Expression of Fas/CD95 and Bcl-2 by Primitive Hematopoietic Progenitors Freshly Isolated From Human Fetal Liver

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The cell-surface expression and the functional status of the CD95/Fas antigen on primitive hematopoietic progenitors (PHPs) freshly isolated from human fetal liver (FL) were studied. PHPs were phenotypically defined as CD34+CD38-/+ cells. The most immature subfractions of PHPs, CD34+-CD38- and CD34+-CD38+ FL cells, expressed CD95, whereas the more mature CD34+CD38+ and CD34+CD38- FL cells displayed low CD95 expression. Combinations of cytokines, such as kit ligand (KL) + interleukin-3 or KL + granulocyte-macrophage colony-stimulating factor (GM-CSF) upregulated the expression of CD95 on PHPs upon in vitro culture. Tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) further increased the CD95 expression induced by KL + GM-CSF. The hematopoietic potential of sorted CD34+ lineage (lin) CD95- versus CD34+lin-CD95+ FL cells was compared by colony-forming unit-culture (CFU-C) assays performed in serum-deprived medium. Lin+ cells were composed of erythrocytes, monocytes, T cells, B cells, and natural killer cells. Our results indicated that both CD95- and CD95+ subsets contained pluripotent progenitors, generating myeloid and erythroid progenitors. The functional status of CD95 and the effects of TNF-alpha and IFN-gamma, cytokines known to induce CD95-mediated apoptosis, were analyzed by incubation of PHPs in the presence of anti-CD95 monoclonal antibodies (MoAbs). The effect of anti-CD95 MoAbs was measured by viable cell counting, flow cytometry, and CFU-C assays. A decrease of CFU-C numbers was observed in the presence of anti-CD95 MoAbs and TNF-alpha and/or IFN-gamma. However, whereas growth factor deprivation induced apoptosis of PHPs, cross-linking of CD95 did not lead to apoptosis of PHPs measured by flow cytometry and viable cell counting. The correlation of increased intracytoplasmic levels of bcl-2 with high levels of cell-surface CD34 and the presence of CD95 on fresh FL cells suggests that bcl-2 may be involved in protecting against CD95-mediated apoptosis of FL PHPs.

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Hematopoiesis is supported by a network of growth factors that promote the expansion and differentiation of primitive hematopoietic progenitors (PHPs) into all the hematopoietic lineages. Nevertheless, increasing evidence supports the notion that normal physiologic activity during hematopoietic cell growth involves not only cell division but also programmed cell death. It has been reported that the in vitro deprivation of growth factors, such as erythropoietin (Epo) or interleukin-3 (IL-3), causes the apoptosis of bone marrow (BM) cells and of growth factor-dependent cell lines. Additional pathways other than growth factor deprivation may also regulate apoptosis during hematopoiesis. CD43-mediated apoptosis of CD34+ BM hematopoietic progenitors has been reported. Recent reports have shown the appearance of CD95 on adult BM hematopoietic progenitors after culture in the presence of tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma), suggesting a possible role of CD95 in the regulation of hematopoiesis.

The growth and differentiation of hematopoietic progenitors are differentially regulated throughout embryonic/fetal ontogeny and postnatal life. Indeed, comparative studies of hematopoietic progenitors isolated from FL, cord blood, and adult BM indicated that FL cells had the greatest hematopoietic potential in serum-deprived cultures stimulated with...
Therefore, it is relevant to address the question of whether growth factors or when grown on BM stromal layers. Furthermore, it is relevant to address the question of whether not only the proliferative potential, but also the susceptibility to undergo apoptosis, is different in FL PHPs compared with adult PHPs. In this report, we characterized the expression of CD95 on freshly isolated FL PHPs at 15 to 22 weeks of gestation and the effects of KL, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, TNF-α, and IFN-γ on the levels of cell-surface CD95 expression on FL PHPs. The functional status of CD95 in PHPs was investigated by measuring the hematopoietic potential of FL hematopoietic progenitors in the presence of anti-CD95 MoAbs in colony-forming unit-culture (CFU-C) assays and by measuring apoptosis using flow cytometry.

MATERIALS AND METHODS

MoAbs. The MoAbs used in this study are summarized in Table 1. Isotype-matched controls were purchased as follows: IgG1 and IgG2a from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA); IgG2b from Caltag (South San Francisco, CA); and IgM from Pharmingen (San Diego, CA). All of these MoAbs were used unconjugated, biotinylated, and conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC), as indicated in the figure legends. Streptavidin (SA) APC was purchased from BDIM. Propidium iodide (PI) was purchased from Molecular Probes, Inc (Eugene, OR).

