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Acquisition of MDR Phenotype by Leukemic Cells Is Associated With Increased Caspase-3 Activity and a Collateral Sensitivity to Cold Stress

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ABSTRACT

The acquisition of a multidrug-resistant (MDR) phenotype by tumor cells that renders them unsusceptible to anti-neoplasic agents is one of the main causes of chemotherapy failure in human malignancies. The increased expression of P-glycoprotein (MDR1, P-gp, ABCB1) in tumor cells contributes to drug resistance by extruding chemotherapeutic agents or by regulating programmed cell death. In a study of MDR cell survival under cold stress conditions, it was found that resistant leukemic cells with P-gp over-expression, but not their sensitive counterparts, are hypersensitive to cold-induced cell death when exposed to temperatures below 4°C. The transfection of parental cells with a P-gp-expressing plasmid makes these cells sensitive to cold stress, demonstrating an association between P-gp expression and cell death at low temperatures. Furthermore, we observed increased basal expression and activity of effector caspase-3 at physiological temperature (37°C) in MDR cells compared with their parental cell line. Treatment with a caspase-3 inhibitor partially rescues MDR leukemic cells from cold-induced apoptosis, which suggests that the cell death mechanism may require caspase-3 activity. Taken together, these findings demonstrate that P-gp expression plays a role in MDR cell survival, and is accompanied by a collateral sensitivity to death induced by cold stress. These findings may assist in the design of specific therapeutic strategies to complement current chemotherapy treatment against cancer. J. Cell. Biochem. 113: 1416–1425, 2012.

KEY WORDS: LEUKEMIC CELLS; MDR; P-GLYCOPROTEIN; APOPTOSIS; COLD STRESS, CASPASE-3

The term "multidrug resistance" (MDR) is used to describe the ability of tumor cells to resist treatment involving a broad range of unrelated antineoplasic drugs after exposure to a single cytotoxic agent. This phenomenon includes several survival strategies, like the over-expression of drug extrusion pumps [P-glycoprotein (P-gp), etc.], increased activity of the DNA repair mechanisms and alterations of the pathways that control apoptosis; nevertheless, resistance to death is the ultimate consequence regardless of the mechanism(s) involved [Shabbits et al., 2003]. Frequently, the development of resistance to a particular therapeutic drug is associated with a greater sensitivity to an alternative agent or stimulus. This phenomenon is referred as to "collateral sensitivity" (CS) and is the consequence of cellular alterations produced during the process of MDR phenotype acquisition. Many molecules have been identified as promoters of CS, demonstrating that cell lines resistant to virtually any clinical therapeutic agent can be induced to develop hypersensitivity to a specific drug and/or stimulus [Szakacs et al., 2006; Hall et al., 2009]. Thus, it is of fundamental importance to study the factors that contribute to the survival of drug-resistant cells and the relationships between them for them to be applied to

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the design of appropriate therapies. In this respect, while it is undeniable that P-gp is able to efflux xenobiotics out of cells, its biological function in the regulation of cancer cell survival remains to be firmly established. Several reports suggest that P-gp may regulate apoptosis, chloride channel activity, cholesterol metabolism, differentiation, proliferation, adhesion, and immune cell function [Mahadevan and List, 2004; Sarkady et al., 2009]. Taken all of this into account, it is necessary to search for new stimuli able to induce cell death in resistant tumor cells and to determine the role of P-gp in controlling the cell death pathways involved in multidrug resistance. Here, we report for the first time, that the acquisition of MDR phenotype by leukemic cells is associated with an increase in pro-apoptotic caspase-3 activity, although, surprisingly, it does not induce the death of MDR cells at physiological temperature (37°C). However, exposure to cold stress triggers the cell death program through a mechanism that can be reversed by using a caspase-3 inhibitor, demonstrating the CS of MDR cells to low temperatures. The hypersensitivity of MDR cells to cold stress is negatively associated with their P-gp drug extrusion activity. These results support the hypothesis that P-gp could contribute, in a fundamental way, to MDR cell survival.

MATERIALS AND METHODS

TUMOR CELLS, CELL CULTURE, AND CELLULAR EXTRACTS

The L1210 leukemic cell line from DBA/2 mice was used as a tumor model. A DNM-resistant (L1210R) subline (~160-fold resistant to DNM) was selected from parental murine leukemia L1210 cells, as previously described [Castro-Galache et al., 2003], and maintained in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% FBS (Gibco), 2 mM L-glutamine, 10 U/ml penicillin G, and 10 μg/ml streptomycin sulphate (Gibco). This resistant subline, L1210R, overexpresses P-gp (MDR-1) protein as previously described [Castro-Galache et al., 2003]. The P-gp-transfected cell line CBMC-6 (stands for *Centro de Biología Molecular y Celular*) was obtained as previously described [Castro-Galache et al., 2003] by transfecting L1210 cells with the plasmid pcDNA 3-mpgp containing the mouse *mdr1a* P-gp cDNA under the control of the cytomegalovirus (CMV) promoter.

To prepare cellular extracts, cells were plated at 3×10^5 cells/ml in six-well culture plates and incubated at 4°C or 37°C from 0 to 24 h. Cell protein extracts were obtained by collecting total cells, washing them with phosphate buffer saline (PBS), and resuspending them in Cell Signaling lysis buffer (Cell Signaling Technologies, Beverly, MA) following the manufacturer's instructions.

MEASUREMENT OF APOPTOSIS AND CELL CYCLE

L1210, L1210R, and CBMC-6 cells were cultured in fresh culture medium, plated at 3×10^5/ml/well on 24-well plates and maintained at 4°C for different times (0–24 h). Then, apoptosis was evaluated by using FITC-Annexin V (BD Pharmingen, Cary, NC) and propidium iodide (PI; BD Pharmingen, Franklin Lakes, NJ) according to the manufacturer's instructions. The analysis was performed in a Flow cytometer (Becton Dickinson) argon laser of 15 mW at 488 nm. Ten thousand events were collected and analyzed using CellQuest software (Beckton Dickinson).

