NEOPLASIA

Triggering Noncycling Hematopoietic Progenitors and Leukemic Blasts to Proliferate Increases Anthracycline Retention and Toxicity by Downregulating Multidrug Resistance

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Expression of the multidrug resistance (MDR) mechanisms P-glycoprotein (Pgp) and MDR-related protein (MRP) decrease cellular retention and consequently cytotoxicity of anthracyclines. MDR is expressed on normal human hematopoietic progenitors and leukemic blasts. Normal CD34$^+$ progenitors showed rhodamine efflux in 20% to 30% of the cells, which could be blocked by verapamil. These cells appeared noncycling, in contrast to the proliferating rhodamine bright (RhoB) cells. We postulated that MDR expression can be downregulated by proliferation induction. Triggering rhodamine dull (RhoD) CD34$^+$ cells to proliferate indeed resulted in a higher rhodamine retention and significantly decreased efflux modulation by verapamil ($P = .04$). Also in acute myeloid leukemia (AML), the proliferation rate (percentage S/G_2+M and Iododeoxyuridine labelings index) was significantly less in the RhoD blasts ($P \leq .008$) and proliferation induction of RhoD blasts resulted in increased rhodamine retention. Anthracycline cytotoxicity was less for RhoD than RhoB cells in both normal progenitors and leukemic blasts. Proliferation induction of the RhoD cells resulted in increased anthracycline sensitivity. We conclude that noncycling progenitors, both normal and leukemic, have a relatively high MDR expression. Triggering these cells into proliferation downregulates MDR expression. These findings can be exploited to overcome MDR in the treatment of AML patients.

DRUG RESISTANCE IN acute myeloid leukemia (AML) has been associated with the expression of the P-glycoprotein (Pgp), the phenotype of the multidrug resistance (MDR)-1 gene.[1-4] Overexpression of the MDR-related protein (MRP) in AML may also play a clinical role.[5-7] Both proteins are active transmembrane transporters, pumping a wide variety of substances including cytotoxic drugs out of the cells, thus lowering the intracellular concentration.

MDR activity can be determined by a functional assay with specific dyes. MDR expression corresponds to a low retention of rhodamine or daunorubicin and its increase by specific modulators like verapamil or cyclosporin A.[8-12] In mice, rhodamine dull (RhoD) hematopoietic progenitors appeared to be primitive noncycling cells.[13] Darzynkiewicz et al[14] and Myc et al[15] showed that rhodamine retention distinguishes between cycling and quiescent cells in peripheral blood lymphocytes (PBL) and leukemic cells. This led to the hypothesis that noncycling cells have a higher MDR expression, which may be downregulated by inducing proliferation. Previous experiments performed in our laboratory showed that proliferation induction of normal hematopoietic progenitors and leukemic blasts resulted in an increased sensitivity to anthracyclines.[16,17] However, MDR expression was not studied in these cells.

Studies on MDR expression during the different cell-cycle phases showed conflicting results. Tarasiuk et al[18] found no differences in MDR-dependent efflux during the different cell-cycle phases in K562 cells, whereas Ramachandran et al[19] showed that P388/R-84 cells in S-phase had a 3-fold higher MDR-1 mRNA content than did G_1- and G_2+M-phase cells. Also, MDR expression, determined by the monoclonal antibody (MoAb), C219, was lower in G_1 cells than in S and G_2+M cells. These experiments were performed with cell lines selected for a high MDR expression. As yet, little is known about MDR expression in cycling and in noncycling hematopoietic progenitors and the effects of proliferation induction.

Normal CD34$^+$ progenitors and leukemic blasts from patients with untreated AML were studied for functional MDR expression in relation to cell-cycle status and proliferation. RhoD CD34$^+$ cells or leukemic blast cells with a relatively high MDR expression appeared to be noncycling cells compared with the more proliferating RhoB cells with a relatively low MDR expression. RhoD cells showed less sensitivity to anthracycline toxicity. Triggering RhoD cells into proliferation by hematopoietic growth factors (HGFs) diminished MDR expression and consequently increased anthracycline toxicity.

MATERIALS AND METHODS

Isolation of Cells

CD34$^+$ cells. Bone marrow was obtained from healthy donors, after informed consent. Mononuclear cells were isolated by Ficoll 1.077 g/mL (Pharmacy Biotech, Uppsala, Sweden). CD34$^+$ cells were isolated using directly conjugated CD34 antibody-coupled immunomagnetic beads (M-450, coated with '561,' a class III epitope anti-CD34; Dynal, Oslo, Norway) according to the manufacturer's instructions (Dynal protocol). Isolation, cryopreservation, and thawing procedures of the CD34$^+$ cells have been described previously.[20]

Leukemic blasts. Bone marrow was aspirated from patients with AML and collected in sterile buffered acid citrate dextrose (pH 7.0). Mononuclear cells were isolated by Ficoll 1.077 g/mL. Cells were cryopreserved in vials containing 5 to 20 $\times$ 10^6 cells. Blast cells were gated on the flow cytometer by forward scatter and the absence of the lymphocyte markers CD3 and CD19.

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Lymphocytes. Normal donor lymphocytes were isolated from heparinized blood using Ficoll 1.077 g/mL.

Propidium Iodide (PI) DNA Staining

PI (Calbiochem, San Diego, CA) was used to measure DNA content. Sorted RhoD and RhoB cells were centrifuged (5 minutes, 1,800 rpm, 4°C). The pellet was dissolved in 300 µL of ice-cold hypotonic PI solution (20 µg/mL PI, 0.1% wt/vol trisodium citrate dihydrate (Merck, Darmstadt, Germany), 10% vol/vol RNA-se solution (RNA-se A; Sigma Chemical Co, St Louis, MO), 1 mg/mL in glucose phosphate-buffered saline (G-PBS), and 0.1% vol/vol Triton X-100 in distilled water (dH_2O). Cells were kept on ice overnight and analyzed for DNA content by flow cytometry.

Ki-67 Labeling

The nuclear antigen Ki-67 discriminates between the cell-cycle phases G_0 and G_1.[21] Sorted $CD34^+$ cells were pelleted (5 minutes, 1,800 rpm, 4°C) and subsequently fixed in 1 mL freshly made paraformaldehyde 0.5% wt/vol (5 minutes, 4°C). After washing with G-PBS 0.5% wt/vol bovine serum albumin (BSA, Fraction V, A-9418, Sigma Chemical Co), 1 mL PBS-Triton 0.1% vol/vol (Merck) was added (5 minutes, 4°C). The cells were then washed and incubated with 5 µL fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human Ki-67 MoAb (Dako, Glostrup, Denmark) (20 minutes, 4°C) in 100 µL pooled human serum (PHS). Rabbit $F(ab')_2$ FITC (Dako) was used as a control. After washing with PBS 0.5% wt/vol BSA, green fluorescence (FITC) was measured on the flow cytometer (standard setting) using a 550 dichroic and 525 band-pass filter. The staining procedure was checked with lymphocytes, as normal lymphocytes are almost all G_0 cells and Ki-67 negative, and a cell-line, which was almost 100% Ki-67 positive.