Cytokines. Recombinant human KL, IL-3, IL-6, GM-CSF, and Epo were purchased from R&D Systems, Inc (Minneapolis, MN), unless otherwise indicated, KL, IL-3, IL-6, and GM-CSF were used at 20 ng/mL and Epo was used at 2 U/mL concentrations determined to generate a maximal number of colonies in CFU-C assays. Recombinant IFN-γ (R&D Systems, Inc) was used at 20 ng/mL and recombinant TNF-α (Boehringer Mannheim Biochemicals [Indianapolis, IN] and R&D Systems, Inc) was used at 100 ng/mL.

Isolation of fetal liver hematopoietic progenitors. Human fetal livers were obtained with informed consent from Advanced Biosciences Resources Inc (Alameda, CA), in compliance with regulations issued by the state and by the federal government. Gestational age was determined by foot length and ranged from 15 to 22 weeks. FL samples were homogenized through a wire mesh in the presence of RPMI-1640 (HyClone, Logan, UT) containing 10% fetal bovine serum (FBS). The isolation of CD34+APC+ lin-FITC− FL cells was performed as previously described.

Briefly, the glycoporphin A (GPA)-depleted FL cell suspension was incubated for 30 minutes at 4°C with the following FITC-conjugated MoAbs: anti-CD3 (to stain T cells), anti-CD14 (to stain monocytes), anti-CD16 and anti-CD56 (to stain NK cells), and anti-CD20 (to stain B cells). After washing, the cells were subjected to negative selection using magnetic beads coated with sheep-antimouse IgG (Dynabeads; Dynal, Oslo, Norway). The resulting lin− population was incubated with anti-CD34−APC MoAb before cell sorting using a FACS Vantage (BDIS). The purity of the sorted CD34+ "lin− PI+ and CD34+ "lin− PI+ FL populations was greater than 99% upon reanalysis. In some experiments, the cells were further stained with anti-CD95-FITC MoAbs to sort the CD95− and CD95+ subsets or with anti-CD38-FITC MoAbs to sort the CD38+ and CD38− subsets, among CD34+ "lin− cells.

High proliferative potential colony-forming cell (HPP-CFC) assay. Myeloid progenitor cells were assayed as previously described in semisolid agarose cultures in the presence of serum-deprived medium.

The serum-deprived medium consisted of Iscove's modified Dulbecco's medium (IMDM; Sigma Chemical Co, St Louis, MO) supplemented with 7.5 × 10−4 mol/L α-thioglycerol (Sigma Chemical Co), 50 µg/mL gentamicin (GIBCO BRL, Grand Island, NY), 2% fraction-V ethanol-extracted bovine serum albumin (BSA; Boehringer Mannheim Biochemicals), 200 µg/mL human iron-saturated transferrin (Boehringer Mannheim Biochemicals), 10 µg/mL recombinant human insulin (Boehringer Mannheim Biochemicals), and 40 µg protein/mL human low-density lipoprotein (Sigma Chemical Co). Triplicate or quadruplicate cultures in the presence of KL + GM-CSF + IL-3 + IL-6 were scored after 3 weeks in culture for the presence of HPP-CFC and low proliferative potential colony-forming cells (LPP-CFC). HPP-CFC were defined as precursors that gave rise to colonies greater than 0.5 mm in diameter and containing at least 1 × 106 cells. LPP-CFC were scored as colonies smaller than HPP-CFC but greater than 50 cells/colony.

Δ-Assay. The proliferative potentials of FL CD95+ and CD95− progenitor subpopulations were measured as previously described. In brief, FL CD95+ and CD95− progenitors were seeded into triplicate 1 mL Δ-cultures in serum-deprived medium supplemented with KL + GM-CSF + IL-3 + IL-6. After 1 week of growth in 24-well tissue culture plates (Falcon; Becton Dickinson, Mountain View, CA), the culture cellularity and the number of secondary CFU-C responsive to the cytokine combination mentioned above were determined. The numbers of primary HPP-CFC and LPP-CFC were determined in parallel clonal cultures stimulated by the same 4-cytokine combination. The numbers of cells and CFU-C (secondary HPP-CFC and LPP-CFC) recovered from Δ-cultures are presented as the calculated total number of secondary cells/1 × 106 primary cells or as the fold increase (Δ-value) over input numbers.

Burst-forming unit-erythroid (BFU-E) assays. Clonal cultures to determine the number of erythroid progenitors were performed in triplicate using serum-deprived medium supplemented with 1.2% human

<p>| Table 1. MoAbs Used in This Study |</p>
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4,000 centipoises methyl cellulose (Sigma Chemical CO) and Epo + KL + GM-CSF. Colonies containing red blood cells were scored as BFU-E after 3 weeks of growth at 37°C in 5% CO₂ in air.

