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Apoptosis Protection by the Epo Target Bcl-X_L Allows Factor-Independent Differentiation of Primary Erythroblasts

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Summary

Background: Erythropoietin (Epo) is required for correct execution of the erythroid differentiation program. Erythropoiesis requires Bcl-X_L , a major late target of Epo-receptor signaling. Mice lacking Bcl-X_L die around embryonic age E12.5, forming normal erythroid progenitors but lacking functional red cells. Recently, serum-free culture conditions for expansion of murine red cell progenitors were developed, yielding cells capable of in vivo-like terminal differentiation into enucleated erythrocytes, in response to Epo/insulin. Here we address whether Epo function during terminal maturation involves a cytokine-independent "default program," requiring only apoptosis inhibition through Epo-dependent upregulation of Bcl-X_L.
Results: Exogenous expression of Bcl-X_L or Bcl-2 in primary murine erythroblasts or clonal erythroblast lines derived from *p53*$^{-/-}$ mice allowed these cells to undergo terminal erythroid maturation, in the complete absence of cytokines. A potential autocrine Epo loop was ruled out by respective neutralizing antibodies. Importantly, sustained proliferation of Bcl-X_L-expressing immature erythroblasts still required respective factors (Epo, stem cell factor [SCF], and the glucocorticoid receptor ligand dexamethasone [Dex]). Epo-independent differentiation in these Bcl-X_L- or Bcl-2-expressing, primary erythroblasts was thus triggered by removal of the renewal factors SCF and Dex. This initiated the maturation-specific expression cascade of erythroid transcription factors, followed by differentiation divisions (characterized by a short G1 phase and decrease in cell size), hemoglobin accumulation, and enucleation.
Conclusions: During erythroid maturation, Epo regulates red cell numbers via apoptosis inhibition, caused by Epo-dependent upregulation of the antiapoptotic protein Bcl-X_L. This allows "default" terminal differentiation of apoptosis-protected, committed erythroblasts, independent of any exogenous signals.

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Introduction

The cytokine erythropoietin (Epo) is the principal regulator of red cell numbers by controlling terminal differentiation of immature erythroid progenitors into mature red cells. Targeted disruption of Epo or its cognate receptor (EpoR) in the mouse demonstrated the strict requirement of Epo signaling for red cell development. Embryos lacking Epo or EpoR die around embryonic day E12.5 from failure of definitive erythropoiesis [1, 2]: although the embryos produced apparently normal BFU-E and CFU-E progenitors, these failed to develop into mature, hemoglobinized erythrocytes due to an enhanced rate of cell death [3, 4]. In line with this, expression of Bcl-X_L, an antiapoptotic member of the Bcl-2 gene family, was strongly upregulated late during red cell maturation in an Epo-dependent fashion [5]. Epo withdrawal caused downregulation of Bcl-X_L and Bcl-2 followed by apoptosis in Epo-dependent erythroid and lymphoid cell lines [6, 7]. This probably reflects an aberration of established cell lines, since primary erythroid progenitors do not proliferate with Epo alone and express no Bcl-2 and little Bcl-X_L [5, 8]. Furthermore, Bcl-2 knockout mice do not exhibit an obvious erythroid phenotype [9]. Bcl-X_L-mediated apoptosis protection, however, is required for erythroid differentiation since full [10] or conditional, hematopoietic-specific Bcl-X_L knockout mice [11] died during embryogenesis from fetal liver hematopoietic defects or displayed severe anemia.

These observations raised the question whether Epo regulates erythroid differentiation solely through apoptosis protection via upregulation of Bcl-X_L or whether additional Epo-dependent target genes are required. The available, established erythroid cell lines were unsuitable to address this question, since they require nonphysiological stimuli to induce maturation and differentiate incompletely. Even when Epo-responsive for proliferation, such cell lines show aberrant patterns of gene expression during maturation [6, 12, 13].

Recently, we developed culture conditions for mouse erythroblasts allowing sustained proliferation without differentiation (= renewal) in serum-free media. These cells undergo terminal differentiation into enucleated erythrocytes in response to the physiological agents Epo and Ins [8, 14]. Characterisation of these cells by expression profiling using cDNA arrays [8] correctly identified genes known to be induced by Epo during differentiation, such as *pim-1* [15] and *bcl-X$_l$* [5].

In this paper, we demonstrate that Bcl-X_L is hardly expressed in renewing primary erythroblasts but is strongly upregulated in maturing cells in a strictly Epo-dependent manner. Retroviral transduction of *bcl-X$_L$* or *bcl-2* into primary or immortalized (*p53*$^{-/-}$) erythroid progenitors did not alter their dependence on renewal factors such as Epo, stem cell factor (SCF), and dexamethasone (Dex) during sustained proliferation but allowed the cells to undergo terminal erythropoiesis in the complete absence of Epo/Ins. This included differentiation divisions as well as induction of the characteristic expres-

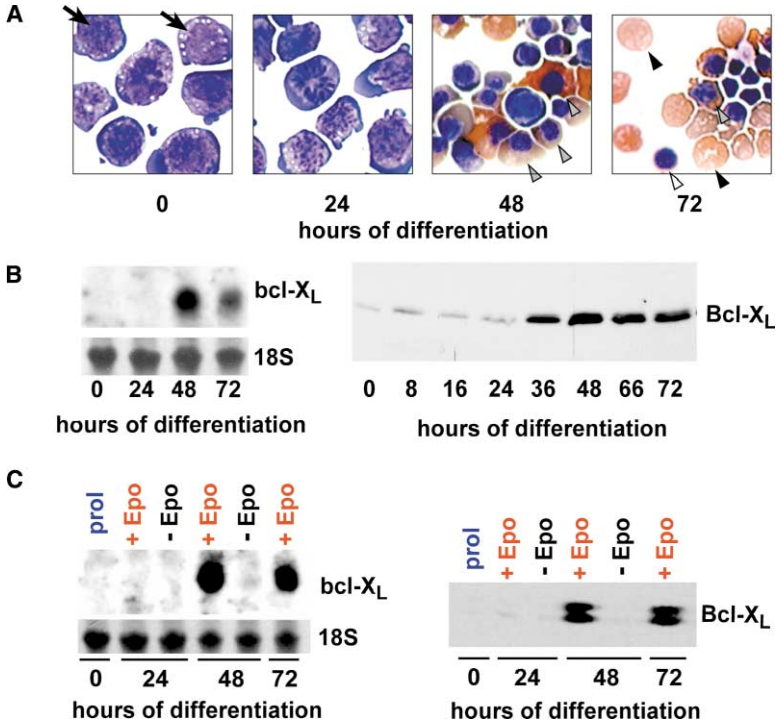

Figure 1. Bcl-X_L Expression during Terminal Erythroid Differentiation Is Induced by Epo

(A) *p53*$^{-/-}$ mouse erythroblasts (clone I/11) were induced to differentiate in standard differentiation medium plus Epo/Ins and cytospins prepared at 24 hr intervals. Black arrows, immature erythroblasts; gray arrowheads, hemoglobinized (brownish stain), partially mature nucleated erythroid cells; black arrowheads, mature, enucleated erythrocytes; white arrowheads, extruded nuclei.

