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Differentiation and Drug Resistance Relationships in Leukemia Cells

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Abstract It is well established that the effectiveness of anticancer drugs may result from combined cytotoxic and differentiation activities on tumor cells. Also, differentiating agents are able to alter the susceptibility of cancer cells to antineoplastic drug therapy. However, the acquisition and/or development of drug resistance that frequently appears in anticancer treatment can impair these interactions between differentiation agents and cytotoxic drugs. In the present study, we report that the acquisition of resistance to anthracyclines in two humans, promyeolocytic leukemia HL-60 and eythroleukemia K562 cell lines, results in a restricted maturation process induced by differentiating agents with respect to that exhibited by their corresponding drug-sensitive counterparts. Interestingly, differentiating agents are able to decrease the overexpression of drug-efflux pumps as it is the case of MRP1 in the resistant HL-60 cells, thus increasing the sensitivity of cells to drug treatment. In addition, susceptibility of the drug-sensitive cells to certain apoptotic stimuli is significantly reduced after differentiation. The results here reported indicate complex interactions between cytotoxic (drug therapy) and non-cytotoxic (differentiation) cancer treatments, which should be taken into account to improve therapeutic efficiency. J. Cell. Biochem. 94: 98–108, 2005.

Key words: differentiation; drug resistance; apoptosis

Anticancer chemotherapy usually results in cellular and molecular alterations leading to the acquisition of resistance, frequently associated with increased tolerance to anticancer drugs and overexpression of active drug-efflux pumps [Gottesmann and Pastan, 1993; Ambudkar et al., 1999; Borst et al., 2000; Gottesmann, 2002]. P-glycoprotein (Pgp) and the multidrug resistance-associated protein known as MRP1, are the two best characterized drug transporters [reviewed in Gottesmann et al., 2002], both being widely expressed in many human tissues and cancers [Ambudkar et al., 1999; Borst et al., 2000].

On the other hand, induction of leukemia cells to differentiation indicates that leukemic development might represent a reversible event of haemotioietic cell differentiation [Sachs, 1984], raising the possibility that treatment with differentiating agents may constitute a non-cytotoxic approach against leukemic cells [Van de Loosdrecht et al., 1994; Sachs, 1996].

It is well established that different anticancer drugs may exert their antineoplastic effects by combining differentiating and cytotoxic actions in several types of cancer including leukemia [Morin and Sartorelli, 1984; Sartorelli et al., 1987; Murate et al., 1991]. It has also been reported that differentiating agents may affect susceptibility of tumor cells to cytotoxic drugs modulating the expression of genes involved in drug resistance [Bates et al., 1989; Mickley et al., 1989; Okabe-Kado et al., 1991; Ishikawa et al., 1994; Marks et al., 1995].

On this basis, we are interested in studying the relationship between differentiation and drug resistance focusing our attention in two complementary aspects: first, to determine if the acquisition of drug-resistance by tumor cells modifies the induction to differentiation with

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respect to drug-sensitive cells and second, to analyze whether cellular maturation by different pathways affects some of the characteristics of drug resistance, specially the expression of drug-efflux pumps responsible for active extrusion of antineoplastic drugs from leukemic cells. In this study, we have utilized human promyeolocytic leukemia HL-60 (HL-60) and human erythroleukemia K562 (K562) cells for being widely models used in differentiation studies [Felsted et al., 1983; Sartorelli et al., 1987; Mollinedo et al., 1993, Murray et al., 1993]. Additionally, we have also derived daunomycin (DNM) (HL-60/R) and adriamycin (Adr) (K562/R)-resistant cell sublines overexpressing MRP1 and Pgp, respectively, to analyze differentiation–drug resistance interactions.

MATERIALS AND METHODS

Materials

DNM, Adr, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), and DMSO were obtained from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Monoclonal antibody MRPm6 was purchased from Monosan (Uden, The Netherlands).

Cells and Culture Conditions

The human promyeolocytic leukemia HL-60 and the human erythroleukemia K562 cell lines were grown in logarithmic phase in defined RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM of L-glutamine, 50 U/ml of penicillin G, and 50 μg/ml of streptomycin, at 37°C in humidified atmosphere and 5% of CO_2. A DNM resistant HL-60 cell subline (HL-60/R) and an Adr resistant K562 cell subline (K562/R) were obtained by stepwise increments of drugs using concentrations from 10 to 320 nM in the former and 10–850 nM in the later. The resulting anthracycline-resistant cell sublines exhibited ~30- and ~90-fold increased tolerance to DNM (HL-60/R) and to Adr (K562/R), respectively, determined by the colorimetric tetrazolium (MTT) proliferation assay as described by Mosmann [1983] using 96-well plates. HL-60 confluent cell cultures were obtained at a cell density of 2×10^6 cells/ml. Cell viability was assessed by the exclusion of 0.1% Trypan blue.

Cell Differentiation

Macrophage/monocytic differentiation of HL-60 cells was induced by a range (1–20 nM) of TPA during 24 h. Likewise, cells were incubated with different DMSO concentrations (0.5–2% (v/v)) during 6–8 days to induce the granulocytic differentiation. The course of differentiation was determined by cell-surface expression of the myeloid differentiation marker integrin Mac-1 molecule (CD11b) using the anti-CD11b antibody (Caltag Lab., Burlingame, CA) by flow cytometry (FCM) and morphology evaluation after Wright–Giemsa staining.

Megakaryocytic differentiation of K562 cells was carried out using a range (1–20 nM) of TPA at different times from 24 to 72 h. Unless specified, selected experimental conditions were 10 nM TPA for 48 h. The course of differentiation was followed by the expression of the megakaryocytic marker CD61 (Caltag Lab.) by FCM.

