rule, drug resistance normally results in resistance to apoptosis induction. By contrast, a paradoxical activity is exerted in this cell model by the cytokine tumour necrosis factor-α (TNF-α), which is capable of inducing apoptosis in P-170-expressing variants better than in wild-type (wt) cells. In the present study we partially address the mechanisms underlying this activity. In fact, the susceptibility of VBL100 cells to TNF-α appears to be specifically due to the depolarization of their mitochondrial membrane, a key factor for apoptotic induction. The same was observed with staurosporine, a specific mitochondrion-mediated proapoptotic chemical probe. Conversely, other proapoptotic stimuli, such as Fas/CD95 or the anti-cancer drug etoposide, did induce significant cell death in wild type cells only. Thus, schematically, mitochondrially dependent stimuli appeared to be more effective in VBL100-cell killing, while 'physiological' stimuli showed the opposite behaviour. Importantly, under steady-state conditions, VBL100 cells displayed *per se* a mitochondrial membrane hyperpolarization that appeared strictly related to their high susceptibility to specific apoptotic stimuli. In conclusion, the study of a well-established cell model such as that represented by the wt/VBL CEM lymphoid cell line seems to suggest that the multidrug resistance phenotype can specifically sensitize cells towards 'unphysiological', mitochondria-associated cell death cascade or, in the same fashion, it could shift cells from type I (mainly plasma membrane-associated) towards type II (mainly mitochondrial membrane-associated) phenotype.

Key words: drug resistance, mitochondrial membrane potential, tumour necrosis factor-α.

INTRODUCTION

It has been hypothesized that the apoptotic process may be a physiological event eliminating damaged or infected cells or regulating the cell number within a tissue [1]. Several lines of evidence have demonstrated that apoptosis can be physiologically modulated by a number of factors or compounds capable of determining cell fate and, in turn, regulate the steady state of a tissue. Among compounds able to modulate cell survival and death by apoptosis are a number of drugs of widespread use in human non-infectious as well as infectious pathological conditions. Of great importance are, for instance, anti-tumour agents such as topoisomerase I (e.g. camptothecin) and topoisomerase II (e.g. etoposide) inhibitors, or other antiproliferative agents such as cisplatinum, ionizing radiations or hyperthermic treatments [2]. Strikingly, in the same manner, some drugs of relevance in the management of various infectious diseases have also been suggested to be capable of modulating cell survival, e.g. HIV-protease inhibitors [3]. Thus, because of the importance of cell survival or death in the chemotherapy of both infectious and non-infectious diseases, apoptosis-regulatory activity exerted by different drugs has been extensively analysed in a number of experimental studies [4,5]. In particular, cell sensitivity to specific apoptotic stimuli resulted in activation of specific apoptotic pathways. In fact, the complex cascade of events involving pro-apoptotic or anti-apoptotic signals, including activation of several apoptosis-specific proteases, i.e. caspases, depend upon (or are associated with) the type of the 'trigger' that activates the process [6]. However, the differing sensitivities of various cell types to apoptosis triggers is still a matter of debate. By contrast, a hypothesis regarding two different apoptotic pathways leading to activation of cell-specific programmes has recently been proposed. These two pathways, called type I and II, are attributed to different initiation patterns [7]. Respectively the activation of caspase 8 or 9 has specifically been suggested as first trigger in the death process in the two pathways. Importantly, however, only the type II apoptotic machinery seems to involve mitochondrial-associated caspase cascade in the early phases.

The emergence of the multidrug resistance (MDR) phenotype was initially described as an important clinical phenomenon in which a tumour cell exposed to a single antiproliferative agent becomes resistant to a large number of other structurally and

Abbreviations used: TNF-α, tumour necrosis factor-α; wt, wild-type; MDR, multidrug resistance; ABC, ATP-binding cassette; MAb, monoclonal antibody; PmI, propidium iodide; CHX, cycloheximide; CyA, cyclosporin A; BA, bongkrekic acid; TRAIL, TNF-related apoptosis-inducing ligand; STS, staurosporine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; IVM, intensified video microscopy; TNFR1, TNF-α receptor 1; $\Delta\Psi$, mitochondrial membrane potential.

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functionally unrelated cytotoxic compounds. In humans, the *mdr1* gene encoding for a 170 kDa transmembrane protein has been found to be functionally associated with the tumour-cell MDR phenotype [8]. This 170 kDa molecule currently named 'P-170' or 'P-glycoprotein' is an ATP-binding-cassette (ABC) protein which acts as an ATP-dependent transporter and pumps hydrophobic drugs out of cells, reducing their intracellular concentration and subsequently their efficacy as chemotherapeutic agents [9,10]. In eukaryotes, most of the members of the ABC protein family function as ATP-dependent active transporters in the plasma membranes and in the membranes of intracellular organelles, including mitochondria [11].

Several studies also evaluated the possible association of MDR with the apoptotic-cell-death programme. It was observed that resistance to different drugs, e.g. due to the expression and function of P-170 protein on the cell surface, lead to parallel resistance to certain apoptotic stimuli [12,13]. With this in mind, we investigated the mechanisms underlying a paradoxical effect previously reported [14], i.e. the high susceptibility to apoptosis mediated by tumour necrosis factor-α (TNF-α) in a lymphoblastoid cell line expressing P-170 (CEM VBL100), compared with the corresponding wild-type (wt) counterpart (wtCEM). We discuss this paradoxical activity of TNF-α cytokine, analysing the mechanisms on the basis of the susceptibility to various apoptotic stimuli. In particular, type I and type II apoptotic processes have been evaluated. In the light of our results, the role exerted by mitochondria in apoptotic triggering of P-170-positive cells appears to be significant. A sort of 'sensitization' of VBL100 cells towards mitochondrion-associated death pathway was in fact found. These data seem to suggest a type I/type II cell shifting associated with (or due to) the presence of P-170.

MATERIALS AND METHODS

Cell lines

The parental drug-sensitive human T-lymphoblastoid CCRF-CEM (wtCEM) cell line and its MDR variant CEM-VBL100 (VBL100) were originally provided by Dr W. T. Beck (St Jude Children's Research Hospital, Memphis, TN, U.S.A.) [14]. Cell lines were grown at 37 °C in RPMI 1640 supplemented with 10 % (v/v) fetal-calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), 1 % non-essential amino acids, 5 mM L-glutamine, penicillin (100 i.u./ml) and streptomycin (100 mg/ml). Cells were subcultured in 25 cm^2 Falcon plastic flasks at a density of approx. 5×10^5 cells/ml. Flasks or wells were placed in 37 °C incubator in air/CO_2 (19:1) atmosphere.

P-170 expression

To evaluate and to control P-170 expression on the cell surface of CEM cells and their derivative MDR variant CEM VBL100, a monoclonal antibody (MAb), named MM4.17, was used. This MAb reacts only with human living MDR cells and recognizes an epitope localized on the fourth loop of the P-170 extracellular domain. MM4.17 specifically binds with MDR1-P-glycoprotein isoform [15]. Antibodies MRK 16 (which recognizes an external domain) [16] and C219 (which recognizes a cytoplasmic domain) [17] were also used as controls. Cells were incubated with specific MAb for 30 min at 4 °C and then washed in PBS. After this time cells were incubated with a FITC-conjugated anti-mouse IgG for 30 min at 4 °C and washed twice in PBS and immediately analysed by flow cytometry. Isotypic control (IgG1; Sigma) were used in all experiments. To exclude dead cells from our analysis, we incubated the samples with propidium iodide (PmI; 40 μg/ml, Molecular Probes Inc. Eugene, OR, U.S.A.). Only VBL100 cells markedly express P-170 (median value of the FACS histogram, 100.9), whereas wtCEM cells were completely negative (median value of the FACS histogram, 3.0) on their surface, as previously demonstrated [14].