(B) Aliquots from differentiating I/11 cells were analyzed for Bcl-X_L mRNA (left; 18S: 18S ribosomal RNA, loading control) and protein (right; total cell lysates, using a mouse-specific Bcl-X_L antibody).

(C) Cultures of I/11 cells were differentiated in the presence (+Epo) or absence (−Epo) of Epo and aliquots analyzed for Bcl-X_L mRNA (left) and protein (right) as above. Cells −Epo after 72 hr could not be analyzed, due to massive apoptosis. Bcl-X_L protein appears as a double band due to phosphorylation, which was not always resolved due to different gel systems; compare (B) and (C).

sion cascade of key erythroid transcription factors. Erythroid progenitors protected from apoptosis by exogenous Bcl-X_L or Bcl-2 executed the erythroid differentiation program even in the complete absence of any exogenous signals, as shown by using fully defined media. This indicates that Epo controls erythroid differentiation via Bcl-X_L-mediated apoptosis protection. In the presence of Bcl-X_L or Bcl-2, terminal erythroid maturation proceeds as a cell-autonomous process, triggered by removal of renewal factors, which seem to repress this intrinsic default pathway in committed erythroblasts.

Results

Epo-Dependent Expression of Bcl-X_L during Synchronous Red Cell Maturation

Recently, we described cultures of immortalized erythroblasts from p53-deficient mice (clone I/11), which closely resemble primary erythroid progenitors with respect to renewal factor dependence and normal erythroid differentiation [8, 14]. We screened for potential Epo target genes by expression profiling [8] during synchronous, terminal erythroid maturation of these cells (Figure 1A).

Of all the apoptotic and antiapoptotic genes represented on the cDNA arrays, the most striking regulation was exhibited by *bcl-X$_L$*, a member of the *bcl-2* gene family with antiapoptotic function. Bcl-X_L mRNA levels remained low during the first 20 hr of erythroid differentiation but were strongly upregulated in the maturing cells after 48 hr, a finding confirmed by Northern blot analysis (Figures 1A and 1B) and previously observed in other systems [5, 16]. Western blot analysis confirmed that self-renewing erythroblasts had low Bcl-X_L protein expression, which did not change during the first 24 hr of differentiation but was strongly induced between 24 and 36 hr of differentiation, remaining nearly constant until the end of the process at 72 hr (Figure 1B, right panel). Expression of Bcl-2 protein could neither be detected in proliferating nor in differentiating erythroid cells (Figure 5B).

Previous work on erythroid cell lines dependent on Epo for proliferation had indicated that signaling via the EpoR caused induction of Bcl-X_L, protecting proliferating cells from apoptosis [7, 16, 17]. We therefore addressed in our model system whether EpoR signaling was required for induction of Bcl-X_L during terminal erythropoiesis. I/11 erythroblasts were induced to differentiate in the presence or absence of Epo and analyzed at different time points for differentiation parameters as well as for expression of *bcl-X$_L$* mRNA and protein. As expected, erythroid cells started to undergo massive apoptosis after 48 hr of culture in the absence of Epo (data not shown) and failed to express Bcl-X_L at any time (Figure 1C) but differentiated normally in the presence of Epo and expressed high amounts of *bcl-X$_L$* mRNA and protein after 48 hr (Figure 1C). In conclusion, Epo is required for the induction of Bcl-X_L late during erythroid differentiation, in line with its role in protection against apoptosis.

Exogenous Expression of Bcl-X_L Rescues Differentiation of Erythroid Cells in the Absence of Epo

Next we analyzed whether expression of exogenous Bcl-X_L could substitute for Epo signaling during erythroid differentiation. For this, I/11 erythroblasts were infected with a retroviral vector expressing human Bcl-X_L, linked to a GFP gene via an internal ribosome entry site (MSCV-GFP-IRES-Bcl-X_L, see Experimental Procedures). FACS analysis revealed that >80% of the cells

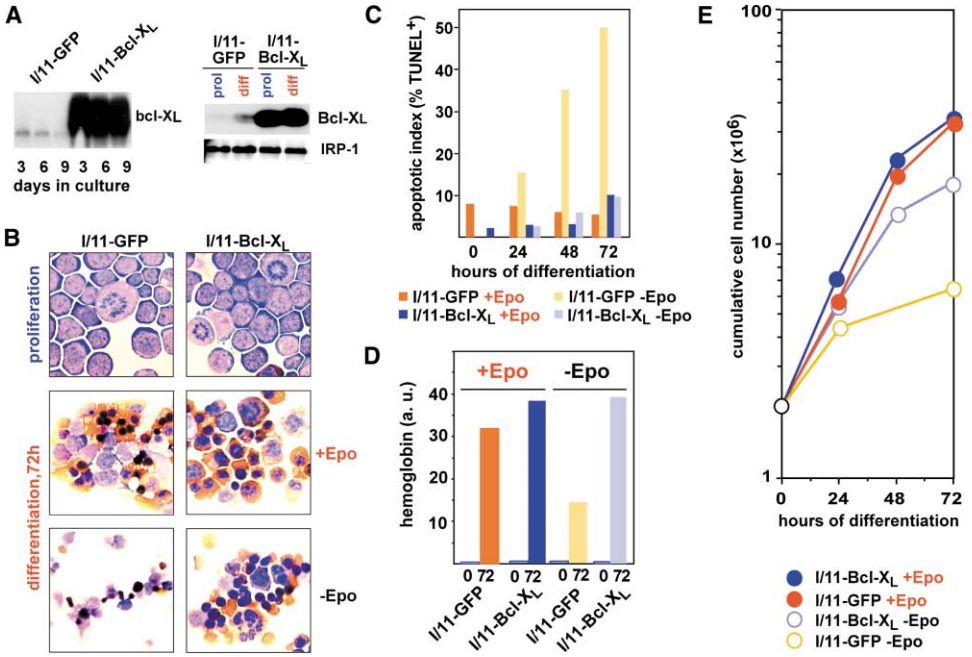

Figure 2. Erythroblasts Overexpressing Exogenous Bcl-X_L Differentiate Normally in the Absence of Epo

(A) I/11 erythroblasts infected with a retrovirus expressing human *bcl-X_L* cDNA (I/11-Bcl-X_L) or empty vector (I/11-GFP) were analyzed for *bcl-X_L* mRNA 3, 6, and 9 days after infection (left) and for Bcl-X_L protein before (prol) or after differentiation induction (diff) for 48 hr (right). Loading control, Iron Regulatory Protein 1 (IRP-1) constitutively expressed during differentiation (E.W.M., unpublished data).

