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Multiparametric analysis of apoptotic and multidrug resistance phenotypes according to the blast cell maturation stage in elderly patients with acute myeloid leukemia

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Background and Objectives. Acute myeloid leukemia (AML) is a heterogeneous group of malignant diseases, often characterized by coexistence of more than one subpopulation of blast cells. Multiparametric flow cytometry immunophenotyping has proven to be a reliable and sensitive approach for the discrimination of myeloid blast cells from residual normal cells present in bone marrow samples from AML patients and, at the same time, allows the identification of different maturation compartments among myeloid blasts. Therefore, it provides a unique tool for assessing apoptotic and multidrug resistance (MDR)-associated phenotypes in individual subsets of leukemic cells.

Design and Methods. The aim of the present study was to explore the simultaneous expression of proteins related to both apoptosis (APO2.7, bcl-2, bax) and multidrug resistance (MDR1, MRP, LRP) in the different blast cell subpopulations detected at diagnosis in a group of 72 elderly patients with AML. In addition, we included 5 bone marrow samples from healthy adult donors in the analysis.

Results. Immature blast cells ($CD34^+$: subset I) showed a significantly higher level of bcl-2 expression (p <0.0001) together with a lower reactivity for APO 2.7 (p=0.02) as compared to the other more mature $CD34^-$ cell subsets. The expression of Bax parallelled that of APO 2.7, although the difference between immature $CD34^+$ blast cells and the mature blast cell subsets did not reach statistical significance (p=0.18). These results translated into a significantly (p<0.0001) higher bcl-2/bax ratio for the $CD34^+$ blast cells as compared to that of the two $CD34^-$ blast cell subpopulations. Regarding the expression of the multidrug resistance-associated proteins Pgp and MRP, $CD34^+$ blast cells displayed a greater expression of both proteins as compared to the more mature $CD34^-$ AML blast cells, but differences according to maturation stage of AML blast cells did not reach statistical significance. In contrast, LRP expression was significantly lower in the more immature $CD34^+$ blast cell subset than in the more mature ones (p=0.01).

Interpretations and Conclusions. As far as normal bone marrow is concerned our results suggest that all blast cell subpopulations are more protected from apoptosis than their normal counterparts. We conclude that in elderly patients with AML the more immature blast cells are more resistant to apoptotic processes, which could explain why, when AML relapses, the blast cells frequently display a more immature phenotype than that observed at diagnosis. Contradictory results in multidrug resistance profile support the hypothesis that failure to respond to chemotherapeutic drugs in AML is a multifactorial phenomenon.

Key words: AML, apoptosis, drug-resistance, immunophenotyping.

Acute myeloid leukemia (AML) is a heterogeneous group of malignant diseases, often characterized by coexistence of more than one subpopulation of blast cells.[1] Although current treatment strategies induce high rates of morphologic complete remission (mCR), a high proportion of patients will eventually relapse. Fur-

thermore, at relapse, it is not uncommon that blast cells display more immature immunophenotypic features than they do at diagnosis.[2,3] These findings suggest that immature blast cells may be more resistant to chemotherapy and more efficiently protected against undergoing apoptosis[4,5] than differentiated blast cells. In this sense, several reports have shown that AML cases positive for CD34 antigen frequently display greater expression of anti-apoptotic proteins, as well as markers of drug resistance.[6-15] However, these reports have not been based on multiparametric analysis, by which individual cells may be simultaneously analyzed for the expression of particular antigens (thereby defining their stage of maturation) and markers of multidrug resistance (MDR) and/or apoptosis-associated proteins.

At present, it is well known that normal cells also express drug resistant associated proteins,[16-20] and their pattern of expression varies according to the cell lineage and maturation stage.[16-20] Therefore, upon analyzing apoptosis and MDR-associated markers in AML patients, it would be mandatory to discriminate between leukemic and normal cells. Multiparametric flow cytometry immunophenotyping has proven to be a reliable and sensitive approach for the discrimination of myeloid blast cells from residual normal cells presented in bone marrow samples from AML patients; at the same time it allows the identification of different maturation compartments among myeloid blasts.[1] Therefore, it provides a unique tool for assessing apoptotic and MDR-associated phenotypes in individual subsets of leukemic cells. Among patients with AML, the elderly usually have an unfavorable prognosis which may be related to a failure in drug-induced apoptosis,[21] representing a unique model for the analysis of apoptosis and MDR-associated phenotypes.

The aim of the present study was to explore the simultaneous expression of proteins related to both apoptosis (APO2.7, bcl-2, bax) and multi-drug resistance (MDR1, MRP, LRP) in the different blast cell subpopulations detected at diagnosis in a group of 72 elderly patients with AML.