Chemicals

Cycloheximide (CHX; 4 μM; Sigma), cyclosporin A (CyA; 10 μM, Sigma), bongkrekic acid (BA; 30 μM; generously provided by Professor A. Toninello, University of Padova, Padova, Italy), TNF-α (50 i.u./ml; Sigma), TRAIL (TNF-related apoptosis-inducing ligand; 100 ng/ml; Alexis, San Diego, CA, U.S.A.), staurosporine (STS; 20 nM; Sigma), etoposide (Sigma; ranging from 2 to 20 μM) or anti-Fas antibodies (125 ng/ml; clone CH11; Upstate Biotechnology, Lake Placid, NY, U.S.A.) were added to the cultures as specified below.

Treatments

To induce apoptosis wtCEM and VBL100 cells were treated as follows: (a) 2 h of CHX and then TNF-α for 1.5 h (short time treatment) or 4 h (long time treatment); (b) etoposide (20 μM) for 8 h (short time treatment) or 18 h (long time treatment); (c) antibody anti-Fas for 3 h (short time) or 6 h (long time); (d) STS for 3 h (short time) or 6 h (long time); (e) 2 h of CHX and then TRAIL for 6 h (short time) and 18 h (long time). We performed specific control experiments by using CHX alone for 6 h and 8 h. This drug did not induce any appreciable effect on either apoptosis and mitochondrial membrane potential. Moreover, ethanol and DMSO (etoposide and STS vehicles respectively) were also considered as experimental controls. No differences were detected between untreated and vehicle-treated cells. To study the specific involvement of mitochondria in apoptotic phenomena, cells were pretreated for 1 h with 10 μM CyA or 30 μM BA and then exposed to different apoptotic stimuli. Cells treated with CyA or BA alone were considered as controls. At the end of treatments cells were analysed for apoptosis quantification and mitochondrial transmembrane potential evaluation as specified. Results obtained with TRAIL completely overlapped those found with anti-Fas antibodies and they have thus been omitted from the results.

To verify the expression of TNF-α receptor 1 (TNFR1) and CD95 on the cell surface of wtCEM and VBL100 cells, MAbs directly conjugated to R-phycoerythrin to human TNFR1 (Caltag Laboratories, Burlingame, CA, U.S.A.) and CD95 (Becton Dickinson, Mountain View, CA, U.S.A.), respectively were used. No significant difference between wt and VBL100 CEM cells was found in the expression of these two receptors.

Evaluation of apoptosis

Quantitative evaluation of apoptosis was performed by using the following flow- and static-cytometry methods: (i) TdT incorporation of labelled nucleotides into DNA strand-breaks (TUNEL-FITC; Boeringher Mannheim, Milan, Italy). Cells fixed with 4 % formaldehyde in PBS for 15 min were washed and then permeabilized with 70 % ice-cold ethanol for 5 min at 4 °C. After washing cells were incubated with TUNEL reaction mixture according to the manufacturer's instructions; (ii) double staining by using annexin V-FITC apoptosis detection kit (Eppendorf s.r.l., Milan, Italy). By using this technique, cells which have lost membrane integrity (and therefore considered as necrotic cells) will show red staining with PmI (40 μg/ml) throughout

the nucleus and then they will be easily distinguishable from the living cells; (iii) staining with chromatin dye Hoechst (Molecular Probes) as previously described [18].

Mitochondrial membrane potential ($\Delta\Psi$)

The $\Delta\Psi$ of control and treated cells was studied by using the probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes). According to the method, cells were stained with 10 μM JC-1. JC-1 is a metachromatic probe able to enter selectively the mitochondria. It exists in a monomeric form (in the green channel, FL1), but, depending on the membrane potential, JC-1 can form J-aggregates that are associated with a large shift in the emission range (in the orange channel, FL2) [19]. JC-1 facililtates both a qualitative (shift from green to orange) and a quantitative ('pure' fluorescence intensity) measure of $\Delta\Psi$. As a methodological control, cells were also treated with increasing concentration (from 0.1 to 10 μg/ml) of the K^+ ionophore valinomycin (Sigma) which dissipates $\Delta\Psi$, but not the pH gradient [20]. After washing samples were immediately analysed by intensified video microscopy (IVM) as previously described [21] or by a flow cytometer. To verify cell viability parallel tubes were incubated with PmI (40 μg/ml for 15 min at 37 °C) before analyses.

Data analysis and statistics

All the samples were analysed with a FACScan flow cytometer (Becton Dickinson) equipped with a 488 argon laser. At least 20000 events were acquired. Data were recorded and statistically analysed on a Macintosh computer using CellQuest Software. All data reported are the means ± S.D. for at least four separate experiments. Statistical analysis of apoptosis and $\Delta\Psi$ data was performed by comparing two groups of samples (represented by wtCEM versus VBL100 exposed to the same treatment) by Student's t test. With regard to the flow-cytometry experiments, calculation of fluorescence (expressed as median value) was carried out after conversion of logarithmically amplified signals into values on a linear scale, and the statistical significance was calculated by using the parametric Kolmogorov–Smirnov test. Only P values of less than 0.01 were considered as significant.

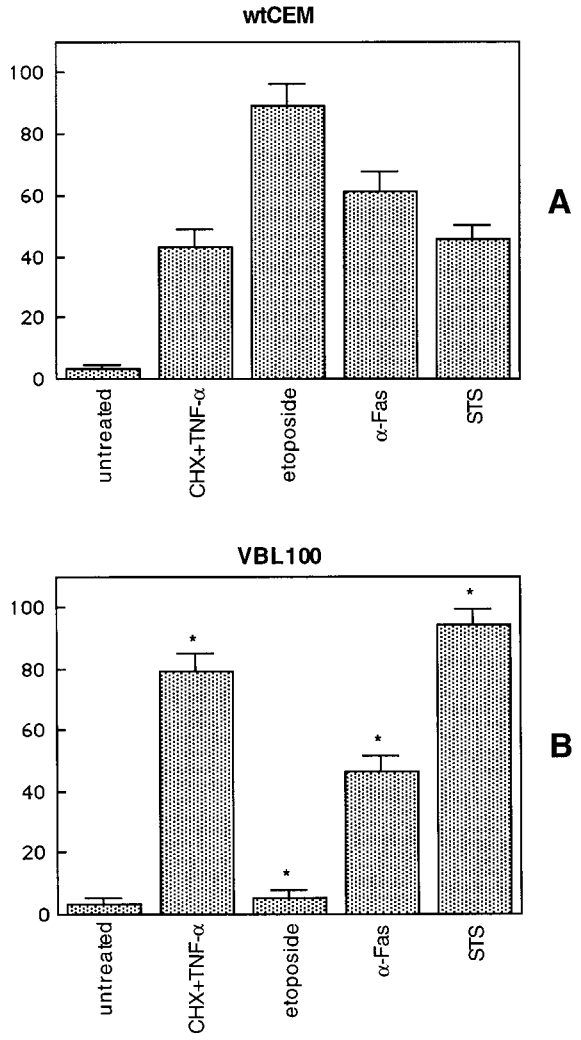

Figure 1 Quantification of apoptosis in wt (A) and VBL100 (B) CEM cells

Values reported were obtained by flow-cytometric analysis by using incorporation of TdT-labelled oligonucleotides into the DNA. Results are means ± S.D. for five different experiments. wt and VBL100 CEM cells exposed to the same treatment were compared. $^*P < 0.01$.