Data presentation and statistical analysis. The data from multiple experiments were pooled and are presented as the mean ± standard error of the mean (SE). Statistical significance was determined on individual experiments using the two-tailed paired Student’s t-test. Differences in the data were considered significant when P ≤ 0.05.

Immunofluorescence and flow cytometry. Cell surface phenotypic analyses were performed as previously described, using a FACSort (BDIS). For intracytoplasmic staining with anti-bcl-2-FITC MoAbs and isotype-matched control MoAbs, cells were fixed overnight at 4°C with phosphate-buffered saline (PBS) containing 1% paraformaldehyde. After treatment of the cells with PBS containing 0.1% Triton X-100 (Sigma Chemical Co) at 4°C for 40 minutes to permeabilize the cell membrane, cells were extensively washed in PBS containing 2% FBS, 0.01% NaN₃ (Sigma Chemical Co), and 0.1% Triton X-100. The cells were then stained for 30 minutes at 4°C with the indicated MoAbs in the presence of the PBS solution used for the washes. After three washes, the cells were resuspended in PBS containing 1% paraformaldehyde and analyzed on the FACSort.

Induction of apoptosis in culture by anti-CD95 MoAbs. The Jurkat and CEM cell lines were used as controls for the anti-CD95 MoAb-induced apoptosis assays in FL hematopoietic cells. The anti-CD95 MoAb (IgM) used in these assays was the clone CH-1 (Upstate Biotechnology Inc, Lake Placid, NY) and was added to the cultures at the concentrations indicated in the figure legends. Both anti-CD95 MoAbs and isotype-matched controls (IgM) were dialyzed overnight against IMDM medium containing 2% FBS at 4°C to eliminate NaN₃. Jurkat and CEM cells were cultured at 0.5 to 1 × 10⁶ cells/mL in the presence of different concentrations (0.1 to 5 µg/mL) of the CH-1 anti-CD95 MoAb and an isotype control for 1, 3, 6, and 12 hours.

Assessment of DNA fragmentation by flow cytometry. Measurements of apoptosis were performed by using the dye Hoechst 33342, which generates fluorescent complexes when bound to DNA. After the exposure to anti-CD95 MoAbs for the indicated periods of time, 0.5 to 2 × 10⁶ cells were harvested, washed twice in PBS containing 2% BSA, and resuspended at 10⁶ cells/mL in serum-deprived medium (in the case of FL hematopoietic progenitors) or in RPMI-1640, 10% FBS (in the case of Jurkat or CEM cell lines). The cells were then stained with 15 mm/mL Hoechst 33342 (Molecular Probes Inc) for 1 hour at 37°C and analyzed using a FACS Vantage flow cytometer with UV excitation at 310 nm and doublet discrimination for single cells, as previously described. Data from 30,000 cells were collected and analyzed by gating the viable (PI-) cells. Cellularity and cell viability were determined by cell counting and trypan blue exclusion, respectively, at each time point using a hemocytometer.

RESULTS

CD95 is expressed on primitive FL hematopoietic progenitors. It has been previously shown that the levels of CD38 expression on CD34+ hematopoietic progenitors gradually increase during their differentiation, regardless of whether the source of hematopoietic progenitors was human FL, fetal BM, or adult BM. The expression of CD95 on FL hematopoietic progenitors at different stages of maturation, as assessed by the gradual acquisition of CD38, was investigated. Freshly isolated FL cells depleted of GPA+ cells were stained with anti-CD34, anti-CD38, and anti-CD95 MoAbs.

Figure 1 shows that the most primitive subset of FL hematopoietic progenitors, CD34+CD38 cells (region R1), expressed CD95 antigen. The next population in the developmental pathway, CD34+CD38 FL cells (region R2), showed a similar profile of CD95 expression compared with the previous population. The more mature CD34+CD38+ FL cells (region R3) as well as CD34+CD38- FL cells (region R4) expressed CD95 at low levels, suggesting that CD95 cell-surface expression is modulated during hematopoietic maturation in the FL.