(B) I/11-Bcl-X_L and I/11-GFP control cells were cytocentrifuged before (proliferation) or after differentiation for 72 hr in standard differentiation medium +/−Epo.

(C) Bcl-X_L-expressing and I/11-GFP erythroblasts induced to differentiate as described in (B) were analyzed for apoptosis at the times indicated by TUNEL staining and flow cytometry (see Experimental Procedures).

(D) Quantitation of hemoglobin accumulation in the cell populations in (B) (see Experimental Procedures).

(E) Cells from the same cultures as in (B)–(D) were counted and cumulative cell numbers calculated (see Experimental Procedures).

were stably infected, thus, no sorting was required, and all experiments were performed with infected mass cultures [18]. As shown in Figure 2A, exogenous *bcl-X_L* mRNA as well as protein was stably expressed at high levels (Figure 2A, left panel). Bcl-X_L expression did not alter the dependence of renewing cells on proliferation factors (Epo/SCF/Dex). In the absence of these factors, Bcl-X_L-overexpressing erythroblasts ceased to proliferate within 24 hr but underwent cell death only after 6–8 days, while control cells died within 24–48 hr (data not shown).

We then induced the Bcl-X_L-expressing I/11 erythroblasts (I/11-Bcl-X_L) to differentiate in the presence or absence of Epo, compared to empty vector control cells (I/11-GFP). With Epo, I/11-Bcl-X_L erythroblasts and I/11-GFP control cells showed no detectable differences in multiple differentiation parameters (e.g., morphology, amounts of hemoglobin, and proliferation rate; Figures 2B–2E). Without Epo, however, control cells underwent nuclear fragmentation and died (Figure 2B), while Bcl-X_L-expressing cells terminally differentiated into erythrocytes, as seen by size reduction, nuclear condensation, enucleation at the end of differentiation, and massive accumulation of hemoglobin (Figures 2B–2E). TUNEL assays confirmed that I/11-Bcl-X_L cells showed almost no apoptosis without Epo (apoptotic index, 3%–10%; Figure 2C), while I/11-GFP control cells exhibited the expected, high rates of apoptosis in the absence of Epo (>50% after 72 hr of differentiation).

Another important feature of normal erythroblast differentiation is that the cells undergo three to four "differentiation divisions," causing a 10- to 20-fold increase in cell number during maturation. These divisions are characterized by a shortened G1 phase and rapid size decrease [14, 19]. Interestingly, in the absence of Epo, I/11-Bcl-X_L cell numbers still increased >10-fold, while I/11-GFP control cells rapidly died and disintegrated (Figure 2E). Taken together, erythroid progenitors protected from apoptosis by exogenous Bcl-X_L apparently undergo normal erythroid maturation in the absence of Epo, including three differentiation divisions, in vivo-like hemoglobin accumulation, size decrease, and enucleation (see Discussion).

Epo-Independent Execution of the Erythroid-Specific Transcriptional Program

As judged from the differentiation parameters described above, Epo was not required for erythroid differentiation in Bcl-X_L-overexpressing erythroblasts. Therefore, we asked whether Epo was still required to trigger the late erythroid transcriptional program, involving upregulation of erythroid-specific transcription factors (Gata-1, EKLF, and NF-E2). Alternatively, simple removal of renewal factors (SCF, Dex) could trigger this program. I/11-Bcl-X_L and I/11-GFP cells were induced to differentiate in the presence or absence of Epo. Total RNA from these cells was hybridized on Northern blots, using

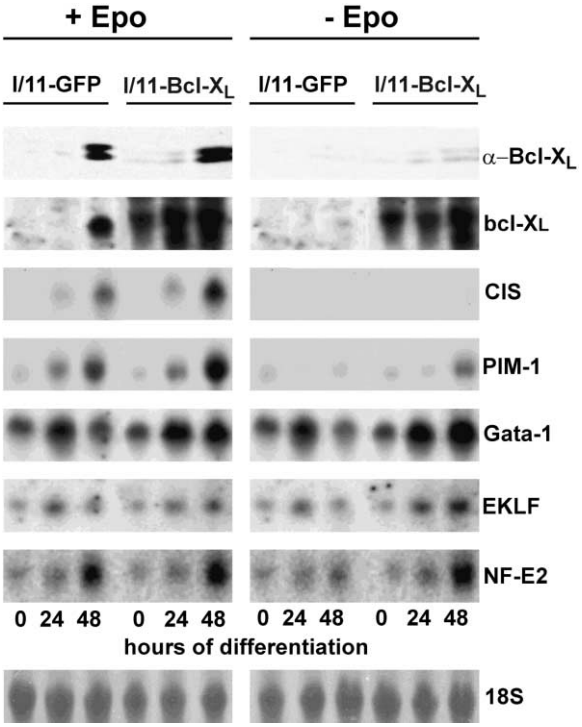

Figure 3. Expression Kinetics of Erythroid Transcription Factors and Epo Target Genes

I/11-Bcl-X_L and I/11-GFP control cells were differentiated for 24–48 hr in standard differentiation medium +/−Epo. Total mRNA from these cells as well as from proliferating controls (0 hr differentiation) was then subjected to Northern blot analysis. Blots were hybridized with cDNA probes specific for the Epo target genes *CIS*, *PIM-1*, and *bcl-X*$_L$ (the latter to show endogenous versus exogenous *bcl-X*$_L$ mRNA expression), as well as with cDNA probes for the erythroid transcription factors NF-E2, Gata-1, and EKLF; 18S ribosomal RNA, loading control (bottom). To demonstrate that exogenous *bcl-X*$_L$ overexpression did not affect regulation of endogenous Bcl-X_L protein, aliquots from the cell populations used for Northern blot analysis were subjected to Western blot analysis, using an antibody for murine Bcl-X_L (α-Bcl-X_L, top).

cDNA probes for different transcription factors. The expression levels and kinetics of early (Gata-1, EKLF) as well as late upregulated transcription factors (NF-E2; [8]) were identical between I/11-Bcl-X_L and I/11-GFP control cells, regardless of Epo administration (Figure 3).