RESULTS

P-170 influences apoptotic susceptibility

In a previous paper [14] we reported that P-170 expression in the human T-lymphoblastoid cell line CEM was associated with an alteration of the apoptotic program. In fact, P-170-expressing CEM cells (VBL100) were more sensitive to TNF-α-induced apoptosis than the parental drug-sensitive cell line (wtCEM) [14,22]. To clarify the role exerted by MDR phenotype in this phenomenon, we report here experiments carried out with some physiological (i.e. TNF-α, anti-Fas) or 'unphysiological' (i.e. etoposide, STS) apoptotic stimuli which involve different pathways: (i) CHX/TNF-α, a protein-synthesis-independent pro-apoptotic stimulus that involves mitochondrial reactive oxygen species [23,24]; (ii) etoposide, a topoisomerase II inhibitor [25]; (iii) Fas-triggering, the major physiological receptor-mediated stimuli for T-lymphocytes [26]; and (iv) STS, a mitochondrial perturbating agent able to induce apoptosis [27]. Results obtained are reported in Figure 1 and can be summarized as follows: (a) a lower apoptosis induction was observed in wtCEM (Figure 1A) cells after exposure to CHX/TNF-α and STS with respect to VBL100 cells (Figure 1B; $\Delta = 49$ and 50 % respectively); (b) by contrast, after Fas-triggering and etoposide administration a higher percentage of apoptosis was detected in wtCEM than in VBL100 P-170 expressing cells ($\Delta = 25$ and 94 %, respectively); (c) there were no significant differences in the percentage of baseline apoptosis between wtCEM and VBL100 cells. No cytotoxic effects or apoptosis induction were revealed in cells treated with CHX alone (results not shown). Results obtained by using TRAIL as apoptotic stimulus (results not shown) completely overlapped those found with anti-Fas antibodies. It was noteworthy that, as expected, VBL100 cells were almost completely resistant to etoposide-induced apoptosis. Statistical analyses of apoptotic cell death was thus performed for five different experiments. Significant differences were found (asterisks above the histogram bars, $P < 0.01$) between VBL100 and wtCEM cells exposed to various apoptotic inducers considered here. These data clearly show that VBL100 cells were more sensitive to those apoptotic stimuli that are known to primarily involve the mitochondrial pathway, such as TNF-α and STS.

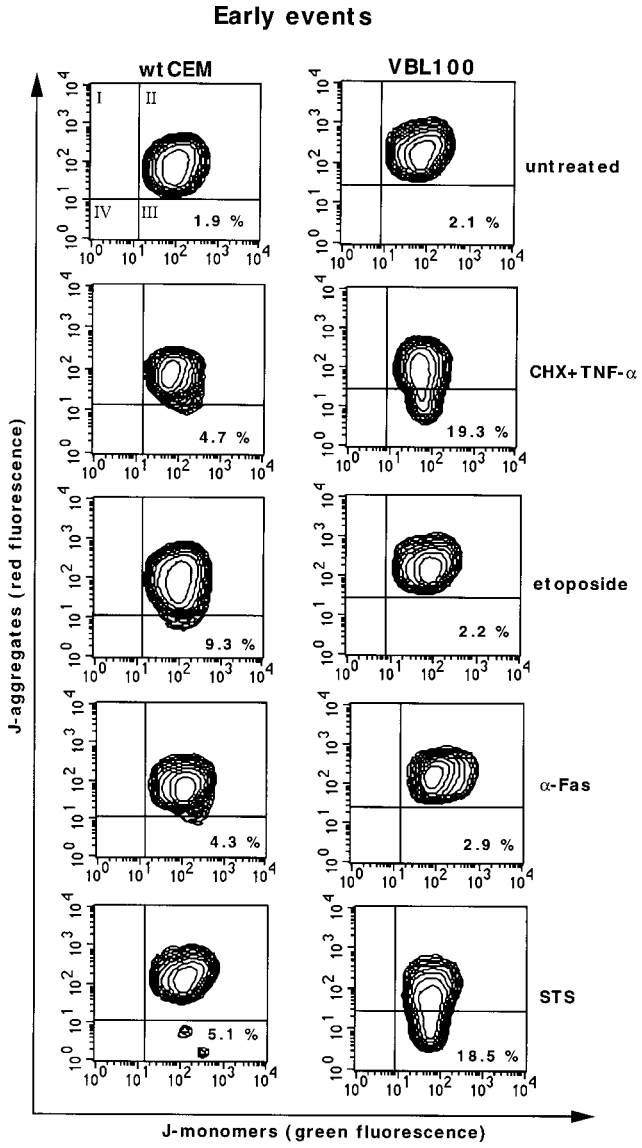

Figure 2 Cytofluorimetric analysis of $\Delta\Psi$ in the early events of apoptosis

wtCEM (left panel) and VBL100 (right panel) cells were stained with JC-1 to analyse $\Delta\Psi$ in short-term experiments. In the quadrant III, which is indicated in the upper-left panel, cells with depolarized mitochondria are visible. Results from a representative experiment (of five) are shown. It is noteworthy that the mitochondrial depolarization occurs as an early event only in VBL100 cells exposed to TNF-α and STS. Percentages in the quadrants refer to the proportion of cells with depolarized mitochondria.

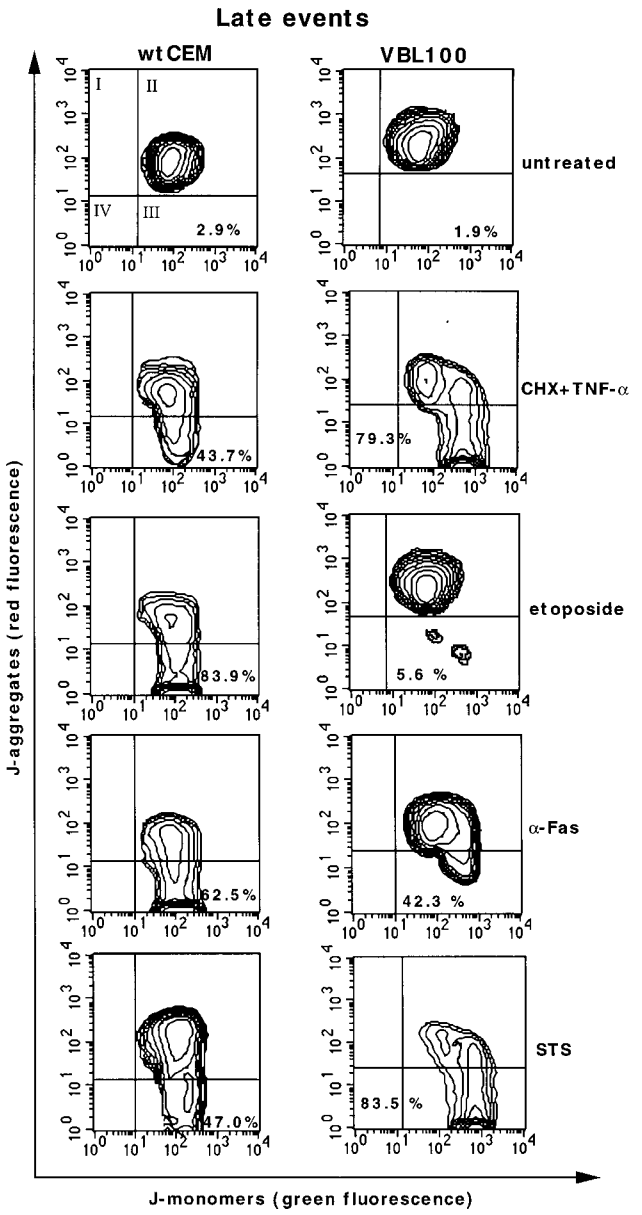

Figure 3 Cytofluorimetric analysis of $\Delta\Psi$ in the late events of apoptosis