CD95 is expressed by both myeloid and lymphoid FL hematopoietic progenitors. To study the distribution of hematopoietic progenitors committed to either the myeloid or the lymphoid lineages among the CD95+ and CD95- subpopulations, three-color analyses of freshly isolated GPA+ PI-lin FL cells were performed. In these experiments, anti-CD19 MoAbs were added to the lin markers used throughout the study (composed of CD3, CD14, CD20, and CD56) to thus enrich in early (CD10+CD19+) lymphoid progenitors. As shown in Fig 2A, an electronic gate was set to contain the CD34+ fraction among GPA+PI-lin FL cells. In Fig 2B, the analysis of the expression of CD95 versus several cell-surface markers is shown on gated CD34+GPA+PI-lin FL cells. Eleven percent of the myeloid progenitors and primitive hematopoietic progenitors, which express CD33 marker, coexpressed CD95. Most of the early lymphoid progenitors (CD34+CD10+CD19+) coexpressed CD95. We also analyzed other cell-surface markers that are present on immature hematopoietic progenitors, such as HLA-DR, CDw90 (Thy-1), CD7, CD45RA, and CD117 (c-kit).

Figure 2B shows that HLA-DR is expressed by most of the CD34+CD95+ FL population. CDw90, CD7, and CD117 were found to be expressed in about one-third of the CD34+CD95+ FL cells. Low levels of CD45RA are expressed by 20% of CD34+CD95- FL cells, whereas 15% of CD34+CD95- FL cells lacked CD45RA expression.

KL, IL-3, and GM-CSF upregulate CD95 expression on hematopoietic progenitors in vitro. We next assessed whether various growth factors known to promote expansion and differentiation of primitive hematopoietic precursor cells might influence CD95 expression on these cells or their progeny. KL, IL-3, and GM-CSF induce a synergistic proliferative response of a heterogeneous array of hematopoietic progenitors, including HPP-CFC and LPP-CFC FL cells. FACs analyses were performed on the progeny of sorted CD34+CD38 lin FL cells that were cultured in the presence of KL + GM-CSF, KL + IL-3 (Fig 3), or KL + IL-6 (data not shown). It was observed that, irrespective of the day of staining or the cytokine combination used to promote the proliferation of FL hematopoietic progenitors, CD95 expression was upregulated (Fig 3). In addition, it has been shown that TNF-α and IFN-γ markedly increase CD95 expression on both mature and immature hematopoietic cells. In Fig 4, CD95 expression on sorted CD34+lin FL cells was determined on freshly isolated cells and after 3 days in culture in the presence of several combinations of KL + GM-CSF, TNF-α, and IFN-γ. The culture of CD34+lin FL cells in the presence of KL + GM-CSF resulted in an increase in the percentage of cells expressing
CD95 compared with the freshly isolated population, although the fluorescence intensity did not substantially increase (Figs 3 and 4). The addition of TNF-α and/or IFN-γ on KL + GM-CSF–stimulated cultures increased both the percentage of cells expressing CD95 and the fluorescence intensity of CD95 expression on FL hematopoietic progenitors over the levels observed in cultures supported by KL + GM-CSF. The maximal induction of CD95 expression was obtained with the combination of TNF-α + IFN-γ (Fig 4).

Interestingly, no induction of CD95 was observed when the CD34<sup>−</sup> FL cells were cultured in serum-deprived medium in the absence of cytokines.

**CD95 defines subpopulations with different hematopoietic potential among the CD34<sup>−</sup> FL cells.** The finding of an heterogeneous expression of CD95 on CD34<sup>−</sup> FL hematopoietic precursors opened the possibility of separating by cell sorting the CD95<sup>−</sup> and the CD95<sup>+</sup> subsets and of comparing their hematopoietic potential in CFU-C assays. One important consideration in the cell separation and subsequent functional study was to use MoAbs against CD95 that did not promote apoptosis of the cells of interest. In our experiments, the anti-CD95 MoAbs of the IgG1 isotype used for the phenotypic characterization and for the cell sorting were unable to induce apoptosis of Jurkat and CEM cell lines, activated peripheral blood lymphocytes, and FL cells (data not shown). Our standard sorting and reanalysis protocol is depicted in Fig 5A and the results of a representative reanalysis of the sorted populations are shown in Fig 5B. We consistently observed an average high purity (≥98%) of CD95<sup>−</sup> cells and a lower purity (≥92%) of CD95<sup>+</sup> cells, which was due to the low level of CD95 expression on CD34<sup>++</sup> cells. CD95<sup>−</sup> and CD95<sup>+</sup> cells displayed a similar FSC versus SSC profile (data not shown), indicating no difference in size between these populations.

