bd-x Prevents Apoptotic Cell Death of Both Primitive and Definitive Erythrocytes at the End of Maturation

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Summary

bd-x is a member of the bd-2 gene family, which regulates apoptotic cell death in various cell lineages. There is circumstantial evidence suggesting that bd-x might play a role in the apoptosis of erythroid lineage cells, although there is no direct evidence. In this study, we used Bcl-X null mouse embryonic stem (ES) cells, and showed that Bcl-X is indispensable for the production of both embryonic primitive erythrocytes (EryP) and adult definitive erythrocytes (EryD) at the end of their maturation. In vivo, bd-x−/− ES cells did not contribute to circulating EryD in adult chimeric mice that were produced by blastocyst microinjection of the bd-x−/− ES cells. bd-x−/− EryP and EryD were produced by in vitro differentiation induction of ES cells on macrophage colony-stimulating factor–deficient stromal cell line OP9, and further analysis was carried out. The emergence of immature EryP and EryD from bd-x−/− ES cells was similar to that from bd-x+/+ ES cells. However, prominent cell death of bd-x−/− EryP and EryD occurred when the cells matured. The data show that the antiapoptotic function of bd-x acts at the very end of erythroid maturation.

Key words: erythrocytes • Bcl-X • apoptosis • embryonic stem cells • cell differentiation

Hematopoiesis is regulated by the orchestration of several processes, including cell proliferation, cell differentiation, and cell death. Apoptosis, also referred to as programmed cell death, regulates the survival of progenitor cells and the turnover of mature elements, which are important for maintaining the homeostasis of hematopoietic cells (1). Growth factors are thought to play important roles in the apoptosis of hematopoietic progenitors. They act as survival factors for hematopoietic precursors, and the hematopoietic progenitors succumb to apoptosis in their absence (2–4). On the other hand, the survival of hematopoietic cells is also controlled by the Bcl-2 protein family (5–8). Family members such as Bcl-2, Mcl-1, A1, and Bcl-X, are expressed in hematopoietic cells and are considered to function as repressors of apoptotic cell death (9).

In mouse embryogenesis, erythropoiesis originates in the yolk sac blood islands, beginning at approximately embryonic day 7.5, then migrates to the fetal liver, spleen, and eventually to the bone marrow (10). Embryonic primitive erythropoiesis in the yolk sac and definitive erythropoiesis in the fetal liver, spleen, and bone marrow produce primitive erythrocytes (EryP)1 and definitive erythrocytes (EryD), respectively. Although the glycoprotein hormone erythropoietin (EPO) was initially characterized as stimulating both the proliferation and differentiation of EryD progenitors, it has been shown that EPO maintains the viability of primarily EryD progenitor cells (11–13). The dependence of the survival of EryD on EPO is reported in various experimental systems, including Friend virus anemia strain–infected murine splenic erythroid progenitors, the erythroleukemia cell line, and murine fetal liver erythroid progenitors (2, 14–16). These studies showed that survival of the late erythroid progenitors (CFU-E) of EryD was dependent on EPO and that the EPO-dependent period lasted from the CFU-E stage to the beginning of hemoglobin synthesis. Gene targeting experiments with EPO and EPO receptor revealed that EPO has important roles in primitive erythropoiesis (12, 13). We found that the survival of immature EryP was also dependent on EPO; as was that of EryD progenitors (our unpublished data). Thus, EPO prevents apoptosis of both EryP and EryD during their immature state.

The protein Bcl-2 prevents apoptosis triggered by various stimuli, including chemotherapeutic drugs, γ-irradiation, viral infections, oxidant stress, and notably growth factor deprivation (6). Bcl-2 and Bcl-XL act as cell death repressors, whereas Bax and the alternatively spliced bd-x gene product, Bcl-XS, act as cell death promoters (6, 17, 18). A family of bd-2 genes participates in the regulation of cell survival in multiple cell lineages, including the hematopoietic system.
Apoptosis of Erythroid Cells

The hemoglobin type of B6 mice and 129/Ola mice are single (H b/b; H bb) and diffuse (H b/b; H bb), respectively, and were used to evaluate the contribution of ES cells in circulating erythrocytes of chimeric mice. These two types of hemoglobin can be distinguished by electrophoresis. Cellulose acetate electrophoresis of cystamine-modified hemoglobin was performed essentially as described (33). W hole blood in PBS containing 50 mM EDTA was layered onto 2 vol of Histopaque-1077 (Sigma Chemical Co.) and centrifuged at 3,500 g for 20 min at room temperature. The pellet, enriched for RBCs, was collected. 10 μl purified RBCs was added to 300 μl cystamine lys+ buffer (12.5 mg/ml cystamine dihydrochloride, 1 mM dithiothreitol, 0.55% ammonium hydroxide) and agitated to lyse the RBCs. The samples were applied to TITAN III cellulose acetate plates and run in TBE buffer (0.18 M Tris, 0.10 M boric acid, 0.002 M EDTA) for 40 min at 300 V. The plates were placed in staining solution (1% Ponceau S, 5% TCA) for 10 min and rinsed in three changes of 5% acetic acid for 10 min each. The percentage contributions of ES cells in adult chimera were examined using the allotype of GPl from various nonhematopoietic organs, such as the liver and kidney. The hemoglobin type analysis data were obtained from the chimera in which the contribution of ES cells to nonhematopoietic organs was >50%.