wtCEM (left panel) and VBL100 (right panel) cells were stained with JC-1 to analyse $\Delta\Psi$ in long-term experiments. In the quadrant III, which is indicated in the upper-left panel, cells with depolarized mitochondria are visible. Results of a representative experiment (of five) are shown. It is noteworthy: (i) that the mitochondrial depolarization generally occurs in late apoptosis of wtCEM cells, (ii) that VBL100 cells exert a different behaviour depending on the proapoptotic stimulus, (iii) that the mitochondrial membrane potential is almost completely altered after TNF-α and STS exposure, and, finally, (iv) that density plots of untreated cells (first row) appear characterized by different morphological features. Percentages in the quadrants refer to the proportion of cells with depolarized mitochondria.

Alterations of $\Delta\Psi$ after apoptosis induction

In light of above results, and in view of the importance of mitochondrial integrity in the apoptotic cell death, we analysed $\Delta\Psi$ values at different times after exposure to the apoptotic stimulus. As a positive control for this series of experiments we used the same cell lines (wtCEM and VBL100) treated with different concentrations (0.1, 1 and 10 μ/ml) of the K^+ ionophore valinomycin as specified in Materials and methods section. According to literature data [19] we observed a dose-dependent decrease in FL2 signals (results not shown) after incubation with valinomycin. Observations on $\Delta\Psi$ performed after short exposure times (Figure 2) revealed in wtCEM cells (left panel) no significant alteration of $\Delta\Psi$ after all the apoptotic stimuli considered. By contrast, in VBL100 cells a significant percentage of cells with depolarized mitochondria was found after TNF-α (19.3%) and STS (18.5%) treatments. As expected, according to the data obtained on apoptosis, no significant alteration of $\Delta\Psi$ after etoposide and Fas-triggering was revealed in these cells (right panel). After prolonged exposure times, corresponding to those considered for apoptosis quantification reported in Figure 1 (as specified in the Materials and methods section), a higher per-

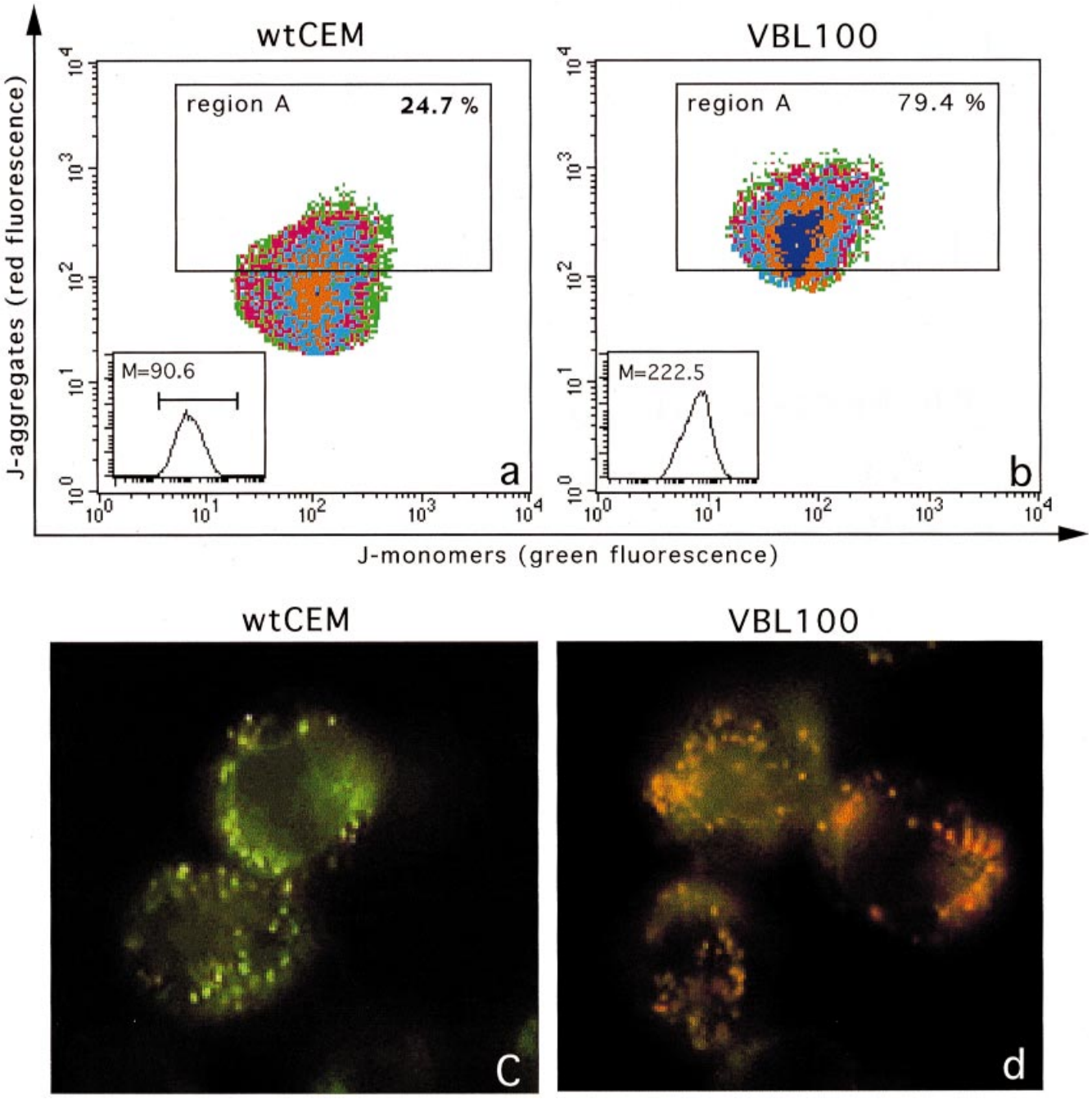

Figure 4 Quantitative and qualitative analyses of $\Delta\Psi$ in untreated cells

Density plot of wtCEM (**a**) and VBL100 (**b**) cells stained with JC-1 and analysed by flow cytometry. A representative experiment of eight is shown. Insets to the Figures represent J-aggregates emission (expressed as median value of fluorescence) which proportionally increase with elevation of mitochondrial membrane polarization. In 'region A' the percentage of cells with hyperpolarized mitochondria is reported. Statistical analysis performed on results from eight independent experiments revealed significant differences ($P < 0.01$) between wtCEM and VBL100 cells. Qualitative analysis of the mitochondrial membrane state of wtCEM and VBL100 cells performed by IVM after staining with JC-1 are shown in (**c**) and (**d**). Noteworthy is the prevalence of red fluorescence emission (corresponding to J-aggregates, which increase when mitochondrial membrane becomes more polarized) in VBL100 cells (**c**) with respect to wtCEM (**b**) cells.