CD34<sup>−</sup>FL CD95<sup>−</sup> and CD34<sup>−</sup>FL CD95<sup>+</sup> FL subpopulations were assayed for the presence of myeloid progenitors capable of generating large colonies (HPP-CFC) and smaller colonies (LPP-CFC). In serum-deprived cultures stimulated
with a combination of cytokines (KL + GM-CSF + IL-3 + IL-6) found to support a high frequency of CFU-C, both CD34++lin CD95- and CD34++lin CD95+ FL gave rise to myeloid progenitors belonging to the HPP-CFC or LPP-CFC type (Fig 6A). The CD95- subpopulation showed a slightly greater myelopoietic potential compared with CD95+ cells. Statistical analyses of the data indicated that CD95+ FL progenitors generated statistically significant higher numbers of LPP-CFC in three of five experiments \((P = .004 \text{ to } .04)\), whereas no statistically significant difference in the numbers of HPP-CFC was observed.

To eliminate the possibility that the anti-CD95 MoAbs used for the sorting affect the in vitro proliferation of FL hematopoietic progenitors, the sorted subpopulations were cultured in parallel liquid cultures in serum-deprived medium supplemented with KL + GM-CSF + IL-3 + IL-6 in the presence of saturating amounts (up to 5 \(\mu\)g/mL) of the unconjugated anti-CD95 MoAbs (IgG\(_1\)) used for the cell sorting or in the presence of an isotype control. The culture cellularity and viability, which were determined daily by cell counting and trypan blue exclusion, indicated no differences in the cultures containing anti-CD95 MoAbs compared with cultures containing an isotype control. The cellular viability typically ranged from 85% to 95% in these cultures.

To further characterize the hematopoietic potential of CD34++lin CD95+ and CD34++lin CD95- FL cells, secondary myeloid CFU-C assays were performed after 7 days of growth in \(\Delta\)-cultures in the presence of KL + GM-CSF + IL-3 + IL-6 (Fig 6A). A consistent observation in all the experiments performed \((n = 4)\) was that the CD34++lin CD95+ FL cells proliferated more extensively than did the CD34++lin CD95- subpopulation. The \(\Delta\)-values for the increase of the culture cellularity indicated an average 282-fold and a 154-fold increase for the CD34++lin CD95+ and CD34++lin CD95- cells, respectively \((n = 4)\). Figure 6A shows that the total number of secondary colonies generated from CD34++lin CD95+ FL cells was again slightly greater than that from the CD95- counterpart, although this difference did not prove to be statistically significant.
Fig 3. Regulation of CD95 expression on FL hematopoietic progenitors by cytokines. Sorted CD34+CD38−lin−PI− FL cells (>98% pure) were cultured at 5 × 10^5 cells/mL in serum-deprived medium stimulated with KL + GM-CSF or KL + IL-3. After the indicated periods of time, cells were harvested and stained with anti-CD95-PE MoAbs (M) and IC MoAbs (O). The X-axis represents the fluorescence intensity in logarithmic scale and the Y-axis represents the relative number of cells. A total of 2 × 10^6 PI− cells were collected using a FACSsort for analysis. Results from one representative experiment of three are shown.

Fig 4. Effects of TNF-α and IFN-γ on CD95 expression of CD34+lin−PI− FL cells. Sorted CD34+lin−PI− FL cells (>99% pure) were cultured at 10^5 cells/mL in serum-deprived medium and stimulated with KL + GM-CSF ± TNF-α, IFN-γ, or both. After 3 days in culture, cells were harvested and stained with anti-CD95-PE MoAbs (M) and IC MoAbs (O). A total of 2 × 10^6 PI− cells were collected. The X-axis represents the fluorescence intensity for CD95 expression in logarithmic scale and the Y-axis represents the relative number of cells. Representative results are shown of three experiments.
cells after pretreatment with the IgG, anti-CD95 MoAb used for the staining and sorting of FL cells. The Jurkat cell line was used as a positive control for anti-CD95–mediated apoptosis. The results of our study are shown in Fig 7A, in which 44% of PI− Jurkat cells underwent apoptosis when cultured for ≥3 hours in the presence of ≥1 μg/mL anti-CD95 MoAbs. The sensitivity of our flow cytometry tech-

The erythroid progenitor potential of CD34+lin−CD95− and CD34+lin−CD95+ FL cells was also determined (Fig 6B) in methyl-cellulose cultures in the presence of KL + GM-CSF + Epo. The numbers of BFU-E were scored in culture and the majority of the red blood cells containing colonies were also observed to contain myeloid cells (CFU-mix). In line with the results obtained in the primary and secondary CFU-C, CD34+lin−CD95− FL cells did show a more extensive erythroid progenitor activity than the CD95+ hematopoietic progenitors (P = .0007 to .0691, n = 3).

