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Quantitative analysis of Fas and bcl-2 expression in hematopoietic precursors

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Background and Objectives. We investigated the expression of bcl-2 and CD95 (Apo1-/Fas) on CD34+ cells obtained from bone marrow (BM), mobilized peripheral blood (MPB), and umbilical cord blood (UCB) samples. The expression of bcl-2 and Fas was then compared with that of other markers usually associated with immaturity; functional tests using the agonistic antibody anti- Fas CH11 were also carried out.

Design and Methods. The analysis was performed by flow cytometry on purified CD34+ cells in a three (CD95 PE, CD34 APC and CD71 FITC) and in a four (CD38 PE, HLA-DR PerCP, CD34 APC and bcl-2 FITC) fluorescence assay.

Results. The results were expressed as mean fluorescence index (MFI); bcl-2 expression was significantly higher (p <0.001) in BM (3.73±0.63) than in MPB (2.47±0.39) and UCB (2.38±0.58); Fas was significantly less expressed (p <0.001) in UCB (1.27±0.78) than in MBP (3.63±2.19) and BM (4.56±1.69). CD34 expression was significantly (p <0.001) brighter in UCB compared to in MBP and BM, while CD38 and CD71 were significantly (p =0.005 and p <0.001, respectively) more expressed in BM than in MPB and UCB. Fas values were directly correlated to CD38; both Fas and bcl-2 were directly related to CD71 and inversely to CD34. Culture assays showed that hematopoietic precursor cells from BM, MPB and UCB had a low susceptibility to undergo Fas-mediated apoptosis.

Interpretation and Conclusions. In conclusion, bcl-2 and Fas are less expressed in UCB than in MPB and BM; early hematopoietic precursor cells are relatively resistant to CD95-triggered apoptosis; the observed correlation between Fas/bcl-2 and markers of immaturity suggests that they may be determinants of commitment in early hematopoietic precursors.

Key words: Fas, bcl-2, bone marrow, mobilized peripheral blood, umbilical cord blood

It is now apparent that programmed cell death plays a pivotal role in the control of the hematopoietic process;[1-3] such a control operationally involves a number of factors including members of the bcl-2 family and the Fas/Fas ligand system.[2] Bcl-2 and other anti-apoptotic proteins such as bcl-x (bcl- X_L and bcl-X_S), bcl-w, MCL-1, and A1 exert their action by protecting cells from the induction of apoptosis;[4] contrariwise, bax, bad and bak proteins promote cell death.[5] All these proteins show a sequence homology and the ability to form heterodimers (in particular, bcl-2/bax and bcl-X_L/bax); for this reason it has been proposed that the susceptibility of cells to apoptosis depends on the ratio between anti-apoptotic and pro-apoptotic proteins.[6] Nevertheless, recent studies suggest that these two different groups of proteins are prone, rather than to dimer formation, to act independently by binding to a common target.[7,8] Primitive CD34+ precursors from bone marrow (BM) were found to be bcl-2 negative by some authors[9,10] and, in contrast, bcl-2 positive by others.[11,12] Recently, bcl-2 has also been reported to be expressed on CD34+ cells from mobilized peripheral blood (MPB) as well as from umbilical cord blood (UCB).[12]

Fas antigen (CD95/Apo-1) is a receptor belonging to the tumor necrosis factor (TNF) receptor family. Once triggered by agonistic antibodies or natural ligand (FasL),[13] it induces apoptosis via direct and sequential activation of caspases. Fas is physiologically expressed on different tissues such as spleen, lymph nodes[13] and on mature hematopoietic cells such as T- and B-cells, natural killers (NK), monocytes and granulocytes.[14]

Studies of the expression of Fas on CD34+ cells from BM also yielded conflicting results.[11,15] By contrast, CD34+ MPB cells display low levels of Fas, which increase under cytokine stimulation *in vitro*.[16] Finally, few data about CD95 expression on UCB cells are available,[15] and comparative studies dealing with quantitative bcl-2 and Fas expression in these three different sources of progenitor cells have not been reported in the literature.