Design and Methods

A total of 72 previously untreated elderly patients (> 65 years of age) with *de novo* AML were included in the present study. In all cases, bone marrow (BM) samples were collected in EDTA anticoagulant and processed within the first 18 hours after collection. The percentage of blast cells ranged from 35% to 97% (median 72%). It should be noted that only cases in which unequivocal discrimination between leukemic blast cells and other cells could be made were included in this study. M3 cases were excluded from the study, and the distribution of the series, in accordance with the FAB classification, was as follows: M0 16%; M1 15%; M2 18%; M4 16%; M5 25%; M6 5%; M7 3%; and not classifiable 2%. In addition, 5 bone marrow samples from healthy adult donors were included in the study as a control.

Whole BM samples (approximately 1×10^6 cells in 100 μL/test) were analyzed using a four-color direct immunofluorescence technique. The following combinations of monoclonal antibodies (MoAb) were used (FITC, PE, PECy5, APC): CD32/ APO2.7/ CD34/CD45; bcl-2/ CD32/ CD34/ CD45; bax/ CD32/ CD34/CD45; CD19/ MDR1/CD34/CD33; MRP/CD34/ CD19/CD33; LRP/ CD34/CD19/CD33). This multiparametric flow cytometric strategy allows us to discriminate between leukemic and normal residual cells present in each sample, as well as to identify the maturation stage of the different blast cell subsets found. Accordingly, three different stages of maturation were defined for myeloid blast cells, according to their pattern of antigen expression: subpopulation I ($CD34^+$) -the most immature-, subpopulation II ($CD34^-$, $CD32^{-/+}$, $CD33^+$), and subpopulation III ($CD34^-$, $CD32^{+/++}$, $CD33^{++}$), the most mature (Figure 1). Regarding the relationship between FAB classification and the three previously defined subpopulations, among the more immature cases (M0 and M1) subpopulation I ($CD34^+$) predominated, either as a single cell component (50% of M0 and 44% of M1 cases), or associated with subpopulation II (30% and 20% of M0 and M1 cases, respectively). In M2 leukemias, subpopulations I and II were equally distributed while in monocytic leukemias population III was strongly represented (60% of cases), and interestingly, subpopulation I was never detected as a single component.

Apoptosis and multidrug resistance-related proteins were analyzed with the following MoAb reagents for the detection of intracellular proteins: anti-APO2.7-PE (clone 2,7A6A3; Beckman-Coulter, Miami, FL, USA) and anti-Bax (clone 4F11; Beckman-Coulter), anti-bcl-2-FITC (clone 124; DAKOPATTS, A/S, Glostrup, Denmark), anti-MRP (clone MRPm6; CHEMICON Temecula, CA, USA) and anti-LRP (clone LRP-56; CHEMICON). MDR1 expression on the cell surface was assessed using an anti-Pg-p (clone 15D3; Becton Dickinson, San José, CA, USA). MoAbs directed against surface antigens used for the identification of blast cells, and their discrimination from