Functional analysis of CD95 on FL hematopoietic progenitors. The functional status of CD95 expressed by FL hematopoietic progenitors was determined by culturing sorted CD34+lin− FL cells in the presence of anti-CD95 MoAbs (CH-11, IgM) known to promote apoptosis of human PBL and cell lines by mimicking the natural CD95 ligand.80 These studies were performed with the total CD34+lin− FL cells to avoid the observed inhibition of CH-11 binding to the

![Fig 5. Summary of our protocol to sort CD34+lin−PI−CD95− or CD95+ FL cells. (A) R1 gate was set to contain most of GPA-lin-FITC−FL cells. R2 includes the viable (PI−) cells and R3 excludes the remaining contaminating lin-FITC+ cells. For the sort, an electronic gate comprising R1 + R2 + R3 was set; subsequently, two gates were set to sort the CD34+lin−CD95+ and the CD34+lin−CD95− subpopulations. In (B), the results of reanalysis of the cells after the sort are shown. Note that the dot plots have been magnified to increase resolution by using the CellQuest Program (BDIS). Representative results are shown of six experiments.](image)

![Fig 6. Myeloid and erythroid progenitor activity in the CD95+ and CD95− fractions of the CD34+lin+PI− FL progenitors. (A) HPP-CFC and LPP-CFC assays were performed as described in the Materials and Methods. The primary (1) and secondary (2) HPP-CFC and LPP-CFC numbers are presented as the median ± SE of five and four results, respectively. (B) BFU-E assays were performed as described in the Materials and Methods, and the numbers of BFU-E are presented as the median ± SE of three experiments.](image)
nique to detect apoptotic cells not only of cell lines, but also of hematopoietic progenitors was shown by 3-day growth factor-induced proliferation (KL + GM-CSF + IL-3 + IL-6 in serum-deprived medium), followed by 3-day growth factor deprivation of sorted CD34\(^+\)FL cells maintained at 0.5 to 1 \(\times\) 10\(^6\) cells/mL (Fig 7B). Under these conditions, the presence of anti-CD95 MoAbs did not augment the level of apoptosis induced by growth factor deprivation after any of the time periods analyzed (1, 2, and 3 days in culture). Freshly isolated CD34\(^++\)lin\(^-\) FL cells were exposed to anti-CD95 MoAbs or to an irrelevant IgM MoAb for 24 and 48 hours and no apoptosis was detected (data not shown). Induction of apoptosis of CD34\(^++\)lin\(^-\) FL cells mediated by anti-CD95 MoAbs was further tested in the presence of TNF-\(\alpha\) and IFN-\(\gamma\), known activators of the CD95 pathway. As shown in Fig 7C, CD34\(^++\)lin\(^-\) FL cells did not undergo apoptosis in the presence of anti-CD95 MoAbs when cultured with TNF-\(\alpha\) and IFN-\(\gamma\) for 3 (Fig 7C) or 5 days (data not shown). Cellularity and cellular viability were not modified in any of the different culture conditions compared with the controls. These data indicate that, similarly to mature lymphocytes, the cross-linking of CD95 with MoAbs known to induce apoptosis is not sufficient to obtain CD95-mediated apoptosis after 3 or 5 days of exposure to anti-CD95 MoAbs.

To study the possible effect of anti-CD95 MoAbs during hematopoietic differentiation, we analyzed the myelopoietic potential of sorted PHPs (CD34\(^++\)CD38\(^-\)FL cells) as well as the more mature CD34\(^++\) CD38\(^-\) FL cells in the presence of anti-CD95 MoAbs or an isotype-matched MoAb and in the presence of combinations of TNF-\(\alpha\) and/or IFN-\(\gamma\). The numbers of total secondary CFU-C were determined in these cultures and in Fig 8 it is shown that the anti-CD95 MoAbs did not significantly modify the number of secondary CFU-C generated by CD34\(^++\) CD38\(^-\)FL cells in the presence of anti-CD95 MoAbs or an isotype-matched MoAb and in the presence of combinations of TNF-\(\alpha\) and/or IFN-\(\gamma\). The numbers of total secondary CFU-C were determined in these cultures and in Fig 8 it is shown that the anti-CD95 MoAbs did not significantly modify the number of secondary CFU-C generated by CD34\(^++\) CD38\(^-\) FL cells in the presence of anti-CD95 MoAbs or an isotype-matched MoAb and in the presence of combinations of TNF-\(\alpha\) and/or IFN-\(\gamma\). The addition of TNF-\(\alpha\) and/or IFN-\(\gamma\) greatly decreased the number of CFU-C compared with the controls obtained in the absence of these factors. These data are in agreement with reports showing the inhibitory effects of TNF-\(\alpha\) and IFN-\(\gamma\) on adult BM hematopoietic progenitors. The presence of anti-CD95 MoAbs on the cultures with