To analyze the cell cycle profile, L1210, L1210R, and CBMC-6 cells, maintained at 4°C, were harvested at selected times, washed with cold 10 mM phosphate buffer, pH 7.4, supplemented with 2.7 mM KCl and 137 mM NaCl (PBS), and centrifuged at 500*g* for 8 min. Pelleted cells were resuspended in 75% cold ethanol, fixed for 1 h at −20°C, centrifuged and resuspended in 0.5 ml of PBS supplemented with 0.5% Triton X-100 (Sigma–Aldrich, St Louis, MO) and 0.05% Ribonuclease A (Serva Electrophoresis, Heidelberg, Germany). Cells were incubated for 30 min at room temperature, stained with PI, and the distribution of the cell DNA content was analyzed by flow cytometry. Non-viable cells were excluded from the analysis on the basis of their abnormal size.

The cells were examined and photographed with a Nikon (Tokyo, Japan) eclipse TE 2000-U inverted microscope using a 20× objective.

ANTIBODIES AND WESTERN BLOT EXPERIMENTS

Equal amounts of cell extract proteins (15 μg/lane) were subjected to polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes (Bio-Rad, Hercules, CA). After blocking (2% BSA-TBS-T or 5% non-fat milk–PBS-T), membranes were incubated with the corresponding primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using the ECL detection system (Amersham Biosciences, Buckinghamshire, UK). The antibodies used in our study were the following: anti-caspase 3 pAb [Baumgart et al., 2010], anti-MDR-1 (clone D-11) [Mumenthaler et al., 2009], anti-ERK1/2 pAb [Domina et al., 2000] (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-GAPDH pAb (Sigma–Aldrich) [Hobbs et al., 2011]. Quantification was carried out by Scion Image Software and normalized to the respective loading control.

CASPASE-3 ACTIVITY ASSAY

Caspase-3 activity was measured using the CaspACE assay system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, L1210, L1210R, and CBMC-6 cells were cultured in fresh culture medium, plated at 3×10^5/ml/well on 24-well plates and maintained at 4°C for different periods of time (0–8 h). The cells were then harvested and resuspended in cell lysis buffer at a concentration of 1×10^8 cells/ml. The cells were lysed by freeze-thawing, the lysates were centrifuged, and the supernatant fractions were collected. Assays were performed in 96-well plates by incubating the cell extracts in reaction buffer containing the caspase-3 colorimetric substrate, acetyl-DEVD-pnitroaniline (Ac-DEVD-pNA). Upon cleavage by caspase-3, pNA produces a yellow colored product that was monitored by a Jupiter ELISA reader (Asys Hytech, Eugendorf, Austria) at 405 nm.

CASPASE INHIBITION ASSAY

To test the involvement of caspase-3 in cold-induced apoptosis, cells were pretreated for 1 h at 37°C with 10 μM of the caspase-3 inhibitor, Z-DEVD-fmk (Calbiochem, Darmstadt, Germany) followed by cold exposure treatment for 24 h. To study the involvement of serine-proteases in hypothermia-induced cell death, cells were treated with 25 μM of the general serine-protease inhibitor AEBSF

[4-(2-aminoethyl)-benzenesulfonyl fluoride] for 1 h at 37°C prior to cold exposure. Control cells were incubated with 1% dimethyl sulfoxide (DMSO), the vehicle in which all the inhibitors were dissolved. Cell viability was determined using PI staining and flow cytometry analysis.

RHODAMINE ACCUMULATION ASSAY

L1210, L1210R, and CBMC-6 cells were incubated with 0.15 μM rhodamine 123 (control) and 5 μM verapamil (VRP; as a reference for the chemosensitizing effect) for 30–45 min at 37°C. Thereafter, cells were incubated at 37°C or 4°C for different times, washed once with PBS. The pellet was resuspended in the same PBS buffer at 1×10^6/ml/sample. After incubation, steady-state intracellular rhodamine accumulation was determined by flow cytometry, as previously described [Castro-Galache et al., 2003].

STATISTICAL ANALYSIS

Data are represented as the mean ± SD or SE. Data were analyzed with Student's *t*-test. Statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

RESULTS

COLD STRESS INDUCES CELL DEATH IN L1210R AND CBMC-6 BUT NOT IN L1210 CELLS

As a model of MDR tumor cells, we have used the mouse leukemic cell line L1210, its daunomycin (DNM)-resistant subline L1210R, which is characterized by the expression of the ATP-binding cassette (ABC) transporter P-gp (MDR-1) and the P-gp-transfected cell line CBMC-6, obtained as described in the Tumor Cells, Cell Culture, and Cellular Extracts Section. First, the effect of an environmental stress such as extreme hypothermia was studied in the three cell lines by light microscopy at 4°C. A large number of damaged cells were observed in L1210R and CBMC-6 cells cultured for 24 h under cold stress conditions but not in the L1210 cells, underlining the hypersensitivity to low temperature of both MDR cell lines compared with their parental cell line. Microscopic examination suggested that L1210R cells are more sensitive than CBMC-6 to cold stress (Fig. 1a). To quantify and determine the time course of the cell death process, the three cell lines were maintained for several hours (0–24 h) at 4°C, and the extent of cell death/survival was evaluated by counting the number of viable cells after staining with PI and by flow cytometry analysis. Figure 1b shows that only 10% of the