Cell Cycle Analysis and Apoptosis

Cell cycle analysis was performed by FCM in an Epics XL instrument (Beckman Coulter Co., Miami, FL) as follows: cells were centrifuged and washed with cold 10 mM phosphate buffer pH 7.4, supplemented with 2.7 mM KCl and 137 mM NaCl (PBS), and centrifuged again. The 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 and 0.05% RNase A. Then, cells were incubated for 30 min at room temperature, stained with propidium iodide, and the distribution of cellular DNA content was analyzed.

Apoptosis was induced by incubation of the cells with 100 μM etoposide for 24 h before (control) or after differentiation with TPA. The extent of apoptosis was measured by FCM by determining the amount of apoptotic cells in the sub-G_1 peak as previously described [Castro-Galache et al., 2003].

DNM Accumulation

Uptake of DNM by HL-60 and K562 cells was carried out by incubation of cell suspensions ($0.5–1 \times 10^6$ cells/ml) with 3 μM DNM in HBS at 37°C. To study the effects of chemosensitizers on intracellular DNM retention, cells were incubated with 5 μM verapamil (VRP) or 200 μM genistein during 20 min before adding DNM. Upon 90 min of incubation (steady-state conditions), aliquots were removed and analyzed by FCM. The concentration of drug associated to each cell is proportional to the

mean fluorescence intensity (mean fluorescence channel) determined in the histograms and was measured by FCM taking advantage of the intrinsic fluorescence of DNM. A total of 10,000 cells were measured during each sample analysis.

Western Blot Analysis

Detection of MRP1 was done by Western immunobloting using MRPm6 as primary antibody. Cells ($5-30 \times 10^6$) were washed in PBS, lysed in Tris-HCl 10 mM pH 7.4 supplemented with 5 mM EDTA, 200 mM NaCl, 5 mM iodoacetamide, 0.5 mM phenylmetylsulfonyl fluoride (PMSF), and disrupted in Potter–Elvehem and Polytron homogenizers. Homogenates were centrifuged at 14,000*g* for 20 min and supernatants were centrifuged at 100,000*g* for 1 h. Pellets were resuspended in the same lysis buffer and protein content was determined by the Bradford method (BioRad Laboratories, Richmond, CA). Then, 20 μg of protein was electrophoresed in 7.5% SDS–polyacrylamide gels (PAGE). Gels were negatively stained with zinc (Zinc destain kit, BioRad Laboratories) and after band visualization transferred onto nitrocellulose blotting sheets. Peroxidase-conjugated antibodies were used as secondary antibodies followed by enhanced chemiluminiscence (ECL) (Amersham International, Buckinghamshire, UK) to develop protein bands.

MRP-1 Determination by Real-Time RT-PCR

To determine the levels of MRP-1 mRNA, total RNA from non-treated or TPA-treated cells was isolated by using the TRI reagent (Sigma-Aldrich Co., St. Louis, MO). To eliminate potential DNA contamination, total RNA was treated with RQ1 DNase (Promega Corp., Madison, WI) for 30 min at 37°C followed by 2 min at 94°C. Reverse transcription of 1 μg of RNA was performed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time quantitative PCR was performed to amplify 20 ng of cDNA. Using the ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Primers and probe to amplify *MRP-1* gene were designed using Primer Express software (Applied Biosystems).

The sequence of the primers for MRP-1 were: MRP-1F (sense) 5′-gcgagtgtctccctcaaacg-3′, and MRP-1R (antisense) 5′-tcctcacggtgatgctgttc-3′. The sequence of the Taqman probe for MRP-1 was 5′-tgacagcatcgagcgacggcc-3′. This probe was labeled with 6-FAM in 5′ end as the reporter dye, and with TAMRA in 3′ end as the quencher dye. The MRP-1 amplicon size was 118 bp and expanded between exons 14 and 15. All seven described MRP-1 isoforms were amplified using this primer set.

Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as endogenous reference in multiplex PCR. Primers and Taqman probe for this housekeeping gene are commercially available from Applied Biosystems (Taqman Pre-Developed Assay Reagents for gene expression). GAPDH Taqman probe was labeled with VIC in 5′ end as the reporter dye, and with TAMRA in 3′ end as the quencher dye.

MRP-1 mRNA relative gene expression was calculated by the comparative C_t method referred to the GAPDH housekeeping gene expression (ABI PRISM 7700 Sequence Detection System: User Bulletin #2, Applied Biosystems).

RESULTS

Characterization of Anthracycline-Resistant HL-60 and K562 Cells

DNM-resistant HL-60/R and Adr-resistant K562/R cells were selected from wild-type drug-sensitive HL-60 and K562 cells, respectively, as described in "Materials and Methods." Both anthracycline-resistant cell sublines behave similarly in terms of the following criteria, therefore only the results corresponding to HL-60 are shown: (i) high tolerance to growth in the presence of the corresponding anthracycline as compared to the drug-sensitive cells (Fig. 1a); (ii) defective intracellular accumulation of DNM which can be reversed by chemosensitizers (Fig. 1b); and (iii) overexpression of the ATP-dependent drug-efflux pumps responsible for the extrusion of the drugs from the cells (Fig. 1c).