Fig 7. Functional status of CD95 on FL hematopoietic progenitors. (A) Jurkat cells, used as controls for anti-CD95 MoAbs mediated apoptosis, were analyzed after 3 hours in the presence of medium, 2 \(\mu\)g/mL of IgM (IC), or 2 \(\mu\)g/mL of anti-Fas MoAb. (B) Sorted CD34\(^++\)lin\(^-\) FL progenitors were cultured in the presence of KL + IL-3 + IL-6 + GM-CSF, washed, cultured 3 days under the indicated conditions, and tested for the appearance of apoptotic cells. (C) Sorted CD34\(^++\)lin\(^-\) FL progenitors were cultured in the presence of the same growth factors indicated above \(\pm\) TNF-\(\alpha\) or IFN-\(\gamma\) and \(\pm\) 2 \(\mu\)g/mL of anti-Fas MoAb or isotype control for 3 or 5 days and tested for the presence of apoptotic cells. Results of day 3 of analysis are shown. The percentage of apoptotic cells, indicated by the marker on the left of the histograms, was less than 5% in all of the cases. The results of three representative experiments are shown.
Fig 8. Effects of anti-CD95 MoAbs on myeloid progenitor activity in the FL. The hematopoietic potential of sorted CD34⁺CD38⁻lin⁻PI⁻ and CD34⁺CD38⁺lin⁻PI⁻ FL cells (>98% pure) was tested using the Δ-assay in the presence of KL + IL-3 + IL-6 + GM-CSF ± 2 μg/mL of anti-Fas MoAb or IC. TNF-α and/or IFN-γ were included, as indicated at the bottom of the figure. The numbers of secondary CFU-C are shown as the median ± SE of triplicate cultures from two independent representative experiments. *Statistical significance (P ≤ .05).
freshly isolated CD34+CD38− and CD34+CD38+ cells, which are considered to comprise the earliest, most primitive hematopoietic progenitors in the FL. We report that the downregulation of CD95 during hematopoietic differentiation correlated with an increase in the expression of CD38 and a decrease in the expression of CD34. In addition, we showed that CD95 is present on both myeloid (CD34−CD33+) and lymphoid (CD34+CD10−CD19−) progenitors. These results suggest that CD95 expression occurs in a defined window during fetal hematopoietic development in which CD95 might play an important role in the regulation of early hematopoiesis.

Our results differ with recent reports in which the freshly isolated total CD34+ BM cells displayed negative or low CD95 expression. In those studies, detectable CD95 expression was observed after in vitro exposure of the hematopoietic progenitors to TNF-α and/or IFN-γ. It should be noted that different intensity of staining was obtained with anti-CD95-FITC versus anti-CD95-PE MoAbs (Fig 2). The anti-CD95-PE MoAbs gave a greater resolution of the FL subpopulations as well as brighter staining (1 decade higher) compared with the anti-CD95-FITC MoAbs, as previously reported. Previous reports showing lack of expression of CD95 on freshly isolated CD34+ BM cells used anti-CD95-FITC MoAbs, which might account for their negative results. Indeed, we have observed similar expression of CD95 on adult BM PHPs compared with FL PHPs by using anti-CD95-PE MoAbs (Bárcena and Muench, manuscript in preparation). In our system, TNF-α or IFN-γ also induced an upregulation of CD95 expression on FL hematopoietic progenitors. In addition, our data indicate that the upregulation of CD95 was a consequence of the exposure of the cells to any of the combinations of cytokines tested (KL + IL-3 or GM-CSF or IL-6), suggesting a general mechanism of CD95 upregulation during proliferation promoted by growth factors.

The presence of CD95 on a subpopulation of PHP led us to explore the hematopoietic potential of CD34+ versus CD34+/− PHPs. Both subpopulations generated mixed CFU-C (exhibiting myeloid and erythroid progenitors) in clonogenic cultures in methyl cellulose, indicating that they indeed contained multipotent progenitors. We conclude from our data that both subpopulations contain fairly similar numbers of myeloid progenitors in terms of primary and secondary HPP-CFC and LPP-CFC. However, the CD95− compartment of hematopoietic progenitors did have