Materials and Methods

Target Disruption of the bcl-x Gene. E14 ES cells derived from strain 129/Ola were used throughout the experiment. ES bd-x+/+, bd-x+/-, and bd-x–/- cell lines were produced as described previously (21). Genomic DNA containing the bd-x locus was isolated from a library of mouse strain 129/Sv DNA. A 1.8-kb XhoI-BamHI fragment containing most of the bd-x coding region was replaced with either a PGK-neo polyadenylated (poly A) cassette or a PGK-hyg poly A cassette. Both targeting vectors contain 6.0-kb 5’ and 1.0-kb 3’ regions of homology with the drug-resistance markers and a PGK-3’ poly A cassette. Transfection and selection were performed as described (31). DNA prepared from ES cells was digested with EcoRV, transferred to a nylon membrane, and then hybridized with the 0.4-kb KpnI-PstI probe that flanked the 3’ homology region. The expected sizes of wild-type bd-x, mutant bd-x with the neo targeting vector, and mutant bd-x with the hyg targeting vector were 9.8, 7.0, and 5.5 kb, and were detected in wild-type, bd-x+/-, and bd-x–/- ES clones, respectively.

Production of Chimeric Mice and Analysis of the Contribution of ES Cells. ES bd-x+/+, bd-x+/-, and bd-x–/- cells were injected into the 3.5-d post-coitum blastocysts from C57BL/6 mice to generate chimeric mice. GPl (glucose phosphoisomerase) isozymes were used to analyze the contribution of ES cells in various organs of chimeric mice. The GPl isozyme of B6 mice and of 129/Ola mice from which E14 ES cells were established are GPl-1A and GPl-1B, respectively. Separation and detection of GPl isoenzymes were performed essentially as described (32). Chimeric mice were perfused with PBS to eliminate blood cell contamination, and dissected tissues were kept at –7.0°C. Frozen tissue samples were thawed and gently homogenized in water, and cells were then lysed by three rounds of freezing and thawing. After centrifugation of the homogenates, the supernatants were diluted with water and then electrophoresed on Titan III Zip Zone cellulose acetate plates (H elena Laboratories) in Tris-glycine buffer (25 mM Tris, 200 mM glycine, pH 8.5) for 4 h at 150 V at 4°C. The stainings were performed by overlaying the mixture consisting of 2 ml of 0.2 M Tris·HCl (pH 8.0), 0.1 ml each of 0.25 M magnesium acetate, 10 mg/ml NADP, and 100 mg/ml fructose 6-phosphate, and 0.2 ml of MTT, 0.05 ml of 2.5 mg/ml phena-zene methylsulfonate, 5 μl glucose 6-phosphate dehydrogenase (140 U/ml; Sigma Chemical Co.), and 5 ml of 2% agarose. The GPl isozyme bands appeared after a few minutes in the dark. Density of the bands was analyzed by densitometer.

The induced cells were trypsinized at day 5, and 106 cells were transferred onto fresh OP9 cells on a 10-cm plate. Nonadherent cells were harvested on day 6, 7, and 8 to obtain EryP. On day 10, all of the cells on individual plates were harvested by vigorous pipetting and transferred to individual 10-cm plates with a fresh OP9 cell layer, and then both adherent and nonadherent cells were harvested to obtain EryD after day 11.

Hematopoietic Colony Formation From Day 8 Hematopoietic Clusters. To determine the differentiation capacity of bd-x+/- and bd-x–/- ES cells, on day 8 of the differentiation induction 30 hematopoietic clusters were picked and transferred to semisolid culture medium containing IL-3 (50 U/ml) and EPO (2 U/ml), which promote erythroid and myeloid cell growth. As previously reported, the day 8 hematopoietic clusters have a clonal origin and can differentiate into erythroid and various myeloid lineages under these conditions (27). 5 d after transfer into this myeloid permissive semisolid medium, individual colonies were picked, cytosin specimens were stained with May-Grunwald-Giemsa, and the emerged blood cells were typed.