In Vivo Iododeoxyuridine Labeling and Staining Procedure

To study the proliferative state of leukemic blasts in vivo, untreated patients with newly diagnosed AML, who all gave their written informed consent, volunteered to be given Iododeoxyuridine (IdUrd) intravenously. IdUrd is a nonradioactive thymidine analogue, incorporated during DNA synthesis. Vials of 200 mg freeze-dried IdUrd were supplied by the National Cancer Institute (NCI) (Bethesda, MD) (NCI-protocol T89-0075). A nontoxic dose of 200 mg IdUrd was dissolved in 30 mL isotonic saline (0.9% wt/vol NaCl) and given as a 15-minute intravenous infusion. Between 4.6 and 7.0 hours (mean value, 5.4) after in vivo IdUrd labeling, bone marrow was aspirated in sterile buffered acid citrate dextrose (pH 7.0).[22] Mononuclear cells were isolated by Ficoll 1.077 g/mL and cryopreserved in vials containing 5 to 20×10^6 cells.

After thawing and staining, 25% of the most RhoD and RhoB blast cells were sorted, pelleted, fixed, and stained as described by van Erp et al.[23] The green fluorescence was measured after a final wash step with G-PBS 0.5% wt/vol BSA. Non-IdUrd–labeled low-density blood cells of a healthy volunteer served as negative controls. The labeling index (LI) is defined as the percentage IdUrd-labeled cells and represents the cells in S-phase of the cell cycle. A correction was made for the cells that left the S-phase because the IdUrd labeling was performed. Subsequently DNA was stained with an isotonic PI solution.

$$LI\ (\%) = \frac{IdUrd^{Nondivided} + (IdUrd^{divided} \times 0.5)}{100 - (IdUrd^{divided} \times 0.5) - (G_2 + M\ phase \times 0.5)} \times 100$$

Proliferation Induction

Sorted RhoD $CD34^+$ cells or RhoD leukemic blasts were diluted in medium A (1×10^6 cells/mL) consisting of Iscove's medium, supplemented with 2 mmol/L glutamin (Flow Laboratories, Irvine, Scotland), 50 µg/mL streptomycin, and 50 U/mL penicillin (Flow Laboratories), 20% vol/vol fetal calf serum, 5% wt/vol BSA, 0.3 mg/mL human transferrin (Sigma), 50 µmol/L 2-β-mercaptoethanol (Sigma), 20 ng/mL recombinant granulocyte colony-stimulating factor (rG-CSF) (Amgen, Thousand Oaks, CA), and 25 ng/mL recombinant stem cell factor (rSCF) (a gift from Amgen), 50 ng/mL interleukin-3 (IL-3) (Sandoz, Basel, Switzerland), 20 ng/mL recombinant granulocyte-macrophage (rGM)-CSF (Sandoz), and 1.5 U/mL recombinant erythropoietin (rEPO; Cilag, Herentals, Belgium). MDR efflux was measured after 48 hours proliferation induction.

Normal lymphocytes were exposed for 24 hours to IL-2 100 U/mL (Glaxo, Geneve, Swizerland) and phytohemagglutinin (PHA) 4 µg/mL (Murex Diagnostics, Dartford, UK) for induction of proliferation.

MDR Expression

MDR was measured in a functional rhodamine efflux assay. Rhodamine (Rh123; Sigma Chemical Co) was dissolved in dH_2O and stored as a sterile stock solution (10 µg/mL). The cells were incubated (200,000 cells/mL) in Iscove's medium supplemented with 5% wt/vol heat-inactivated fetal calf serum (FCS, Hyclone, Logan UT) with a final rhodamine concentration of 0.1 µg/mL (60 minutes, 37°C). After centrifugation, the cells were diluted in dye-free Iscove's medium with 0.5% wt/vol FCS with or without 10 µmol/L verapamil (Knoll AG, Ludwigshaven, Germany) to block efflux (2 hours, 37°C). Putting the cells on ice stopped efflux. A similar efflux assay was performed with daunorubicin (Rhone-Poulenc Rorer BV, Amstelveen, The Netherlands). This anthracycline exhibits intrinsic fluorescence and is also expelled by the MDR. Rhodamine and daunorubicin fluorescence was quantitated by flow cytometry.

Efflux modulation was expressed as rhodamine ratio (RR), the fluorescence intensity of the cellular rhodamine content in the presence of verapamil divided by the fluorescence intensity of the cellular rhodamine content in the absence of verapamil. This ratio is independent of cell size, which increases after proliferation induction.

Flow Cytometric Measurements and Single-Cell Sorting

A Coulter Epics Elite Flow cytometer, equipped with an autoclone device (Coulter, Miami, FL), was used for PI, Ki-67, and IdUrd measurement, dye retention, as well as to sort single cells for clonogenic assay. Cells were excitated with a single Argon ion laser emitting at 488 nm, running at 15 mW (standard setting). Gating on forward and right angle scatter was used to exclude dead cells and debris. Fluorescence intensity of cellular rhodamine or daunorubicin was measured using a 515 nm and a 550 nm long pass filter, respectively. After 2 hours of efflux in dye-free medium without verapamil, the distinct population of RhoD cells and 25% of the most RhoB cells of normal $CD34^+$ cells or leukemic blast were sorted to study MDR expression, proliferation (induction), or cytotoxicity. In case no distinct RhoD population could be recognized (in leukemic blasts), 25% of the most RhoD blasts were sorted.

DNA content was analyzed after staining with PI by flow cytometry using a 610-nm long pass filter. The area and peak of the red fluorescence signal was recorded in list mode. The ratio area: peak was used to discriminate artifacts due to doublets of diploid cells.