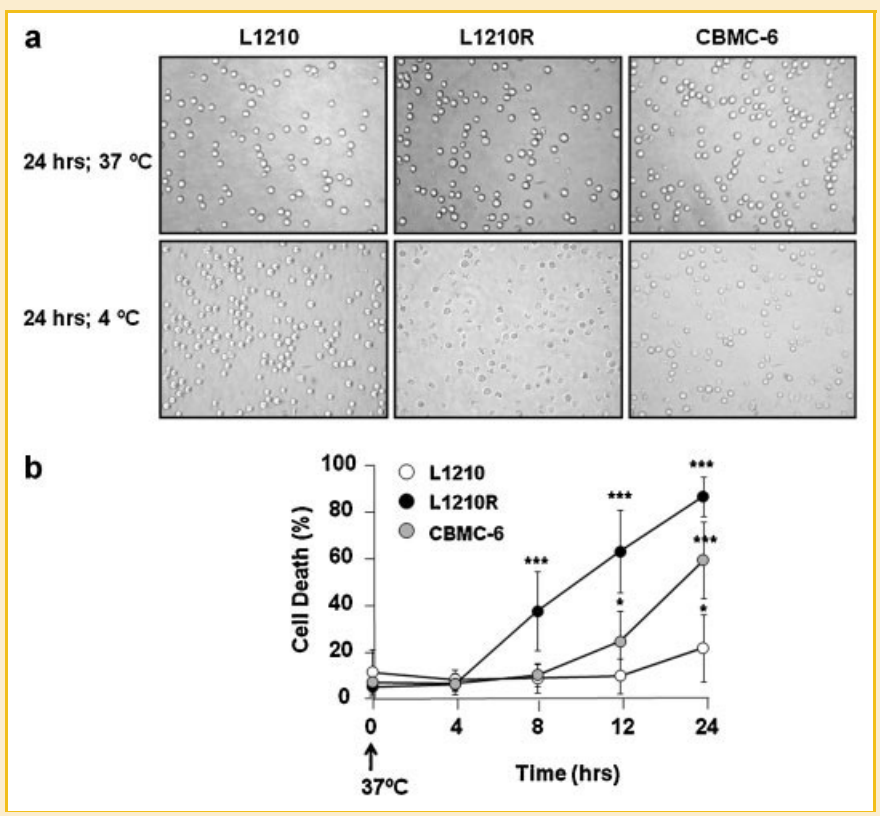

Fig. 1. Cold exposure induces death of L1210R and CBMC-6, but not of L1210 cells. a: Cell lines were incubated at 37°C or 4°C for 24 h and examined and photographed with a Nikon eclipse TE 2000-U inverted microscope using a 20× objective. b: Cell lines were incubated at 37°C or 4°C for up to 24 h; collected at different times and assessed for cell death by propidium iodide staining and flow cytometry analysis. Data are presented as the mean ± SD of five separate experiments. Asterisks represent statistical significance with respect to control cells (cultured at 37°C; $^{*}P < 0.05$, $^{***}P < 0.001$, *t*-test).

parental cells die after 12 h at 4°C, while death of the resistant and P-gp-transfected cell population increased drastically to reach 70% in the case of L1210R cells and 25% in the case of CBMC-6 cells. This trend was even more evident after 24 h of cold-exposure: ≈80–90% cell death in L1210R cells and ≈60–70% in CBMC-6 compared with the 20–25% of dead L1210 cells.

This death-effect was not observed when the cells were incubated at the milder temperatures of 10 and 20°C (data not shown).

These data demonstrate that acquisition of MDR phenotype by leukemic cells increases their susceptibility to cold stress-induced cell death. Furthermore, these results strongly suggest that P-gp over-expression is involved in this process, since significant differences in cell death were observed between P-gp transfectant cells (CBMC-6) and their susceptible counterparts after being exposed to low temperatures (4°C) for a prolonged period of time.

DIFFERENTIAL DISTRIBUTION OF THE DNA CONTENT IN L1210 VERSUS L1210R OR CBMC-6 CELLS UNDER COLD STRESS CONDITIONS

The effect of low temperature on the cell cycle profiles was studied. To this end, we analyzed the cell cycle distribution of L1210, L1210R, and CBMC-6 cells maintained at 4°C for 0–24 h, staining the cells with PI and analysing them by flow cytometry as described in Materials and Methods Section. The results showed that L1210R and CBMC-6 cells maintained at 4°C undergo an initial arrest in the G1 phase (Fig. 2; 12 h at 4°C), suggesting an apoptotic process, decreasing as cell death begins to occur after 24 h (Fig. 2; 24 h at 4°C). Of note is the fact that a DNA content shoulder lower in G1, which is characteristic of relatively late apoptotic stages (sub-G1 peak; Fig. 2; 12 h at 4°C), was already evident in L1210R and CBMC-6 cells after 12 h of cold exposure. As the experiment at 4°C progressed, there was an apoptotic L1210R cell increase from 4.6% in basal conditions (Fig. 2; 37°C) to 17.4% after 24 h of cold exposure, parallel to the decrease and disappearance of cells in the G1 phase at the end point. As regards the CBMC-6 cells, the percentage of cells in the sub-G1 peak increased from 5.2% in basal conditions (Fig. 2; 37°C) to 22.1% after 24 h. On the other hand, L1210 cells tended to accumulate during the G1–S phase (Fig. 2; 12–24 h at 4°C) and did not show a sub-G1 peak after exposure to low temperature for 24 h (5.4% of the cells at sub-G1 peak).

These data indicate that the progression of cells into and through the G1 phase of the cell cycle may precede the apoptosis induced by low temperatures in MDR and P-gp-transfected cells.

COLD STRESS INDUCES PHOSPHATIDYLSERINE EXPOSURE ON L1210R AND CBMC-6 CELLS

To confirm that extreme hypothermia induces apoptosis in MDR cells, we analyzed the exposure of phosphatidylserine on the cell surface, an early event in the apoptotic process. To this end, the cells were maintained at 4°C for 24 h and then labeled with annexin V-FITC-conjugated and PI. As shown in Figure 3, cold exposure significantly induced higher levels of apoptosis (as defined by the