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**Fig 9.** Intracytoplasmic expression of bcl-2 on sorted subpopulations of FL progenitors. Anti-bcl-2-FITC MoAbs (thick lines) were used as well as IgG1-FITC IC (thin lines) to stain the indicated subpopulations of hematopoietic progenitors. (A) and (B) indicate independent cell sorting experiments representative of three and two experiments, respectively. A total of 1 × 10⁶ cells were collected and analyzed using a FACSort.
a tendency of generating greater numbers of myeloid progenitors than the CD95− counterpart population. In agreement with this observation was the observed higher number of BFU-E obtained from CD95− cells compared with CD95+ cells and the acquisition of CD95 expression by CD95− after culture in the presence of cytokines. These data suggest that the CD95− subpopulation might precede the CD95+ subpopulation during early hematopoietic development. A consistent observation was that, in all the Δ-assays performed, the CD34++lin−CD95+ FL cells proliferated more extensively than the CD34++lin−CD95− subpopulation. One possible interpretation is that the activation or proliferative status is different in these subpopulations, although no difference in size was observed between CD34++lin−CD95+ and CD34++lin−CD95− cells. We hypothesize that the CD95+ FL progenitors might represent a rapidly dividing compartment among CD34++lin− FL cells, whereas the CD34++lin−CD95− subpopulation might be composed of slowly proliferating or quiescent cells. Future experiments will test this hypothesis.

Recent reports have shown that anti-Fas MoAbs are able to induce apoptosis of CD34+ BM progenitors as well as to decrease the number of myeloid and erythroid CFU-C in the presence of TNF-α and/or IFN-γ. In agreement with these findings, we did not find any measurable effect of anti-CD95 MoAbs on the apoptosis of FL PHP in the presence of growth factors. Furthermore, we observed no diminution of the number of secondary colonies that FL PHP generated in these conditions. Our data indicate that anti-CD95 MoAbs have an inhibitory effect on the number of CFU-C generated by both CD34++CD38−lin− and CD34++CD38lin− FL progenitors when TNF-α and/or IFN-γ were present in the cultures, which is in agreement with the findings of previous reports. However, and in contrast with these reports, we were unable to detect DNA fragmentation of hematopoietic progenitors incubated for 3 and 5 days with anti-CD95 MoAbs and TNF-α or IFN-γ. In this study, measurement of DNA fragmentation on viable cells (PI−) was performed by flow cytometry, which has been shown to be a powerful technique as sensitive as the agarose gel electrophoresis of nucleosomal DNA ladder. The explanations for this discrepancy can be several. First, it has been reported that apoptosis can occur in human polymorphonuclear cells and in cell lines with low DNA fragmentation. It has been shown that FL cells had greater hematopoietic potential in serum-deprived cultures stimulated with growth factors or when grown on BM stromal layers in comparison with adult BM progenitors. Therefore, a differential susceptibility of FL PHP compared with adult BM PHP to undergo apoptosis is plausible. This difference might be due to intracellular events located downstream of the CD95-mediated signaling cascade and to a different rate of the apoptotic cascade. Second, a decrease of CFU-C numbers when PHPs are cultured in the presence of anti-CD95 MoAbs and TNF-α and/or IFN-γ due to inhibition or accelerated differentiation of hematopoietic progenitors rather than apoptosis is a possibility consistent with the data obtained.

In this report, we established that there is a clear correlation between the levels of expression of bcl-2 and CD34 in FL hematopoietic progenitors. Furthermore, we observed a higher level of expression of bcl-2 in the CD95+ subset than in CD95− cells among CD34++lin− hematopoietic progenitors. Our interpretation of these data is that the expression of bcl-2 (and possibly of other members of the bcl-2 family) is regulated during hematopoietic differentiation and is likely to play a protective role against apoptotic signals received by hematopoietic progenitors. The presence of bcl-2 and bcl-xL proteins in the majority CD34lin− BM cells has been recently investigated and the expression of bcl-xL was detected in PHPs defined as CD34+CD38− BM cells. Interestingly, this population is extremely rare in the FL, in which most of the CD34+CD38− cells express CD33. Functional analysis of the two subsets CD33+ and CD33− among CD34++CD38− FL cells indicated a far greater myelopoietic potential of the CD33− subpopulation compared with the CD33+ subset, again suggesting that BM and FL PHP might be phenotypically and functionally different.

Finally, we conclude that the functional analysis of molecules expressed on hematopoietic precursors during human hematopoietic development might shed light on the underlying mechanisms responsible for the regulation of hematopoiesis. The role of apoptosis as a regulator of hematopoiesis and the involvement of molecules such as CD95 and CD43 has just begun to be explored. Knowledge gained from these studies may lead to a better understanding of the early events responsible for the physiological regulation of fetal and postnatal hematopoiesis and may potentially aid in the understanding of the mechanisms involved in the generation of some hematological disorders.

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Expression of Fas/CD95 and Bcl-2 by primitive hematopoietic progenitors freshly isolated from human fetal liver

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