In contrast, bona fide target genes of Epo-induced Stat5 signaling, such as *CIS* and *pim-1* [15, 20], were induced in a strictly Epo-dependent fashion, irrespective of the presence or absence of exogenous Bcl-X_L (Figure 3). Similarly, Epo still induced endogenous Bcl-X_L protein in both cell types during differentiation, regardless of the presence or absence of exogenous Bcl-X_L (Figure 3, α-Bcl-X_L).

In conclusion, Epo mainly controls survival of differentiating erythroid cells, which execute an autonomous, Epo-independent default program, including transcriptional and developmental changes. Thus, Epo most likely does not directly cause the early/late switch in gene transcription which accompanies and probably specifies the erythroid differentiation program [21]. Rather, this cell-autonomous process may be triggered when repression events, such as maintenance of renewal by c-Kit/GR signaling, are abrogated (see Discussion).

Terminal Red Cell Differentiation of Bcl-X_L-Expressing Erythroblasts Does Not Require Any Exogenous Signals

The data presented so far did not rule out that medium components (in serum or serum supplements) or Epo produced in an autocrine fashion (shown to occur in various erythroid cell lines) [22–24] might substitute for exogenously added Epo in differentiating I/11-Bcl-X_L cells. To exclude such an autocrine Epo-loop, saturating doses of Epo-neutralizing antibody were added to cultures of I/11-Bcl-X_L cells differentiating in serum-containing differentiation medium. Maturation was similarly efficient in the absence of Epo plus Epo-neutralizing antibody as in cells plus or minus Epo (Figure 4A, left part). Also other differentiation parameters, e.g., reduction of cell size and formation of erythrocytes (as judged by cytospin analysis) remained unaffected (data not shown). The same neutralizing antibody prevented differentiation of parental I/11 cells at reduced Epo concentrations (Figure 4A, right panel), causing cell death instead (data not shown) and inhibited Epo-dependent proliferation of I/11 cells [14].

These results still did not exclude that (a) factor(s) from fetal calf serum or the serum supplement indispensable for renewal of immature erythroblasts might substitute for Epo in I/11-Bcl-X_L cells. In addition, insulin, which at least in chicken cooperates with Epo in sustaining erythroid differentiation [19], was present in all differentiation assays. To exclude such effects from Ins and/or other, nondisclosed proteinaceous components present in complete StemPro34™ medium, we induced erythroid differentiation in the "basal" StemPro medium plus human transferrin and >99% pure bovine serum albumin. This medium permitted terminal, strictly Epo-dependent erythroid differentiation of I/11 erythroblasts similar to control medium plus serum, yielding the same hemoglobin levels and only slightly lower numbers of mature, partially enucleated cells (data not shown). Erythroid differentiation of I/11-Bcl-X_L cells in this medium, withdrawing Epo, Ins, or both, showed that maturation proceeded similarly well under all conditions tested, as analyzed by hemoglobin accumulation (Figure 4A), erythrocyte numbers (Figure 4B), and decrease in cell size (data not shown).

Bcl-X_L Prevents Apoptosis but Does Not Substitute for Proliferation Factors in Immature Erythroblasts Undergoing Renewal

For sustained proliferation, I/11 erythroblasts require Epo, SCF, and Dex [14]. In addition, they either need serum or the "nutrient supplement" of serum-free medium (Stem Pro34™) and stop to proliferate if exposed to basal Stem Pro34™ alone. As described above, however, the latter medium plus ultra-pure BSA permitted I/11-Bcl-X_L erythroblast maturation, including two to three differentiation divisions [19] in the complete absence of cytokines. We therefore analyzed the behavior of immature, proliferating cells in this defined medium.