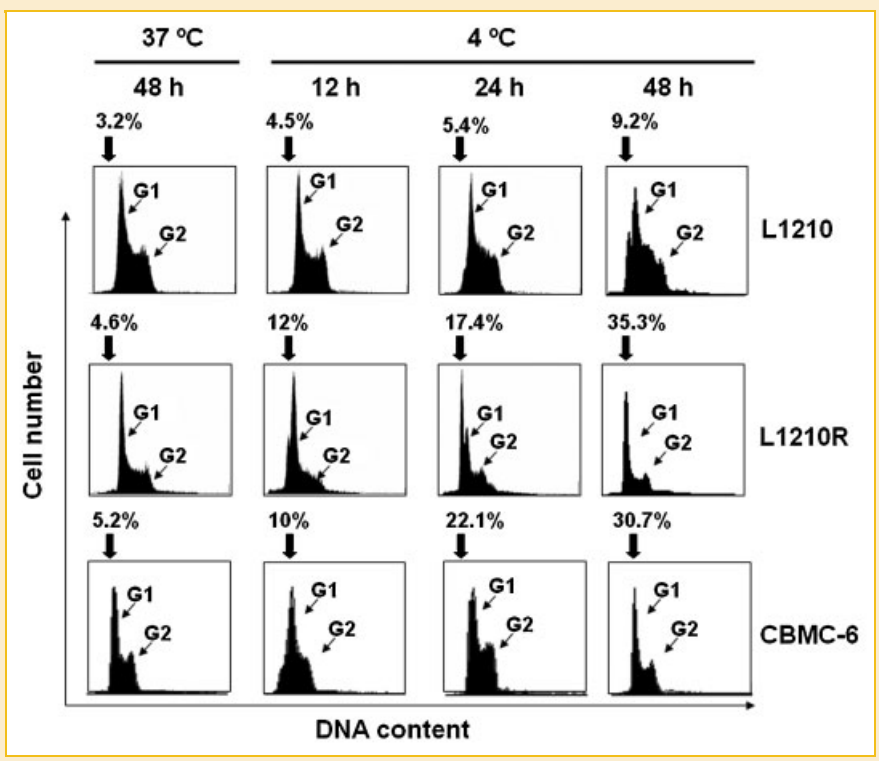

Fig. 2. Flow cytometry analysis of the distribution of cellular DNA content in L1210, L1210R, and CBMC-6 cells maintained for 12, 24 and 48 h at 4°C. The first column corresponds to non-treated cells (controls at 37°C). Percentages of apoptotic cells corresponding to the sub-G1 peak are presented, and the peaks are indicated by a bold arrow. This figure is representative of four different experiments that gave similar results.

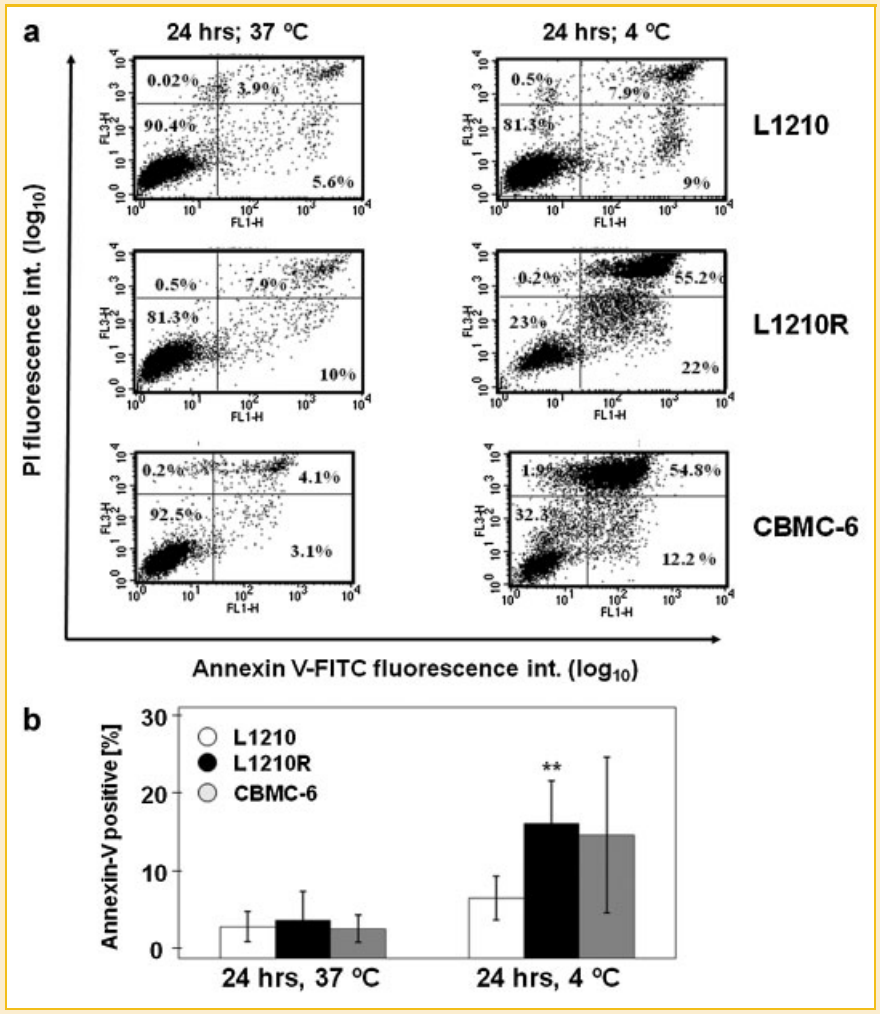

Fig. 3. Cold-induced phosphatidylserine exposure in L1210, L1210R, and CBMC-6 cells. Cells were incubated for the indicated periods of time at 4°C, stained with Annexin-V-FITC and propidium iodide (PI), and analyzed by flow cytometry. A total of 10,000 cells were analyzed and percentages of the different cell populations were calculated. a: Data show a representative flow cytometry dot-plot out of four assays performed with similar results. b: Quantitation of early apoptosis of L1210, L1210R, and CBMC-6 cells after 24 h of cold treatment. Data represent mean ± SD from four independent experiments. Asterisks represent statistical significance with respect to control cells (cultured at 37°C; $^{**}P < 0.01$, *t*-test).

annexin V-positive/PI-negative ratio) in CBMC-6 and L1210R cells than its parental L1210 cells. After 24 h the ratio (apoptosis) was approximately 18% and 15% for L1210R and CBMC-6 cells, respectively, compared with 3–9% for L1210 cells (Fig. 3b). In addition, as shown in Figure 3a, after 24 h of cold exposure, most of the L1210R and CBMC-6 cells also stained with PI (55.2% and 54.8%, respectively), which is indicative of late apoptosis or necrosis, while the percentage of L1210 cells stained with PI was only 7.9%.