In Vitro Anthracycline Cytotoxicity

Inhibition of clonogenic capacity by anthracyclines in $CD34^+$ cells and leukemic blasts with a relatively low (RhoB) or high (RhoD) MDR expression before and after prestimulation with HGFs was quantified using a single-cell clonogenic assay. A single cell was sorted in each well from round bottom 96-wells plates (Costar no. 3799, Cambridge, MA) prefilled with 75 µL liquid medium A to which daunorubicin or idarubicin (Pharmacia & Upjohn, Milano, Italy) were added at increas-

ing concentrations (range, 0.0001 to 0.1 μg/mL) and cultured in a fully humidified atmosphere (37°C, 5% CO_2). Wells were analyzed by counting the cells at days 4, 11, and 18 providing information on the clonogenic capacity and duration of proliferation. At day 18, colonies (>50 cells/well) of $CD34^+$ cells were classified as small size (50 up to 500 cells/well), medium size (500 up to 5,000 cells/well), or large size (>5,000 cells/well). In normal $CD34^+$ cells, the relative number of colonies (>50 cells/well) was plotted, in AML, the relative number of clusters and colonies (>10 cells/well). The 50% inhibitory concentration (IC50) of normal $CD34^+$ cells and leukemic blasts was calculated from the plotted dose-response curves.

Colonies (>50 cells/well) derived from normal CD34 cells were registered as granulocytes/monocytes, erythrocytes, or mixed. Morphologic analysis of large colonies was checked by immunostaining with the MoAb's glycophorine-Pe (Coulter) to identify erythrocytes, CD-14 PE (Becton Dickinson [BD] BV, Etten-Leur, The Netherlands) to identify monocytes and CD15-FITC (BD) to identify granulocytes.

Statistics

The Wilcoxon Mann Witney U-test or Student's *t*-test was applied for statistical analysis of the results.

RESULTS

Cell-Cycle Status of RhoD Versus RhoB Cells

Normal blood lymphocytes were used to show that the technique PI staining and Ki-67 labeling is adequate. DNA staining of lymphocytes with PI showed that greater than 90% of the lymphocytes were in G_0/G_1 and less than 1% were positive for the proliferation marker Ki-67. After proliferation induction, the fraction of Ki-67 positive lymphocytes increased to greater than 95%, while greater than 40% of cells were in S/G_2+M.

DNA staining of $CD34^+$ cells showed that 5% of the RhoD cells were in S/G_2+M-phase (4.5% ± 2.2%, n = 5), compared with 22% of the RhoB fraction (21.5% ± 4.4%, n = 5). An example of DNA analysis of $CD34^+$ cells is shown in Fig 1. Rhodamine and daunorubicin staining showed a similar distribution. A total of 1.6% of the daunorubicin dull cells and 29.9% of the daunorubicin bright cells were in S/G_2+M. The proliferation marker Ki-67 was higher in the RhoB than in the RhoD $CD34^+$ cells, 75% and 43%, respectively, but differences were much less extreme compared with lymphocytes.

In leukemic blasts, PI staining also showed that the percentage S/G_2+M was significantly higher in the RhoB leukemic blasts (31.1% ± 11.7%) (n = 8) compared with the RhoD blasts (10.6% ± 3.2%) (P = .008). The relevance of these in vitro findings was confirmed by in vivo data. After in vivo administration of IdUrd, the LI of the RhoD blasts (4.8% ± 2.1%) was significantly lower (P = .001) compared with the RhoB blasts (15.0% ± 6.0%) (Fig 2).

MDR Expression on RhoD and RhoB Cells

MDR expression reduces cellular rhodamine content by efflux. A low rhodamine retention was observed in a distinct population of 20% to 30% of normal $CD34^+$ cells. Blocking the efflux by verapamil confirmed the presence of functional MDR (Fig 3A).

Rhodamine retention was studied in 10 different samples of AML patients. Patient characteristics are summarized in Table 1. Rhodamine retention in AML blasts was heterogeneous, and a more or less distinct RhoD population could be recognized in most samples. Figure 3B shows an example (patient no. 3) with a large distinctive RhoD population, whereas Fig 3C shows an example (patient no. 1) with only a small RhoD population. Increased rhodamine retention in the presence of verapamil confirmed MDR expression by these blast cells.

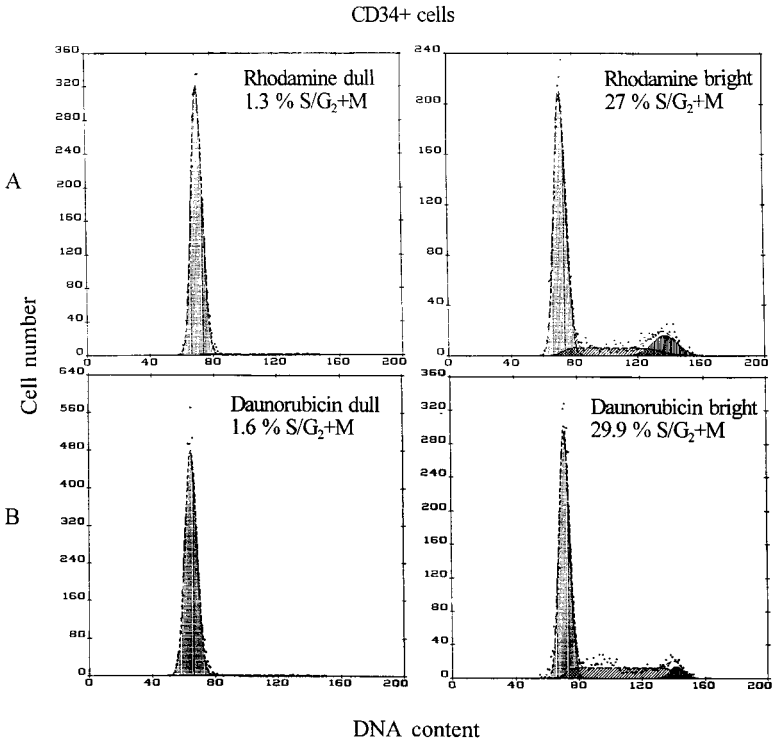

Fig 1. After the rhodamine (A) or daunorubicin (B) efflux assay rhodamine or daunorubicin dull and 25% of the most rhodamine or daunorubicin bright $CD34^+$ cells were sorted and stained with PI. A representative example of the DNA content of sorted subfractions is presented.

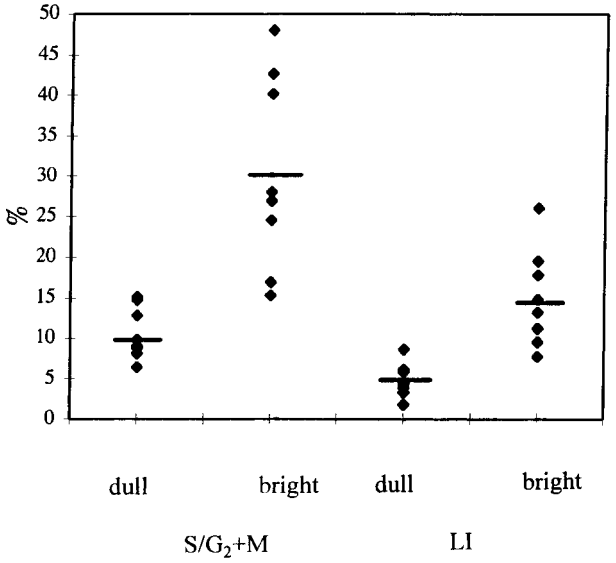

Fig 2. Patients with newly diagnosed AML received intravenously IdUrd. After the rhodamine efflux assay, 25% of the most RhoD and RhoB leukemic blasts were sorted. The sorted subfractions were stained and the IdUrd LI could be calculated. PI was used to measure DNA content. Percent S/G_2+M and the IdUrd of the RhoD cells was significantly lower ($P = .008$ and $P = .001$, respectively) compared with the RhoB cells.