Differentiation of HL-60 and K562 Cells

Macrophage/monocytic lineage differentiation of wild-type HL-60 cells was achieved by treatment with 1 nM TPA and followed by expression of the myeloid differentiation marker integrin Mac-1 molecule (CD11b), as indicated in Figure 2a. Increasing the TPA concentration up to 20 nM did not change the levels of CD11b expression. Therefore, further experiments were carried out using 1 nM TPA. In addition, differentiated cells exhibited

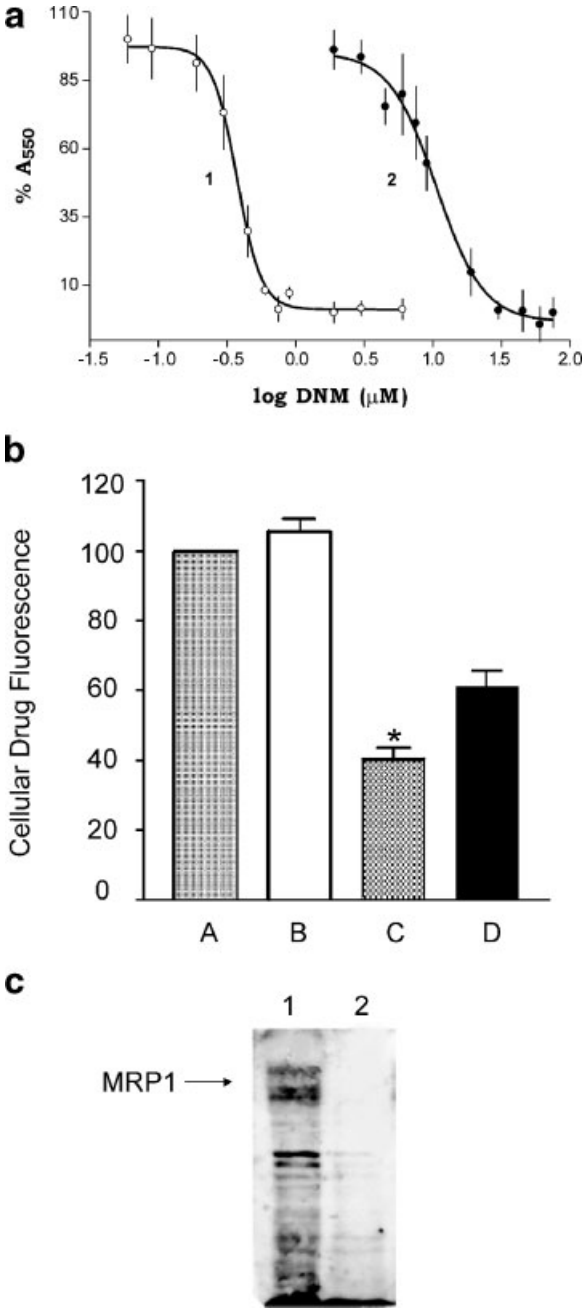

Fig. 1. **a**: Cell proliferation of HL-60 (1) and HL-60/R (2) cells in the presence of increasing daunomycin (DNM) concentration, determined by the MTT reduction assay [Mosmann, 1983]. Both curves were fitted using Graphpad software ($r \geq 0.992$) and IC50 was calculated as the mean of three experiments. Bar ± SEM ($n \geq 4$). **b**: Long-term uptake of DNM by the HL-60 (**A**–**B**) and HL60/R (**C**–**D**) cells, in the absence (A and C) or in the presence (B and D) of 5 μM verapamil (VRP). The Y-axis indicates the normalized fluorescence of DNM (100% refers to the fluorescence of DNM in the HL-60 cells in the absence of VRP). Bar ± SD ($n \geq 8$). *$P < 0.0003$ between samples C and D (*t*-test). **c**: Immunodetection of MRP1 in HL-60/R cells (**1**) using the Mab MRPm6 as indicated in ''Materials and Methods.'' (**2**) Control HL-60 cells.

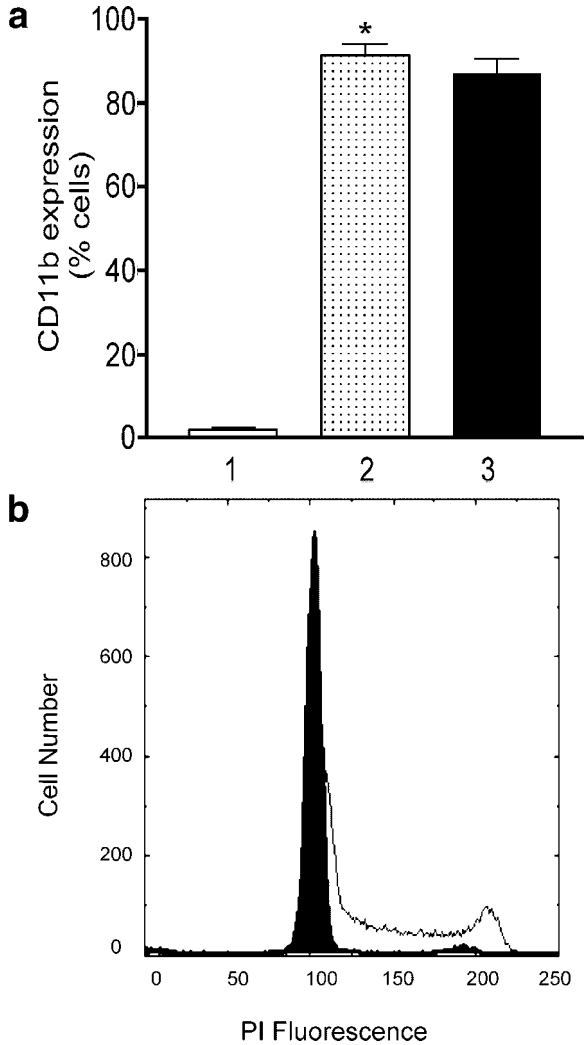

Fig. 2. **a**: Differentiation of HL-60 cells estimated by CD11b expression after incubation with 1.3% (v/v) DMSO during 8 days (**2**) or 1 nM 12-*o*-tetradecanoylphorbol-13-acetate (TPA) for 24 h (**3**). (**1**) Control (undifferentiated) HL-60 cells. Bar ± SEM ($n \geq 5$); *$P < 0.85$ between samples 2 and 3 (*t*-test). **b**: Distribution of cellular DNA in the HL-60 cells (black) induced by treatment with 1 nM TPA for 24 h. Control HL-60 cells (white).