L1210R CELLS DISPLAY HIGHER LEVELS OF CASPASE-3 ACTIVITY AT PHYSIOLOGICAL TEMPERATURE THAN PARENTAL CELLS

Next, we investigated the mechanism/s involved in the process of cold stress-induced apoptosis of MDR cells by measuring the activation of caspase-3. Cell lysates were analyzed first by Western blot, using an antibody against caspase-3 and its cleaved form (Fig. 4a,b). Active caspase-3 levels differed among the three cell lines assayed. For example, we observed high basal levels of caspase-3 activity in L1210R cells, followed by lower levels in CBMC-6 cells. In contrast, a low basal level of caspase-3 activation was observed in L1210 cells. As shown in Figure 4, when the three cell lines were maintained at 4°C, the activity of caspase-3 in L1210R and CBMC-6 remained constant during cold exposure. In the case of L1210 cells, there was a slight but significant increase of caspase-3 activity after 4–8 h of cold exposure. Further confirmation was obtained by using a colorimetric assay that measures the enzymatic activity of caspase-3 (Fig. 4c).

PHARMACOLOGICAL INHIBITION OF CASPASE-3 SIGNIFICANTLY BLOCKS COLD-INDUCED APOPTOSIS IN MDR CELLS

To determine the contribution of effector caspase-3 to the cell death mechanism induced by cold, cells were treated with Z-DEVD-FMK, a selective inhibitor of this protease. As can be seen in Figure 5, parental cells (L1210) treated with Z-DEVD-FMK showed a slight

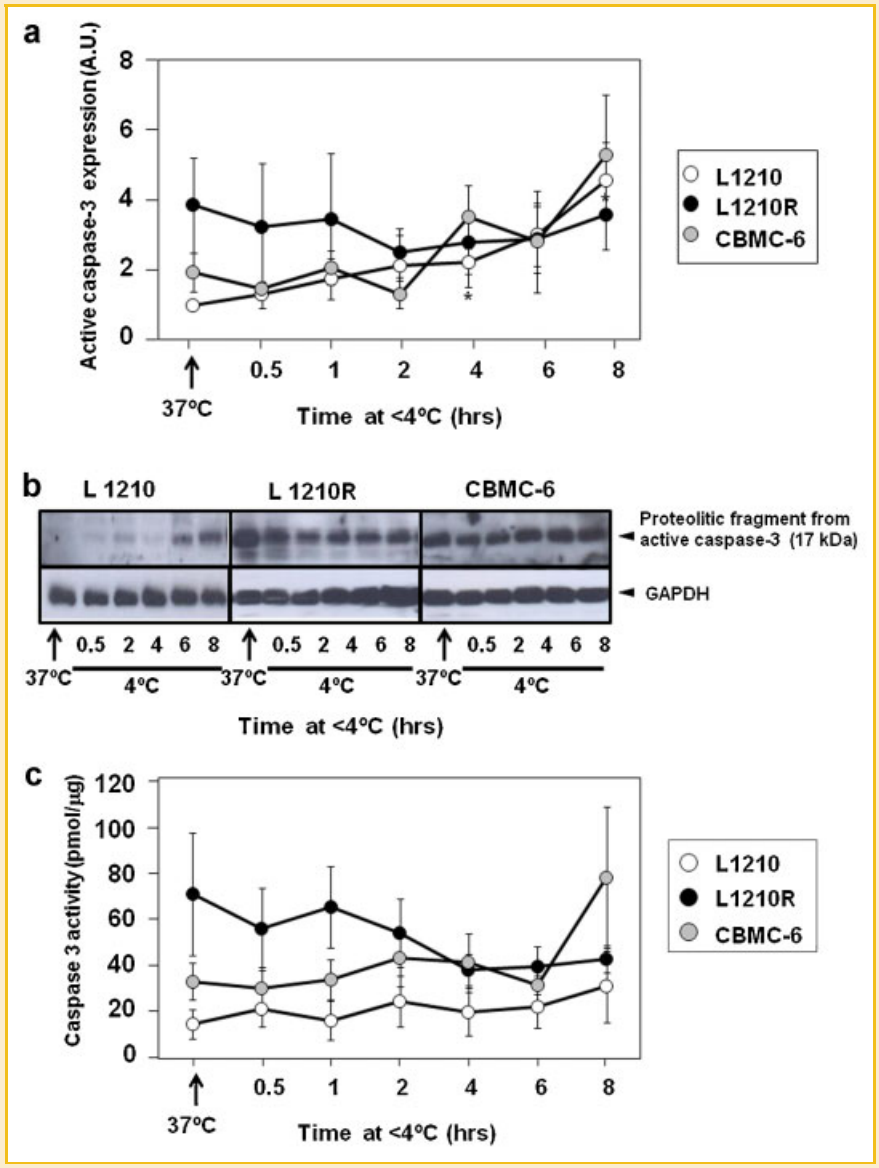

Fig. 4. Cold-induced caspase-3 activation in L1210, L1210R, and CBMC-6 cells. Cells were incubated for the indicated periods of time at 37°C or 4°C, collected to obtain cellular extracts and analyzed by Western blot or by colorimetric assay to determine caspase-3 proteolytic fragments or caspase-3 enzymatic activity, respectively. a: Graph shows levels of the proteolytic fragment of active caspase-3 from L1210, L1210R, and CBMC-6 cells incubated at 37°C or after cold treatment. b: Figure shows a representative Western blot, out of four assays performed with similar results, with caspase-3 proteolytic fragment from L1210, L1210R, and CBMC-6 cells cultured at 37°C or after cold treatment. c: Graph shows levels of caspase-3 proteolytic activity from L1210, L1210R, and CBMC-6 cells cultured at 37°C or after cold treatment. Data represent mean ± SD from four independent experiments. Asterisks represent statistical significance with respect to control cells (cultured at 37°C; $^{*}P < 0.05$, *t*-test).