MDR Expression After Proliferation Induction

Exposure of RhoD and RhoB $CD34^+$ cells to HGFs for 48 hours resulted in an increase of the proportion of S/G_2+M in both fractions. The fraction of S/G_2+M increased from 4% to 40% in RhoD and 17.5% to 39% in RhoB cells. After proliferation induction of the RhoD cells (Fig 4A1), a second rhodamine efflux assay was performed. The cellular rhodamine content (Fig 4A2) was higher than before the proliferation induction. Again, the most RhoD and RhoB cells after stimulation with HGFs were sorted. In the example shown in Fig 4A2, 15% of the RhoD and 32.2% of the RhoB cells were in S/G_2+M.

Table 1. Patients' Characteristics

No.	Morphology	Cytogenetics	% Blasts	CD34*	% RhoD	Clinical Response†
1.	AML-M1	Normal	59	neg	<10	CCR
2.	AML-M2	Normal	78	neg	<10	Rel within 6 mo
3.	AML-M4	inv (3), −7	71	pos	33	PRes
4.	AML-AUL	num ab	63	neg	27	PRes
5.	AML-M4	Normal	57	neg	<10	PRes
6.	AML-M2	t(7;11)	29	pos	<10	CCR
7.	AML-M3	t(15;17)	64	neg	17	CCR
8.	AML-M4	t(8;21)	62	pos	14	Rel after 2 yr
9.	AML-M5b	Normal	64	neg	18	Rel within 1 yr
10.	AML-M2	t(8;21)	87	pos	11	Rel after 2 yr
11.	AML-M4	num ab	76	ND	24	PRes

Abbreviations: CCR, continued complete remission; PRes, primary resistant; Rel, relapse; num ab, numerous aberrations; pos, positive; neg, negative; ND, not done.

*Positive: >30% CD34 expressing cells.

†Clinical response after anthracycline-containing regimen.

Staining with daunorubicin showed a less distinct daunorubicin dull $CD34^+$ population (Fig 4B1). The daunorubicin dull cells were sorted and exposed to HGFs. In agreement with the results after rhodamine staining, the cellular daunorubicin content was higher after stimulation with HGFs (Fig 4B2). To exclude the effect of stimulation with HGFs on the cellular size and thus fluorescence, a relative rhodamine ratio (RR) was calculated. RR was defined as the fluorescence intensity of the cellular rhodamine content in the presence of verapamil divided by the fluorescence intensity of the cellular rhodamine content in the absence of verapamil. The RR was significantly higher ($P = .009$, $n = 6$) in the RhoD cells compared with the RhoB cells. After triggering RhoD cells to proliferate with HGFs, the RR was significantly lower ($P = .04$, $n = 6$) compared with the nonstimulated RhoD cells (Fig 5).

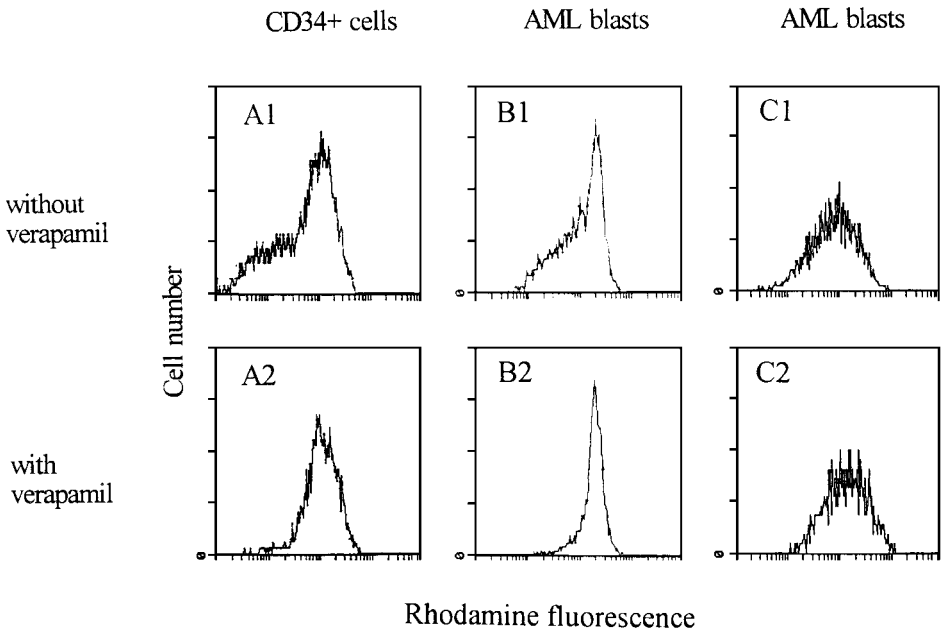

Fig 3. After a 1-hour incubation with rhodamine, cells were exposed for 2 hours to dye-free medium without (1) or with (2) verapamil to allow rhodamine efflux. Distribution of rhodamine content of normal $CD34^+$ cells (A) and AML blasts (B and C) was measured by flow cytometry. Efflux inhibition by verapamil (2) resulted in an increase of rhodamine content in cells with MDR expression.