Purification and Counting Viability of the Induced Cells. More than 75% of the differentiation-induced cells between days 6 and 8 were EryP, and the same proportion of cells between days 11 and 13 were EryD. In some experiments, the purification of EryP and EryD was carried out with metrizamide step gradient centrifugation. The cells were washed once with Tyrode's buffer containing 0.1% gelatin. 1–5 × 106 cells in 1 ml of the Tyrode's buffer were layered on a step gradient of 2.0 ml of 30% wt/vol metrizamide (N-acetal Tetense) and 2.0 ml of 15% wt/vol metrizamide.
The cells were centrifuged at room temperature for 20 min at 400 g at the interface between the 15% metrizamide and the 30% metrizamide. The cells remaining at this interface were collected and washed three times with α-MEM with 20% FCS. After the purification, >98% of the cells were dianisidine-positive erythroid cells, with a viability of 95–98%.

Hemoglobin-containing cells were confirmed with dianisidine staining as reported previously (35). To examine EPO responsiveness (the experiment shown in Fig. 3), 3.0 × 10⁶/ml dianisidine-positive differentiation-induced cells were cultured in 6-well plates containing 20% FCS supplemented with α-MEM in the absence or presence of 2 U/ml EPO without the OP9 cell layer. The viability of the cells was examined using the trypan blue dye exclusion method and calculated by counting >200 cells. May-Grunwald Giemsa staining of cytopsin specimens was also carried out to examine the morphological changes of apoptotic EryP.

The number of hemoglobin-containing cells and the percentage of viable cells are reported as mean ± SD. The t test was used for statistical analysis, using StatView software.

Electrophoretic analysis of DNA Fragmentation. After culture for 18 h in the presence or absence of 2 U/ml EPO, 10⁶ cells were harvested by centrifugation at 200 g for 10 min. Low molecular weight DNA was extracted following the method of Sellins and Cohen (36). One quarter of the extracted DNA was electrophoresed in a 2.0% agarose gel and stained with ethidium bromide. The viability of the cells was examined using the trypan blue dye exclusion method and calculated by counting >200 cells. May-Grunwald Giemsa staining of cytopsin specimens was also carried out to examine the morphological changes of apoptotic EryP.

The number of hemoglobin-containing cells and the percentage of viable cells are reported as mean ± SD. The t test was used for statistical analysis, using StatView software.

Results

No contribution of bd-x null ES cells to circulating adult definitive erythrocytes. ES cells of bd-x⁺⁺, bd-x⁻⁻, and bd-x⁻⁻ genotypes were injected into the blastocysts of C57BL/6 mice to assess their ability to differentiate into various organs in vivo. There were no differences in the growth of parental bd-x⁺⁺, bd-x⁻⁻, and bd-x⁻⁻ ES cells (data not shown). Chimeric mice of >80% chimerism by coat color were analyzed for the contribution of the injected ES cells in various organs based on the activity of GPI-1 isozymes. E14 ES cell-derived cells express the GPI-1A isozyme, which is easily distinguishable from the GPI-1B isozyme of the C57BL/6-derived cells (37). As for heart, kidney, and muscle, there were no differences in the contribution of parental bd-x⁺⁺, bd-x⁻⁻, and bd-x⁻⁻ ES cells (Table I). On the other hand, the contribution of bd-x⁻⁻ ES cells to lymphoid organs such as spleen and thymus was significantly lower than that of bd-x⁻⁻ or bd-x⁻⁻ ES cells. This result is compatible with a previous report on the shortened life span of bd-x⁻⁻ immature lymphocytes (21).

Two bd-x⁻⁻ ES cell lines (clones 18 and 3a) were analyzed for the function of the bd-x gene in hematopoiesis. Host blastocysts from the strain C57BL/6 are homozygous for the Hbββ-β-globin haplotype (corresponding to the "single" band in Fig. 1, lane 1). In contrast, 129/Ola mice, from which the ES cell line of this study was established, are homozygous for the Hbββ+ haplotype (corresponding to the "major" and "minor" bands in Fig. 1, lane 2). The proportion of major and minor hemoglobin shows the contribution of the injected ES cells to mature circulating EryD in the chimeric mice. When bd-x⁺⁺ or bd-x⁻⁻ ES cells were used for chimera production, the contribution of the

| Organs | Genotype |
|--------|----------|
|        | +/+     | +/-     | -/-     |
| Heart  | 62 ± 22 | 63 ± 15 | 52 ± 13 |
| Kidney | 60 ± 15 | 60 ± 27 | 55 ± 18 |
| Muscle | 99 ± 2  | 100 ± 0 | 99 ± 38 |
| Thymus | 38 ± 15 | 47 ± 21 | 21 ± 14*|
| Spleen | 71 ± 9  | 63 ± 15 | 33 ± 21*|

*P < 0.05 by t test with the groups of +/+ and +/- mice.