characteristics of mature cells such as cellular aggregation, increased cytoplasm/nucleus ratio, etc. (not shown). As expected, TPA promoted the blockade of HL-60 cells in the G_1 phase of the cell cycle, with 91% of the cells in the G_1 phase, as compared to 46% that was observed in exponentially growing cells (Fig. 2b). DMSO was also used to promote granulocytic differentiation of HL-60 cells and treated cells exhibited similar characteristics to those described in the case of TPA (Fig. 2a). Since regardless of granulocytic (DMSO) or macrophage/monocytic (TPA) differentiation both agents induced similar effects on

HL-60 cells, we will describe, unless specified, the results obtained with the latter.

In the case of K562 cells, TPA-induced megakaryocytic differentiation was followed by measuring cell proliferation and appearance of CD61 positive cells [Liu et al., 2004]. Three different experiments indicate that after 48 h of incubation with 10 nM TPA, K562 cells underwent severe inhibition of proliferation with cell arrest in the G_1 phase and significant CD61 expression (30–40% of cells).

Does Differentiation Affect Susceptibility of Cells to Drug-Resistance?

It has been reported that differentiating agents may, in addition to promote cellular maturation, alter the drug resistance pattern of tumor cells [Bates et al., 1989; Mickley et al., 1989; Okabe-Kado et al., 1991; Ishikawa et al., 1994; Marks et al., 1995]. On this basis, we investigated whether TPA and DMSO treatment could also induce cellular alterations associated with the drug-resistance phenotype. In this regard, HL-60 cells were able to grow at higher concentrations of DNM after being treated (and differentiated) with TPA, Figure 3a. Also, intracellular accumulation of DNM was reduced in differentiated cells, as compared to wild-type HL-60 cells (Fig. 3b). These two observations resemble some properties of tumor cells bearing the drug resistance phenotype [Gottesmann and Pastan, 1993; Ambudkar et al., 1999], which is usually characterized by overexpression of different drug-efflux pumps, as molecular mechanisms of defense against drug injury [Borst et al., 2000; Gottesmann, 2002; Gottesmann et al., 2002]. Since the multidrug resistance-associated protein (MRP1) [Cole et al., 1992] is overexpressed in the HL-60/R cells (Fig. 1c), we have analyzed the possible induction of MRP1 expression by differentiation treatments. Hence, we used a functional assay with the chemosensitizers, VRP and genistein, which were able to increase the intracellular accumulation of antineoplastic drugs in resistant cells by blocking the drug efflux activity of Pgp and MRP1 [Versantvoort et al., 1994; Feller et al., 1995; Castro and Altenberg, 1997]. No changes in the intracellular steady-state level of DNM were observed in TPA-treated HL-60 cells with any of the two chemosensitizers, indicating that expression of MRP1 was not responsible for: (i) increased tolerance of differentiated HL-60 cells to DNM and (ii) lower accumulation of the drug after TPA treatment. Why do differentiated HL-60 cells behave in some aspects as drug-resistant cells? To answer this question, and taking into account that the most apparent effect induced by TPA was the G_1 phase arrest (Fig. 2b), we forced HL-60 cells to grow under confluent conditions. Confluent HL-60 cells were arrested in the G_1 phase of the cell cycle and under these conditions, a significant reduced accumulation of DNM with respect to exponentially growing HL-60 cells was observed, Figure 4. Expression of CD11b in confluent cells was undistinguishable from exponentially growing cells indicating