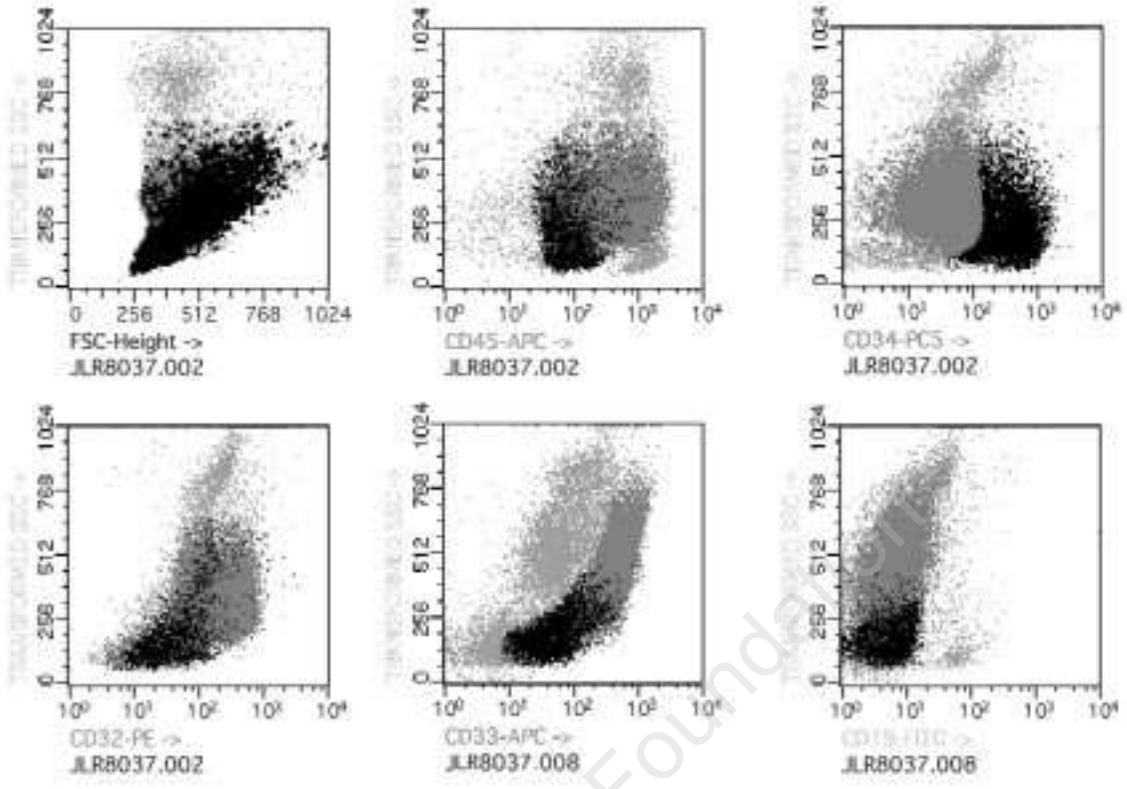

Figure 1. AML case in which previously defined subsets I and III were detected among the myeloid blast cells. Subset I (in black), accounting for 30% of total blast cells, was $CD34^{+}CD45^{dim+}$ $CD32^{-/dim+}$ $CD33^{-/dim+}$ and $CD19^{-}$; and subset III (in dark grey), accounting for 60% of total blast cells, was $CD34^{-}CD45^{+/++}$ $CD32^{dim/++}$ $CD33^{++}$ and $CD19^{-}$ (normal residual cells in light grey).

normal residual cells included: CD32-FITC and CD32-PE from SEROTEC (Oxford, UK); CD19-FITC, CD34-PE, and CD33-APC from Becton Dickinson; CD19-PECy5, and CD34-PECy5 from Beckman-Coulter, and CD45-APC from Caltag Laboratories (San Francisco, CA, USA).

For surface stainings, BM samples were processed as previously described.[1,14] Briefly, 100μL of sample/tube were incubated for 30 min with the appropriate combination of MoAb at room temperature (RT) in the dark (for up to 30'). Once this incubation period was finished, 2mL of FACS lysing solution (Becton Dickinson) diluted 1:10 (vol/vol) in distilled water was added, in order to lyse erythrocytes. After another 10 min incubation in the dark (RT), cells were centrifuged (5 min at 540 g) and the cell pellet washed with 4 mL of PBS. Finally, cells were resuspended in 0.5 mL of PBS until analyzed in the flow cytometer.

Intracellular detection of APO 2.7 and bcl-2 proteins was performed after staining for surface proteins using a direct immunofluorescence technique. For this purpose, the FIX & PERM reagent kit (Caltag Laboratories) was used, strictly following the recommendations of the manufacturer. After staining for the cytoplasmic antigens, cells were washed and resuspended in 0.5 mL of PBS until analysis in the flow cytometer.

Cytoplasmic expression of Bax, MRP and LRP was assessed using an indirect immunofluorescence technique after cell fixation/permeabilization (FIX & PERM reagent kit) followed by staining for surface antigens. In this technique rabbit anti-mouse immunoglobulins conjugated with FITC ($F(ab')_2$ fragments; DAKO) were used as a second layer. After staining, cells were washed with PBS and resuspended in 0.5mL of PBS until analysis in the flow cytometer.

Appropriate isotype-matched negative controls were used for each of the techniques described above. In all cases, data acquisition was performed in a FACScalibur flow cytometer (Becton Dickinson), using the CellQuest software program (Becton Dickinson). A total of 20,000 BM events were