ES cells to the circulating EryD was proportional to the contribution of ES cells to the other organs. However, when bd-x⁻⁻ ES cells were used, no contribution of the ES cells to circulating EryD was detected, despite their significant contribution to the other nonlymphohematopoietic organs (Fig. 1). These data clearly show that bd-x has an essential role for the in vivo production of EryD. In addition, the results from the chimeric mice demonstrate that the contribution of bd-x to EryD production is cell autonomous, since the hematopoietic microenvironment in the chimeric animal could not complement the defective EryD production from bd-x⁻⁻ ES cells.

The process of definitive erythroid lineage cell production can be divided into two stages. The earlier stage is commitment and involves differentiation from multipotential progenitor cells to committed erythroid lineage cells. The later stage is proliferation and maturation of the committed EryD progenitors. Two possibilities might account for the failure of EryD production by bd-x⁻⁻ ES cells. One is the commitment failure of the multipotential hematopoietic cells to mature definitive erythrocytes (chimeric organs (Fig. 1)). These data clearly show that bd-x has an essential role for the in vivo production of EryD. In addition, the results from the chimeric mice demonstrate that the contribution of bd-x to EryD production is cell autonomous, since the hematopoietic microenvironment in the chimeric animal could not complement the defective EryD production from bd-x⁻⁻ ES cells.

The proportion of the injected ES cells to mature circulating EryD in the chimeric mice. When bd-x⁺⁺ or bd-x⁻⁻ ES cells were used for chimera production, the contribution of the

Figure 1. Contribution of ES-derived cells to mature adult definitive erythrocytes of chimeras. Hemoglobin was analyzed in the peripheral blood of C57BL/6 mice (lane 1) and 129/Ola mice (lane 2). The Hbββ haplotype (single) is specific for host blastocysts of strain C57BL/6, and the Hbββ haplotype (diffuse; major and minor) is specific for strain 129/Ola from which the ES cell line used in this study was established. Peripheral blood samples of chimeras made with bd-x⁺⁺ ES cells (lanes 3 and 4), bd-x⁻⁻ ES cells (lanes 5 and 6), bd-x⁻⁻ ES cells, clone 18 (lanes 7–9), and bd-x⁻⁻ ES cells, clone 3a (lanes 10–12) were analyzed to examine the contribution of ES cells.
Various Hematopoietic Lineages In Vitro

Day 13 Definitive 8.6
Day 12 Definitive 3.2

| Colony type | +/+ | −/− Clone 18 | −/− Clone 3a |
|-------------|-----|-------------|-------------|
| nmEM        | 5   | 4           | 2           |
| nmE         | 9   | 7           | 4           |
| mEM         | 1   | 2           | 2           |
| mE          | 11  | 9           | 13          |
| E           | 2   | 1           | 1           |
| nm          | 1   | 4           | 3           |
| m           | 1   | 3           | 5           |

Day 8 hematopoietic clusters were picked and transferred into semisolid media containing IL-3 and EPO. Colonies that appeared 8 d after the transfer were picked and stained with May-Grunwald Giemsa. No significant differences between the groups by χ² test with the group of bcl-x−/− ES cells was carried out using OP9 stromal cells. The differentiation capacity of day 8 in vitro differentiation–induced ES cells was examined. The day 8 hematopoietic progenitor cells was examined. The day 8 hematopoietic clusters were of clonal origin, and most of them could differentiate into multiple hematopoietic lineages, including the definitive erythroid lineage (27). There were no differences in the number of day 8 hematopoietic clusters induced from bcl-x−/− or bcl-x−/+ ES cells (data not shown). As shown in Table II, there were also no significant differences in the types of colonies that developed from the day 8 hematopoietic clusters in methylcellulose semisolid media containing IL-3 and EPO as growth factors. These data show that bcl-x is not necessary for the differentiation of the definitive erythroid lineage from multipotential hematopoietic progenitors. Since Bcl-X is essential for the production of fully mature EryD, bcl-x seemed to play important roles during the maturation of EryD after commitment to the erythroid lineage.