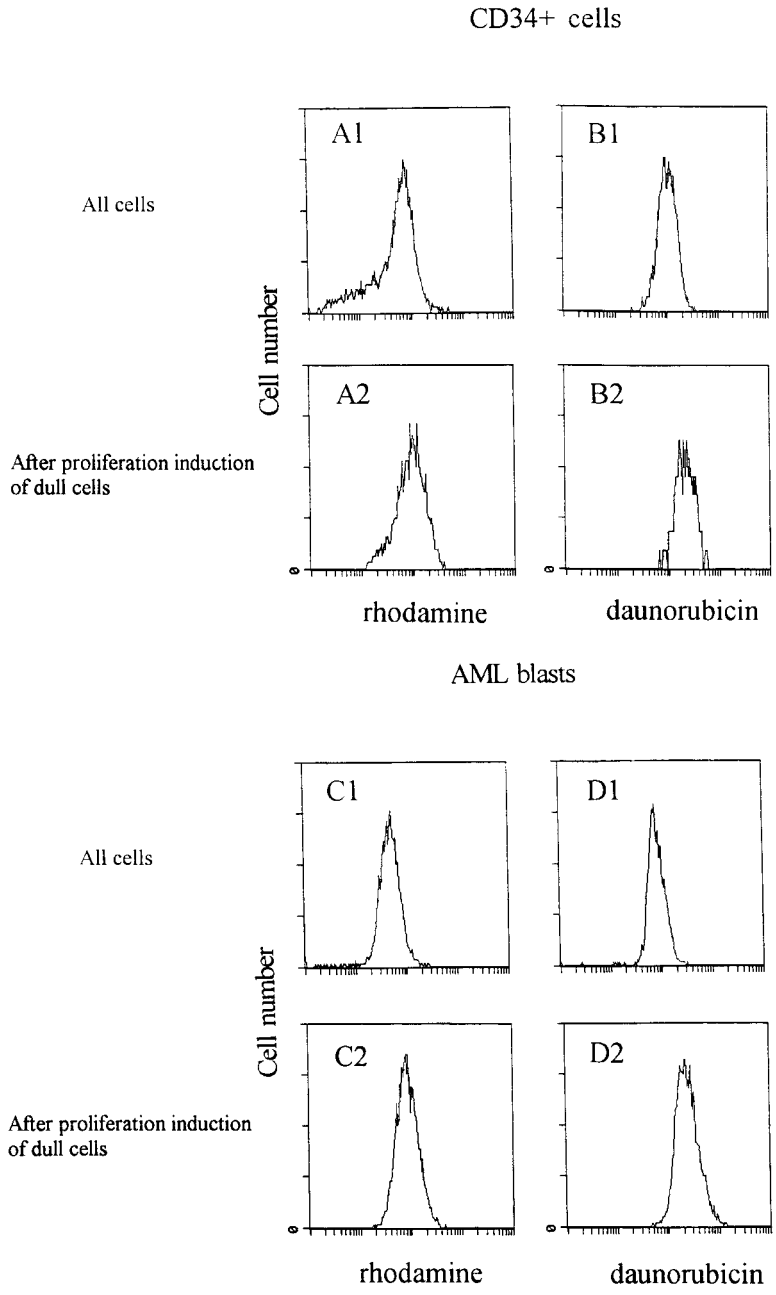

Fig 4. Distribution of rhodamine (A and C) or daunorubicin (B and D) content in normal CD34+ cells or AML blasts after the rhodamine/daunorubicin efflux assay (1). The 25% most rhodamine/daunorubicin dull cells were sorted and triggered to proliferate by exposure to HGFs for 48 hours (2). After proliferation, induction cellular rhodamine/daunorubicin was higher compared with the sorted rhodamine/daunorubicin dull cells.

The RhoD population in AML blasts varied from patient to patient sample. The example depicted in Fig 4 shows a small rhodamine (Fig 4C1), respectively, daunorubicin (Fig 4D1) dull fraction. After proliferation induction of the rhodamine/daunorubicin dull cells, an increased cellular rhodamine/daunorubicin content was seen in the efflux assay (Fig 4C2 and D2).

The effect of verapamil on rhodamine retention in AML blasts with a large and small RhoD population, respectively, is illustrated in Fig 6A and C. In the presence of verapamil, cellular rhodamine content increased in the RhoD fraction. After sorting and exposure of the RhoD cells to a cocktail of HGFs, an increase in rhodamine content was seen only in a subfraction of the stimulated cells (Fig 6A2 and C2). This subfraction of cells with an increased rhodamine content after proliferation induction showed a decrease to negligible efflux modulation (Fig 6B2 and D2). In contrast, cells with a low rhodamine content after proliferation induction maintained their susceptibility to efflux modulation. Sorting the cells with a relatively low rhodamine content after proliferation induction (Fig 6A2) showed that 4.2% were in S/G_2+M-phase, in contrast to 21.2% of the cells with the relatively high rhodamine content.

Anthracycline Toxicity in RhoD and RhoB Cells and After Prestimulation of RhoD Cells With Growth Factors

To confirm MDR downregulation after induction of proliferation, anthracycline toxicity of normal CD34+ RhoD cells before and after prestimulation with HGFs was studied. Prestimulation

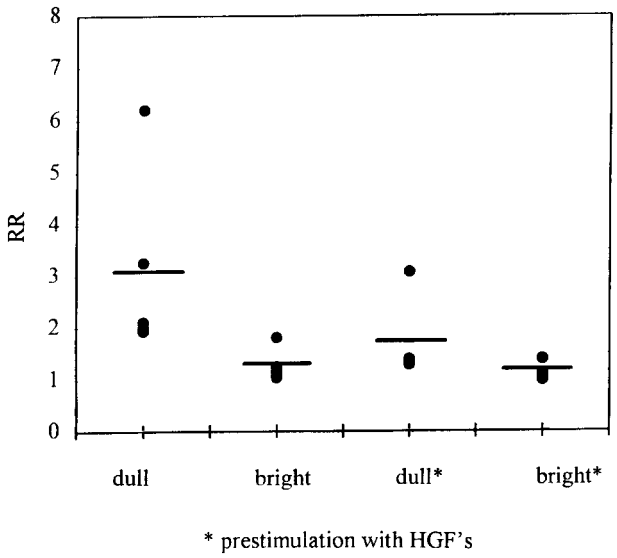

Fig 5. Efflux modulation of rhodamine by verapamil is quantified by the RR. RR = the fluorescence intensity of the cellular rhodamine in the presence of verapamil: the fluorescence intensity of the cellular rhodamine content in the absence of verapamil. The RR was measured in RhoD and RhoB CD34$^+$ cells and after prestimulation of these cells for 48 hours in vitro with HGFs. The RR of RhoD CD34$^+$ cells was significantly higher (P = .009) compared to the RR of RhoB cells. The RR of RhoD CD34$^+$ cells was also significantly higher (P = .04) compared to RhoD cells prestimulated with HGFs.