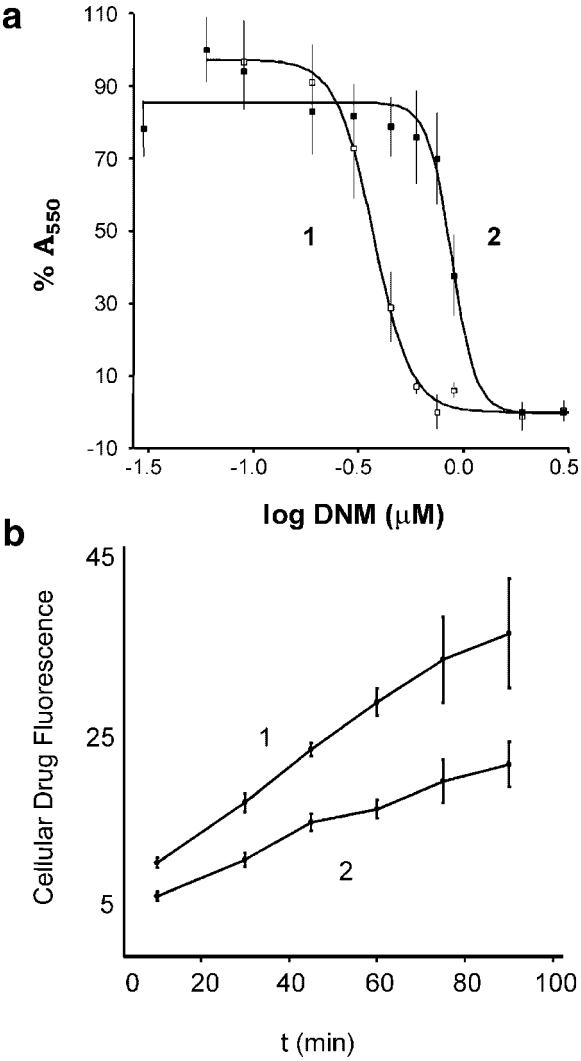

Fig. 3. **a**: Increased tolerance to growth in the presence of DNM of HL-60 cells after treatment with TPA (**2**) estimated by the MTT assay. Control of untreated HL-60 cells (**1**). Bar ± SEM ($n \geq 3$). **b**: Time-course of intracellular accumulation of DNM in the HL-60 control (**1**) and TPA-treated cells (**2**) followed by flow cytometry (FCM). Bar ± SEM ($n \geq 4$). $P < 0.002$ (*t*-test).

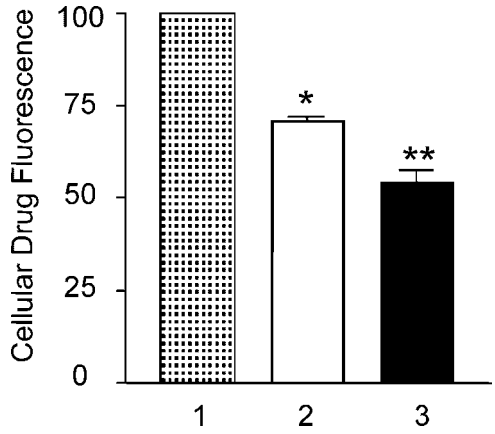

Fig. 4. Steady-state intracellular accumulation of DNM of (**1**) control, (**2**) confluent, and (**3**) DMSO-treated HL-60 cells determined by FCM. The Y-axis indicates the normalized fluorescence of DNM (100% refers to control HL-60 cells). Bar ± SD ($n \geq 3$). $^*P < 0.02$ between samples 1 and 2; $^{**}P < 0.113$ between samples 2 and 3 (*t*-test).

that confluency did not induce maturation of HL-60 cells. Thus, it seems that cell cycle arrest in the G_1 phase may, at least in some extent, reproduce the apparent cellular characteristics of drug resistance observed in the TPA-treated HL-60 cells.

Influence of the Drug Resistance Pattern on Cell Differentiation

An additional aim of this work was to evaluate whether drug-sensitive and drug-resistant leukemia cells behave differently to differentiating agents. Figure 5a shows that HL-60/R cells are restricted to monocyte-like differentiation by TPA when compared to drug-sensitive HL-60 cells, as estimated by the expression of the CD11b antigen. Giemsa staining (not shown) and cell cycle analysis confirmed that HL-60/R cells underwent limited differentiation upon TPA treatment. This can be visualized in Figure 5b in which the G_1 phase accounts for 71% of the cells versus 91% previously observed in the case of drug-sensitive TPA-treated HL-60 cells, Figure 2b. On the other hand, CD61 expression in the K562/R cells was undistinguishable from the one observed in TPA-untreated K562/R cells, indicating that K562/R cells were more insensitive to TPA differentiation effects than HL-60/R cells.

In spite of this, differentiating agents affect some characteristics associated with drug resistance in HL-60/R cells and, unlike drug-sensitive HL-60 cells, intracellular accumulation of DNM is somewhat increased with respect to

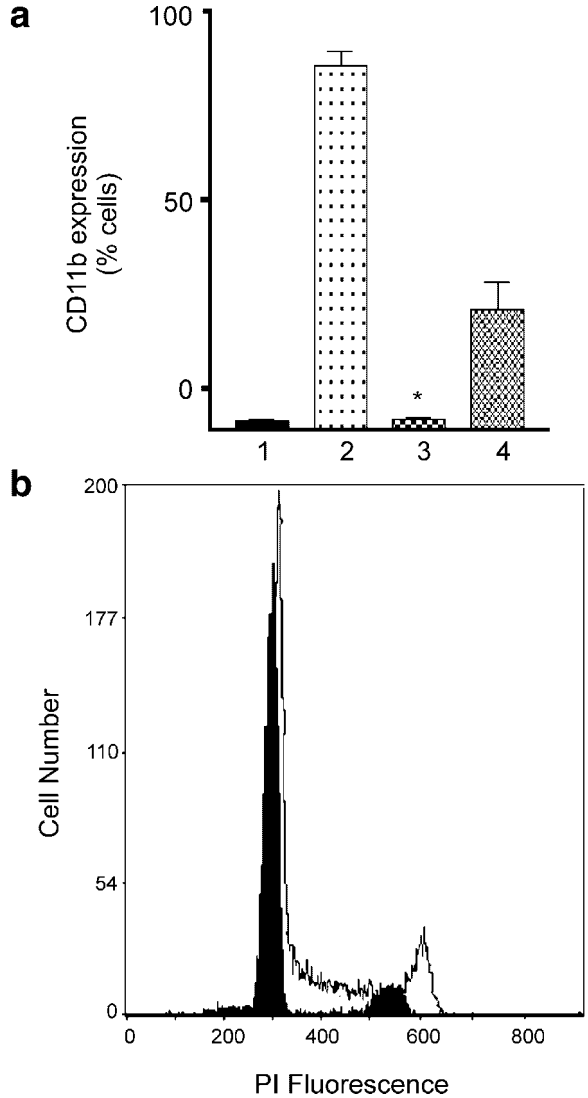

Fig. 5. **a**: Differentiation of HL-60/R cells by treatment with 1 nM TPA for 24 h (**4**) estimated by FCM as the number of cells (%) expressing CD11b. Note the large difference with differentiated HL-60 (**2**) cells. Histograms (**1**) and (**3**) correspond to untreated HL-60 and HL-60/R cells, respectively. Bar ± SD ($n \geq 5$); $^*P < 0.02$ between samples (3) and (4). **b**: Distribution of cellular DNA in the TPA-treated HL-60/R cells (black) overlapping with that corresponding to control HL-60/R cells (white).