Table II. Differentiation of bcl-x−/+ and bcl-x−/− ES Cells into Various Hematopoietic Lineages In Vitro

| Colony type | +/+ | −/− Clone 18 | −/− Clone 3a |
|-------------|-----|-------------|-------------|
| nmEM        | 5   | 4           | 2           |
| nmE         | 9   | 7           | 4           |
| mEM         | 1   | 2           | 2           |
| mE          | 11  | 9           | 13          |
| E           | 2   | 1           | 1           |
| nm          | 1   | 4           | 3           |
| m           | 1   | 3           | 5           |

Day 6 Primitive 4.0
Day 7 Primitive 8.4
Day 8 Primitive 8.4

Table III. Numbers of Diанизidine-positive Cells Induced from bcl-x−/+ and bcl-x−/− ES Cells

| Day of induction | Type of erythroid cells | +/+ | −/− Clone 18 | −/− Clone 3a |
|------------------|-------------------------|-----|-------------|-------------|
| Day 6            | Primitive                | 4.0 ± 0.5 | 4.0 ± 0.4 | 4.0 ± 0.5 |
| Day 7            | Primitive                | 9.6 ± 0.7 | 9.5 ± 1.9 | 7.9 ± 1.2* |
| Day 8            | Primitive                | 8.4 ± 1.7 | 0.7 ± 0.2* | 0.8 ± 0.5† |
| Day 12           | Definitive               | 3.2 ± 0.5 | 3.4 ± 0.4 | 3.0 ± 0.3 |
| Day 13           | Definitive               | 8.6 ± 0.5 | 2.8 ± 0.3* | 3.2 ± 0.5† |
| Day 14           | Definitive               | 16.8 ± 1.0 | 4.5 ± 0.9* | 3.8 ± 0.9* |

10⁵ of the day 5 induced cells were cultured on the OP9 cell layer in the presence of EPO (2 U/ml). Data (×10⁴ of primitive and ×10⁴ of definitive erythroid cells) are shown as mean ± SD of six dishes. The data shown are representative of three independent experiments. *P < 0.05 by t test with the group of +/+ mice; †P < 0.001 by t test with the group of +/+ mice.
of the cells harvested between days 7 and 8, and >90% of the cells harvested between days 12 and 14 were EryP and EryD, respectively, when the bcl-x	errorthroid cells were induced for differentiation. The percentage of viable bcl-x\textsuperscript{-} EryP seems relatively high for the very low number of bcl-x\textsuperscript{+} EryP (Table IV). This apparent discrepancy was probably due to the removal of dead bcl-x\textsuperscript{-} EryP by adherent macrophages. Electron microscopic features at about day 8 of the differentiation induction showed macromphages with prominently phagocytosed dead EryP (data not shown).

C\textsuperscript{el}D\textsuperscript{eath} of In Vito D\textsuperscript{ifferentiation}-\textsuperscript{duced} Erythroid Lineage Cells. The morphological features and DNA fragmentation of the induced cells were analyzed to confirm that the decreased number and viability of bcl-x\textsuperscript{-} cells were due to apoptosis. Immature EryP and EryD were purified by metrizamide density gradient separation on days 6.5 and 11.5 of the differentiation induction, immature EryP and EryD were purified. These erythroid cells were not considered to be late-stage erythroid progenitors, but rather immature erythroid cells, because hemoglobinization had already begun but the cells still showed an immature morphology. After purification on days 6.5 and 11.5, the bcl-x\textsuperscript{+} and bcl-x\textsuperscript{-} erythroid lineage cells were cultured without OP9 stromal cells in the presence or absence of EPO. 1 and 1.5 d after the culture, the viability of the cells was examined (Fig. 3). Deprivation of EPO at this stage affected the viability of EryP much more severely than EryD; however, the results of the examination of EryP and EryD were essentially the same. In the presence of EPO, there were significant differences in the viability of bcl-x\textsuperscript{+} and bcl-x\textsuperscript{-} erythroid cells (P < 0.0005 by t test). Even in the absence of EPO, the differences in the viability were significant (P < 0.0005 by t test). Furthermore, EPO deprivation decreased the viability of both EryP and EryD even in the context of bcl-x null (P < 0.0001 by t test). Taken together, EPO deprivation and the bcl-x null mutation affected cell death additively.

**Table IV. Percentages of Viable Cells during the Differentiation Induction from bcl-x\textsuperscript{+} and bcl-x\textsuperscript{-} ES Cells**

| Day of induction | +/- | +/- Clone 18 | +/- Clone 3a |
|-----------------|-----|--------------|--------------|
| Day 7           | 90 ± 3 | 89 ± 2 | 89 ± 2 |
| Day 7.5         | 91 ± 3 | 88 ± 3 | 84 ± 3* |
| Day 8           | 89 ± 2 | 75 ± 3* | 78 ± 3* |
| Day 12          | 82 ± 3 | 81 ± 3 | 80 ± 1 |
| Day 13          | 87 ± 3 | 53 ± 7* | 52 ± 3* |
| Day 14          | 85 ± 2 | 48 ± 2* | 47 ± 2* |

10\textsuperscript{5} of the day 5 induced cells were cultured on the OP9 cell layer in the presence of EPO (2 U/ml). Data are shown as mean ± SD of six dishes. The data shown are representative of three independent experiments. *P < 0.005 by t test with the group of +/- mice; †P < 0.0001 by t test with the group of +/- mice.