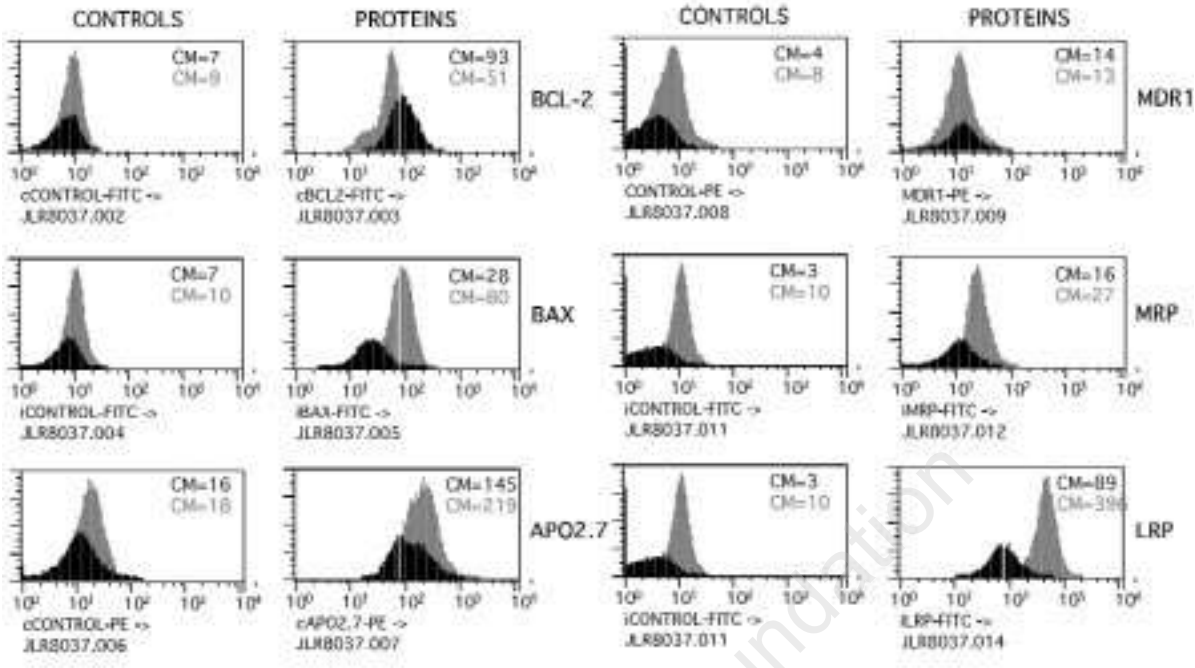

Figure 2. Histograms of expression of apoptosis (bcl-2, bax, APO2.7) and MDR (MDR1, MRP, LRP)-associated proteins in the AML case defined in Figure 1, in which subset I (black) and subset III (dark grey) were detected among the myeloid blast cells.

acquired for each 4-color staining. The PAINT-A-GATE software program (Becton Dickinson) was used for data analysis.

For each individual apoptosis and MDR-associated marker, antigen expression was analyzed and reported as its relative fluorescence intensity (RFI), calculated as the ratio between the mean fluorescence intensity (MFI) of the tested cells for the corresponding protein and MFI of corresponding controls. For all proteins, except APO 2.7, the control was based on the MFI of the corresponding blast cell subpopulation in the isotype-matched negative control tube, which included all the other surface MoAb for each combination, thereby allowing the identification of blast cell subpopulations. For APO 2.7, MFI of lymphocytes was used as the internal control due to the high autofluorescence of this particular combination of apoptosis associated proteins.[22-24]

Statistical analysis

The statistical analysis was performed using the SPSS software program (SPSS 8.0.15 Inc. Chicago, IL, USA). For each variable its median, mean, standard deviation, and range were calculated. The Kruskal Wallis test was used in order to assess the statistical significance of the differences observed between groups. *p* values lower than 0.05 were considered to be of statistical significance.

Results

Figures 1 and 2 illustrate how the analysis of the expression of APO 2.7, bcl-2, bax, P-gp, MRP and LRP was performed in an AML patient for the different blast cell subpopulations present in the sample.

In most cases (50/72, 65%), immature blast cells ($CD34^+$: subset I) were identified, and the mature subpopulations ($CD34^-$ $CD32^{-/+}$, $CD33^+$: subset II; and $CD34^-$ $CD32^{+/++}$, $CD33^{++}$: subset III) were identified in 38 cases (52%) and in 26 cases (36%), respectively. Interestingly, the most immature subpopulation (subset I) showed a significantly higher level of bcl-2 expression (p <0.0001) together with a lower reactivity for APO 2.7 (p=0.02) as compared to the other more mature $CD34^-$ cell subsets ($CD34^-$, $CD32^{-/+}$, $CD33^+$; and $CD34^-$, $CD32^{+/++}$, $CD33^{++}$) (Table 1). The expression of bax parallelled that of APO 2.7, although the difference

Table 1. Expression of bcl-2, bax, and APO2.7 apoptosis-related proteins in different maturation-associated subpopulations of blast cells in elderly AML patients.

Subpopulation of blast cells	*bcl-2 RFI*	*Bax RFI*	*APO2.7 RFI*
I: $CD34^+$	13.13±6.1	1.38±0.81	2.65±2.58
(n=47)	12.00	1.14	1.93
II: $CD34^-$ and $CD32^{-/+}$	8.52±5.76	1.42±0.49	3.15±3.00
(n=38)	7.42	1.28	2.18
III: CD34 and $CD32^{+/++}$	5.55±3.65	2.13±1.98	3.20±1.38
(n=26)	4.78	1.27	3.03
p	<0.0001	NS	0.02

Results expressed as mean ± standard deviation and median of relative fluorescence intensity (RFI: mean fluorescence intensity (MFI) of the analyzed cells divided by the MFI of corresponding controls). NS: not significant.

Table 2. Expression of P-gp, MRP, and LRP multidrug resistance-associated proteins in different maturation-related subpopulations of blast cells in elderly AML patients.

Subpopulation of blast cells	*P-gp RFI*	*MRP RFI*	*LRP RFI*
I: $CD34^+$	3.51±3.3	2.10±0.97	6.29±5.97
(n=50)	2.40	1.86	4.47
II: CD34-and $CD33^+$	2.79±3.1	1.90±0.61	8.91±6.12
(n=34)	1.78	1.87	6.52
III: CD34- and $CD33^{++}$	2.15±0.85	1.78±0.64	11.12±9.56
(n=21)	1.83	1.42	7.91
p	NS	NS	0.01

Results expressed as mean ± standard deviation and median of relative fluorescence intensity (RFI: mean fluorescence intensity (MFI) of the analyzed cells divided by the MFI of corresponding controls). NS: not significant.