untreated DNM-resistant cells, Figure 6a. However, such enhancement represents approximately 50% of the level of intracellular DNM corresponding to drug-sensitive cells, suggesting that some functional drug efflux mechanisms remain operative. In support of this, there is a small but significant increase in the intracellular DNM accumulation after incubation of TPA-treated HL-60/R cells with the chemosensitizer genistein, Figure 6a. Also, differentiated HL-60/R cells exhibit increased sensitivity to

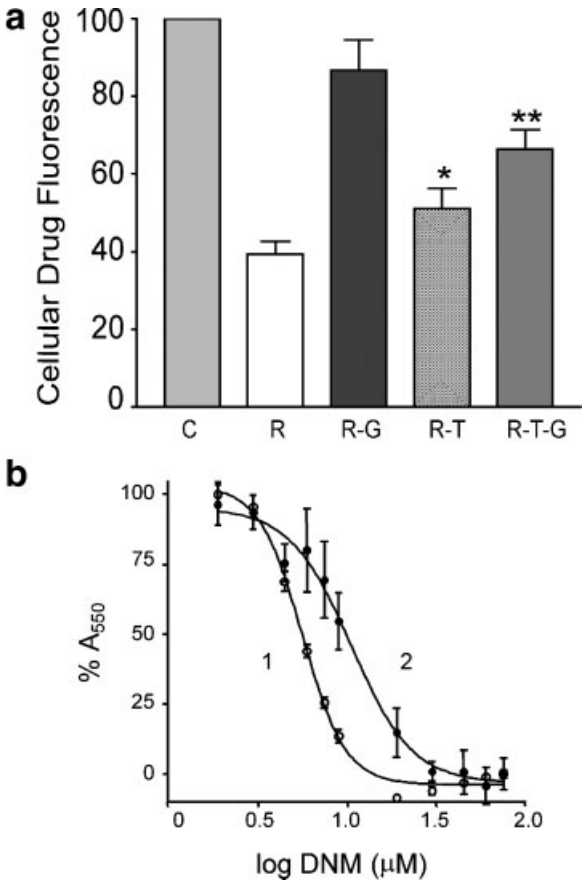

Fig. 6. **a**: Intracellular DNM accumulation of HL-60/R and TPA-treated HL-60/R cells in the absence (R) and (R-T) or in the presence of 200 μM genistein (R-G) and (R-T-G), respectively. DNM accumulation of HL-60 cells (C) is included as reference (100% in the Y-axis). Bar ± SD ($n \geq 3$); *$P < 0.01$ between R and R-T samples; **$P < 0.05$ between R-T and R-T-G samples. **b**: Cell proliferation of DMSO-treated HL-60/R (**1**) and HL-60/R (**2**) cells in the presence of increasing DNM concentration. Bar ± SEM ($n \geq 4$).

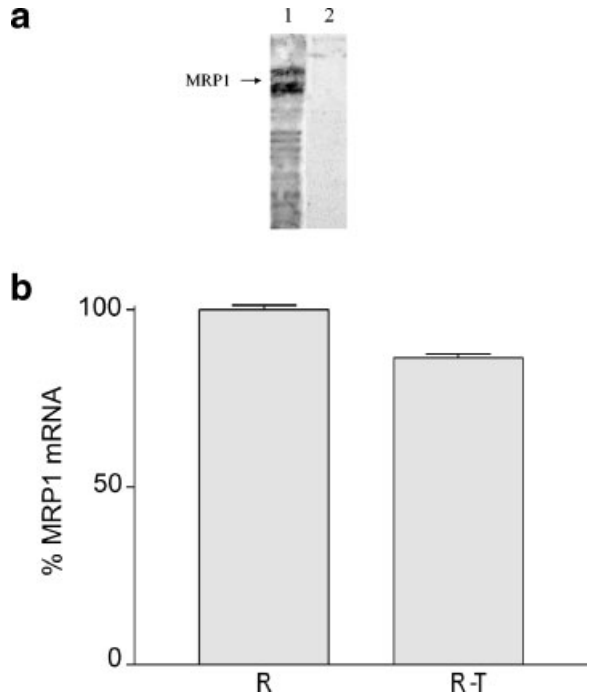

Fig. 7. **a**: Immunoblotting with the anti-MRP1 monoclonal antibody MRPm6 showing undetectable MRP1 expression in HL-60/R cells after treatment with 1 nM TPA for 24 h (**2**). Cell extracts from HL-60/R cells are included as control of MRP1 expression (**1**). **b**: Comparative MRP1 mRNA levels in the TPA-treated HL-60/R (R-T) cells and HL-60/R cells analyzed by RT-PCR, as described in ''Materials and Methods.'' Bar ± SD.

DNM with respect to non-treated HL-60/R cells, Figure 6b.