of RhoD cells with HGFs resulted in an increased sensitivity to anthracyclines. IC50s for daunorubicin (Fig 7A) and idarubicin (Fig 7B) of RhoD cells decreased from 0.006 μg/mL to 0.004 μg/mL and from 0.006 μg/mL to 0.003 μg/mL, respectively, after prestimulation. After prestimulation of RhoD cells with HGFs for 48 hours, these cells become RhoB. Nevertheless, these RhoB cells are different from the RhoB cells in the initial sample. Clonogenic capacity of the RhoD, RhoB, and prestimulated RhoD normal progenitors were compared to discriminate between the effects of proliferation induction and possible differentiation into RhoB cells after exposure to HGFs. Single RhoB cells developed significantly fewer and smaller colonies ($P < .005$) compared with both RhoD cells and RhoD cells after prestimulation with HGFs. The mean values of single-cell–derived colonies/96-wells plate for RhoD, RhoB, and RhoD cells prestimulated with HGFs were, respectively, 5.7, 12, and 11 for small; 7.8, 14.2, and 15.5 for medium; and 21.5, 5, and 13.8 for large colonies. Furthermore, the RhoD cells and prestimulated RhoD cells showed significantly more colonies with mixed differentiation ($P < .05$) than RhoB cells. Differentiation into mixed colonies was similar for RhoD cells before and after prestimulation with HGFs. Prestimulation of RhoD cells with HGFs shifted only the differentiation pattern of the colonies from granulocyte/monocyte to more erythroid colonies ($P < .005$). The mean values of erythroid and granulocyte/monocyte colonies/96 wells plate were, respectively, 6.8 and 21.8 for RhoD; 5 and 24.7 for RhoB cells; and 25.3 and 20 for RhoD cells after prestimulated with HGFs. Proliferation induction of RhoD cells did not influence the outgrowth of mixed colonies. The mean values of mixed colonies of RhoD, RhoB, and RhoD cells after proliferation induction were 6.3, 1.5, and 9, respectively (Table 2). Immunostaining of colonies with MoAbs confirmed the morphologic analysis after May-Grünwald-Giemsa staining (data not shown).

The outgrowth of leukemic blasts was highly variable, as known from agar cultures. Because the number of colonies was too low, cell aggregates with more than 10 cells were counted to analyze differences in anthracycline cytotoxicity between RhoD, RhoB, and stimulated RhoD blasts. The percentage RhoD cells in the tested AML samples varied from less than 10% to 33% (Table 1) and proliferation induction of RhoD blasts was not always complete (Fig 6). We selected 2 patients with sufficient outgrowth for these experiments. Figure 8A and B show the difference in daunorubicin cytotoxicity between RhoD and RhoB leukemic blasts: the IC50 was 0.008 μg/mL and 0.006μg/mL of RhoD cells and 0.004 μg/mL and 0.003 μg/mL of RhoB cells of patient no. 1 and patient no. 5, respectively. The effect of proliferation induction on anthracycline toxicity in leukemic blasts is illustrated in Fig 8B. The daunorubicin IC50 of RhoD cells decreased from 0.006 μg/mL to 0.003 μg/mL after prestimulation with HGFs.

DISCUSSION

In this study, we analyzed the relation between MDR expression and cell cycle and the effect of proliferation induction on MDR expression. Our experiments show that RhoD blasts, both normal and leukemic, have a higher MDR expression, are less sensitive to anthracycline toxicity, and more in G_0/G_1-phase of the cell cycle than RhoB blasts. Triggering these RhoD cells into proliferation increased rhodamine retention and anthracycline sensitivity and decreased efflux modulation, confirming MDR downregulation.

The functional rhodamine efflux assay with and without a MDR modulator has proven to be the most sensitive method to detect MDR expression, especially in small subpopulations.[20,24] A relatively high efflux was observed in 20% to 30% of the normal CD34$^+$ hematopoietic progenitor cells. These RhoD CD34$^+$ cells appeared to be predominantly in G_0/G_1-phase. In contrast, the RhoB cells were cycling with greater than 20% cells in S/G_2+M. To discriminate G_0 and G_1 cells, cells were labeled with the nuclear proliferation marker Ki-67. In normal bone marrow CD34$^+$ cells, Ki-67 labeling in RhoD and RhoB cells was 43% versus 75%, respectively. Thus, the percentage G_0 cells in the RhoD population was higher (57%) than that in the RhoB population (25%), although differences were less extreme compared with nonstimulated versus stimulated lymphocytes. Less than 1% of the lymphocytes were Ki-67 positive before and more than 95% of the cells were Ki-67 positive after proliferation induction. Similar problems with Ki-67 labeling of bone marrow cells have also been observed by van Bockstaele et al,[25] who suggested that the relatively low percentage Ki-67 positivity observed in the RhoB cells might be explained by differentiation of the cells and a lower Ki-67 expression during the S-phase. Other proliferation markers, such as MIB and proliferating cell nuclear antigen (PCNA), also did not distinguish clearly between G_0 and G_1 (data not shown).

Triggering noncycling CD34$^+$ cells to proliferate resulted in a higher cellular retention of rhodamine and daunorubicin. Proliferation induction is followed by increase in cell size, number, and activity of organelles, such as mitochondria and

