RAPID COMMUNICATION

Erythropoietin Can Promote Erythroid Progenitor Survival by Repressing Apoptosis Through Bcl-XL and Bcl-2

By Maite Silva, Didier Grillot, Adalberto Benito, Carlos Richard, Gabriel Nunez, and Jose Luis Fernandez-Luna

Erythropoietin (Epo), the hormone that is the principal regulator of red blood cell production, interacts with high-affinity receptors on the surface of erythroid progenitor cells and maintains their survival. Epo has been shown to promote cell viability by repressing apoptosis; however, the molecular mechanism involved is unclear. In the present studies we have examined whether Epo acts as a survival factor through the regulation of the bcl-2 family of apoptosis-regulatory genes. We addressed this issue in HCD-57, a murine erythroid progenitor cell line that requires Epo for proliferation and survival. When HCD-57 cells were cultured in the absence of Epo, Bcl-2 and Bcl-X, but not Bax were downregulated, and the cells underwent apoptotic cell death. HCD-57 cells infected with a retroviral vector encoding human Bcl-XL or Bcl-2 rapidly stopped proliferating but remained viable in the absence of Epo. Furthermore, endogenous levels of bcl-2 and bcl-xL were downregulated after Epo withdrawal in HCD-57 cells that remained viable through ectopic expression of human Bcl-XL, further indicating that Epo specifically maintains the expression of bcl-2 and bcl-xL. We also show that HCD-57 rescued from apoptosis by ectopic expression of Bcl-X, can undergo erythroid differentiation in the absence of Epo, demonstrating that a survival signal but not Epo itself is necessary for erythroid differentiation of HCD-57 progenitor cells. Thus, we propose a model whereby Epo functions as a survival factor by repressing apoptosis through Bcl-XL and Bcl-2 during proliferation and differentiation of erythroid progenitors. © 1996 by The American Society of Hematology.

Erythropoietin (Epo) is the principal growth factor that promotes the survival, proliferation, and differentiation of erythroid progenitor cells. Epo binds to a specific cell-surface receptor that is expressed on immature erythroid progenitor cells. Analysis of mutant mice has clearly established an essential role for Epo and its receptor (Epo-R) in erythropoiesis in vivo. In embryos of mice deficient in Epo or the Epo-R, definitive erythropoiesis is completely impaired and liver tissue contains increased numbers of nucleated erythroid cells undergoing apoptosis. However, neither Epo nor the Epo-R are required for erythroid lineage commitment or for proliferation and differentiation of burst-forming unit-erythroid (BFU-E) to colony-forming unit-erythroid (CFU-E) progenitors, because both differentiation stages are present in mice carrying null mutations in the Epo or Epo-R genes. Thus, genetic analysis as well as in vitro studies have established an essential role for Epo in the survival and maturation of committed CFU-E progenitors and erythroblasts. Beyond the late basophilic erythroblast stage, it is also known that the level of the Epo-R is downregulated, and the cells are no longer dependent on Epo for their maturation.

In the absence of Epo, erythroid progenitors die and their genomic DNA is degraded into oligonucleosomal fragments, a feature of apoptotic cell death. Thus, a model has been proposed whereby the concentration of circulating Epo controls the number of erythroid progenitors that survive by repressing apoptosis. However, the mechanism by which Epo suppresses apoptosis of erythroid progenitor cells is unknown. Several genes of the bcl-2 family have been identified that function as inhibitors of apoptosis in hematopoietic cells. Of these, bcl-2 and bcl-x are expressed in the erythroid lineage and may be involved in the regulation of erythroid survival. Using a mouse erythroleukemia cell line, HCD-57, which requires Epo for proliferation and survival, we show herein that in the absence of Epo, two apoptosis-regulatory proteins, Bcl-XL and Bcl-2, and their corresponding mRNAs are rapidly downregulated and this is accompanied by activation of an apoptotic process. We also show that HCD-57 cells transduced with a retroviral vector encoding bcl-xL or bcl-2 remain viable in the absence of Epo, indicating that Bcl-XL and/or Bcl-2 are sufficient for HCD-57 survival. Finally, we demonstrate that bcl-xL–transduced HCD-57 cells can be induced to undergo erythroid differentiation when stimulated with hemin in the absence of Epo, indicating that survival signals but not Epo are required for erythroid differentiation of HCD-57 cells. These findings indicate that Epo regulates the expression of both Bcl-XL and Bcl-2 in the erythroid progenitor cell line HCD-57, and this seems to be a major mechanism by which Epo maintains the viability of erythroid progenitor cells.