increase in cell death rates. Interestingly, treatment of L1210R and CBMC-6 cells with a caspase-3 inhibitor significantly diminished cold-induced cell death, which reached a level similar to that observed in the parental line. To discount whether serine proteases were involved in the cell death process, we used a general serine protease inhibitor, AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], which effectively inhibits serine proteases but not other types of proteases. As shown in Figure 5, AEBSF slightly inhibits cell death induced by cold in L1210R (10–15%) but does not inhibit cell death in CBMC-6 cells, suggesting that the cell death mechanisms induced by cold stress in L1210R are serine-protease and caspase-3-dependent, while in CBMC-6 cells, which differ from the parental cell line in their P-gp expression, the cell death mechanism involved is caspase-3-dependent, suggesting an association between P-gp expression and caspase-3 activity.

EXPRESSION OF P-GLYCOPROTEIN INCREASES IN RESPONSE TO COLD STRESS IN L1210R AND CBMC-6 CELLS

To analyze whether P-gp expression in L1210R and CBMC-6 cells was affected by cold exposure, its expression in L1210R and CBMC-6 cells cultured at 37°C and at low temperatures was analyzed by Western blot. As shown in Figure 6a,b, basal P-gp expression was

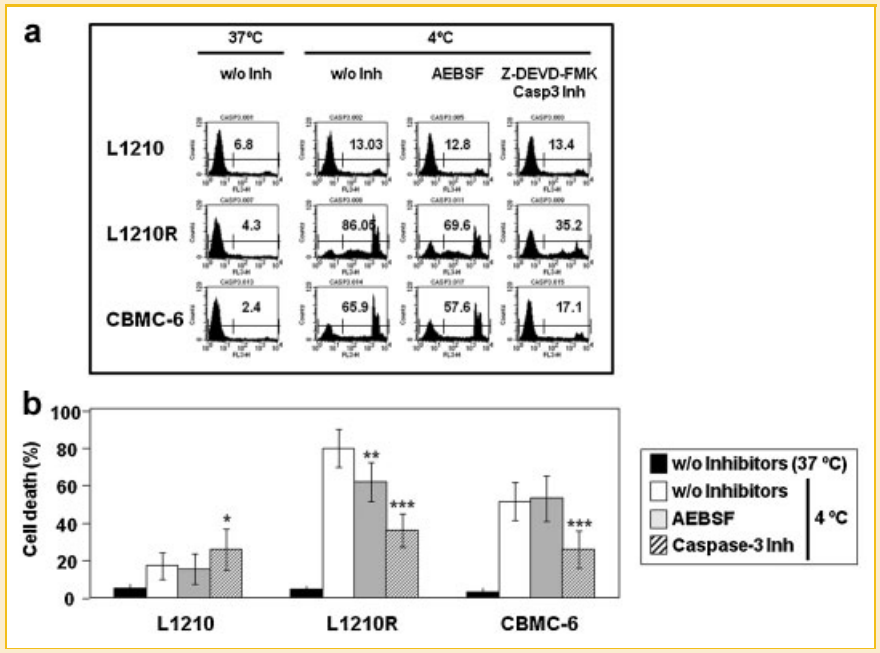

Fig. 5. Cold-induced cell death of L1210R and CBMC-6 cells is partially inhibited by a caspase-3 inhibitor. Cells were treated with 10 μM of the caspase-3 inhibitor Z-DEVD-FMK and with 25 μM of the general serine-protease inhibitor AEBSF for 1 h before exposure to cold treatment for 24 h. Cells treated with 1% DMSO were used as a control. Dead cells were detected by staining with propidium iodide (PI), and analyzed by flow cytometry. a: Figure shows a representative flow cytometry experiment out of the three that were performed. b: Data represent the mean of percentages of PI positive cells from three independent experiments. Asterisks represent statistical significance with respect to death levels in control cells (cells cultured at 4°C w/o caspase inhibitor; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *t*-test).

higher in L1210R compared to CBMC-6 cells, while no expression was detected in L1210 cells. After 12–24 h of cold exposure, P-gp protein expression increased in both L1210R and CBMC-6 cell lines (Fig. 6c) over basal protein levels (cells incubated at 37°C). This result shows that under cold stress, these cells up-regulate P-gp expression, probably as part of a general response to any kind of stress [Seo et al., 2010].

P-GLYCOPROTEIN DRUG EFFLUX ACTIVITY DECREASES UNDER COLD EXPOSURE

P-gp efflux activity has been described to be strongly affected by temperature, functioning optimally at 37°C but becoming inactive at 4°C [Seo et al., 2010]. To test this in our model, the accumulation of the P-gp substrate, rhodamine 123 [Legrand et al., 2001] was analyzed in L1210, L1210R, and CBMC-6 cells incubated at both 37°C and 4°C for different times. The levels of rhodamine fluorescence displayed by L1210 cells, which do not express P-gp, and the three cell lines incubated with rhodamine and VRP (P-gp extrusion activity inhibitor), were used as controls of maximum dye uptake. As expected, at 37°C, L1210 cells accumulated high levels of rhodamine compared with L1210R or CBMC-6 cells, which more readily extruded the dye, and displayed a lower degree of fluorescence. However, when the cells were maintained at 4°C, the levels of intracellular drug decreased drastically in L1210 cells due to the loss of permeability of the cell membrane (data not shown), but remained almost constant in the other two cell lines assayed (L1210R and CBMC-6). Thus, on one hand, P-gp expression increased in these cell lines under cold stress, and on the other hand, there was no decrease in rhodamine accumulation, as would be expected if P-gp maintained its drug efflux activity at 4°C. In fact, if we compare the fluorescence mean intensity of MDR cells relative to L1210 fluorescence over a period of 8-h exposure at 4°C, a sustained and significant increase of dye uptake can be observed in MDR cells, which indicates an effective inhibition of their extrusion pump activity (Fig. 7a). When the cells were incubated at 37°C with rhodamine and the P-gp extrusion activity inhibitor VRP, the inhibitor was seen to induce an increase in rhodamine accumulation in L1210R and CBMC-6 cells compared with control cells (incubated with rhodamine but not VRP). However, treatment with VRP at 4°C, did not lead to any increase in rhodamine accumulation (compared with control cells, without VRP), confirming the fact that P-gp extrusion activity becomes inactive at 4°C (Fig. 7b).