centage of cells with depolarized mitochondria was detected (Figure 3). In fact, we observed that, after both CHX-TNF-α and STS treatments, VBL100 cells (right panel) underwent a dramatic decrease in mitochondrial membrane polarization. This is represented by the alteration of the contour plots as shown in the specific parts of the Figure. With the above method, the cells with depolarized mitochondria are those moving from quadrant II to quadrant III as they lose greenish-orange fluorescence (in FL2). This effect was significantly ($P < 0.01$) reduced in wtCEM cells (left panel) which did not express P-170 ($\Delta = 45$ and 44 % respectively). By contrast, a higher percentage of cells with depolarized mitochondria was revealed in wtCEM cells after etoposide exposure and Fas-triggering. It is noteworthy that although no significant change in the percentage of cells with

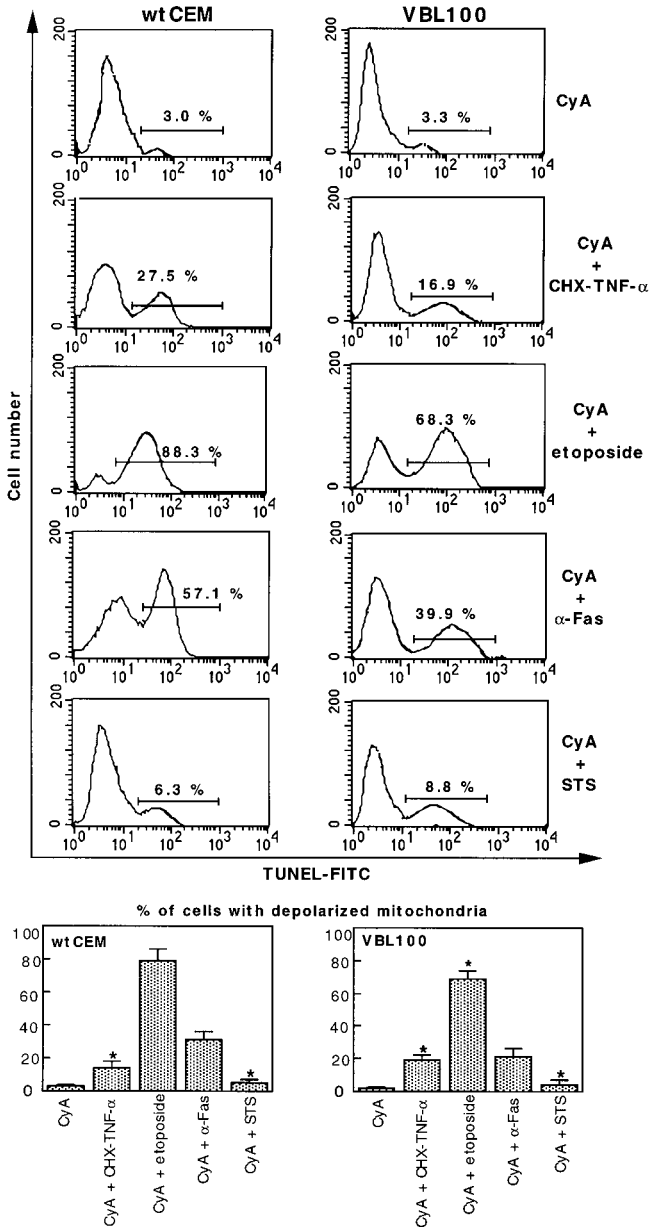

Figure 5 Quantitative evaluation of early apoptotic events in the presence of the mitochondrial membrane permeability transition inhibitor CyA

CyA powerfully protects VBL100 CEM cells from apoptosis induced by both CHX/TNF-α (second row) and STS (fifth row) when compared with values reported in Figure 1. CyA is very effective in 'sensitizing' VBL100 cells to etoposide-induced apoptosis (third row). Treatment with CyA alone did not induce any appreciable effect in both wt and VBL100 CEM cells (first line). Bottom row, cytofluorimetric evaluation of $\Delta\Psi$. A significantly lower percentage of cells with depolarized mitochondria ($P < 0.01$ compared with the data reported in Figure 3) were found when apoptotic treatments with CHX/TNF-α or STS were performed in the presence of CyA rather than in its absence (see Figure 3).

depolarized mitochondria was revealed between wtCEM and VBL100 untreated cells (2.9 and 1.9 % respectively), a marked difference in $\Delta\Psi$ was found. Conceivably, this was a hyperpolarization of the mitochondrial membrane in VBL100 cell line (see the first row in Figure 3). In fact, an increase of fluorescence emission in the FL2 channel, corresponding to J-aggregates, which increase when mitochondrial membrane becomes more polarized [24], was revealed. In light of these observations, specific quantitative and qualitative analyses of this particular phenomenon were performed (Figure 4). Whereas only 24.7 % of wtCEM untreated cells (Figure 4a) were detectable in the region A of the plot (high red fluorescence), in VBL100 cells (Figure 4b) this percentage increased up to 79.4 %. This highly significant difference ($P < 0.01$) was confirmed by IVM analyses, which revealed a predominance of green fluorescence emission in wtCEM cells (Figure 4c) when compared with VBL100 cells (Figure 4d). In fact, in these cells the emission in the orange channel prevailed. Considering that mitochondrial hyperpolarization phenomenon has been described as an event occurring during the very early apoptotic phases [28–30], we can hypothesize a direct correlation between the increased susceptibility of VBL100 cells to undergo apoptosis after mitochondrial-dependent apoptotic stimuli and the 'physiological' state of their mitochondria.

Role of the permeability transition pore

Previous studies indicated a mechanistic linkage between sustained mitochondrial membrane depolarization and formation of the permeability transition pore [31]. 'Megapore' formation is a very important event for those stimuli, such as for instance STS and TNF-α, whose apoptosis mainly involves mitochondrial pathway [23,24]. To verify the importance of these mitochondrial events in our experimental system we used two different agents, CyA and BA, able to modulate mitochondrial homoeostasis and, consequently, apoptotic phenomenon. The two compounds are in fact capable of inhibiting or reversing pore opening, consequently preventing mitochondrial-membrane-permeability transition, by using different mechanisms. In particular, the binding of CyA to cyclophilin D prevents its interaction with the translocator, whereas BA is a direct antagonizing ligand of the adenine-nucleotide translocator [32]. Our results clearly indicated that both CyA (Figure 5) and BA (Figure 6), were capable of significantly affecting apoptotic process. More precisely, results shown in Figures 5 and 6 should be compared with those shown in Figure 1. They clearly indicate that CyA exerted an anti-apoptotic activity in both cell lines only in the case where they were treated with CHX/TNF-α or STS. However, although a lower percentage of apoptotic cells was shown by wtCEM cells exposed to TNF-α or STS when compared with the values reported in Figure 1, in the VBL100 cells this difference was higher, and a highly significant apoptotic hindering was detected ($\Delta = 79.2$ % for TNF-α and 90.2 % for STS; $P < 0.01$ with respect to results obtained in absence of CyA and reported in Figure 1). Importantly, CyA was also very effective in 'sensitizing' VBL100 cells to etoposide-induced apoptosis. The percentage of apoptosis varied in fact from 6.1 % (without CyA, see Figure 1) to 68.3 % (with CyA, Figure 5). This activity could be related to the previously reported effects specifically exerted by CyA on P-170 pump function (see the Discussion in [33]). For this reason we decided to evaluate further the mitochondrial 'modulation' of apoptosis by using BA as a pore-opening inhibitor. The results obtained partially overlap those obtained with CyA, that is, only 'type II stimuli' were counteracted. An impressive and highly significant apoptotic hindering was in fact detected in VBL100 cells exposed to TNF-α and STS ($\Delta =$ 84.4 % for TNF-α and 95.4 % for STS; $P < 0.01$ with respect to results obtained in absence of BA and reported in Figure 1). Conversely, BA was substantially ineffective towards Fas- and etoposide-induced cell death. As expected, treatments with CyA or BA alone did not induce any appreciable apoptotic cell death for both wt and VBL100 CEM cells (first rows of the Figures 5