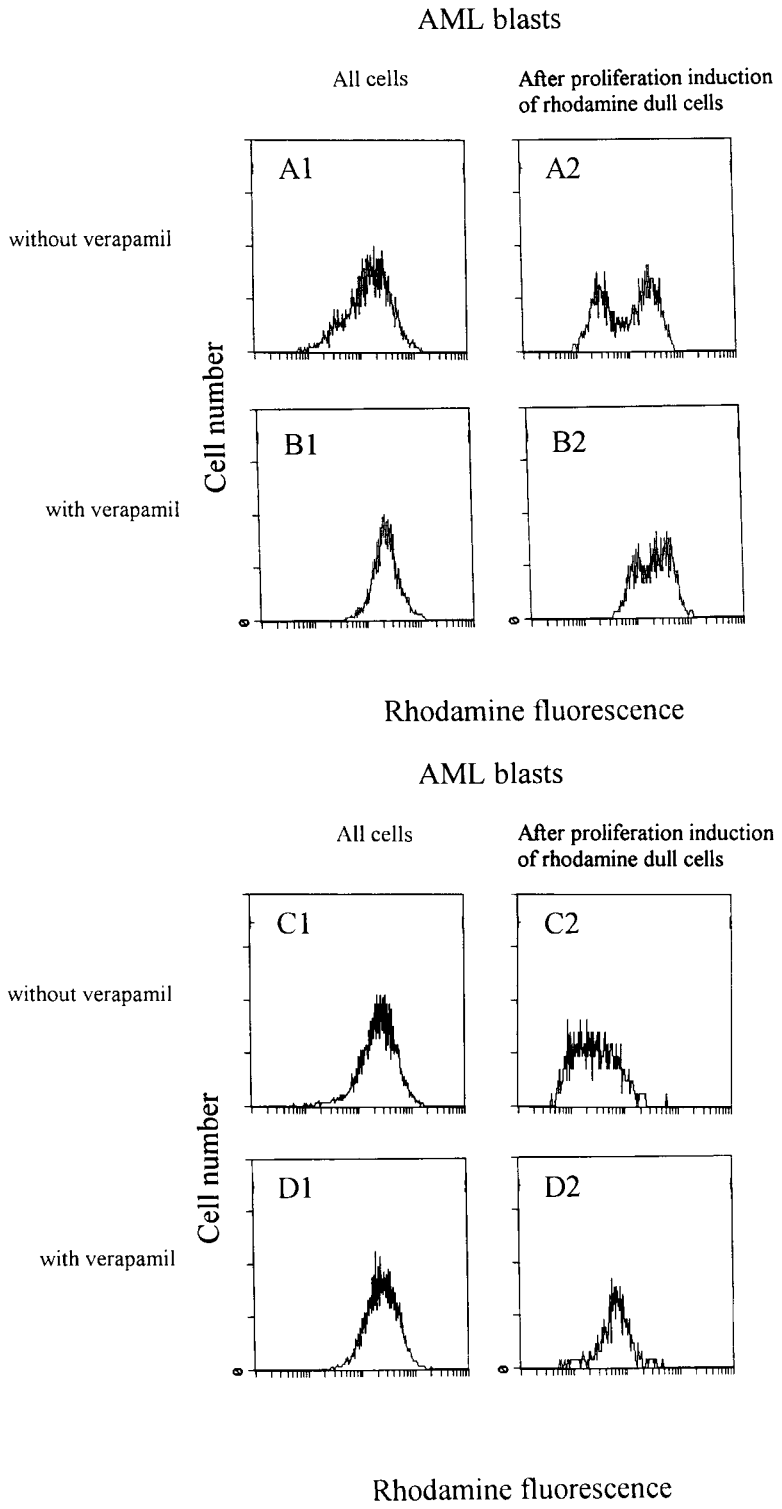

Fig 6. Two examples of rhodamine content (A and C) in AML blasts after the rhodamine efflux assay with (2) or without (1) verapamil in the efflux medium. Efflux inhibition by verapamil caused a higher cellular rhodamine content predominantly in the RhoD cells. RhoD blasts were sorted and triggered to proliferate by exposure to HGFs for 48 hours. Effective proliferation induction resulted in an increase of cellular rhodamine content (A2 and C2). In these samples, only a subpopulation of the blasts became rhodamine bright after proliferation induction. Efflux modulation by verapamil was negligible in the rhodamine bright cells. The persisting rhodamine dull cells kept their sensitivity to efflux modulation by verapamil (B2 and D2).

proteins, and therefore may increase cellular fluorescence signal on the flow cytometer independent of a change in intracellular concentration. Darzynkiewicz et al[14] postulated that rhodamine retention discriminates between cycling and quiescent cells due to increased mitochondria binding. However, activity and membrane potential of the mitochondria do not influence rhodamine efflux. Furthermore, the higher retention of anthracyclines after induction of proliferation cannot be explained by mitochondrial activity. To quantify MDR expression independent of cell size, we calculated a ratio of MDR-mediated efflux (RR). Proliferation induction resulted both in a higher cellular rhodamine content and a significantly lower RR.

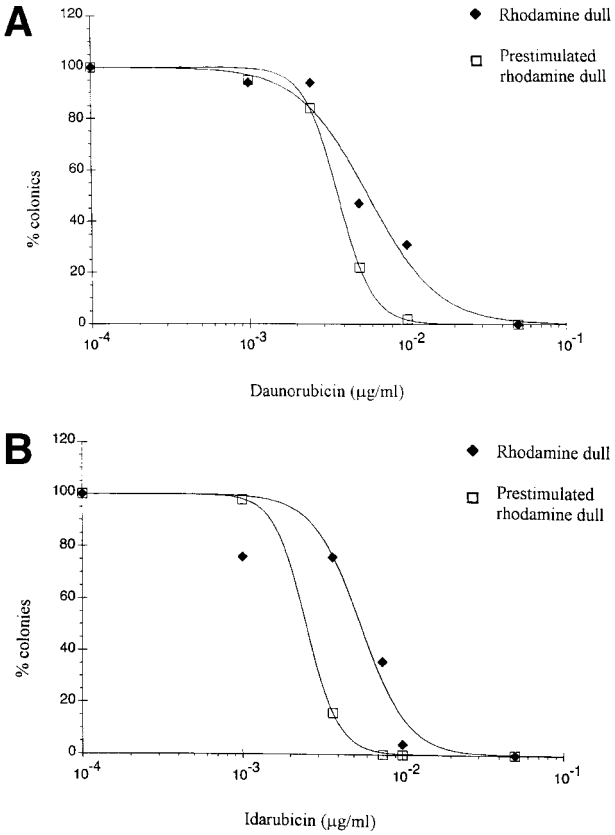

Fig 7. Dose-response curve of daunorubicin (A) and idarubicin (B). In a single-cell clonogenic assay, RhoD CD34+ cells before and after prestimulation in vitro with HGFs for 48 hours were exposed to increasing concentrations of anthracycline. Proliferation was expressed as the percentage colonies (>50 cells/well) in medium A with daunorubicin/idarubicin compared with the control (medium A without daunorubicin/idarubicin). The IC50s of daunorubicin (A) and idarubicin (B) of RhoD cells were 0.006 μg/mL and 0.006 μg/mL before and 0.004 μg/mL and 0.003 μg/mL, respectively, after proliferation induction.