**Discussion**

bd-x, a member of the bd-2 family of apoptosis regulatory genes, can be alternatively spliced to produce two protein isoforms, Bcl-X\textsubscript{L} and Bcl-X\textsubscript{S} (6, 17, 18). Bcl-X\textsubscript{L} exhibits remarkable structural homology with Bcl-2 and inhibits apoptotic cell death. Evidence from studies of cell lines and transgenic mice suggests that the bd-2 gene family plays a role in the survival of erythroid lineage (22, 23, 25). The expression pattern of bd-x obtained from primary human erythroid cells and mouse erythroblasts infected with

![Figure 2](image-url)
the anemia-inducing strain of Friend virus (FVA) suggests that bcl-x among bcl-2 gene family members is the principal antiapoptotic regulator during late erythroid differentiation (24). Bcl-X is strongly increased during the terminal differentiation stages of human and mouse erythroblasts in the presence of EPO, reaching maximum transcript and protein levels at the time of maximum hemoglobin synthesis. This increase in Bcl-X expression leads to an apparent level ∼50 times greater than the level in proerythroblasts before EPO stimulation. In contrast, neither mouse nor human erythroblasts express Bcl-2 transcript or protein. The levels of other bcl-2 family members, Bax and Bad proteins, remain relatively constant throughout differentiation, but diminish at the end of terminal differentiation near the time of enucleation. These data on the expression pattern of the bcl-2 gene family products imply that bcl-x is the critical member of the bcl-2 family during erythroid differentiation. Furthermore, the increased apoptotic cell death of hematopoietic cells in bcl-2−/− fetal liver and the absence of defects in the fetal liver of bcl-2−/− mice support the hypothesis that Bcl-X, not Bcl-2, is the important factor in erythropoiesis (21, 38–40). However, there is no direct evidence for the role of bcl-x in erythropoiesis, despite this circumstantial evidence.

To examine the critical physiological roles of the bcl-x gene on hematopoiesis, chimeric mice production and OP9 in vitro differentiation induction were carried out using bcl-x−/− ES cells. There was no contribution by bcl-x−/− ES cells to the circulating EryD in the chimeric mice, demonstrating that bcl-x is indispensable for the full maturation of EryD. Defects in erythropoiesis were analyzed in detail using in vitro differentiation induction from ES cells by coculturing the cells on the macrophage colony-stimulating factor–deficient OP9 stromal cell line (the OP9 system [28]). Two waves of erythroid cell production were observed when ES cells were cocultured with OP9 stromal cells. The development of hematopoietic cells in this OP9 system is very similar to that observed in developing mouse embryos (27, 29, 41). The first wave of erythropoiesis, appearing between days 6 and 8 of the induction, and the second wave, appearing after day 10 of the induction, correspond to primitive and definitive erythropoiesis, respectively, by morphological and biochemical criteria (29). Our data clearly show that apoptotic cell death of bcl-x−/− erythroid lineage cells was observed only at the end of maturation in both primitive and definitive erythropoiesis.

Bcl-x−/− mice die at about embryonic day 13 (21). Extensive apoptotic cell death is evident in hematopoietic cells in fetal liver. There is a threefold increase in TUNEL (for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling)–labeled apoptotic nuclei in histologically identifiable hematopoietic cells in embryonic day 12.5 bcl-x−/− liver compared with wild-type tissue. These data suggest that erythropoiesis in the fetal liver of bcl-x−/− mice is impaired because the vast majority of fetal liver hematopoietic cells at this gestational stage are erythroid lineage cells. These data on defective EryD production by the OP9 system are consistent with these in vivo data. During in vitro differentiation, although significant numbers of bcl-x−/− erythroblasts survived, almost no enucleated erythrocytes could be detected. This result shows that the pivotal function of bcl-x is expressed at the late stage of erythroid maturation. The critical role of bcl-x seems to be brought about by the remarkable increase of Bcl-X protein at the end of erythroid maturation. In vitro differentiation induction shows that apoptotic cell death of EryP also occurred at the late stage of maturation, which is consistent with primitive erythropoiesis in the bcl-x−/− mice. The effect of bcl-x on primitive erythropoiesis was not examined extensively because of the difficulty counting EryP numbers correctly in tiny mouse embryos. However, the following two lines of evidence strongly suggest that EryP production in bcl-x−/− mice was impaired to some extent. First, bcl-x−/− mice were paler than the control mice at day 12.5 of gestation.
defective erythropoiesis from analyzing the cells quantitatively (26, 27). To analyze the differentiation into fully mature blood cells and the feasibility of being able to differentiate from ES cells, the OP9 system has several remarkable advantages, among which are their potential to differentiate from ES cells while the hematopoietic microenvironment is provided by OP9 stromal cells. It is concluded from the defective EryD production in the chimeric mice that this defect is cell autonomous. In addition, the defective erythropoiesis of the bd-x<sup>-/-</sup> genotype with the OP9 system strongly supports this conclusion.