Based on these premises, we compared bcl-2 and Fas expression on CD34+ cells obtained from BM, MPB and

UCB. The degree of immaturity of CD34+ stem cells from the three sources was also investigated by evaluating the expression of CD38, CD71, HLA-DR and CD34; finally, functional tests were performed with the agonistic antibody CH11 to explore the sensitivity of HPC to CD95-triggered apoptosis.

Design and Methods

Samples

BM samples were obtained from 5 normal adult volunteers and 8 patients with acute lymphoid leukemia (ALL) in complete remission (CR) for more than a year and whose blasts did not express CD34 antigen at the time of diagnosis. MPB samples were collected from 6 patients with non-Hodgkin's lymphoma (NHL), 4 with ALL, 3 with multiple myeloma (MM), 1 with chronic lymphocytic leukemia (CLL) , and 1 with multiple sclerosis (MS). In all patients, hematopoietic progenitor cells (HPC) were mobilized by high-dose chemotherapy (cyclophosphamide 7 g/m^2) followed by RmetHuG-CSF (5 μg/kg daily, subcutaneously).

UCB cells were obtained from 16 full term deliveries by drainage of blood into sterile collection tubes containing citrate-phosphate-dextrose after clamping and cutting off the cord. Cord blood samples were stored at room temperature and processed within 24 hours.

Cell preparation and CD34+ cell purification

Mononuclear cells were separated by gradient centrifugation at 400 g for 30 min at room temperature using Histopaque1077 g/mL (Sigma Chemical, St. Louis, MO, USA) and were washed twice in phosphate-buffered saline (PBS); CD34+ cells were purified by positive selection, using the Vario Macs magnetic cell separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.[17] Briefly, 10^8 mononuclear cells were suspended in 300 μL of PBS supplemented with 5 mM EDTA and 0.5% bovine serum albumin PBSA and incubated with 100 μL human IgG FcR blocking antibody and 100 μL monoclonal microbead-conjugated CD34 antibody (clone QBEND/10; Miltenyi Biotec) for 30 min at 4°C. Thereafter, the cells were washed and passed through a 30-mm filter and separated in a column exposed to a magnetic field. The column was washed four times with PBSA (3 mL aliquots) and removed from the separator. This procedure achieved highly purified CD34+ populations (mean 90±5.8%, range 84-96%) as demonstrated by staining the collected cells with HPCA-2 phycoerythrin (PE) (Becton Dickinson, Mountain View, CA, USA) which targets a CD34 antigen epitope distinct from that recognized by QBEND/10 used in the purification process.

Monoclonal antibodies (MoAbs)

CD71 fluorescein isothiocyanate (FITC)-conjugated, CD38 PE-conjugated, HLA-DR peridinin chlorophyll protein (PerCP)-conjugated, CD34 allophycocyanin (APC)-conjugated MoAbs and isotype-matched controls were purchased from Becton Dickinson (Mountain View, CA, USA); CD95 PE and isotype matched control were purchased from Pharmingen (San Diego, CA, USA); anti-Bcl-2 protein FITC MoAb was purchased from DAKO (Glostrup, Denmark). Anti-Bax pure MoAb was purchased from Calbiochem (San Diego, CA, USA).

Anti-human Fas mAb CH11, purchased from Upstate Biotechnology (Lake Placid, NY, USA) was used to induce apoptosis of Jurkat cells and CD34+ cells obtained from BM, MPB and UCB.

Flow cytometric immunophenotyping

For bcl-2 and bax expression, the cells were first incubated with CD38 PE, HLA-DR PerCP and CD34 APC for 15 min at room temperature in the dark. After two washings in phosphate-buffered saline (PBS), the cells were fixed and permeabilized using a commercially available kit (Fix & Perm Permeabilization Kit, CALTAG, Burlingame, CA, USA) and then incubated with bcl-2 FITC for 15 min at room temperature in the dark and washed twice in PBS. For indirect bax staining, the cells were further incubated with the goat anti-mouse-IgG1 FITC for 15 min at room temperature in the dark and washed twice in PBS. For Fas expression, the cells were incubated with CD71 FITC, CD95 PE and CD34 APC for 15 min at room temperature in the dark and then washed twice in PBS. The samples were then run on a FACSCalibur flow cytometer (BD, Mountain View, CA, USA), equipped with an argon laser emitting at 488 nm. A minimum of 10,000 cells was acquired in a list mode file format and analyses were performed with CELLQuest software (BD). The results were obtained as mean fluorescence index (MFI), calculated as the ratio of sample mean channel: control mean channel, and the data were presented as mean±standard deviation (SD).