It seems reasonable to assume that inhibition of the MRP1 drug efflux protein could be involved in the above two observations, i.e., increased intracellular DNM accumulation and sensitivity in the HL-60/R cells upon differentiation treatment. To explore this possibility, we have determined changes on the overexpression of MRP1 in the differentiated cells at both, mRNA and protein levels. Western blot experiments demonstrate that MRP1 expression was undetectable in TPA-treated resistant cells, Figure 7a. Also, RT-PCR assays demonstrate a decrease of MRP1 mRNA levels in HL-60/R cells after TPA treatment, Figure 7b. Nevertheless, Figure 7b also indicates that TPA does not completely abolish MRP1 transcription, since considerable MRP1 mRNA levels were still observed in TPA-treated HL-60/R cells. This would be consistent with the suggested remaining functionality of the MRP1 protein, as already indicated in the DNM accumulation experiments (Fig. 6a). In this context, a question arises as to whether the observed restricted differentiation in the HL-60/R cells could be assigned to the TPA efflux by the MRP1 protein. Such a possibility was ruled out because the level of differentiation reached when the HL-60/R cells were treated with TPA, was similar to that obtain upon blocking the MRP1 function with VRP.

Differentiation and Apoptosis Relationships

The above observations show a certain modulation of differentiation on drug-resistance, which in turn, can affect the programmed cell death [Castro-Galache et al., 2003]. On this basis, we have studied how TPA differentiation might impair apoptosis in anthracycline-sensitive and anthracycline-resistant cells by treatment with 100 μM etoposide for 24 h

(HL-60) or 48 h (K562). Control experiments indicate that while etoposide induces considerable apoptosis in HL-60 and K562 cells (~60 and 48% of the cells, respectively, appear in the sub-G_1 phase as estimated by FCM), it is fairly inefficient in the drug-resistant cells. However, and after TPA differentiation, there is a significant reduction of the number of wild-type cells entering apoptosis, while TPA-treated anthracycline-resistant cells remain insensitive (HL-60/R) or become refractory to etoposide (in K562/R cells the percentage of apoptotic cells falls from 15 to 5%). These results are summarized in Table I.

DISCUSSION

The human promyelocytic leukemia HL-60 and erythroleukemia K562 cell lines have been widely used as cellular models in differentiation experiments [Collins, 1987; Sartorelli et al., 1987; Ahmed et al., 1991; Murray et al., 1993]. In addition, these cells can be selected with antitumor drugs to acquire a drug-resistance phenotype, frequently characterized by the overexpression of different drug-efflux pumps, responsible for lowering the intracellular concentration of anticancer drugs [Mickley et al., 1989; Ishikawa et al., 1994; Jönson et al., 1995; Ferrao et al., 2003; Kotaki et al., 2003]. Thus, using HL-60 and K562 cells we can establish possible differentiation-drug resistance relationships taking into account that: (1) differentiating agents can modulate the drug-resistance pattern [Bates et al., 1989; Mickley et al., 1989; Okabe-Kado et al., 1991; Ishikawa et al., 1994; Marks et al., 1995] and (2) chemotherapeutic agents may promote maturation of tumor cells [Morin and Sartorelli, 1984; Sartorelli et al., 1987].

Our aim of the present study was to elucidate whether cell differentiation occurs in drug-sensitive and drug-resistant HL-60 cells in a similar fashion. We have observed that while drug-sensitive HL-60 and K562 cells are induced to differentiation by common differentiating agents like DMSO or TPA, anthracycline-resistant HL-60/R and K562/R cells become more refractory to maturation. This situation does not appear to be related to possible differences in preliminary maturation stages between the two cell types, since both display a similar starting differentiation state, as indicated by the CD11b (HL-60) or CD61 (K562) expression levels. Neither the poor differentiation observed in the drug-resistant cells can be ascribed to the extrusion of TPA by the characteristic drug-efflux pumps associated to resistance since, as observed in the HL-60/R cells, blockade of the MRP1 function by VRP does not change the extent of TPA-induced differentiation.

Complementary, we have also analyzed whether TPA treatment can modulate any of the cellular characteristics associated with the occurrence of the drug-resistance pattern. In the case of HL-60 cells, maturation took place concomitant to cell cycle arrest in the G_1 phase. Interestingly, maturation was accompanied by two characteristics of tumor cells bearing a drug-resistance phenotype: (i) defective intracellular drug accumulation with respect to drug-sensitive cells and (ii) increased tolerance to growth in the presence of drugs. Related to the later, it could be argued that differentiating agents could modulate the susceptibility of the cells to apoptosis impairing mitochondrial activity (which constitutes the basis of the MTT assay), rather than increasing drug tolerance. In fact, wild-type HL-60 cells decrease their susceptibility to apoptosis after differentiation and apparently, this could contribute to a tolerance increase to DNM (Fig. 3a). However, HL-60/R cells, which remain fairly insensitive to apoptosis after TPA treatment, decrease their tolerance to DNM (Fig. 6b) indicating that the MTT assay, in addition to reflecting alterations

TABLE I. Effect of 12-*o*-Tetradecanoylphorbol-13-Acetate (TPA) on Etoposide-Induced Apoptosis in HL-60 and K562 Cells

	HL-60 cells[a]		K562 cells[b]	
Treatment	wt HL-60	HL-60/R	wt K562	K562/R
Etoposide (1)	60.7 ± 3.3	2.89 ± 0.42	48.8 ± 2.7	15.0 ± 1.2
TPA + etoposide (2)	10.1 ± 0.9	2.81 ± 0.44	30.2 ± 1.6	5.1 ± 0.4

Data are expressed as percentage of the cells ± SEM in the sub-G_1 phase estimated by FCM (n = 3).
[a]Cells treated with: (1) 100 μM etoposide 24 h or (2) 1 nM TPA 24 h + 100 μM etoposide 24 h.
[b]Cells treated with: (1) 100 μM etoposide 48 h or (2) 10 nM TPA 48 h + 100 μM etoposide 48 h.

in the susceptibility of the cells to apoptosis, is also detecting changes in the tolerance of the cells to anticancer drugs.