MATERIALS AND METHODS

Cell culture. The murine Epo-dependent erythroleukemia cell line, HCD-57 (provided by Dr David Hanksins, Johns Hopkins School of Medicine, Baltimore, MD) was maintained at 2.5 to 5 × 10⁶ cells/mL in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO, Grand Island, NY), supplemented with 30% fetal calf serum, 2 × 10⁻³ mol/L β-mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.1 U/mL of recombinant murine Epo...
(Boehringer Mannheim, Indianapolis, IN). Viability and total cell counts were determined by trypan blue exclusion and counting of at least 200 cells from each individual culture. In some experiments, HCD-57 cells were cultured in the presence of 100 μm/L hemin (Sigma Chemical Co, St Louis, MO) with or without Epo. At the indicated times, aliquots of 5 × 10⁴ cells were stained with May-Grünwald-Giemsa solution for cytologic analysis.

Assay for DNA synthesis. Cells were cultured with or without Epo and every 24 hours, 2.5 × 10⁴ cells were incubated in a microtiter plate in the presence of 1 μCi [3H]-TdR (83 Ci/mmol) (Amersham Life Sciences, Arlington Heights, IL) per well for an additional 4 hours. Cells were obtained on prewet cell harvester strips (Whatman, Maidstone, UK), washed with water and 95% ethanol, air-dried, and counted in a liquid scintillation counter.

HCD-57 retroviral transduction. Exponentially growing HCD-57 cells were cocultured with fibroblasts producing the ecotropic retrovirus LXSNa-Hu-bcl-x (D. Grillot, G. Nunez, in preparation) or amphotropic LXSNa-Hu-bcl-2 retrovirus, for 24 hours and nonadherent cells were serially isolated to eliminate virus producing cells. After retrovirus transduction, HCD-57 cells were plated at 1 cell per well in the presence of Epo and G418 (1 mg/mL), and clones were selected for further analysis based on the expression of Bcl-xL or Bcl-2 by Western blot analysis (see below).

mRNA expression analysis. Total RNA was prepared by the guanidinium thiocyanate method. To assess mRNA expression, a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method was used by assessing PCR products during the exponential phase of cDNA amplification. The generated cDNA was amplified by using primers for murine bcl-2 (5’GATGTCCACGTACCTGACCATAGCTG3’ and 5’AGGTATCACCACGATGATGACTGAGGCC3’), bcl-x (5’CCGGAAGCCTTCAATGATC3’ and 5’CAAGGAACCAGGTGTTAGAG3’), bax (5’CTGCGACATTACATGGAACGTG3’ and 5’GTCGACATCTGCGGACATTTAGG3’) and β-actin (5’AGGATGTCATCTACTCTCC3’ and 5’TGGTGGTGTGAGACTGAGC3’). In some experiments primers for human bcl-xL were also used. A 50-μL PCR mixture containing 1 μL of the RT reaction was amplified with the following profile: 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. After 25 to 26 amplification cycles, the expected PCR products (366 bp [bcl-2], 344 bp [bcl-xL], 155 bp [bcl-x], 194 bp [bax], and 220 bp [β-actin]) were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

DNA fragmentation analysis. Cells (1 to 3 × 10⁴) were washed with phosphate-buffered saline and pelleted by centrifugation at 200g for 5 minutes. Genomic DNA was isolated from cell pellets as described previously. Samples were suspended in 15 μL of water containing 0.25 mg/mL RNase A. DNA samples were electrophoresed on a 2% agarose gel and stained with 0.1% ethidium bromide.