Clonogenic (CFU-Mix and CFU-GM) assays

Semisolid culture assays were used to grow HPC; nuclear cells (NC) obtained from BM, MPB and UCB were cultured in a standard growth factor supplemented methylcellulose medium (MethoCult GF H4434), provided by Stem Cell Technologies (Vancouver, Canada) which contained recombinant human (rh) GM-CSF (10 ng/mL), rh-SCF (50 ng/mL), rh-IL3 (10 ng/mL), rh-erythropoietin (3 U/mL) and 25% fetal bovine serum. Since the percentage of CD34+ cells was significantly higher in MPB and BM than in UCB samples (3.1±2.97 and 3.8±1.93 vs 0.48±0.29, respectively, p=0.02), the absolute number of NC plated per dish was adjusted according to the percentage of CD34+ cells in each source.

The cells were incubated for 14 days at 37°C in a humidified atmosphere containing 5% CO_2 air and, by observation with an inverted microscope, cell aggregates containing more than 50 cells were scored as granulocyte-macrophage colony-forming units (CFU-GM), red cell aggregates containing more than 500 cells were scored as erythroid burst-forming units (BFU-E) and cell aggregates containing at least erythroid and granulocytic cells were scored as mixed colony-forming units (CFU-Mix).

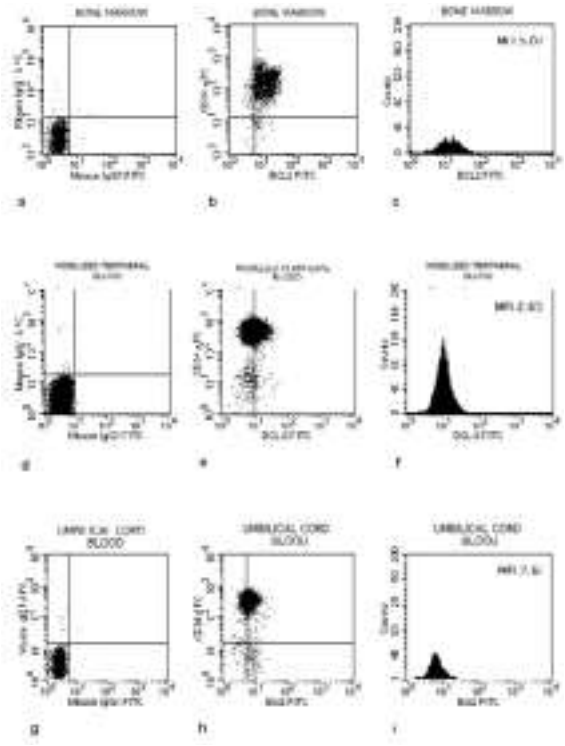

Figure 1. Expression of bcl-2 on hematopoietic progenitors from BM, MPB and UCB; CD34+ cells isolated by positive selection using the Vario Macs magnetic cell separator were stained with anti CD34 APC and anti bcl-2 FITC or the appropriate isotype controls. The quadrants were set to include >95% on the negative control, constituted by the cells stained with isotype-matched controls conjugated with APC and FITC (a, d, g). Bcl-2 was more expressed in BM (b, c) than in MPB (e, f) and UCB (h, i) cells.