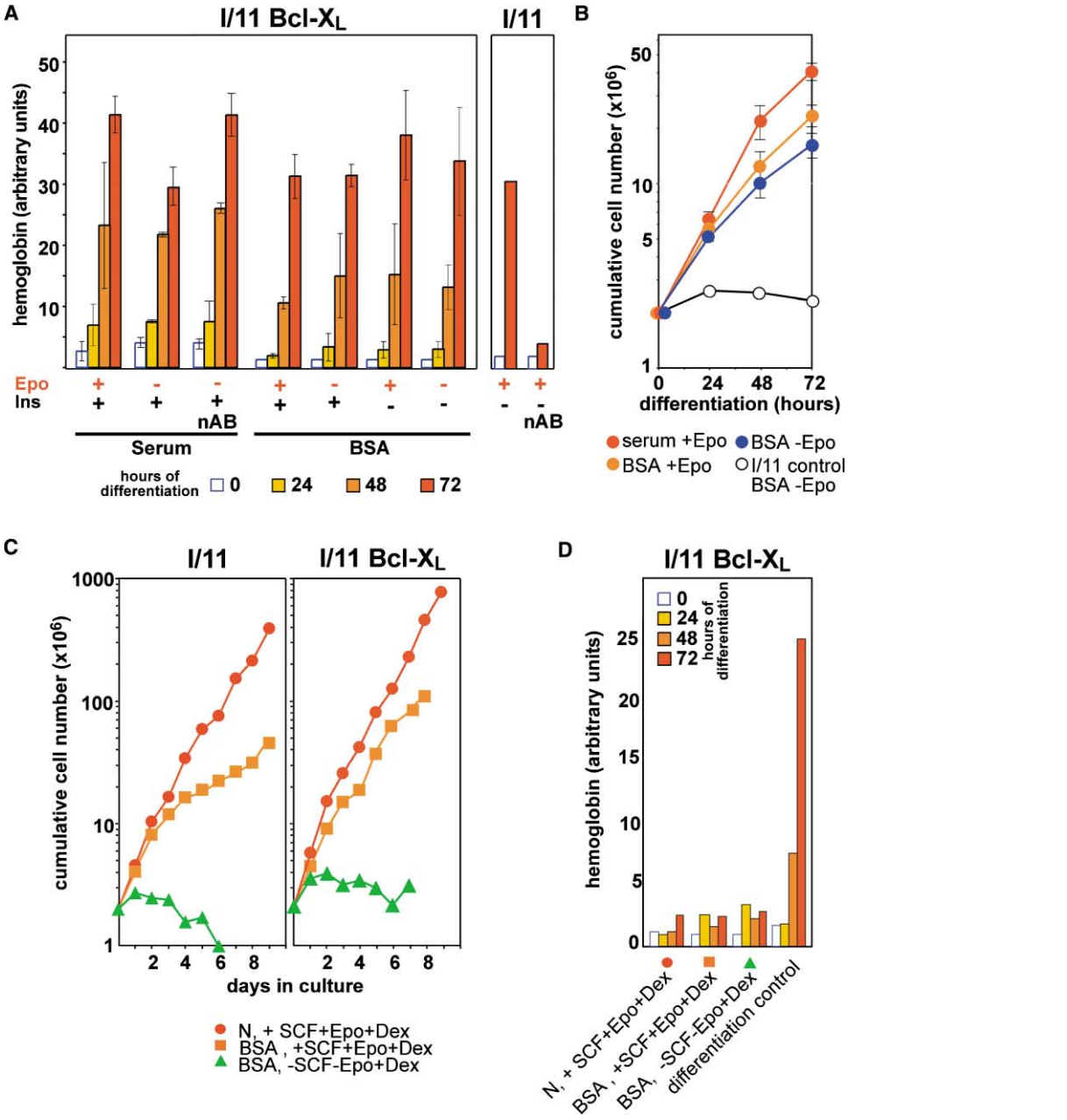

Figure 4. Normal Differentiation of Erythroblasts Expressing Retrovirally Transduced bcl-X_L Requires No Exogenous Factors

(A) Left: I/11-Bcl-X_L erythroblasts were induced to differentiate either in standard differentiation medium (serum) +/−Epo or the latter plus a saturating dose of a neutralizing Epo antibody (nAB; 1.50 dilution) (left four data sets) or in fully defined differentiation medium (BSA) +/−Epo and/or Ins (right four data sets). Shown are the mean values and standard deviations of hemoglobin determinations from three independent experiments at 0, 24, 48, and 72 hr of differentiation. Right: To confirm efficient action of the Epo-neutralizing antibody, uninfected I/11 cells were differentiated in standard medium at reduced Epo concentrations (1 U/ml) +/− antibody and analyzed for hemoglobin accumulation after 72 hr.

(B) Increase in cell numbers during differentiation of I/11-Bcl-X_L erythroblasts, performed in defined medium (BSA) +/−Epo and compared to proliferation of the same cells under optimum conditions (standard differentiation medium +Epo (serum+Epo). I/11-GFP controls did not proliferate at all in defined medium −Epo.

(C) I/11-Bcl-X_L and I/11-GFP control cells cultivated in standard StemPro34™ plus renewal factors (N, +Epo+SCF+Dex), fully defined medium plus the same factors (BSA, +SCF+Epo+Dex), or the same medium lacking Epo and SCF (BSA, −SCF−Epo+Dex). Proliferation kinetics were determined by daily counting and calculation of cumulative cell numbers.

(D) Hemoglobin levels of I/11-Bcl-X_L cells used in (C) were measured during the first 4 days of culture. As a positive control, hemoglobin accumulation during standard differentiation of I/11-Bcl-X_L is shown at the right.

As expected, I/11 control cells rapidly died when kept in Dex alone (= absence of Epo and SCF). In the presence of renewal factors, they continued to proliferate at a clearly reduced rate (Figure 4C), indicating the requirement of additional, unknown components for maximal proliferation. More interestingly, the Bcl-X_L-expressing I/11 erythroblasts maintained in this medium plus Epo/SCF/Dex proliferated almost as fast as control cells (Figure 4C) and survived as stationary, cell cycle-arrested cells for up to 7 days if deprived of Epo/SCF but not Dex. These cells were able to resume proliferation after readdition of Epo/SCF and did not initiate differentiation, as depicted by constantly low hemoglobin levels, which were the same as in cells proliferating under full factors (Figure 4D). These results demonstrated that forced expression of Bcl-X_L does not abolish the dependence of erythroid progenitors on Epo/SCF/Dex for "renewal divisions," while in the same cells differentiation divisions can proceed cell-autonomously, in the complete absence of cytokines.

Primary, Bcl-X_L-Expressing Wild-Type Erythroblasts Execute the Same Default Erythroid Differentiation Program as Respective, Immortal *p53*$^{-/-}$ Cells

Despite the close similarity of I/11 cells to primary wild-type erythroblasts [8, 14], we could not exclude that the lack of p53 in combination with constitutive Bcl-X_L expression might be sufficient to trigger autonomous, factor-independent erythroid differentiation. To address this question, we established serum-free culture conditions allowing medium-term (about 20 days) expansion (about 10^5-fold) of primary, fetal liver-derived erythroblasts (E.M.D. and H.D., submitted). Using these cells, we analyzed whether primary, wild-type erythroblasts expressing exogenous Bcl-X_L also would undergo factor-independent, cell-autonomous erythroid differentiation.

Introduction of the Bcl-X_L gene into wild-type cells was performed by using the same MSCV-based retroviral vector previously used for infection of I/11 cells. This construct yielded reproducible infection rates of more than 80% (data not shown; see [18]). In serum-containing medium plus Epo, batch cultures of primary wild-type (WT) erythroblasts expressing either GFP alone (WT-GFP) or Bcl-X_L (WT-Bcl-X_L) could both be successfully differentiated into enucleating erythrocytes (Figure 5A, left panels). In defined medium (basal StemPro34™ medium plus ultra-pure BSA, see above) plus Epo, a similarly complete maturation of both cell types could be obtained (data not shown). In defined medium lacking Epo/Ins, however, only WT-Bcl-X_L cells were able to accumulate hemoglobin and undergo complete maturation (Figure 5A, bottom right). As expected, WT-GFP control cells died under these conditions (Figure 5A, top right). These results were confirmed (1) by quantitative evaluation of cytospins for enucleated erythrocytes, nucleated maturing cells, and dead cells (Figure 5A, pie diagrams below photographs); (2) by photometric hemoglobin determinations; and (3) by determining cell proliferation during differentiation (data not shown). With respect to all these parameters, differentiation of WT-Bcl-X_L cells in defined medium lacking cytokines was only marginally less efficient than maturation of control WT-GFP erythroblasts under optimal conditions (serum-containing medium plus Epo).

In addition, WT-GFP and WT-Bcl-X_L erythroblasts were analyzed for CFU-E colony formation in presence and absence of Epo (Figure 5B). With Epo, both cell types gave rise to CFU-E colonies of comparable size and with the same frequency. In the absence of Epo, WT-GFP cells did not form colonies at all, whereas WT-Bcl-X_L cells yielded slightly reduced numbers of smaller colonies. Cytospin preparations indicated that the colonies consisted of mature erythroid cells, except for WT-GFP cells cultivated without Epo, where only dead cells could be detected.