Table 3. Expression of bcl-2, bax, and APO2.7 apoptosis-related proteins in normal cells from bone marrow of healthy donors.

Type of cells	*bcl-2 RFI*	*Bax RFI*	*APO2.7 RFI*
$CD34^+$	8.27±1.9	0.99±0.06	17.33±5.85
(n=5)	8.36	1	17.01
Granulo-monocytic lineage	1.96±0.46	1.12±0.07	6.26±0.75
(n=5)	1.99	1.09	6.30

Results expressed as mean±standard deviation and median of relative fluorescence intensity (RFI: mean fluorescence intensity (MFI) of the analyzed cells divided by the MFI of corresponding controls).

between its expression on immature $CD34^+$ blast cells and that on the mature blast cell subsets did not reach statistical significance (p=0.18). These results translated into a significantly (p<0.0001) higher bcl2/bax ratio for the $CD34^+$ blast cells as compared to that for the two $CD34^-$ blast cell subpopulations (the median bcl-2/bax ratio was: 7.42 in subset I; 4.72 in subset II; and 1.81 in subset III; p<0.0001). Interestingly, similar results were also found when we restricted analysis to those AML cases in which only subpopulations I and II (21 cases), or I and III (11 cases), or II and III (12 cases) were detected. Upon comparing the results of blast cells with those of normal bone marrow cells, it was observed that the normal $CD34^+$ cells displayed lower levels of bcl-2 (p=0.04) and higher levels of APO 2.7 (p=0.04) than those observed in the $CD34^+$ blast cells. In parallel, granulo-monocytic cells from healthy controls (Table 3) expressed lower levels of bcl-2 (p=0.003) and higher levels of APO 2.7 (p=0.005) than did the mature blast subpopulations (II and III).

Regarding the expression of the multidrug resistance-associated proteins P-gp and MRP, it should be noted that $CD34^+$ blast cells displayed a greater expression of both proteins as compared to the more mature $CD34^-$ AML blast cells, but differences according to maturation stage of AML blast cells did not reach statistical significance (median RFI for P-gp of 2.4, 1.8, and 1.8 for the subsets I, II, and III, respectively p=0.21; and median RFI for MRP of 1.9, 1.9, and 1.4 for the subsets I, II, and III, respectively p=0.36). In contrast, LRP expression was significantly lower in the more immature $CD34^+$ blast cell subset than in the more mature ones: median RFI of 4.5, 6.5, and 7.9 for the subsets I, II, and III, respectively (p=0.01) (Table 2).

Similarly to that which occurred with the apoptotic proteins, the results observed with multidrug resistance-associated proteins were also consistent when we restricted the analysis to those AML cases in which two subpopulations were detected. Regarding normal bone marrow samples no significant differences were observed in the expression of drug resistance-associated proteins between normal bone marrow $CD34^+$ cells and $CD34^+$ myeloid blast cells from AML. However, granulo-monocytic cells from healthy controls expressed higher levels of these proteins than the more mature blast subpopulations (II and III), especially in the case of LRP protein (p=0.04) (Table 4).

Table 4. Expression of P-gp, MRP, and LRP multidrug resistance-associated proteins in in normal cells from bone marrow of healthy donors.

Type of cells	P-gp RFI	MRP RFI	LRP RFI
CD34+	3.06±0.62	2.89±0.9	5.15±2.55
(n=5)	3.08	2.95	4.42
Granulo-monocytic lineage	2.45±0.58	6.35±3.97	16.67±6.94
(n=5)	2.51	3.84	15.35

Results expressed as mean±standard deviation and median of relative fluorescence intensity (RFI: mean fluorescence intensity (MFI) of the analyzed cells divided by the MFI of corresponding controls).

Discussion

Acute myeloid leukemia is a heterogeneous group of malignant diseases, and when it develops in elderly patients has a particularly unfavorable prognosis frequently related to both a lower response rate and a higher incidence of relapses as well as lower tolerance to and higher toxicity of chemotherapy. It has been suggested that the expression of members of the bcl-2 family (e.g. bcl-2, bax)[25,26] and other proteins related to multi-drug resistance (e.g P-gp, MRP, LRP)[14, 27-29] are involved in the cellular mechanisms contributing to chemotherapeutic resistance in AML. In line with these observations particularly low levels of *in vitro* drug-induced apoptosis have been shown in blast cells from elderly AML patients.[21] On the other hand, sequential studies of AML patients at diagnosis and relapse indicate that phenotypic changes may frequently occur and that they are associated with the expression of a more immature immunophenotype at relapse.[2,3] This may be due either to the fact that immature blast cells are more resistant to chemotherapy or/and that they are protected from undergoing apoptosis.[4,5]

It is now well established that proteins associated with apoptosis and multidrug resistance are also expressed by normal cells, and differences in the level of expression of these proteins have been found within a given cell type according to its maturation stage.[17-20] Therefore, the analysis of expression of proteins involved in apoptosis and multidrug resistance should focus particularly on the different blast cell subpopulations found at diagnosis in AML patients, since this might provide new insights into the mechanisms of chemo-resistance in AML.