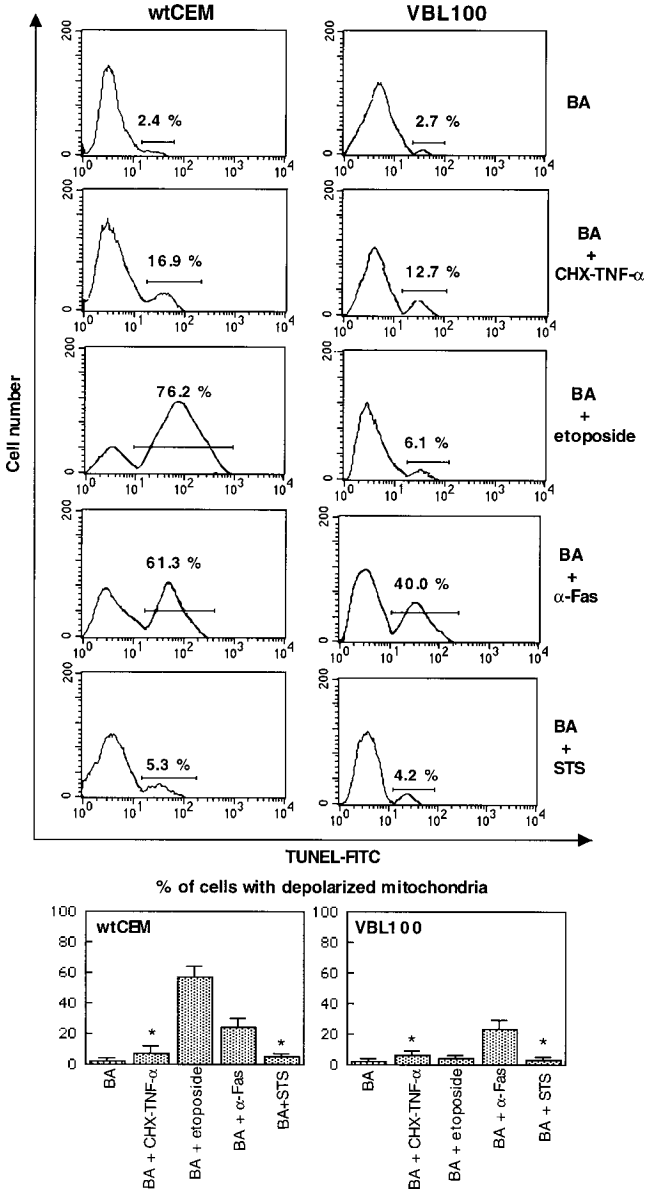

Figure 6 Quantitative evaluation of early apoptotic events in the presence of mitochondrial membrane permeability transition inhibitor BA

BA strongly decreases VBL100 CEM cell apoptosis (compare with Figure 1) induced by both CHX/TNF-α (second row) and STS (fifth row). Note that BA is not as effective as CyA in 'sensitizing' VBL100 cells to etoposide-induced apoptosis (third row). Treatment with BA alone did not induce any appreciable effect in both wt and VBL100 CEM cells (first row). Bottom row, cytofluorimetric evaluation of $\Delta\Psi$. A significantly lower percentage of cells with depolarized mitochondria ($P < 0.01$ compared with the data reported in Figure 3) was found when apoptotic treatments with CHX/TNF-α or STS were performed in the presence of BA rather than in its absence (see Figure 3).

and 6). Finally, we also monitored the mitochondrial membrane polarization, i.e. the percentage of cells with depolarized mitochondria (last rows of Figures 5 and 6). Our results indicated a significantly ($P < 0.01$) lower percentage of cells with depolarized mitochondria after TNF-α and STS in the presence of both CyA or BA than in the absence of these megapore transition inhibitors (compare values in Figures 5 and 6 with values in the plots of Figure 3; asterisks indicate a $P < 0.01$ with respect to those values).

DISCUSSION

The expression of P-170 has been widely described by a series of works indicating its importance in both infectious as well as non-infectious diseases [34,35]. In fact the presence and the function of P-170 cationic pump was reported for a plethora of studies regarding chemotherapic intervention either in tumour growth control or in bacterial and viral pharmacological management [34,35]. The importance of this 'non-specific' pump was thus thoroughly investigated by using both *in vivo* and *in vitro* approaches. In the present work, confirming the widespread importance of P-170 pump, we analysed some possible mechanisms underlying the effects of various stimuli on cell susceptibility to apoptosis by using a paradigmatic and well known cell model represented by a human lymphoblastoid cell line, the CEM cell line, and its P-170-expressing counterpart, the VBL100 cell line. We found: (i) that P-170 function is associated with a sort of 'sensitization' to mitochondrially bound apoptotic stimuli and (ii) that the mechanism for this increased susceptibility might be consistent with a specific $\Delta\Psi$ modification, i.e. hyperpolarization. Thus, according with recent literature data on ABC proteins [36], we hypothesize a specific activity exerted by P-170 glycoprotein on mitochondrial membrane homoeostasis.

It is known that 'normal' non-tumour cells depend on mitochondria for ATP synthesis, while cancer cells, even in the presence of oxygen, exert a high rate of anaerobic glycolysis [37,38]. This is an important survival factor, allowing cancer cells, chiefly in solid tumours, to proliferate under low-oxygen conditions. However, there are very few data on leukaemic cells regarding this matter in the literature [39]. These cells are in fact in oxygen-rich conditions, so that they do not seem to need anaerobic glycolysis. However, a few, but nevertheless, significant indications seem to emerge from literature data suggesting a key role for mitochondrial activity in leukaemic cells [40]. For instance, mitochondrial DNA amplification has been found in cells from acute leukaemia [41]. More interestingly, with respect to P-170-expressing cells, an enhanced activity of mitochondrial electron-transport chain has been described in multidrug-resistant VBL100 CEM cells with respect to their wt counterpart [42,43]. This could imply that increased ATP demand of leukaemic P-170-positive cells is mainly supplied by mitochondrial activity (and more than by anaerobic glycolysis). Accordingly, it was recently shown by Jia and co-workers that CEM/VBL100 cells show more active mitochondrial respiration and display a higher mitochondrial DNA content with respect to the parental cell line [39]. Finally, it was found that a decrease in mitochondrial electron-transport chain activity renders leukaemic cells resistant to apoptotic induction by TNF-α [44]. This means that a positive correlation might exist between 'high' mitochondrial activity and cell susceptibility to TNF-α or other pro-apoptotic stimuli (i.e. STS) directly involving mitochondria. Altogether these findings suggest a 'sensitization' of VBL100 cells to mitochondrially mediated apoptotic stimuli.