Reduced intracellular drug accumulation and growth in the presence of high drug concentrations are two characteristics associated with the overexpression of drug-efflux pumps. In this context, previous studies have reported regulation of the expression of Pgp by differentiating agents [Bates et al., 1989; Mickley et al., 1989; Okabe-Kado et al., 1991; Ishikawa et al., 1994; Marks et al., 1995]. Thus, induction of Pgp has been described in some colon carcinoma and neuroblastoma cells treated with sodium butyrate [Mickley et al., 1989] or retinoic acid [Bates et al., 1989], respectively. In contrast, we have shown absence of drug efflux activity in DMSO or TPA-treated HL-60 cells when VRP or genistein were used as chemosensitizers in drug accumulation experiments. Possible explanations for the discrepancies obtained with the HL-60 cell line are: (i) neither all colon cell lines underwent induction of Pgp by differentiating agents nor all differentiating agents promoted induction of Pgp in the report by Mickley et al. [1989]. TPA was not included in that study, and (ii) the effects of cellular maturation on the modulation of the expression of the drug-efflux pumps, could be dependent on the cell type. Thus, it appears that alterations other than the emergence of a drug resistance phenotype should account for the enhanced tolerance to DNM and reduced drug accumulation observed in HL-60 cells after treatment with differentiating agents. In fact, drug sensitive HL-60 and K562 cells, which are initially susceptible to etoposide-induced apoptosis become resistant to the toxin upon TPA differentiation. In the case of HL-60 cells, some of these alterations can be partly reproduced upon arresting the cells in the G_1 phase in confluent cell cultures, despite confluent cells show identical level of differentiation that exponentially growing, TPA-untreated HL-60 cells.

On the other hand, TPA and DMSO decrease the MRP1 protein levels in HL-60/R cells while poorly affecting the MRP1 mRNA. In our opinion, such a situation can be achieved if after differentiation, the amount of protein falls below the sensitivity of Western blot, which is lower than that of RT-PCR. In fact, it seems that despite MRP1 protein is undetectable after differentiation, there exists a remaining functional drug efflux activity (see "Results"). Also, the occurrence of a translational control of MRP1 would account for a lack of correlation between the decrease of protein and MRP1 mRNA levels.

The decrease of MRP1 protein after differentiation in the HL-60/R cells, is in contrast with the study by Mickley et al. [1989], but agrees with that reported by Okabe-Kado et al. [1991], describing inhibition of Pgp by the differentiation factor activin-A in human K562 leukemia cells. The fact that diverse differentiating agents could modulate the expression of individual drug-efflux pumps in a different manner, suggests the possibility of tissue-specific regulation as recently discussed by Castro-Galache et al. [2003].

Maturation induction apparently affects the characteristics of drug resistance in HL-60 and HL-60/R cells in opposite ways: while HL-60/R cells accumulate in the G_1 phase, the intracellular drug accumulation increases with respect to non-differentiated HL-60/R cells and MRP1 expression is lost, differentiated HL-60 cells (not R) accumulate in the G_1 phase, but intracellular drug accumulation decreases with respect to control HL-60 cells. Neither HL-60 cells nor differentiated HL-60 cells express detectable levels of MRP1. In our interpretation, we claim that the G_1 arrest accounts for the decrease of drug accumulation in the HL-60 cells when compared with confluent HL-60 cells. In the case of differentiated HL-60/R cells, a decrease in drug accumulation would be expected because they are also arrested in the G_1 phase, but on the contrary, we found that DNM accumulation is increased. It must be taken into account that differentiated HL-60/R cells have lost MRP1 expression (Fig. 7a) and this should increase the accumulation of DNM in the cells. Therefore, in the case of differentiated HL-60/R cells, two opposite effects must be considered: first, a G_1 arrest that as in the HL-60 cells would result in a decrease of intracellular drug accumulation, and second, loss of MRP1 that would cause an increase in intracellular drug accumulation. The balance between both effects results in a net increase in DNM accumulation, indicating that the decrease associated with the G_1 arrest ($\sim$25%, Fig. 4) is quantitatively inferior to the increase due to the loss of MRP1 ($\sim$60%), as determined when DNM accumulation in MRP1 expressing (HL-60/R) and MRP1 non-expressing (HL-60) cells is compared (Fig. 1b).

In conclusion, we report that leukemia cells tend to decrease their susceptibility to certain apoptotic stimuli like etoposide after treatment with differentiating agents. The effect appears more pronounced in drug-sensitive cells, which in turn, are more vulnerable to differentiation than their corresponding drug-resistant cells. Especially important is the observation that effectiveness of the treatment of leukemia cells with differentiating agents can be influenced by their susceptibility to antineoplastics: drug-sensitive cells can differentiate into mature cells while drug-resistant cells are restricted to incomplete stages of differentiation. Accordingly, differentiation therapy should precede the treatments with antineoplastics to prevent the acquisition of drug-resistance by anticancer drugs. Even in the case of drug-resistant cells, treatment with differentiating agents could be beneficial if, as in HL-60/R cells, it could inhibit the expression of drug efflux mechanisms allowing cells to regain susceptibility to anticancer drugs. In this case, sequentially combined differentiation-antineoplastic protocols should improve individual therapies.

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