Exogenous Bcl-2 Can Substitute for Bcl-X_L in Apoptosis Protection during Differentiation

Finally, we asked if other Bcl family antiapoptotic proteins such as Bcl-2 would play a role during erythroid differentiation. Neither primary nor I/11 cells expressed endogenous Bcl-2 during proliferation or differentiation. In contrast, Friend murine leukemia virus transformed erythroblasts used as a positive control showed a clear Bcl-2 signal (Figure 5C, top) [25]. To test whether exogenous Bcl-2 could replace Bcl-X_L in allowing cytokine-independent differentiation, primary erythroblasts were infected with a retroviral construct harboring human *bcl-2* cDNA (pMSCV-Bcl-2-IRES-GFP). As expected, the resulting WT-Bcl-2 cells expressed high levels of exogenous Bcl-2 under all conditions tested (Figure 5C, top right). When the cells were induced for differentiation, they behaved virtually indistinguishable from their pMSCV-Bcl-X_L counterparts: efficient maturation was observed in the absence of Epo (Figure 5C, bottom).

Discussion

In this paper, erythroid progenitors faithfully recapitulating all major in vivo steps of terminal erythroid maturation were used to address an unsolved question: does Epo regulate erythroid cell numbers by controlling survival of differentiating erythroid progenitors (which then mature in the absence of additional, exogenous signals), or does it trigger both erythroblast differentiation and protection from apoptosis by regulating the expression of multiple downstream target genes (like *Gata-1*, *CIS*, *pim-1*, or *bcl-X$_L$*)? By ectopic expression of a single target gene of Epo-dependent signaling, the antiapoptotic protein Bcl-X_L, in both immortalized *p53*$^{-/-}$ and primary wild-type erythroblasts, we provide strong evidence for the first alternative: the antiapoptotic effect of Epo-induced Bcl-X_L upregulation alone was sufficient to allow cell-autonomous execution of the terminal erythropoiesis program, including sequential expression of erythroid-specific transcription factors. This also suggests that no other known Epo target genes are essential for this maturation process.

Erythroid Differentiation—A Default, Intrinsic Pathway

An important finding of this paper is that terminal erythroid differentiation depends on apoptosis protection

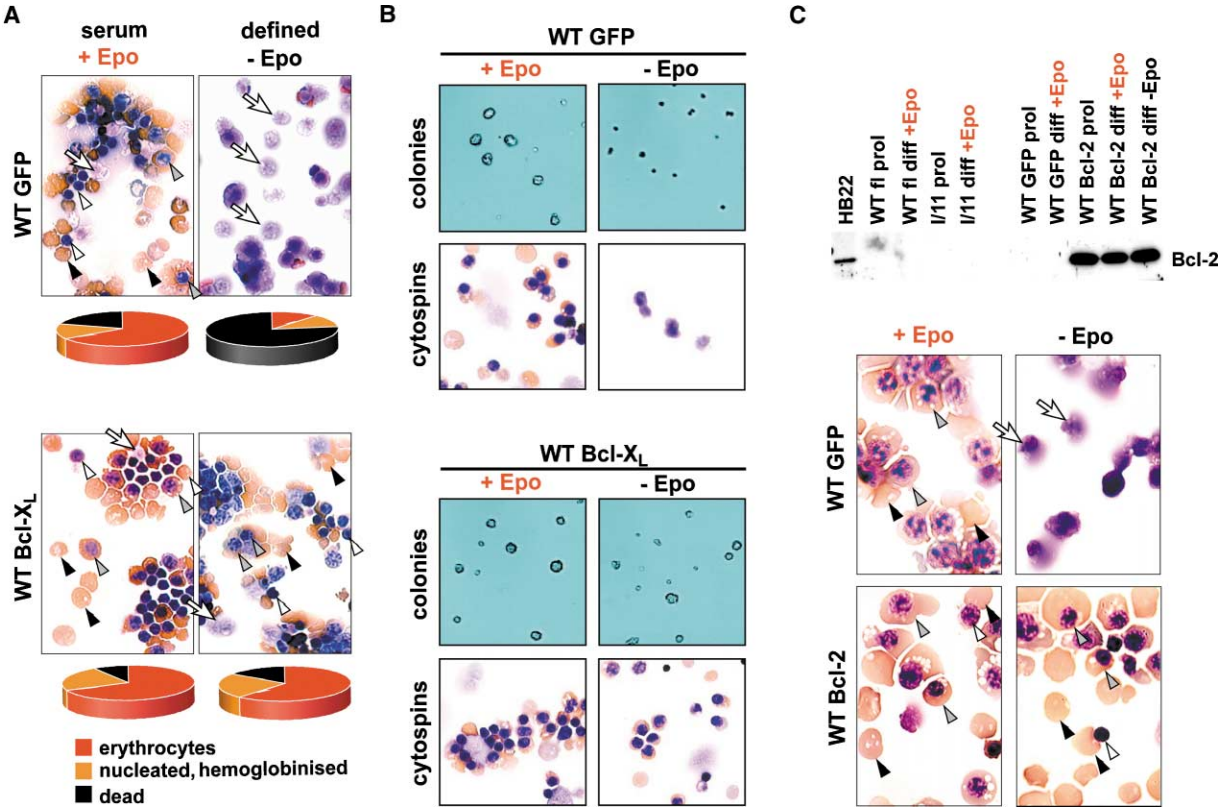

Figure 5. Primary Fetal Liver-Derived Erythroblast Ectopically Expressing Bcl-X_L or Bcl-2 Differentiate in the Absence of Factors

(A) Primary erythroblasts expressing GFP (top, WT-GFP) or GFP-Bcl-X_L (bottom, WT Bcl-X_L) were differentiated in standard (serum +Epo) or defined medium without Epo (defined −Epo). Cytospins prepared after 48 hr of differentiation were stained and photographed or evaluated quantitatively (pie diagrams) for enucleated erythrocytes (red), nucleated mature cells (orange), or apoptotic/disintegrated cells (black). White arrows, apoptotic cells; gray arrowheads, hemoglobinized, partially mature erythroid cells; black arrowheads, mature, enucleated erythrocytes; white arrowheads, extruded nuclei.

(B) Cells from (A) were seeded in CFU-E methocel +/−Epo and analyzed for colonies 2 days later. Top, photomicrographs; bottom, cytospin preparations from isolated colonies.