A high number of studies have been reported in which expression of bcl-2, MDR1, MRP, LRP, and even other apoptosis or multidrug resistance-associated proteins have been analyzed in AML patients.[6-15] However, so far, little is known about the different expression of these proteins in individual AML blast cell subpopulations defined according to their stage of maturation, and discrepant results have been reported concerning the correlation between the expression of some of these proteins and CD34 antigen expression in AML blast cells.[6-9,14,30,31] These discrepancies may be due to the fact that most of the studies considered only whether the AML was CD34 positive or negative,[6-9,31] according to a predefined threshold (>10% or 20% positive cells), without taking in account that in AML patients, CD34+ and CD34- blast cells frequently coexist.[1]

Multiparametric flow cytometry immunophenotyping, based on multiple MoAb staining, allowed us not only to discriminate myeloid blast cells from the residual normal cells present in each sample, but also to identify the different stages of maturation in myeloid blast cell subsets.[1] In addition, this was combined with the simultaneous analysis of the expression of proteins associated with either apoptosis (APO 2.7, bcl-2, bax) or drug resistance (P-gp, MRP and LRP), thus permitting their specific assessment in different subsets of myeloid blast cells previously identified and defined according to their stage of maturation.

Bcl-2 is probably the most widely studied apoptosis-related protein.[9-11, 16-17, 20-21, 25, 26, 32, 33] Although discrepant results have been published concerning its expression in different AML FAB subtypes,[9-11,17] higher levels of bcl-2 expression have been reported for CD34+ AML cases,[9-11] but simultaneous measurement of both proteins has not been carried out. Our present findings, based on the analysis of specific blast cell subsets support previous reports. Regarding the expression of APO 2.7, which detects the mitochondrial 7A6 antigen particularly expressed by cells undergoing apoptosis, and bax, the information so far available is scanty, and restricted almost exclusively to the bax protein whose expression has not been associated with a particular FAB subtype.[11,32] Our results show that the immature CD34+ myeloid blast cells have a lower expression of both bax and APO2.7, indicating that these cells could be protected from entering into apoptosis. This finding was particularly significant when the bcl-2/bax ratio was considered as a marker of apoptosis. In addition, we compared the results obtained in these blast cell subpopulations with those of their normal cell counterpart. Our results show that CD34+ blast cells are more protected from apoptosis than the normal CD34+ cells (the blast cells displayed higher bcl-2 and lower APO 2.7 levels). The same pattern was

observed when granulo-monocytic cells from healthy controls were compared with the more mature blast subpopulations.

Resistance to chemotherapy is a major factor limiting successful treatment of AML. One of the best-characterized drug resistance mechanisms is extrusion of drugs by the energy-dependent multidrug resistance (MDR1) transport protein or P-gp. A large number of studies have been published on P-gp expression and prognosis in AML.[4,5,7,11,27-29,32] However, a major drawback of the interpretation not only of P-gp but also of MRP and LRP data is the lack of standardization in the results obtained by different analytical assays,[13,22,23,34] and consensus has been difficult to reach. In our study, we followed the consensus recommendations for staining and analysis of these proteins.[22-24] With respect to the potential relationship between the maturation stage of AML blast cells and P-gp activity, previous studies have shown that both the more immature FAB subtypes[11,15] and the $CD34^+$ AML cases[7,12,13] usually display a higher P-pg expression and/or activity. However, these results have not been confirmed in all studies.[14] Controversial results have been obtained regarding MRP and LRP according to the CD34 expression in AML - positive or negative cases.[6-8] In the present study the expression of P-gp and MRP proteins tended to be higher in $CD34^+$ blast cell populations than in $CD34^-$ ones, but differences did not reach statistical significance. These results are in line with the high bcl-2 and low Apo 2.7 expression in this subset of myeloid blast cells, and could also explain why these cells are more resistant to both apoptosis and chemotherapy. Surprisingly, our results show that LRP expression was significantly lower in the more immature blast cell subset ($CD34^+$), suggesting that myeloid maturation might be associated with different patterns of MDR-associated phenotypes and/or that several drug resistance mechanisms are concomitantly implicated in AML. With respect to the expression of drug resistance-associated proteins in normal bone marrow, no clear differences were noted between normal $CD34^+$ cells and $CD34^+$ myeloid blast cells from AML. However, granulo-monocytic cells from healthy controls express higher levels of these proteins than do the more mature blast subpopulations, especially in the case of LRP protein. These apparently contradictory results could be explained by the great complexity of the mechanism involving drug resistance.