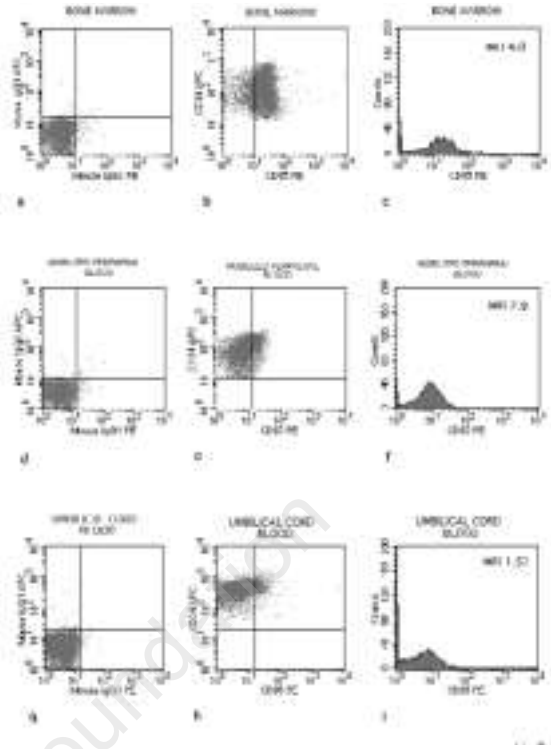

Figure 2. Expression of Fas/CD95 on hematopoietic progenitors from BM, MPB and UCB; CD34+ cells isolated by positive selection using the Vario Macs magnetic cell separator were stained with anti CD34 APC and anti CD95 PE or the appropriate isotype controls. The quadrants were set to include >95% on the negative control, constituted by the cells stained with isotype-matched controls conjugated with APC and PE (a, d, g). CD95 expression was higher in BM (b, c) and MPB (e, f) than in UCB (h, i) cells.

Cell lines

Jurkat cells were kept in culture in RPMI containing 10% fetal calf serum (FCS) at 37°C and CO_2 in air.

Cell culture experiments and identification of apoptotic cells

Jurkat cells and purified CD34+ cells ($1x10^6$/mL) from BM, MPB and UCB were cultured for 6 and 24 hours at 37°C and 5% CO_2 in air in RPMI 1640 (GIBCO, Grand Island, NJ, USA) containing 10% FCS to reduce background apoptosis; anti-Fas CH11 1 μg/mL or an irrelevant IgM moAb was added to the cultures. Cell death was demonstrated by the Apoptosis Detection Kit (R&D systems, UK) according to manufacturer's instructions; briefly, CD34+ cells were harvested, washed and then incubated for 15 minutes at room temperature with annexin V-FITC and propidium iodide, and subsequently analyzed by flow cytometry. The combination of annexin V-FITC and propidium iodide allowed for the identification of early apoptotic cells (annexin V-FITC positive), late apoptotic and/or necrotic cells (annexin V-FITC positive and propidium iodide positive) and viable cells (unstained).

Statistical analysis

Differences between UCB, MPB and BM with regard to all the phenotypic markers were analyzed with the two-tailed t-test and a p value <0.05 was accepted as statistically significant. Spearman's rank correlation (r) was used to assess the relationship between Fas, bcl-2 and the other surface antigen MFI values.

Results

Representative distributions of CD34 vs. bcl-2 or CD95 expression on BM, MPB and UCB progenitor cells are shown in Figure 1 and 2, respectively. Bcl-2 MFI was significantly higher (p<0.001) in BM (3.73±0.63) than in MPB (2.47±0.39) and UCB cells (2.38±0.58). Fas expression was significantly (p <0.001) weaker in UCB (1.27±0.78) than in MPB (3.63±2.19) and BM (4.56±1.69) cells. Differentiated myeloid BM cells, as identified by light-scattering properties and CD33 positivity, showed the lowest bcl-2 (1.52±0.17) and the highest CD95 MFI (9±4.3) expression (Figure 3). The bcl-2 and Fas expression on BM cells obtained from normal volunteers and from ALL patients in complete remission