Results obtained by using two different drugs capable of inhibiting mitochondrial membrane transition, i.e. CyA and BA, before apoptotic stimulus seem to confirm our hypothesis. In fact, both CyA and BA were capable of significantly inhibiting apoptosis induced by TNF-α and STS, but not by anti-Fas or etoposide in wtCEM cells and, overall, in VBL100 cells. Notably, both CyA and BA were unable to prevent mitochondrial membrane depolarization (as well as apoptosis) in etoposide treated cells. According to literature [45] it is conceivable that this could be a non-specific toxic activity of etoposide on mitochondrial homoeostasis as a possible final result of apoptotic cell death process more than a target effect of the drug on

mitochondrial pore-opening function. On the other hand, results obtained with CyA and etoposide in VBL100 cells suggest a different role for CyA in these cells. A remarkable increase of susceptibility to etoposide-induced cell death was in fact found when P-170-expressing cells were pretreated with CyA. This could be of relevance in the understanding of the specific activity exerted by CyA on P-170 pump function [33]. We can in fact hypothesize that CyA could reverse P-170 function, thus modifying the etoposide pro-apoptotic cascade.

Our results strongly support such a hypothesis and clearly indicate that VBL100 CEM cells could represent a specific model for the type II apoptotic-cell-death pathway [46]. Moreover, wtCEM cells seem to represent a prototype for type I cells. It was in fact hypothesized that apoptosis could be mediated by two different pathways that, although strictly intertwined, may represent different ways to reach the same result, i.e. the death of a cell [46]. The first pathway is consistently used by those cells that follow the CD95/Fas-mediated apoptotic pathway initiated by active caspase 8 and finally leading to execution phase cascade, such as, for instance, the cleavage of caspase 3 and poly-ADP-ribose polymerase. This pathway involves mitochondria only in the late phases of apoptotic cascade. By contrast, type II cells, although sharing the final steps of the cell-death pathway, appear to behave in a different manner, so that small amounts of active caspase 8 stimulate the apoptogenic activity of mitochondria, causing, in turn, the final caspase cascade which involves apoptosome structure [43]. In fact, type II cells, depending on the mitochondrial branch of the apoptotic pathway, can be physiologically blocked by overexpression of the apoptosis regulatory molecules bcl2 and bcl_{XL} and experimentally by BA and CyA administration [32]. In the light of these hypotheses, our results seem to indicate that the CEM/VBL cell model is particularly representative of the above-described apoptotic cell types I and II. A sort of shift from the first to the second pathway appears in fact to occur depending on the expression and function of P-170 glycoprotein.

This hypothesis seems to be supported by the following favourable experimental points: (i) the higher susceptibility of VBL100 cells to those apoptotic stimuli that follow the mitochondrial pathway, e.g. STS, while wtCEM cells appear to be more sensitive to the plasma-membrane-associated cascade, e.g. by Fas; (ii) the fact that CyA and BA, mitochondrial pore-opening inhibitors [32], significantly block apoptosis induced by TNF-α and STS; (iii) the 'level' of mitochondrial membrane potential in 'steady state' resting conditions, i.e. the hyperpolarization observed in VBL100 cells. This is supported by some recent literature data [46] and can partially explain high VBL 100 cell susceptibility to mitochondria-mediated triggering; iv) the fact that different levels of bcl2, bad and bcl_{xs} have been found in mitochondria of CEM VBL 100 cells with respect to wt cells [47]. Thus a specific role for P-170 pump activity in the cell model system described here should be taken into consideration. It could be hypothesized that the high metabolic challenge in P-170-positive cells might lead to the loss of functional integrity of mitochondrial membranes, possibly resulting in an increased sensitivity to those stimuli which involve cytochrome *c*-mediated apoptotic signalling [48].

In summary, these are the first data that, although obtained in a well established but oversimplified cell system such as CEM/VBL100 cells, clearly indicate an additional role for P-170 glycoprotein. The presence of this glycoprotein, although representing a 'resistance factor' towards certain apoptotic stimuli, can as well represent a 'risk factor' towards mitochondrially bound apoptotic stimuli. It could imply that the presence of P-170 might shift type-I cells, such as wtCEM cells, in such a way as to make them behave like type-II cells. This could also be of some relevance in the understanding of the phenomena of drug resistance and in the management of different human diseases.

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REFERENCES

1 Evan, G. and Littlewood, T. (1998) A matter of life and cell death. Science **281**, 1317–1321
2 Isaacs, J. T. (1994) Advances and controversies in the study of programmed cell death/apoptosis in the development of and therapy for cancer. Curr. Opin. Oncol. **6**, 82–89
3 Phenix, B. N., Angel, J. B., Mandy, F., Kravcik, S., Parato, K., Chambers, K. A., Gallicano, K., Hawley-Foss, N., Cassol, S., Cameron, D. W. and Badley, A. D. (2000) Decreased HIV-associated T cell apoptosis by HIV protease inhibitors. AIDS Res. Hum. Retroviruses **16**, 559–567
4 Compagni, A. and Christofori, G. (2000) Recent advances in research on multistage tumorigenesis. Br. J. Cancer **83**, 1–5
5 Gougeon, M. L. and Montagnier, L. (1999) Programmed cell death as a mechanism of CD4 and CD8 T cell deletion in AIDS. Molecular control and effect of highly active anti-retroviral therapy. Ann. N. Y. Acad. Sci. **887**, 199–212
6 Hengartner, M. O. (2000) The biochemistry of apoptosis. Nature (London) **407**, 770–776
7 Schmitz, I., Walczak, H., Krammer, P. H. and Peter, M. E. (1999) Differences between CD95 type I and type II cells detected with the CD95 ligand. Cell Death and Differ. **6**, 821–822
8 Higgins, C. F. (1993) The multidrug-resistance P-glycoprotein. Curr. Opin. Cell. Biol. **5**, 684–687
9 Mansouri, A., Henle, K. J., Nagle, W. A. and Moss, A. J. (1990) Tumor cell drug resistance and its reversal. SAAS Bull. Biochem. Biotechnol. **3**, 91–96
10 Huisman, M. T., Smit, J. W. and Schinkel, A. H. (2000) Significance of P-glycoprotein for the pharmacology and clinical use of HIV protease inhibitors. AIDS **14**, 237–242
11 Leighton, J. and Schatz, G. (1995) An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast. EMBO J. **14**, 188–195
12 Lotem, J. and Sachs, L. (1993) Regulation by bcl-2, c-myc and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and defective myeloid leukemic cells. Cell. Growth Differ. **4**, 41–47
13 St. Croix, B. and Kerbel, R. S. (1997) Cell adhesion and drug resistance in cancer. Curr. Opin. Oncol. **9**, 549–556
14 Malorni, W., Rainaldi, G., Tritarelli, E., Rivabene, R., Cianfriglia, M., Lehnert, M., Donelli, G., Peschle, C. and Testa, U. (1996) Tumor necrosis factor α is a powerful apoptotic inducer in lymphoid leukemic cells expressing the P-170 glycoprotein. Int. J. Cancer. **67**, 238–247
15 Cianfriglia, M., Willingham, M. C., Tombesi, M., Scagliotti, G. V., Frasca, G. and Ghersi, A. (1994) P-glycoprotein-epitope mapping. I. Identification of linear human-specific epitope in the fourth loop of the P-glycoprotein extracellular domain by MM.4.17 murine monoclonal antibody to human multi-drug-resistant cells. Int. J. Cancer **56**, 153–160
16 Hamada, H. and Tsuruo, T. (1986) Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. Proc. Natl. Acad. Sci. U.S.A. **83**, 7785–7789
17 Kartner, N. and Ling, V. (1989) Multidrug resistance in cancer. Sci. Am. **160**, 26–33
18 Malorni, W., Rivabene, R., Santini, M. T. and Donelli, G. (1993) *N*-Acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells. FEBS Lett. **327**, 75–78
19 Cossarizza, A., Franceschi, C., Monti, D., Salvioli, S., Bellesia, E., Rivabene, R., Biondo, L., Rainaldi, G., Tinari, A. and Malorni, W. (1995) Protective effect of *N*-acetylcysteine in tumor necrosis factor alpha-induced apoptosis in U937 cells: the role of mitochondria. Exp. Cell. Res. **220**, 232–240
20 Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G. and Franceschi, C. (1993) A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem. Biophys. Res. Commun. **197**, 40–45
21 Parlato, S., Giammarioli, A. M., Logozzi, M., Lozupone, F., Matarrese, P., Luciani, F., Falchi, M., Malorni, W. and Fais, S. (2000) CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway. EMBO J. **19**, 5123–5134