Western blotting analysis. The expression of Bcl-xL, Bcl-2, and Bax proteins was determined by Western blotting as previously described. Blots were incubated with rabbit anti-Bcl-X antisemum (provided by D. Grillot, University of Chicago, Chicago, IL), 6C8 monoclonal antibody against human Bcl-2, or rabbit anti-Bax (provided by D. Brain Leber, McMaster University, Hamilton, Ontario, Canada) and then incubated with goat anti-rabbit or goat anti-hamster IgG conjugated to horseradish peroxidase (Amersham) as described. Bound antibody was detected by chemiluminescence using the ECL Western blot kit (Amersham).

RESULTS

Downregulation of Bcl-xL and Bcl-2 but not Bax is accompanied by apoptosis in HCD-57 cells cultured in the absence of Epo. The murine HCD-57 cell line is a useful model for erythroid progenitors in that it requires Epo for survival and proliferation and can differentiate into mature erythroblasts. When HCD-57 cells were cultured in the absence of Epo, the levels of Bcl-xL and Bcl-2, two proteins that function as inhibitors of apoptosis, were clearly downregulated after 24 hours (Fig 1A). Bcl-2 was undetected by 24 hours while the amount of Bcl-xL was downregulated by 24 hours and greatly diminished by 72 hours after Epo withdrawal, as assessed by immunoblot analysis. In contrast, the expression of Bax, another Bcl-2 family member that promotes apoptosis, remained relatively constant within 72 hours after Epo deprivation. To further verify these findings, the levels of bcl-2 and bcl-xL mRNA were assessed by semi-quantitative RT-PCR analysis. In agreement with the protein results, both bcl-xL and bcl-2 were downregulated but expression of bax was maintained within 72 hours of Epo deprivation (Fig 1B). These results were also confirmed by Northern blot analysis (data not shown).

The loss of expression of Bcl-2 and Bcl-xL was accompanied by loss of cell viability caused by the activation of an apoptotic process. Figure 1C shows that genomic DNA from HCD-57 cells cultured in the absence of Epo was degraded into oligonucleosomal fragments that are characteristic of apoptosis. The DNA degradation pattern was detected at 48 hours and was clearly evident by 72 hours after Epo withdrawal (Fig 1C).

Constitutive expression of human Bcl-xL or Bcl-2 in HCD-57 cells confers protection against apoptosis induced by Epo withdrawal. Because both Bcl-xL and Bcl-2 were downregulated in HCD-57 cells after Epo withdrawal, we examined whether ectopic expression of Bcl-xL or Bcl-2 could protect HCD-57 cells from apoptosis. HCD-57 cells were transduced with retrovirus vectors expressing Bcl-xL or Bcl-2 and G418-resistant stable clones were selected by limiting dilution. HCD-57 clones expressing high (HCD-57 BcL-XL#D8), intermediate (HCD-57 Bcl-xL#D9), and low or undetectable (HCD-57 Bcl-xL#D3) levels of human Bcl-xL, as assessed by Western blot analysis were selected for study (Fig 2A). Clone HCD-57 Bcl-xL#D5 and control HCD-57 Neo displayed similar kinetics of cell death after Epo deprivation and no surviving cells could be detected after 3 to 4 days of Epo withdrawal (Fig 2B). In contrast, clone HCD-57 Bcl-xL#D9 that expressed intermediate levels of human Bcl-xL exhibited greater viability than HCD-57 Neo or HCD-57 Bcl-xL#D5 cells (Fig 2B). At day 2 after Epo withdrawal, 85% of the HCD-57 Bcl-xL#D9 cells were viable whereas only 34% of the HCD-57 Neo cells remained viable (P = .008, Student’s t-test). At day 4 of incubation without Epo, 34% of HCD-57 Bcl-xL#D9 cells were still viable and by day 6, essentially all HCD-57 Bcl-xL#D9 cells were dead (Fig 2B). Conversion of high levels of Bcl-xL in clone HCD-57 Bcl-xL#D8 consistently delayed the rate of cell death. At day 4 after Epo withdrawal, 87% of the HCD-57 Bcl-xL#D8 cells were viable whereas only 32% of the HCD-57 Bcl-xL#D5 cells remained viable (P = .001, Student’s t-test). Even after 10 days in the absence of Epo, about 60% of the HCD-57 Bcl-xL#D8 cells remained viable (Fig 2B).