The production of definitive erythroid lineage cells is controlled by EPO (11). EPO induces the proliferation and prevents the apoptotic cell death of EryD. The antiapoptotic effect of EPO on EryD was observed from late erythroid progenitors (CFU-E) until the onset of hemoglobinization (2, 14–16). In other words, EPO-deprived apoptotic cell death is hardly at all observed at the end of maturation when maximal hemoglobin synthesis occurs. On the other hand, massive apoptotic cell death of bd-x<sup>-/-</sup> EryD was observed after day 13 of differentiation induction. It is reasonable to consider that the accumulation of Bcl-X (probably Bcl-X<sub>L</sub>) resulting from EPO stimulation prevents the apoptotic cell death of terminally differentiated erythroid cells. However, the accumulation of Bcl-X cannot be the only way to explain the antiapoptotic effect of EPO, because EPO prevents apoptotic cell death to some extent even in the absence of Bcl-X (Fig. 3). Taken together, it is likely that EPO has dual roles to prevent apoptotic cell death at different differentiation stages.

Of the various methods of in vitro hematopoietic differentiation from ES cells, the OP9 system has several remarkable advantages, among which are their potential to differentiate into fully mature blood cells and the feasibility of analyzing the cells quantitatively (26, 27). To analyze the defective erythropoiesis from bd-x<sup>-/-</sup> ES cells, quantitative analysis of the fully mature erythroid cells was necessary. However, such analysis is almost impossible by the conventional in vitro differentiation induction method with embryoid body formation. The other substantial advantage of the OP9 system is that hematopoietic microenvironment and hematopoietic cells can be analyzed separately by this method. It is well known that hematopoiesis is maintained by the hematopoietic microenvironment, such as stromal cells. By the conventional embryoid body formation method, both hematopoietic microenvironment and hematopoietic cells are induced from ES cells and are unseparable. But with the OP9 system, hematopoietic cells are induced from ES cells while the hematopoietic microenvironment is provided by OP9 stromal cells. It is concluded from the defective EryD production in the chimeric mice that this defect is cell autonomous. In addition, the defective erythropoiesis of the bd-x<sup>-/-</sup> genotype with the OP9 system strongly supports this conclusion.
9. Nunez, G., and M.F. Clarke. 1994. The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol. 4:399–402.

10. Rugh, R. 1968. The Mouse. Oxford University Press, Oxford.

11. Ihle, J.N., F.W. O'uelle, and O. Miura. 1993. Signal transduction through the receptor for erythropoietin. Semin. Immunol. 5:375–389.

12. Wu, H., X. Liu, R. Jaenisch, and H.F. Lodish. 1995. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 83:59–67.

13. Lin, C.-S., S.-K. Lim, V. D'Agati, and F. Costantini. 1996. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. Genes Dev. 10:154–164.

14. Spivak, J.L., T. Pham, M. Isaacs, and W.D. Hankins. 1991. Erythropoietin and hematopoiesis in murine fetal blood. J. Exp. Med. 173:1191–1199.

15. Yu, H., B. Bauer, G.K. Lipke, R.L. Phillips, and G. Van Zant. 1993. Apoptosis and hematopoiesis in murine fetal liver. Blood. 81:373–384.

16. Koury, M.J., L.L. Kelley, and M.C. Bondurant. 1994. The fate of erythroid progenitor cells. Ann. N.Y. Acad. Sci. 718:259–267.

17. Boise, L.H., M. Gonzalez-Garcia, C.E. Postema, L. Ding, T. Lindsten, L.A. Turka, X. Mao, G. Nunez, and C.B. Thompson. 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 74:597–608.

18. Gonzalez-Garcia, M., R. Perez-Ballestero, L. Ding, L. Duan, L.H. Boise, C.B. Thompson, and G. Nunez. 1994. bcl-X(L) and bcl-2. Blood. 84:1506–1510.

19. Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes hematopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. 335:440–442.

20. Nunez, G., L. London, D. Hockenberg, M. Alexander, J.P. MCKearn, and S.J. Korsmeyer. 1990. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hematopoietic cell lines. J. Immunol. 144:3602–3610.

21. Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, et al. 1995. Mosaic cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science. 267:1506–1510.

22. Silva, M., D. Grillot, A. Benito, C. Richard, G. Nunez, and J.L. Fernandez-Luna. 1996. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-X(L) and Bcl-2. Blood. 88:1576–1582.

23. Benito, A., M. Silva, D. Grillot, G. Nunez, and J.L. Fernandez-Luna. 1996. A apoptosis induced by erythroblast differentiation of human leukemia cell lines is inhibited by Bcl-X(L). Blood. 87:3837–3843.

24. Gregori, P.A., and M.C. Bondurant. 1997. The roles of Bcl-X(L) and apopain in the control of erythropoiesis by erythropoietin. Blood. 90:630–640.

25. Lacroon, V., P. Varlet, P. Mavez, A. Porteu, S. Giselle-Brecht, A. Kahn, and C. Lacombe. 1997. Bcl-2 targeted overexpression into the erythroid lineage of transgenic mice delays but does not prevent the apoptosis of erythropoietin-deprived erythroid progenitors. Blood. 90:3050–3056.

26. Wiles, M.V. 1993. Embryonic stem cell differentiation in vitro. Methods Enzymol. 225:900–918.

27. Nakano, T., H. Kodama, and T. Honjo. 1994. Generation of lymphohematopoietic stem cells from embryonic stem cells in culture. Science. 265:1098–1101.

28. Nakano, T. 1995. Lymphohematopoietic development from embryonic stem cells in vitro. Semin. Immunol. 7:197–203.

29. Nakano, T., H. Kodama, and T. Honjo. 1996. Development of primitive and definitive erythrocytes from different precursors in culture. Science. 272:722–724.

30. Weiss, M.J., and S.H. Orkin. 1996. In vitro differentiation of murine embryonic stem cells. New approaches to old problems. J. Clin. Invest. 97:591–595.

31. Wurst, W., and A.L. Joyner. 1993. Production of targeted embryonic stem cell clones. In Gene Targeting: A Practical Approach. A.L. Joyner, editor. IRL Press at Oxford University Press, Oxford. 33–61.

32. Bradley, A. 1987. Production and analysis of chimaeric mice. In Teratocarcinomas and Embryonic Stem Cells. E.J. Roberston, editor. IRL Press at Oxford University Press, Oxford. 113–152.

33. Williams, B.O., E.M. Schmitt, L. Remington, R.T. Bronson, D.M. Albert, R.A. Weinberg, and T. Jacks. 1994. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J (Eur. Mol. Biol. Organ.) 13:4251–4259.

34. Kodama, H., M. Nose, S. Nuida, S. Nishikawa, and S.-I. Nishikawa. 1994. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. Exp. Hematol. 22:979–984.

35. Cooper, M.C., J. Levy, L.N. Cantor, P.A. Marks, and R.A. Rifkind. 1974. The effect of erythropoietin on colonial growth of erythroid precursor cells in vitro. Proc. Natl. Acad. Sci. U.S.A. 71:1677–1680.

36. Sellins, K.S., and J.J. Cohen. 1987. Gene induction by y-irradiation leads to DNA fragmentation in lymphocytes. J. Immunol. 139:3199–3206.

37. Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. Manipulating the Mouse Embryo. 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY. 376–377.

38. Veis, D.J., C.M. Sorensen, J.R. Shutter, and S.J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell. 75:229–240.

39. Nakayama, K., I. Negishi, K. Kuida, H. Sawa, and D.Y. Loh. 1994. Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. U.S.A. 91:3700–3704.

40. Kamada, S., A. Shimono, Y. Shinto, T. Suirimura, T. Takahashi, T. Noda, Y. Kimata, H. Kondoh, and Y. Tsujimoto. 1995. bcl-2 deficiency in mice leads to pleiotropic abnormalities. accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. Cancer Res. 55:354–359.

41. Era, T., T. Takahashi, K. Sakai, K. Kawamura, and T. Nakano. 1997. Thrombopoietin enhances proliferation and differentiation of murine yolk sac erythroid progenitors. Blood. 89:1207–1213.

42. Brotherton, T.W., D.H. Chui, J. Gauldie, and M. Patterson. 1979. H emoglobin ontogeny during normal mouse fetal development. Proc. Natl. Acad. Sci. U.S.A. 76:2853–2857.

43. Mucenski, M.L., K. McLaIn, A.B. Kier, S.H. Szerdow, C.M. Schreiner, T.A. Miller, D.W. Priesty, W.J. Scott, Jr., and S.S. Potter. 1991. A functional c-myc gene is required for normal murine fetal hepatic hematopoiesis. Cell. 65:677–689.