DISCUSSION

We show for the first time the increased susceptibility of P-gp over-expressing cells (L1210R and CBMC-6 cells) to cold stress-induced cell death compared with the parental cell line (L1210 cells, which do not express P-gp), probably through a caspase-3-dependent mechanism.

The "CS" refers to a process through which the acquisition of the MDR phenotype by cancer cells is frequently accompanied by cellular alterations that could render these cells more susceptible to agents others than the drug responsible for the MDR phenotype. These cellular alterations vary depending on the cell line and the drug used in the chemotherapeutic process in a way that it is not

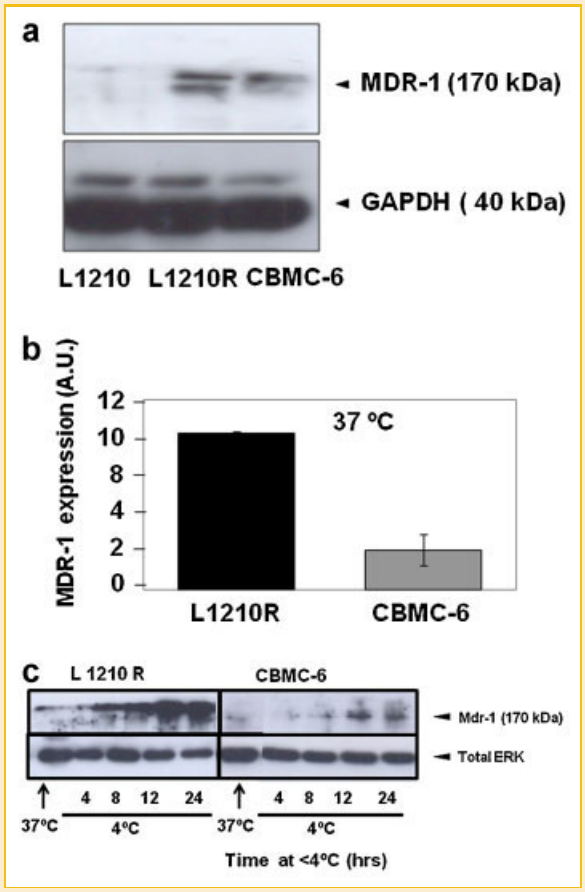

Fig. 6. Differential expression of P-glycoprotein in L1210, L1210R, and CBMC-6 cells under standard conditions (cultured at 37°C) and in response to cold stress. Cells (3×10^5 cells/ml) were incubated at 37°C and 4°C for 4, 8, 12, and 24 h. Cells were harvested and whole-cell lysates were obtained (10 μg of each) and subjected to Western blotting using the indicated specific antibodies to detect P-gp expression. a: P-gp expression in basal conditions (37°C). b: Mean expression levels of P-gp in basal conditions (cells cultured at 37°C) in L1210R and CBMC-6 cells. c: Western blot showing the trend of P-gp expression in L1210R and CBMC-6 cells cultured at 37°C or under cold stress conditions. At least three experiments were performed with similar results.

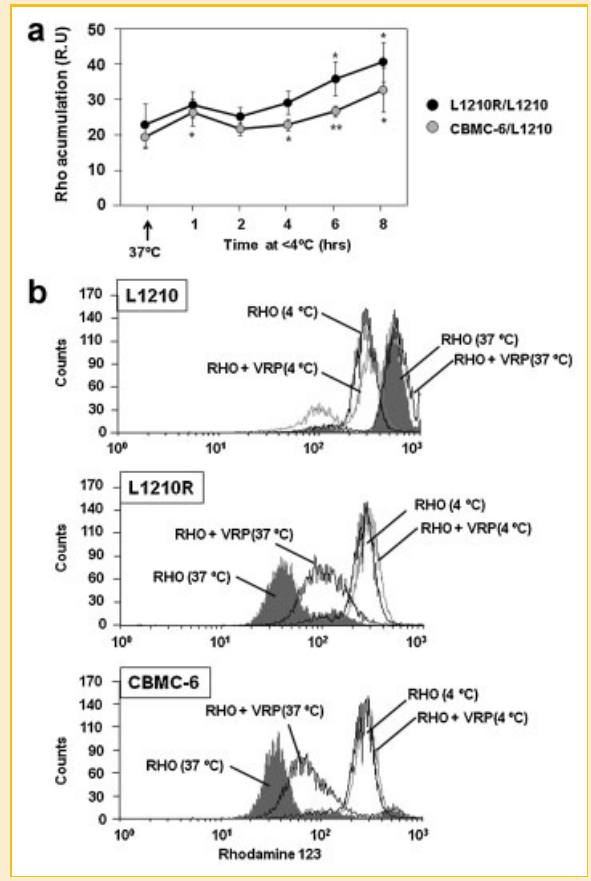

Fig. 7. Time course for rhodamine accumulation in L1210, L1210R, and CBMC-6 cells. Cells were cultured in standard conditions (37°C) or at low temperatures (4°C) for different periods of time. Cell cultures were incubated with 0.15 μM rhodamine for 1 h prior analysis. The samples were collected by centrifugation, resuspended in cold phosphate-buffered saline, and subjected to flow cytometry analysis as described in Materials and Methods Section. a: Figure represents time trend of mean fluorescence of L1210R and CBMC-6 cells relative to fluorescence data of L1210 cells when the three cell lines were incubated for 8 h at 4°C. b: Flow cytometry assay of P-gp efflux activity in L1210, L1210R, and CBMC-6 cells incubated for 6 h at 37°C or 4°C, in the presence of the fluorescent P-gp substrate, rhodamine 123 (Rho123), and treated (or not) with the P-gp extrusion activity inhibitor, verapamil. At least three experiments were performed with similar results. Asterisks represent statistical significance with respect to control cells (cultured at 37°C; $^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test).

always predictable [Hall et al., 2009]. Here, we describe for the first time, the CS of P-gp expressing cells to a physical stress, in this case extreme cold. The relevance of this finding is based not only on its potential therapeutic application, but also on its potential use as a tool to further study the cellular alterations that take place in MDR cells and thus to better understand the complexity of the drug resistance process.