Similarly, three HCD-57 clones were derived by infection with a bcl-2 retrovirus that expressed human Bcl-2. Analysis of these clones showed that expression of human Bcl-2 in-
Epo Deprivation (hrs)  

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Fig 1. Analysis of Bcl-2 family members and apoptosis in HCD-57 cells before and after Epo withdrawal. (A) Western blot analysis of Bcl-XL, Bel-2, and Bax in cells cultured in the presence or absence of Epo for 24, 48, and 72 hours. Lysate from FL5.12 cells transfected with human bcl-xL, bcl-2, and bax expression plasmids (labeled as Bcl-XL, Bcl-2, and Bax, respectively) is shown as a positive control. (B) Semi-quantitative RT-PCR analysis of bcl-xL, bel-2, and bax mRNA at 0, 24, 48, and 72 hours after Epo withdrawal. After 25 cycles, PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide. Amplification of β-actin mRNA was used as an internal control. (C) DNA fragmentation analysis in cells cultured with or without Epo. HCD-57 cells were incubated in the absence of Epo for the indicated time points and genomic DNA fragmentation was monitored by electrophoresis onto a 2% agarose gel and stained with ethidium bromide.

increased the viability of HCD-57 in the absence of Epo, although the effect of Bcl-2 was not as pronounced as that observed with Bcl-XL in the HCD-57 Bcl-XL#D8 clone (data not shown). Hence, expression of Bcl-XL or Bcl-2 can promote the survival of HCD-57 cells in the absence of Epo.

Epo maintains the endogenous levels of bcl-xL and bcl-2 in HCD-57 cells infected with the human bcl-xL retrovirus. It was conceivable that the loss of expression of Bcl-XL and Bcl-2 observed in HCD-57 cells cultured in the absence of Epo was caused by nonspecific reduction of gene expression, because under these conditions HCD57 cells undergo apoptotic cell death. We approached this issue by analyzing the expression of endogenous bcl-xL and bcl-2 mRNAs in clone HCD-57 Bcl-XL#D8 (high levels of exogenous Bcl-XL) at different times after Epo withdrawal. During the first 2 days of culture without Epo, viability of HCD-57 Bcl-XL#D8 cells was above 95% (Fig 2B). However, the expression of both murine bcl-xL and bcl-2 mRNA was clearly decreased by 48 hours, as assessed by semi-quantitative RT-PCR analysis (Fig 2C). In contrast, the expression of bax and β-actin mRNA remained unaltered in the absence of Epo (Fig 2C). In addition, endogenous levels of bcl-2 and bcl-xL were increased to those of HCD-57 Bcl-XL#D8 cells maintained in Epo when the hormone was added again at 48 hours of Epo deprivation (data not shown). Importantly, the levels of retrovirally encoded human bcl-xL remained constant throughout the period of culture in the presence or absence of Epo. Thus, the results indicate that Epo can maintain the levels of bcl-2 and bcl-xL mRNA in HCD-57 cells even under conditions in which apoptosis is suppressed by ectopic expression of Bcl-XL.

Bcl-XL maintains the survival HCD-57 cells but not their proliferative capacity in the absence of Epo. Epo acts both as a growth and survival factor for HCD-57 erythroid progenitors. We asked next whether HCD-57 cells rescued from apoptosis by Bcl-XL could proliferate in the absence of Epo. Figure 3 shows that the proliferative capacity of HCD-57 Bcl-XL#D8 cells dramatically declined by 24 hours after Epo withdrawal at a rate similar to that of HCD-57 Neo cells, although complete inhibition of 3H-thymidine uptake was not achieved until 7 to 8 days of Epo deprivation (Fig 3). These results indicate that in the absence of Epo, Bcl-XL promotes survival with little or no effect on cell proliferation. Similar results were obtained when bcl-2-transduced HCD-57 cells were cultured in the absence of Epo (data not shown).