We conclude that in elderly AML patients the more immature blast cells are more resistant to the apoptotic process, which could explain why, when AML relapses, the blast cells frequently display a more immature phenotype than the one observed at diagnosis. In minimal residual disease studies we have previously reported that different blast cell subpopulations may coexist in AML patients at diagnosis, and a minor one, frequently the most immature, may be responsible for the relapse.[1,2] The present study sheds further light onto this finding, since the more immature blast cells were the more resistant to the apoptotic process, and would have a greater probability of survival. Contradictory results in multidrug resistance profiles support the hypothesis that failure to respond to chemotherapeutic drugs in AML is a multifactorial phenomenon, and more information is needed to understand the resistance of blast cells to chemotherapy.

Contributions and Acknowledgments

LS was responsible for the techniques, analyzed data and drafted the article; AL collaborated in the techniques; MBV was responsible for the analysis and interpretation of data, and revising the article critically for important intellectual content; JGL, RM, AO and JFSM were responsible for the conception and design of the study, and the final approval of the version to be submitted; MVMR, MT, JDGSM and EL were responsible for including patients in the study. We would also like to thank all centers participating in the PETHEMA-LAM99>65 trial for their recruitment of patients included in the present study: Ramón and Cajal Hospital, Madrid; San Carlos University Hospital, Madrid; Puerta del Mar Hospital, Cádiz; Clinic University Hospital, Valencia; Insular Hospital, Las Palmas; Xeral Hospital, Lugo; General Hospital, Castellón; General Hospital, Alicante; Clinic University Hospital, Málaga; University Hospital, Alcalá de Henares; Asturias Central Hospital, Oviedo; General Hospital, Murcia; Virgen de la Concha Hospital, Zamora; Ciudad de Jaén Hospital, Jaén; Clinic University Hospital, Valladolid; Montecelo Hospital, Pontevedra; General Hospital, Albacete; Virgen de la Arraixaca Hospital, Murcia; Dr. Pesset Hospital, Valencia; Galdakano Hospital, Vizcaya; University Hospital, Salamanca; University Hospital, Pamplona. Finally, we would like to thank M. Anderson for his help with the English language of this paper.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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Potential implications for clinical practice

The treatment of older individuals with AML remains difficult and of still limited success.[35] This study provides biological explanations for this poor outcome.

References

1. Macedo A, Orfao A, Gonzalez M, et al. Immunological detection of blast cell subpopulations in acute myeloblastic leukemia at diagnosis: implications for minimal residual disease studies. Leukemia 1995; 9:993-8.
2. Macedo A, San Miguel JF, Vidriales MB, et al. Phenotypic changes in acute myeloid leukaemia: implications in the detection of minimal residual disease. J Clin Pathol 1996; 49:15-8.
3. Thomas X, Campos L, Archimbaud E, et al. Surface marker expression in acute myeloid leukaemia at first relapse. Br J Haematol 1992; 81:40-4.
4. Willman CL. Immunophenotyping and cytogenetics in older adults with acute myeloid leukemia: significance of expression of the multidrug resistance gene-1 (MDR1). Leukemia 1996; 10 (Suppl 1):S33-5.
5. Poulain S, Lepelley P, Cambier N, Cosson A, Fenaux P, Wattel E. Assessment of P-glycoprotein expression by immunocytochemistry and flow cytometry using two different monoclonal antibodies coupled with functional efflux analysis in 34 patients with acute myeloid leukemia. Adv Exp Med Biol 1999; 457:57-63.
6. van der Kolk DM, de Vries EG, Koning JA, van den Berg E, Muller M, Vellenga E. Activity and expression of the multidrug resistance proteins MRP1 and MRP2 in acute myeloid leukemia cells, tumor cell lines, and normal hematopoietic CD34$^+$ peripheral cells. Clin Cancer Res 1998; 4:1727-36.
7. Legrand O, Simonin G, Perrot JY, Zittoun R, Marie JP. Pgp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. Blood 1998; 91:4480-8.
8. List AF, Spier CS, Grogan TM, et al. Overexpression of the major vault transporter protein lung-resistance protein predicts treatment outcome in acute myeloid leukemia. Blood 1996; 87:2464-9.
9. Bradbury DA, Zhu YM, Russell NH. Bcl-2 expression in acute myeloblastic leukaemia: relationship with autonomous growth and CD34 antigen expression. Leuk Lymphoma 1997; 24:221-8.
10. Cascavilla N, Melillo L, D'Arena G, et al. Minimally differentiated acute myeloid leukemia (AML M0): clinico-biological findings of 29 cases. Leuk Lymphoma 2000; 37:105-13.
11. Wuchter C, Karawajew L, Ruppert V, et al. Clinical significance of CD95, Bcl-2 and Bax expression and CD95 function in adult de novo acute myeloid leukemia in context of P-glycoprotein function, maturation stage, and cytogenetics. Leukemia 1999; 13:1943-53.
12. Leith CP, Chen IM, Kopecky KJ, et al. Correlation of multidrug resistance (MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: identification of discordant MDR$^-$/efflux$^+$ and MDR1$^+$/efflux$^-$ cases. Blood 1995; 86:2329-42.
13. Pall G, Spitaler M, Hofmann J, Thaler J, Ludescher C. Multidrug resistance in acute leukemia: a comparison of different diagnostic methods. Leukemia 1997; 11:1067-72.
14. Martinez A, San Miguel JF, Valverde B, et al. Functional expression of MDR-1 in acute myeloid leukemia: correlation with the clinical-biological, immunophenotypical, and prognostic disease characteristics. Ann Hematol 1997; 75:81-6.
15. Lamy T, Drenou B, Grulois I, et al. Multi-drug resistance (MDR) activity in acute leukemia determined by rhodamine 123 efflux assay. Leukemia 1995; 9: 1549-55.
16. Bradbury DA, Russell NH. Comparative quantitative expression of bcl-2 by normal and leukaemic myeloid cells. Br J Haematol 1995; 91:374-9.
17. Porwit-MacDonald A, Ivory K, Wilkinson S, Wheatley K, Wong L, Janossy G. Bcl-2 protein expression in normal human bone marrow precursors and in acute myelogenous leukemia. Leukemia 1995; 9: 1191-8.
18. Drach D, Zhao S, Drach J, et al. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. Blood 1992; 80:2729-34.
19. Legrand O, Perrot JY, Tang R, et al. Expression of the multidrug resistance-associated protein (MRP) mRNA and protein in normal peripheral blood and bone marrow haematopoietic cells. Br J Haematol 1996; 94:23-33.
20. Andreeff M, Jiang S, Zhang X, et al. Expression of Bcl-2-related genes in normal and AML progenitors: changes induced by chemotherapy and retinoic acid. Leukemia 1999; 13:1881-92.
21. Garrido SM, Cooper JJ, Appelbaum FR, Willman CL, Kopecky K, Banker DE. Blasts from elderly acute myeloid leukemia patients are characterized by low levels of culture- and drug-induced apoptosis. Leuk Res 2001; 25:23-32.
22. Beck WT, Grogan TM. Methods to detect P-glycoprotein and implications for other drug resistance-