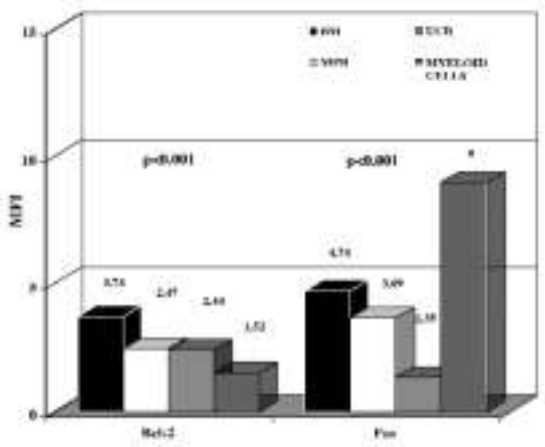

Figure 3. Comparison of bcl-2 and Fas / CD95 expression on BM, MPB and UCB samples; bcl-2 MFI was significantly (p <0.001) higher in BM samples than in MPB and UCB; Fas MFI was significantly (p <0.001) less expressed in UCB samples than in BM and MPB. Bcl-2 was downregulated and Fas upregulated on myeloid differentiated BM cells identified by light scattering properties and CD33 positivity.

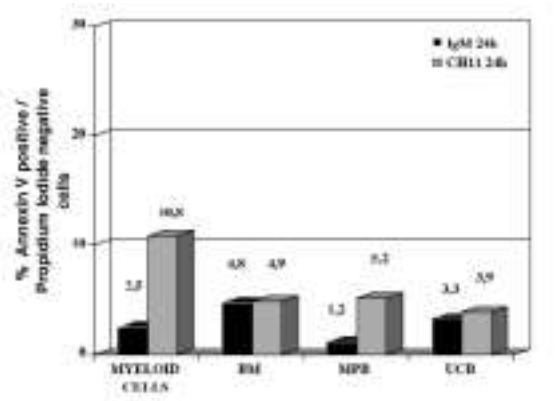

Figure 4. CD95-induced cell death in CD34$^+$ cells from BM, MPB and UCB. Percentage of annexin V positive / propidium iodide negative cells is given for CD34$^+$ cells from BM, MPB and UCB and myeloid differentiated BM cells (SSC^{low} / FSC^{high} / CD33$^+$) cultured in medium plus an irrelevant IgM Ab or anti-Fas CH11 (1μg / mL) for 24 hours. One representative experiment of three with similar results is shown.

did not differ significantly (data not shown).

We also tested the expression of bax protein in 4 BM, 4 MPB and 4 UCB samples and found that BM progenitor cells had brighter positivity than MPB and UCB cells (18.5±11.7, 7.6±4.4, 10±2.5, respectively).

When the degree of immaturity of HPC from the three different sources was investigated, we observed that CD34 MFI was significantly higher in UCB than in MPB and BM (136.76±38.38 vs. 102.23±41.55 and 70.26±18.10, p <0.001, respectively). By contrast, both CD38 MFI (32.02±16.75 vs. 23.21±12.70 and 14.20±2.36, p =0.005, respectively) and CD71 MFI (13.43±4.38 vs. 5.50±2.80 and 5.56±1.57, p <0.001, respectively) were significantly more expressed in BM than in MPB and UCB. No differences in HLA-DR expression were found among BM, MPB and UCB cells, since, as reported by others,[18,19] it is inversely related to HPC maturity on UCB, whereas it is directly related on BM and MPB. Clonogenic assays, also, confirmed the presence of a significantly higher number of early and committed progenitor cells in UCB than in MPB and BM samples (Table 1). Considering all samples, Spearman's test demonstrated a significant correlation between Fas and bcl-2 MFI expression (r = 0.58, p =0.001).

Moreover, we found that Fas and bcl-2 MFI were inversely correlated to the expression of CD34 (r= -0.54, p =0.002, and r= -0.41, p = 0.008, respectively), and directly correlated to that of CD38 (r= 0.53, p =0.002, and r= 0.30, p= 0.093, respectively) and CD71 (r= 0.45, p =0.032, and r= 0.59, p = 0.002, respectively).