Bcl-XL–transduced HCD-57 cells undergo erythroid differentiation in the absence of Epo. The role of Epo and other growth factors in the control of hematopoietic cell differentiation is controversial and difficult to study largely due to the fact that in the absence of growth factor, the cells rapidly undergo apoptosis. Because HCD-57 expressing deregulated Bcl-XL can survive in the absence of Epo, these cells provide a useful experimental approach to study whether Epo is required for erythroid differentiation of progenitor cells. In the absence of hemin, no differentiation was observed in HCD-57 Bcl-XL#D8 cells (Fig 4A through C). Incubation of these cells with hemin for 72 hours induced erythroid...
Epo REGULATES Bcl-XL AND Bcl-2

Fig 2. Bcl-XL maintains HCD-57 survival in the absence of Epo. (A) Western blot analysis of Bcl-XL, Bcl-2, and Bax expression in HCD-57-Bcl-XL clones derived by infection with a human bcl-xL retrovirus. A HCD-57 clone infected with a control retrovirus encoding the neomycin resistance gene (HCD-57 Neo) was shown for comparison. Lysate from FL5.12 cells transfected with human bcl-xL, bcl-2, and bax expression plasmids (labeled as Bcl-XL, Bcl-2, and Bax, respectively) is shown as a positive control. Notice that all clones expressed similar amounts of Bcl-2 and Bax. (B) Viability of HCD-57 Bcl-XL#D5, HCD-57 Bcl-XL#D9, HCD-57 Bcl-XL#D8, and HCD-57 Neo cells after Epo withdrawal. Cells were cultured in the absence of Epo for the indicated times. Viability was measured by trypan blue dye exclusion. All data points represent the mean of triplicate cultures ± SD. (C) Semi-quantitative RT-PCR analysis of murine (endogenous) bcl-xL, bcl-2, bax, and β-actin mRNAs in HCD-57 Bcl-XL#D8 cells incubated in the presence or absence of Epo. Analysis of human bcl-xL (ectopic) mRNA is shown for comparison. After 23 cycles, PCR products were electrophoresed onto a 2% agarose gel and stained with ethidium bromide. The viability of HCD-57 Bcl-XL#D8 cells was greater than 95% at all time points.

Fig 3. 3H-thymidine uptake by HCD-57 Bcl-XL#D8 cells after Epo withdrawal. Cells were cultured with and without Epo for the indicated time points and incorporation of 3H-thymidine was measured as described in Materials and Methods. 3H-thymidine uptake by HCD-57 Neo cells deprived of Epo is shown for comparison. Data are presented as the mean of triplicate cultures ± SD.

DISCUSSION

Several studies have shown that Epo is essential for the survival of erythroid progenitors and their differentiation into erythrocytes. This Epo dependence for erythroid progenitor survival forms the basis for a model of erythroid production in vivo. The model predicts that during normal erythropoiesis Epo levels are insufficient for the survival of
Fig 4. Erythroid differentiation of HCD-57 Bcl-XL#D8 cells cultured in the presence or absence of Epo. (A) Northern blot analysis of β-globin mRNA from HCD-57 Bcl-XL#D8 cells treated with hemin for 72 hours in the presence (lane 2) or absence (lane 3) of Epo. As controls, mRNA from HCD-57 Neo cells cultured with Epo and treated with hemin for 72 hours was also analyzed (lane 4). In lane 1, mRNA from HCD-57 Bcl-XL#D8 cells untreated with hemin is shown for comparison. Ribosomal RNA (rRNA) staining with ethidium bromide was used as loading control. (B) HCD-57 Bcl-XL#D8 cells incubated for 72 hours with or without Epo in the presence of hemin were stained with May-Grünwald-Giemsa solution (original magnification ×1,000). Notice the increase in smaller cells with picnotic nuclei and acidophilic cytoplasm in the absence of Epo. (C) Percentage of mature erythroblasts (polychromatophilic and orthochromatic) in HCD-57 Bcl-XL#D8 cells incubated with or without Epo in the presence of hemin at the indicated time points. As control, HCD-57 Neo cells cultured with Epo and hemin were also analyzed. Notice that in the absence of hemin, no mature erythroblasts were detected at time 0. Data are presented as the mean of triplicate cultures ± SD.
a majority of the Epo-dependent progenitors. Thus, a minority of these progenitors are responsible for the normal erythrocyte production. When Epo levels are increased because of anemia or hypoxia, many of the Epo-dependent progenitors that would ordinarily die, survive and increase erythrocyte production. In the present studies, we present evidence that Epo maintains survival and represses apoptosis of HCD-57 erythroid progenitor cells through the expression of Bcl-2 and Bcl-XL. These two proteins are highly homologous at the amino acid level and have been shown to inhibit apoptosis of hematopoietic progenitor cells induced by growth factor withdrawal including that of interleukin-3 (IL-3), IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-7. A major role for Bcl-XL in maintaining the survival of hematopoietic cells is supported by recent studies of mutant mice in which bcl-x has been disrupted by homologous recombination. Mice deficient in Bcl-x develop massive apoptosis of neuronal and hematopoietic progenitor cells and die at day 13 or 14 of embryonic development. In contrast, mice deficient in Bcl-2 exhibit increased spontaneous apoptosis of lymphoid cells after birth but no apparent abnormalities of the erythroid compartment. Together with our results, these observations suggest that Bcl-XL is a critical factor by which Epo maintains the survival of erythroid progenitors. Bcl-2 appears dispensable for erythroid survival but it may play a role in maintaining cell viability by cooperating with Bcl-XL. For example, lymphoid cells from mice that express both bcl-2 and bcl-x transgenes exhibit increased survival compared with that of mice expressing bcl-2 or bcl-x transgenes alone. Hence, it is possible that Epo promotes survival of erythroid progenitors through both Bcl-2 and Bcl-XL by an additive effect. Finally, these studies cannot rule out factors other than Bcl-XL and Bcl-2, still to be identified, also contribute to the mechanism by which Epo maintains the survival of progenitor cells.