associated proteins. Leukemia 1997; 11:1107-9.
23. Legrand O, Simonin G, Zittoun R, Marie JP. Lung resistance protein (LRP) gene expression in adult acute myeloid leukemia: a critical evaluation by three techniques. Leukemia 1998; 12:1367-74.
24. Marie JP, Legrand O, Perrot JY, Chevillard S, Huet S, Robert J. Measuring multidrug resistance expression in human malignancies: elaboration of consensus recommendations. Semin Hematol 1997; 34 (Suppl 5):63-71.
25. Campos L, Sabido O, Viallet A, Vasselon C, Guyotat D. Expression of apoptosis-controlling proteins in acute leukemia cells. Leuk Lymphoma 1999; 33:499-509.
26. Ong YL, McMullin MF, Bailie KE, Lappin TR, Jones FG, Irvine AE. High bax expression is a good prognostic indicator in acute myeloid leukaemia. Br J Haematol 2000; 111:182-9.
27. Michieli M, Damiani D, Ermacora A, et al. P-glycoprotein, lung resistance-related protein and multidrug resistance associated protein in de novo acute non-lymphocytic leukaemias: biological and clinical implications. Br J Haematol 1999; 104:328-35.
28. Borg AG, Burgess R, Green LM, Scheper RJ, Yin JA. Overexpression of lung-resistance protein and increased P-glycoprotein function in acute myeloid leukaemia cells predict a poor response to chemotherapy and reduced patient survival. Br J Haematol 1998; 103:1083-91.
29. Legrand O, Simonin G, Zittoun R, Marie JP. Both P-gp and MRP contribute to drug resistance in AML. Leukemia 1998; 12:1327-8.
30. Izquierdo MA, Scheffer GL, Flens MJ, et al. Broad distribution of the multidrug resistance-related vault lung resistance protein in normal human tissues and tumors. Am J Pathol 1996; 148:877-87.
31. Lewis NR, Pallis M, Russell NH. Fas receptor-Fas ligand system is independent of both CD34 status and chemosensitivity in acute myeloid leukemia. Exp Hematol 2000; 28:535-42.
32. Campos L, Oriol P, Sabido O, Guyotat D. Simultaneous expression of P-glycoprotein and BCL-2 in acute myeloid leukemia blast cells. Leuk Lymphoma 1997; 27:119-25.
33. Kornblau SM, Vu HT, Ruvolo P, et al. BAX and PKCα modulate the prognostic impact of BCL2 expression in acute myelogenous leukemia. Clin Cancer Res 2000; 6:1401-9.
34. Marie JP, Huet S, Faussat AM, et al. Multicentric evaluation of the MDR phenotype in leukemia. French network of the Drug Resistance Intergroup, and Drug Resistance Network of Assistance Publique-Hopitaux de Paris. Leukemia 1997; 11:1086-94.
35. Löwenberg B.Therapeutic approaches to the older patient with acute myeloid leukaemia. Haematologica 2000, EHA 5 Educational Book, p. 61. Available from URL: http://www.haematologica.it/free/eha5_edu_lowenberg.pdf