22 Penning, L. C., Schipper, R. G., Vercammen, D., Verhofstad, A. A., Denecker, T., Beyaert, R. and Vandenabeele, P. (1998) Sensitization of TNF-induced apoptosis with polyamine synthesis inhibitors in different human and murine tumor cell lines. Cytokine **10**, 423–431

23 Larrick, J. W. and Wright, S. C. (1990) Cytotoxic mechanism of tumor necrosis factor-alpha. FASEB J. **4**, 3215–3223

24 Mehlen, P., Kretz-Remy, C., Briolay, J., Fostan, P., Mirault, M. E. and Arrigo, A. P. (1995) Intracellular reactive oxygen species as apparent modulators of heat-shock protein 27 (HSP27) structural organization and phosphorylation in basal and tumor necrosis factor-α-treated T47D human carcinoma cells. Biochem. J. **312**, 367–375

25 Kaufmann, S. H. (1998) Cell death induced by topoisomerase-targeted drugs: more questions than answers. Biochim. Biophys. Acta **1400**, 195–211

26 Walczak, H., Bouchon, A., Stahl, H. and Krammer, P. H. (2000) Tumor necrosis factor-related apoptosis-inducing capacity on Bcl-2 or Bcl-xL-overexpressing chemotherapy-resistant tumor cells. Cancer Res. **60**, 3051–3057

27 Nicotera, P., Leist, M. and Ferrando-May, E. (1999) Apoptosis and necrosis: different execution of the same death. Biochem. Soc. Symp. **66**, 69–73

28 Banki, K., Hutter, E., Gonchoroff, N. J. and Perl, A. (1999) Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspase in Fas signalling. J. Immunol. **162**, 1466–1479

29 Li, P., Dietz, R. and Von Harsdorf, R. (1999) p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome *c*-independent apoptosis blocked by Bcl-2. EMBO J. **18**, 6027–6036

30 Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R. Y. and Reed, J. C. (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat. Cell Biol. **2**, 318–325

31 Kroemer, G., Zamzami, N. and Susin, S. A. (1997) Mitochondrial control of apoptosis. Immunol. Today. **18**, 44–51

32 Desagher, S. and Martinou, J. C. (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol. **10**, 369–377

33 Chan, H. S., Lu, Y., Grogan, T. M., Haddad, G., Hipfner, D. R., Cole, S. P., Deeley, R. G., Ling, V. and Gallie, B. L. (1997) Multidrug resistance protein (MRP) expression in retinoblastoma correlates with the rare failure of chemotherapy despite cyclosporine for reversal of P-glycoprotein. Cancer Res. **57**, 2325–2330

34 Lucia, M., Cauda, R., Landay, A. L., Malorni, W., Donelli, G. and Ortona, L. (1995) Transmembrane P-glycoprotein (Pgp/P-170) in HIV infection: analysis of lymphocyte surface expression and drug-unrelated function. AIDS Res. Hum. Retroviruses **11**, 893–901

35 Fojo, A. T., Ueda, K., Slamon, D. J., Gottesman, M. M. and Pastan, I. (1987) Expression of a multidrug-resistance gene in human tumors. Proc. Natl. Acad. Sci. U.S.A. **84**, 265–269

36 Mithsuashi, N., Miki, T., Senbongi, H., Yokoi, N., Yano, H., Miyazaki, M., Nakajima, N., Iwanaga, T. and Yokoyama, Y. (2000) MTABC3, a novel mitochondrial ATP-binding cassette protein involved in iron homeostasis. J. Biol. Chem. **9**, 17536–17540

37 Burstein, D. E., Reder, I., Weiser, K., Tong, T., Prisker, A. and Haber, R. S. (1998) GLUT1 glucose transporter: a highly sensitive marker of malignancy in body cavity effusions. Modern Pathol. **11**, 392–396

38 Cavalli, L. R. and Liang, B. C. (1998) Mutagenesis, tumorigenicity, and apoptosis: are the mitochondria involved? Mutat. Res. **398**, 19–26

39 Jia, L., Liu, K. Z., Newland, A. C., Mantsch, H. H. and Kelsey, S. M. (1999) Pgp-positive leukaemic cells have increased mtDNA but no increased rate of proliferation. Br. J. Haematol. **107**, 861–869

40 Jia, L., Allen, P. D., Macey, M. G., Grahn, M. F., Newland, A. C. and Kelsey, S. M. (1997) Mitochondrial electron transport chain activity. but not ATP synthesis, is required for drug-induced apoptosis in human leukaemic cells: a possible novel mechanism of regulating drug resistance. Br. J. Haematol. **98**, 680–698

41 Boultwood, J., Fidler, C., Mills, K. I., Frodsham, P. M., Kusec, R., Gaiger, A., Gale, R. E., Linch, D. C., Littlewood, T. J., Moss, P. A. H. and Wainscoat, J. S. (1996) Amplification of mitochondrial DNA in acute leukaemia. Br. J. Haematol. **95**, 426–431

42 Jia, L., Kelsey, S. M., Grahn, M. F. and Newland, A. C. (1996) Increased activity and sensitivity of mitochondrial respiratory enzymes to tumor necrosis factor α-mediated inhibition is associated with increased cytotoxicity in drug-resistant leukemic cell lines. Blood **87**, 2401–2410

43 Lyon, R. C., Cohen, J. S., Faustino, P. J., Megnin, F. and Charles, E. M. (1988) Glucose metabolism in drug-sensitive and drug-resistant human breast cancer cells monitored by magnetic resonance spectroscopy. Cancer Res. **48**, 870–877

44 Jia, L., Dourmashkin, R. R., Allen, P. D., Gray, A. B., Newland, A. C. and Kelsey, S. M. (1997) Inhibition of autophagy abrogates tumor necrosis factor α induced apoptosis in human T-lymphoblastic leukaemic cells. Br. J. Haematol. **98**, 673–685

45 Robertson, J. D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2000) Distinct pathways for stimulation of cytochrome *c* release by etoposide. J. Biol. Chem. **275**, 32438–32443

46 Walczak, H. and Krammer, P. H. (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp. Cell Res. **256**, 58–66

47 Jia, L., Macey, M. G., Yin, Y., Newland, A. C. and Kelsey, S. M. (1999) Subcellular distribution and redistribution of Bcl-2 family proteins in human leukemic cells undergoing apoptosis. Blood **93**, 2353–2359

48 Ushmoroy, A., Ratter, E., Lehmann, V., Droge, W., Schirrmacher, V. and Umansky (1999) Nitric oxide-induced apoptosis in human leukemic lines requires mitochondrial lipid degeneration and cytochrome *c* release. Blood **93**, 2342–2352

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