To investigate the sensitivity of CD34$^+$ cells to CD95 triggered apoptosis, we performed *in vitro* cultures using the agonistic antibody CH11 (IgM). As a positive control we used Jurkat cells. The MFI of Fas expression was higher on Jurkat and BM myeloid cells (27.9 and 15.6, respectively) than on CD34$^+$ cells from BM (9.1), MPB (11.5) and UCB (2.2). Activation of apoptosis was measured by flow cytometric assessment of annexin V on the surface of the cultured cells; annexin V is a calcium-dependent phospholipid protein with a high affinity for phosphatidyl serine expressed by apoptotic cells.[20] In Jurkat cells apoptosis was already detectable after 6 hours (26.8% annexin V positive cells), but the maximum number of apoptotic cells was found after 24 hours (>90%) of incubation with CH11. CD95-induced apoptosis of CD34$^+$ cells was measured in duplicate and compared with control IgM-induced apoptosis (Figure 4). There was less than 5% specific apoptosis measured in the CH11-treated BM, MPB and UCB cells; in contrast, both Jurkat cells and

Table 1. Comparison of clonogenic efficiency of CD34$^+$ cells from BM, MPB and UCB; the number of CFU-Mix and CFU-GM was significantly (p=0.006 and p=0.001, respectively) higher in UCB than in MPB and BM. The results are expressed as the mean±SD of the number of CFU-Mix, CFU-GM and BFU-E colonies per 10^3 CD34$^+$ cells plated.

Sample	*CFU-MIX*	*p*	*CFU-GM*	*p*	*BFU-E*	*p*
BM	1.5±0.5		34±17.1		32±22.4	
MPB	3.0±2.3	0.006	95.1±33.9	0.001	120.4 ±118.9	0.074
UCB	23.5±17.7		137±36.5		177±57.3	

differentiated myeloid BM cells showed highly efficient apoptosis. These results show that while HPC are relatively resistant, mature cells are more sensitive to Fas-induced programmed cell death.

Discussion

UCB is currently considered as the richest source of very immature hematopoietic precursors based on the prevalence of $CD34^{++}$ $CD38^{-/low}$ $CD71^{-/low}$ phenotype,[21-23] and, from a functional point of view, because of the higher frequency of CFU-Mix and CFU-GM.[24-26] In our study, this observation was further expanded in terms of phenotypic analysis, and UCB characteristics were also compared to those of BM and MPB. In particular, we investigated the pattern of distribution of bcl-2 and Fas on HPC from these three distinct sources, and their relationship with markers such as CD34, CD38 and CD71. We found that bcl-2 was significantly more expressed on BM than on MPB and UCB cells, and that its expression was directly related to the stage of maturation. It has been demonstrated that bcl-2 is downregulated as cell differentiation and maturation progresses: in fact, $CD34^+$ BM cells expanded *in vitro* with different growth factors gradually lose bcl-2[11] and mature peripheral blood cells are negative for bcl-2 expression.[14] By contrast, few data exist on bcl-2 expression in early HPC; Peters *et al.*[12] studied bcl-2 expression on undifferentiated precursors obtained from BM, MPB, and UCB and found that bcl-2 showed a bimodal expression, being less expressed on the most primitive precursor ($CD34^{+++}$/$CD38^{-/low}$) than on the more differentiated ones ($CD34^+$/$CD38^+$); moreover, they detected a strong bcl-XL expression on the immature population with low bcl-2 positivity. These data are in agreement with ours and, together with the observation that bcl-x is predominantly expressed during embryonic development,[9] suggest that in an early phase HPC are protected from apoptosis mostly by bcl-x and subsequently by bcl-2 which, in turn, is lost with differentiation.

We found that Fas was strongly expressed on BM $CD34^+$ cells; previous studies have reported controversial results[11,15,27-29] which may be explained in part by technical details, since different staining intensities are obtained using PE or FITC-conjugated anti-CD95 MoAbs.