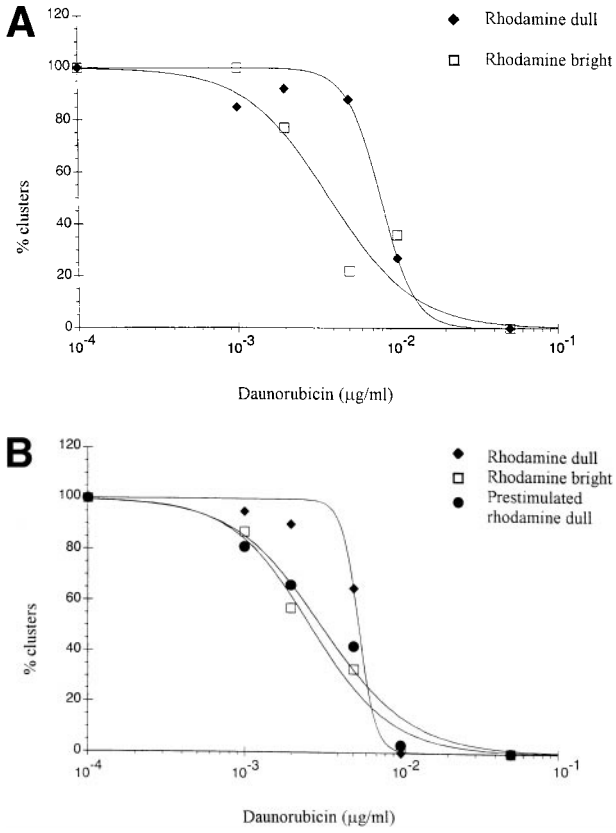

Fig 8. Dose-response curves of daunorubicin. In a single-cell clonogenic assay, RhoD and RhoB leukemic blasts and prestimulated RhoD cells with HGFs for 48 hours were exposed to increasing concentrations of anthracycline. Proliferation was expressed as the percentage clusters (>10 cells/well) in medium A with daunorubicin compared with the control (medium A without daunorubicin). The IC50 of daunorubicin of RhoD and RhoB cells of patient no. 1 (A) were 0.008 μg/mL and 0.004 μg/mL and of patient no. 5 (B) 0.006 μg/mL and 0.003 μg/mL, respectively. The IC50 of daunorubicin of RhoD cells of patient no. 5 decreased from 0.006 μg/mL to 0.003 μg/mL after prestimulation with HGFs (B).

A high percentage of the clonogenic leukemic myeloblasts are non or slowly proliferating cells.[26,27] In AML blasts, the LI of IdUrd and the percentage of cells in S/G_2+M was correlated with rhodamine retention. Similar to normal progenitors, triggering RhoD leukemic blasts to proliferate induced an increase of rhodamine retention and decreased efflux modulation.

Table 2. Clonogenic Capacity of CD34+ Cells

	Distribution of Colony Size and Morphology of Single Cell-Derived Colonies*		
Colony Size	Dull %	Bright %	Stimulated Dull %
<500 cells	16.2	38.2†	17.3
500-5,000 cells	23	46.2†	27.3
>5,000 cells	60.8	15.7†	55.4
Mixed colonies	17.5	5.3‡	14.7
Erythrocytes	19	18.4	45.8†
Granulocytes/monocytes	63.5	79.3	39.5‡

*n = 6.
†$P < .005$.
‡$P < .05$.

We previously reported that normal RhoD CD34+ cells appeared more resistant to anthracycline toxicity in a clonogenic assay than RhoB cells.[20] Triggering RhoD CD34+ cells to proliferate resulted in an increased anthracycline toxicity, confirming MDR downregulation.

Butturini et al[27] and studies performed in our laboratory also demonstrated a cell-cycle–dependent anthracycline toxicity.[16,17] Minderman et al[17] triggered leukemic blasts into proliferation by stimulation with human placental conditioned medium (HPCM) for 48 hours. Doxorubicin IC50 was 0.103 μg/mL before preincubation with HPCM and 0.055 μg/mL after preincubation. The enhancement of cytoxicity after preincubation with HPCM showed a strong trend ($P < .06$) in the matched-pair analysis. Because the percentage RhoD blasts was limited in the tested AML samples, we used a single-cell assay to study anthracycline toxicity in RhoD and RhoB cells. Clonogenic capacity of the tested AML samples showed great variation, as known from agar cultures. We could demonstrate that similar to the results in normal CD34+ cells, RhoD leukemic blasts are protected against anthracycline toxicity.

Prestimulation of RhoD blasts with HGFs resulted in an increased anthracylcline sensitivity.

The size of the RhoD population appeared to be highly variable in the different AML patients. A small subpopulation with high MDR expression may be responsible for treatment failure and relapse. te Boekhorst et al[28] have shown that resistant cells in AML have a high proliferative capacity and these cells may determine the response to therapy.

In 2 patients with AML, Drach et al[29] reported that IL-3 and G-CSF induced downregulation of MDR expression in vivo. Multiple studies showed that HGFs, particularly IL-3, GM-CSF, and G-CSF, can stimulate the proliferation of leukemic myeloblasts.[30-33] van der Lely et al[34] showed that induction of proliferation in AML blasts in vitro by HGFs was effective, but variable in subsets of leukemic blasts. Drach et al failed to observe any effect of cytokines on MDR expression in normal $CD34^+$ cells. MDR expression was studied after exposure for 24 hours to single HGFs by means of a semiquantitative polymerase chain reaction (PCR) in the overall population of $CD34^+$ cells. We used the functional rhodamine efflux assay, which enables identification of a small MDR expressing subpopulation. MDR downregulation might become obscured by analysis of the whole $CD34^+$ population. Moreover, Drach et al[29] did not study the effect of cytokines on proliferation or cell cycle. Our results show that a combination of cytokines resulted in an increase in S/G_2+M. The optimal cocktail of HGFs for proliferation induction in AML blasts is not known. Therefore, we used a cocktail of HGFs to induce normal and leukemic blasts into proliferation and evaluated the effect on proliferation and rhodamine efflux. Proliferation and rhodamine retention already increased after 24 hours, but was more obvious after 48 hours. The cocktail of HGFs was rather effective in normal progenitors; in some AML blasts, the cocktail was not optimal. This explains that in AML, it is more difficult to show the effect of proliferation induction and MDR downregulation.

To answer the question whether MDR downregulation after 24 to 48 hours growth factor exposure was due to induction of proliferation or differentiation into RhoB progenitors, we cultured the RhoD cells after prestimulation with HGFs for 48 hours. In terms of proliferation, this means 1 or 2 cell divisions.[35] A primitive RhoD progenitor, which gives rise to large colonies, will not have undergone extensive differentiation after 1 to 2 cell divisions. The proliferative capacity of these prestimulated RhoD cells remained unchanged, comparable to the nonprestimulated RhoD cells and higher than that of the RhoB cells. We did observe a change in the differentiation pattern to more erythroid colonies and less granulocyte/monocyte colonies after prestimulation of RhoD cells.

Several biologic mechanisms may contribute to anthracycline resistance in AML. Therefore, a multifactorial approach to the treatment of resistant AML seems rational. Specific MDR modulators have been tested in therapeutic schedules in an attempt to improve the efficacy of anthracyclines without any improvements of therapeutic results.[36-40] MDR modulators are currently being investigated for their efficacy in randomized trials in AML. Our results suggest that antileukemic therapy might be even more effective when MDR modifiers are combined with HGFs. Some preliminary data support the relevance of HGFs in clinical studies.[41-44] However, the sensitivity of AML blasts in vivo for HGFs can be variable. The persistence of a RhoD subfraction after proliferation induction of AML blasts might still be responsible for treatment failure. Because the primitive $CD34^+$ hematopoietic progenitors are also triggered and will become more sensitive to anthracyclines, bone marrow hypoplasia might also be made extensive.

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