(C) Top: Bcl-2 protein expression in I/11 cells and primary erythroblasts under proliferation or differentiation conditions +/−Epo, analyzed before and after infection with pMSCV-Bcl-2-IRES-GFP or pMSCV-GFP (see Experimental Procedures). HB22, positive control for endogenous Bcl-2, Friend-MLV-transformed erythroblasts [25]. Bottom: Primary WT-Bcl-2 or WT-GFP control erythroblasts (see top panel) were differentiated as described in (A) and cytospins prepared.

by Bcl-X_L, upregulated by EpoR signaling. In the absence of Epo, *bcl-X_L* mRNA and protein remain at basal levels, insufficient to protect maturing cells from cell death. It was, however, unclear whether EpoR signaling would also trigger the erythroid differentiation program, i.e., initiate activation of early and late erythroid-specific transcription factors. Our results clearly indicate that both *p53*$^{-/-}$ and primary, wild-type erythroblasts could undergo a cell-autonomous, default differentiation program if protected from apoptosis by expression of exogenous Bcl-X_L or Bcl-2. Cytokine-independent, terminal erythropoiesis of Bcl-X_L-expressing cells was indistinguishable from the Epo-induced maturation of control cells with respect to multiple differentiation parameters.

Importantly, the expression pattern of erythroid-specific transcription factors (Gata-1, EKLF, NF-E2) [8, 19] was similar between Bcl-X_L-expressing erythroblasts differentiating in the absence of cytokines and control cells maturing under optimal conditions. Importantly, the earlier steps of this transcription factor cascade (i.e., within the first 36–48 hr) were also induced in Epo-deprived control cells devoid of exogenous Bcl-X_L. This indicates that Epo cannot be the agent initiating this transcription factor cascade (see below).

Using a fully defined medium (containing only low MW chemicals plus ultra-pure BSA and iron-loaded transferrin), we also demonstrated that Bcl-X_L-expressing cells required no other exogenous factor (e.g., substituting for Epo/Ins) to complete execution of the erythroid differentiation program. With an Epo-neutralizing antibody, we could exclude an Epo autocrine loop [23, 24] as a reason for factor-independent differentiation. This identifies Bcl-X_L as the Epo-target gene both essential and sufficient for erythroid maturation.

Our findings contrast previous observations that retrovirally mediated expression of Bcl-X_L (or Bcl-2) in isolated erythroid progenitors could not substitute for Epo in CFU-E colony formation [26]. In this study, Bcl-X_L protein expression levels were not determined, relying on GFP coexpression alone. Together with the fact that our Bcl-X_L-expressing primary erythroblasts readily formed normal numbers of CFU-E colonies, this could

mean that Bcl-X_L expression was too low to allow Epo-independent colony formation in the above report. In another study, erythroid progenitors from mice carrying a *bcl-2* transgene were much less sensitive to reduced Epo levels in CFU-E assays, although they did not form colonies in the complete absence of Epo [27]. Together with recent findings that primary chicken erythroid progenitors overexpressing *bcl-2* differentiated terminally without Epo [25], we conclude that apoptosis inhibition by *bcl-2* family antiapoptotic proteins is sufficient for terminal erythropoiesis to occur, without requirement for additional factors. The relevant in vivo target of Epo-signaling, however, clearly is Bcl-X_L, since neither primary avian nor murine erythroid progenitors express Bcl-2. In addition, broad range caspase inhibitors or ablation of caspase-2 prevented normal erythropoiesis in our primary cell model. Apparently, caspase activity is required for apoptosis-independent protein degradation processes during terminal maturation (S. Pilat, A. Kolbus, H.B., and M. Baccharini, submitted). This represents another argument for our idea that apoptosis protection by Bcl-X_L is physiologically relevant in erythroid differentiation.

How then is maturation and upregulation of erythroid-specific transcription factors triggered in our cell models? The observations suggest that withdrawal of renewal factors (SCF and Dex) initiates erythroid differentiation. Based on preliminary results from expression profiling, SCF and Dex may prevent activation of a "dormant" but preexisting program required to initiate erythroid differentiation. Interference of an active GR with terminal differentiation would explain why Bcl-X_L-expressing cells undergo normal maturation in differentiation media (where Dex is replaced by its antagonist ZK 112.993), while they arrest in cell cycle without maturation in the defined medium lacking Epo/SCF but not GR ligands (Figure 4C). Thus, we hypothesize that renewal factors such as SCF and Dex cooperate with Epo to maintain erythroblast proliferation and to repress a default erythroid differentiation program, which is initiated by withdrawal of SCF and Dex and then only requires apoptosis protection by Epo-induced Bcl-X_L for its completion.

Renewal Signals Preventing Cell-Autonomous Differentiation: A General Principle in Leukemia?

The concept that renewal-inducing signals have to be completely removed in order to allow triggering of the erythroid differentiation program also applies to other systems. Cell-autonomous differentiation was suppressed by proliferation/renewal factors (PDGF and FGF) in oligodendrocyte/astrocyte progenitors, which undergo a fixed number of cell divisions when protected from apoptosis by the combined action of neurotrophins and hormones [28, 29]. Thus, cell-autonomous differentiation programs triggered by the *removal* of signals favoring proliferation/differentiation arrest may be a more generally applicable paradigm.

Renewal factor-induced differentiation arrest may also bear relevance for understanding the development of (erythro-)leukemia. Leukemic cells often differ from their primary counterparts in that they require no or fewer cytokines for renewal, (over)express antiapoptotic proteins, and differentiate only partially or abnormally. Known or unknown oncogenes with their mutations and amplifications (e.g., v-ErbA, v-ErbB, sea, c-Kit, or flk-2) [30–33] provide these erythro-leukemic cells with constitutive renewal signals, which cannot be turned off and thus prevent triggering of terminal maturation. This may explain why only partial differentiation occurs in such cell lines, often combined with ongoing proliferation. This idea is supported by oncogenic mutations inducing proliferation and interfering with maturation in other hematopoietic lineages or even multipotent progenitors [34, 35]. Importantly, the leukemia-inducing protooncogene Bcl-2 or its close relative Bcl-X_L did not contribute to renewal of primary erythroblasts at all, suggesting that antiapoptotic signals by *bcl* family proteins require cooperation with other oncogenes to cause leukemia.