We also observed the presence of Fas on $CD34^+$ HPC from UCB; however, CD95 was expressed in a significantly less bright fashion on this source than on than MPB and BM cells. In addition, we found that it was directly related to CD38 and CD71, but inversely to CD34 expression, suggesting that Fas upregulation is linked to stem cell differentiation and proliferation. Some indirect observations are in agreement with this hypothesis: in fact, the majority of BM and MPB $CD34^+$ cells are cycling,[30] being in S and G_1-phase, respectively, whereas, as reported by Lucotti *et al.*,[31] most (68.4±7%) of the freshly harvested UCB-derived $CD34^+$ cells are in the G_0 phase of the cell cycle.

A recent study[29] reported that soluble FasL (sFasL) is able to induce programmed cell death of mature HPC ($CD34^+CD38^+$) or peripheral blood cells (i.e. granulocytes) expressing a bright CD95. By contrast, in the most immature progenitor cells ($CD34^+CD38^-$) with low or absent CD95 expression, sFasL acts as a growth factor, promoting cell survival. The authors, therefore, assumed that sFasL and membrane FasL (mFasL) shift their function from suppression to activation of apoptosis during early hematopoiesis; in a similar way, the CD95/CD95L system has been found to be involved in the regulation of erythropoiesis[32] and in the control of lymphocyte homeostasis.[33]

Following these observations, we carried out *in vitro* assays using the agonistic antibody CH11 to investigate the sensitivity of $CD34^+$ cells to CD95-triggered apoptosis. We found that $CD34^+$ HPC from BM, MPB and UCB were less prone to undergo programmed cell death than mature myeloid BM cells. This observation could be explained with the hypothesis that only an extensive aggregation of Fas molecules may activate CD95-mediated apoptosis efficiently; in fact, it has been demonstrated[34] that a wide Fas aggregation allows the recruitment of the FADD (Fas-associated death domain) protein and pro-caspase-8 and, then, the generation of the Fas/FADD/pro-caspase-8 complex. This latter is essential for the activation of the caspases, the terminal executors of apoptosis; the higher sensitivity of Jurkat cells and myeloid BM cells compared to HPC may reflect increased receptor density or expression of molecules that promote Fas aggregation on the cell surface.

As we found a significatly different expression of Fas among UCB, MPB and BM $CD34^+$ cells, we wondered whether they also had a different susceptibility to CD95-induced apoptosis; interestingly, peripheral blood $CD34^+$ stem cells mobilized with G-CSF treatment *in vivo* showed increased susceptibility to CD95-triggered apoptosis compared to BM and UCB cells; this result suggests that in early HPC, cytokines inducing cell proliferation, such as G-CSF, lead to upregulation of CD95 and increase the sensitivity of the cells to Fas-induced apoptosis.

We observed that Fas and bcl-2 in early HPC are not inversely balanced, as previously reported;[11,27] in fact these proteins seem to be expressed independently of one another, as a consequence of positive and negative stimuli induced by cytokines and adhesion molecules.

In conclusion, although our results require confirmation in a larger series of samples, they suggest that: 1) within the stem cell compartment bcl-2 and Fas expression is dimmer in UCB than in MPB and BM; 2) UCB, MPB and BM cells are characterized by a low susceptibility to undergo CD95-triggered apoptosis; 3) both bcl-2 and Fas are independently expressed as a part of the differentiation program of early HPC; 4) Fas expression could be regarded as a marker of HPC activation and differentiation.

Contributions and Acknowledgments

All investigators contributed to the design of the study. LM collected the data and wrote the article. GDP, AV and

FB revised the study critically for important intellectual content. AB performed functional tests with anti-CD95. SS and TC were responsible for clonogenic assays. AME and BDM performed the cell separation and flow cytometric analyses. AT, AP, GS and AB collected the umbilical cord samples. GC was responsible for the data analysis. SA revised the manuscript and gave final approval for its submission. All investigators approved the final version of the paper. The order in which the authors appear reflects their contribution to the study.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

The finding that normal CD34+ cells are insensitive to CD95-triggered apoptosis provides the rationale for development of anti-Fas-R antibodies which may be a novel approach for leukemia immunotherapy. The expression of pro-apoptotic and anti-apoptotic proteins needs to be investigated during *ex vivo* expansion of CD34+ cells to define optimal cell culture conditions.

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