In fact, the above observation supports the results obtained by other authors, who have suggested a role for P-gp in cell fate. Hence, transfection of parental cells (L1210) with a plasmid that contains murine *mdr1a* (P-gp) is sufficient to induce cold hypersensitivity in these cells (CBMC-6), which demonstrates the involvement of P-gp in this CS.

Several observations support the possibility that P-gp promotes cell survival through efflux-independent pathways, including the inhibition of caspase-dependent apoptosis [Johnstone et al., 1999; Ruefli et al., 2000, 2002ab] or reduction of ceramide levels [Mizutani et al., 2008]. In fact, it has been described that P-gp protects MDR cells from apoptosis by inhibiting caspases-8 and -3, as P-gp-positive cells have been found to resist the death induced by both UV irradiation and ligation of the cell surface death receptors Fas and TNFR [Johnstone et al., 1999; Ruefli et al., 2000, 2002ab; Martin-Orozco et al., 2005]. Nevertheless, the inhibition of caspase-3-dependent apoptosis has been described as being dependent on the cell lines and the apoptotic stimulus used, since certain stimuli, like curcumin or UV irradiation, induce caspase-3-dependent apoptosis in MDR HL-60 cells over-expressing P-gp [Bielak-Zmijewska et al., 2004; Turella et al., 2006]. In our leukemic cell model, the acquisition of the MDR phenotype is associated with high levels of

caspase-3 activity without affecting cell viability. In this respect, our results also support those obtained by other authors who reported that MDR cells with P-gp over-expression are protected against caspase-3-mediated cell death [Johnstone et al., 1999; Ruefli et al., 2000, 2002ab]. Surprisingly, besides the potential protective role of P-gp at physiological temperature of 37°C, our findings demonstrate hypersensitivity of MDR cells (L1210R and CBMC-6) to low temperatures through a caspase-3-related mechanism. These observations suggest that the protective role of P-gp is temperature-dependent [Wartenberg et al., 2005; Callaghan et al., 2008]. In fact, our results show that although P-gp expression increases at low temperatures, probably as the result of a general mechanism of stress response [Seo et al., 2010], there is a sustained increase of rhodamine accumulation in P-gp-expressing cells, which is indicative of a loss of P-gp efflux activity. Nevertheless, the role of P-gp in controlling caspase-3 activity could not be definitively confirmed by experiments with siRNA oligonucleotides specific for P-gp, since the partial silencing of P-gp protein expression had no effect on L1210R and CBMC-6 cell survival (data not shown). However, taking into account the diversity of the intrinsic factors involved in the regulation of apoptotic pathways in different cancer cells, we cannot exclude the possibility that there may be other factors, apart from P-gp expression, that block the caspase-3 enzymatic activity. Indeed, Campone et al. [2001] have shown that P-gp-expressing HL-60 cells are resistant to caspase-3-dependent apoptosis due to the presence of XIAP which belongs to the family of inhibitor of apoptosis proteins (IAP), and which inhibits caspase-3 by binding to its predomain, thereby preventing the activation of procaspase-3. Additionally, at present it cannot be ruled out that the induction of 170-kDa P-gp expression has other effects on key cellular metabolic pathways, which could influence the cellular sensitivity to apoptosis inducers. Further experiments should be made to clarify this issue.

In view of these findings, it may be speculated that, as a consequence of drug treatment, several L1210 clones evolve to express high levels of P-gp (MDR resistant phenotype), which would act not only by extruding the drug out of the cells but by controlling the increasing caspase-3 activity induced by drug exposure. Under cold stress, P-gp extrusion activity becomes inactive, leaving caspase-3 activity uncontrolled and, as a consequence, able to induce cold stress-induced cell death (supplementary material). Thus, it may be speculated that one strategy used by MDR cells to survive in the presence of a drug could be to control, by means of P-gp or proteins associated with it, the high levels of pro-apoptotic molecules (caspase-3) induced by pharmacological treatment. These proteins may play a role controlling the balance of pro-apoptotic and anti-apoptotic proteins at physiological but not at low temperatures.

More recently, the over-expression of an ATPase mutant P-gp has shown that such cells could not efflux chemotherapeutic drugs, but remained relatively resistant to apoptosis. Therefore, it seems that P-gp may inhibit apoptosis by means of a dual action, which involves both ATPase-dependent drug efflux and the ATPase-independent inhibition of apoptosis [Tainton et al., 2004]. However, these findings have been questioned by others who showed that cells with high levels of P-gp are paradoxically more sensitive to several apoptotic inducers, acting either through the mitochondrial (intrinsic) or the death receptor-mediated (extrinsic) apoptotic pathways [Matarrese et al., 2001; Cenni et al., 2004]. At this moment, we cannot confirm whether this occurs in our system, since our findings support a strong association between the lost of P-gp drug efflux activity and cell death at low temperatures. Further studies should be performed to ascertain whether cold stress-induced cell death is ATP-dependent or -independent.

In conclusion, this work describes the existence of a collateral susceptibility of MDR leukemic cells to extreme low temperatures, and the involvement of P-gp in this cell death process. Our results also suggest a role for the pro-apoptotic caspase-3 in the cell death mechanism induced by cold stress. The identification of new inducers of cell death in leukemic cells, and a study of the mechanisms and molecules affected by acquisition of MDR phenotype may help in the development of new strategies to eliminate chemoresistant cancer cells.

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