Epo Signaling and Bcl-X_L Upregulation in Erythroid Differentiation as Analyzed in Genetically Modified Mice

The idea that *bcl-X_L* is an essential target gene of Epo and required for erythropoiesis in vivo is strengthened by phenotypes of mice with a disrupted *bcl-X_L* gene. Constitutive elimination of *bcl-X_L* caused death at E12.5 due to severe anemia and brain defects [10]. Mice with a conditional disruption of *bcl-X_L* in the hematopoietic system were born but showed massive hemolytic anemia [11]. In both cases, the *bcl-X_L*-defective animals lacked mature erythrocytes but produced large numbers of immature erythroid progenitors, such as BFU-E and CFU-E cells as well as nucleated proerythroblasts. These findings are in excellent agreement with the low or absent *bcl-X_L* expression in immature erythroblasts and its late upregulation during erythrocyte formation, as initially described by others and seen in our in vivo-like differentiation systems.

Conclusions

Our results establish that erythroid differentiation can take place as a cell-autonomous program, including differentiation divisions, in the absence of exogenous signals, provided that the antiapoptotic protein Bcl-X_L is upregulated by Epo. This erythroid maturation process requires the antiapoptotic activity of Bcl family proteins rather than Bcl-X_L-specific functions. During progenitor renewal, execution of the intrinsic erythroid differentiation program is suppressed by the proliferation-promoting factors SCF and Dex and maturation is triggered by their *removal*.

Experimental Procedures

Cells and Culture Conditions

Immortal mouse erythroblasts (clone I/11; p53 deficient [8]) were maintained in serum-free medium (StemPro-34™; Life Technologies) supplemented with human recombinant erythropoietin (Epo; 2 units/ml; Erypo, Cilag AG), murine recombinant stem cell factor (SCF, 100 ng/ml; R&D Systems), dexamethasone (Dex; 10^{-6} M; Sigma), and insulin-like growth factor 1 (IGF-1; 40 ng/ml; Promega) as described [14]. Primary erythroblasts were obtained from fetal livers of wild-type mice (C57/Bl-6/129 mixed) at E11.5–E13.5 as described in detail elsewhere (E.M.D. et al., submitted) and cultivated in the same medium. Proerythroblast cultures were expanded by daily partial medium changes and addition of fresh factors, keeping cell densities

between 1.5 to 4 $\times$ 10^6 cells/ml. For some experiments, proliferation was carried out in fully defined medium (basal StemPro34™ medium lacking Nutrient supplement), containing 6.6 mg/ml highly purified BSA (>99% pure, Sigma, #A0281) plus renewal factors (Epo, SCF, Dex, IGF-1) and iron-saturated transferrin (300 μg/ml; Roche). Cell numbers and size distribution were determined in an electronic cell counter (CASY-1, Schärfe-System); proliferation kinetics and cumulative cell numbers were calculated as described [14].

Differentiation Induction

For standard terminal differentiation, wild-type or I/11 erythroblasts were washed twice in PBS and reseeded at 2 to 3 $\times$ 10^6 cells/ml in S-13 differentiation medium containing 12% FCS; (FCS; Life Technologies) [36], supplemented with Epo (10 units/ml), insulin (Ins; 4 $\times$ 10^{-4} IE = 10 ng/ml, Actrapid HM), the Dex antagonist ZK112.993 (3 $\times$ 10^{-6} M) [37], and iron-saturated human transferrin (1 mg/ml; Sigma). Differentiating erythroblasts were maintained at densities between 2 to 6 $\times$ 10^6 cells/ml. For differentiation in the fully defined medium mentioned above, renewal factors were replaced with differentiation factors/hormones. Where indicated, a rabbit polyclonal antibody neutralizing the biological activity of human Epo (Genzyme #1541-01, 1:50 dilution) was added. For CFU-E colony assays, self-renewing cells (2 $\times$ 10^5/35 mm dish) were plated in duplicate into 1% methyl-cellulose (MethoCult™; StemCell Technologies), supplemented with Epo (3 U/ml) and transferrin. Numbers of small and compact colonies were scored at day 2.

Cell Morphology, Histological Staining, and Determination of Hemoglobin Content

Cytospin preparations and stainings were performed as described [38]. Nucleated and enucleated erythrocytes (brown or yellow small cells), partially mature or immature cells (larger, with gray/blue cytoplasm), and dead cells (fragmented/condensed nuclei, disintegrated cells) were counted after visual inspection in the microscope as described earlier [38], evaluating >600 cells on multiple, randomly selected fields/sample. Hemoglobin content was analyzed photometrically as described [39]. Results are the average of triplicate measurements after normalization for both cell number and volume.

Retroviral Infections

For infection of I/11- and primary wild-type erythroblasts, gpE86 ecotropic producer cell lines were used, expressing a bicistronic retroviral vector (pMCSV) harboring human Bcl-X_L [18] or human Bcl-2 followed by an IRES sequence and GFP. Both producer cell lines had similar titers and were generated as described [40]. Infection efficiency was assessed by flow cytometry 3 days after infection.

Western Blot Analysis

Erythroblasts were washed twice with ice-cold PBS and resuspended in 2xSDS loading buffer. Since hemoglobin interferes with protein determination, samples were carefully normalized to both cell number and size and subjected to polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes. Transfer quality as well as equal loading was verified by Ponceau staining. Antibodies used were for mouse Bcl-X_L (Transduction Laboratories; #B22620), recognizing both human and mouse Bcl-X_L (sc-7195; Santa Cruz), and Bcl-2 (sc-783: Santa Cruz).

Northern Blotting

Total RNA was prepared from 2 to 5 $\times$ 10^7 cells, using Trizol™ (Life technologies). Ten micrograms of RNA per sample were separated in 1% formaldehyde agarose gels, transferred to nylon membranes (Gene Screen, NEN), fixed by UV irradiation, and hybridized sequentially with ^{32}P-labeled cDNA probes for human *bcl-X_L* [41] (cross-hybridizing with murine *bcl-X_L* mRNA); murine *CIS* [42], *pim-1* [43], *EKLF* [44], and *p45 NF-E2* [45]. After washing, filters were analyzed by phosphoimaging (Molecular Dynamics).

Apoptosis Assay

Cells (2 to 5 $\times$ 10^6) were fixed in 0.5 ml of 4% paraformaldehyde in PBS for 30 min at room temperature and stored in 70% ethanol at −20°C. Cells were washed in PBS and incubated in 50 μl TUNEL reaction buffer (Roche), 0.5 nM FluoroLink Cy5-dCTP (Amersham), and 12.5 U TdT (Roche) for 1 hr at 37°C. Cells were washed once with HBS and resuspended in HBS containing 10 μg/ml DAPI to determine DNA content and subjected to flow cytometry.

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