Combinations of cytokines are required for the optimal proliferation and differentiation of multipotent cells in vitro, but other agents such as hemin can potentiate the maturation of these cells. Some growth factor-dependent hematopoietic cell lines can undergo differentiation in response to physiologic stimuli. For example, G-CSF promotes granulocytic differentiation of IL-3-dependent 32D cells and another IL-3-dependent cell line, LyD9, can develop into neutrophilic, macrophage, or erythroid cells depending on the cytokine added. Similarly, when the multipotent FDCP-Mix A4 cell line is cultured with IL-3 at low concentration plus Epo and heme these cells undergo erythroid differentiation. Because growth factors are required for hematopoietic cell survival, it is unclear whether Epo is necessary for differentiation of erythroid progenitors. HCD-57 cells that express deregulated Bcl-XL remained viable in the absence of Epo. Using this approach, we have provided evidence that HCD-57 progenitor cells can undergo heme-induced erythroid differentiation in the absence of Epo, indicating that Bcl-XL is sufficient for maturation of HCD-57 progenitors. Furthermore, erythroid maturation seems to be facilitated when these cells were cultured without Epo. Hence, these studies suggest a model whereby Epo is not required for the induction of erythroid differentiation but rather promotes differentiation by providing survival signals, a function that is essential for maturation of erythroid progenitors. This model is consistent with that described with IL-3-dependent FDCP-Mix myeloid progenitor cells transduced with a bcl-2 retrovirus. FDCP-Mix cells acquired the ability to undergo myeloid differentiation in the absence of IL-3, arguing that IL-3 promotes proliferation and survival, but it is not directly implicated in the induction of differentiation.

In summary, these studies provide evidence that Epo maintains the survival of erythroid progenitors through the expression of Bcl-2 and Bcl-XL. Epo appears to regulate these survival proteins via transcriptional mechanisms or message stabilization because changes in Bcl-2 and Bcl-XL were associated with those of their corresponding mRNAs. These studies also show that proliferation and survival, two activities promoted by Epo, can be dissociated by Bcl-XL or Bcl-2 and that terminal erythroid differentiation can be achieved in the absence of Epo. The signal transduction pathway engaged through Epo as well as the precise gene regulatory mechanism involved in the regulation of Bcl-2 and Bcl-XL are presently unresolved and need to be addressed in future studies.

ACKNOWLEDGMENT

We thank Dr David Hankins for HCD-57 cells, Drs Craig Thompson and Brian Leber for generous supply of antibodies, Dr David Hockenbery for the LXSN-Hu-bcl-2 retrovirus producing line, Dr Dan Wechsler for critical review of the manuscript, and Diana Miller for secretarial help.

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Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2

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