

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

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## **Summary**

*bcl-x* is a member of the *bcl-2* gene family, which regulates apoptotic cell death in various cell lineages. There is circumstantial evidence suggesting that *bcl-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, *bcl-x*<sup>-/-</sup> ES cells did not contribute to circulating EryD in adult chimeric mice that were produced by blastocyst microinjection of the *bcl-x*<sup>-/-</sup> ES cells. *bcl-x*<sup>-/-</sup> 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 *bcl-x*<sup>-/-</sup> ES cells was similar to that from *bcl-x*<sup>+/+</sup> ES cells. However, prominent cell death of *bcl-x*<sup>-/-</sup> EryP and EryD occurred when the cells matured. The data show that the antiapoptotic function of *bcl-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<sub>L</sub> 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)<sup>1</sup> 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,  $\gamma$  irradiation, viral infections, oxidant stress, and notably growth factor deprivation (6). Bcl-2 and Bcl-X<sub>L</sub> act as cell death repressors, whereas Bax and the alternatively spliced *bcl-x* gene product, Bcl-X<sub>S</sub>, act as cell death promoters (6, 17, 18). A family of *bcl-2* genes participates in the regulation of cell survival in multiple cell lineages, including the hemato-

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<sup>1</sup>Abbreviations used in this paper: CFU-E, colony forming unit-erythroid; EPO, erythropoietin; EryP, embryonic primitive erythrocyte(s); EryD, adult definitive erythrocyte(s); ES, embryonic stem; GPI, glucose phosphoisomerase.

poietic lineage. Constitutive overexpression of Bcl-2 suppresses apoptosis in hematopoietic precursors by growth factor withdrawal, and overexpression of Bcl-X<sub>L</sub> also suppresses apoptosis (19, 20). Both Bcl-2 and Bcl-X<sub>L</sub> have recently been reported to be involved in regulating erythroid progenitors and survival (21–25). However, all of the evidence is circumstantial, and it is uncertain how *bcl-x* functions during erythroid differentiation under physiological conditions. In this study, we analyzed the function of *bcl-x* in erythropoiesis using mouse embryonic stem (ES) cells in which both alleles of *bcl-x* were disrupted (26–30). The production of immature EryP and EryD by *bcl-x*<sup>-/-</sup> ES cells was normal. Unexpectedly, however, prominent apoptotic cell death of both EryP and EryD occurred at the very end of erythroid maturation. These data clearly show that Bcl-X is essential in the late erythroid maturation stage.

## Materials and Methods

**Target Disruption of the *bcl-x* Gene.** E14 ES cells derived from strain 129/Ola were used throughout the experiment. ES *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> cell lines were produced as described previously (21). Genomic DNA containing the *bcl-x* locus was isolated from a library of mouse strain 129/Sv DNA. A 1.8-kb XhoI-BamHI fragment containing most of the *bcl-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-*tk* 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 *bcl-x*, mutant *bcl-x* with the *neo* targeting vector, and mutant *bcl-x* with the *hyg* targeting vector were 9.8, 7.0, and 5.5 kb, and were detected in wild-type, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> ES clones, respectively.

**Production of Chimeric Mice and Analysis of the Contribution of ES Cells.** ES *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> cells were injected into the 3.5-d post-coitum blastocysts from C57BL/6 mice to generate chimeric mice. GPI (glucose phosphoisomerase) isozymes were used to analyze the contribution of ES cells in various organs of chimeric mice. The GPI isozyme of B6 mice and of 129/Ola mice from which E14 ES cells were established are GPI-1A and GPI-1B, respectively. Separation and detection of GPI isoenzymes were performed essentially as described (32). Chimeric mice were perfused with PBS to eliminate blood cell contamination, and dissected tissues were kept at -70°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 (Helena 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 phenazine methosulfate, 5 μl glucose 6-phosphate dehydrogenase (140 U/ml; Sigma Chemical Co.), and 5 ml of 2% agarose. The GPI isozyme bands appeared after a few minutes in the dark. Density of the bands was analyzed by densitometer.

The hemoglobin type of B6 mice and 129/Ola mice are single (*Hbb<sup>s</sup>/Hbb<sup>s</sup>*) and diffuse (*Hbb<sup>d</sup>/Hbb<sup>d</sup>*), 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 hemoglobins was performed essentially as described (33). Whole 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 lysis 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 GPI 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%.

**ES Cells and Their Differentiation Induction.** ES *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> cells were cultured on embryonic fibroblasts as feeder cells in the presence of a saturated dose of leukemia inhibitory factor using the standard procedure (31). The culture of OP9 stromal cells and the differentiation induction method were carried out as described (27). OP9 stromal cells were maintained in α-MEM (Life Technologies, Inc.) supplemented with 20% FCS (Summit) and standard antibiotics (27, 34). 10<sup>5</sup> ES cells were transferred onto confluent OP9 stromal cells in 10-cm culture dishes (Nunc). After day 3 of the induction, human recombinant EPO (provided by Kirin Brewery Co. Ltd.) was added at a final concentration of 2 U/ml during the differentiation induction. The induced cells were trypsinized at day 5, and 10<sup>6</sup> cells were transferred onto fresh OP9 cells on a 10-cm plate. Nonadherent cells were harvested on day 6, 7, or 8 to obtain EryP. On day 10, all of the cells on individual 10-cm 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 *bcl-x*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> 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 media, individual colonies were picked, cytospin 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 × 10<sup>6</sup> cells in 1 ml of the Tyrode's buffer were layered on a step gradient of 2.0 ml of 30% wt/vol metrizamide (Nacalai Tesque) 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  $\alpha$ -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),  $3.0 \times 10^5$ /ml dianisidine-positive differentiation-induced cells were cultured in 6-well plates containing 20% FCS supplemented with  $\alpha$ -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 cytospin 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  $\pm$  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,  $10^6$  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.

## Results

**No Contribution of *bcl-x* Null ES Cells to Circulating Adult Definitive Erythrocytes.** ES cells of *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> 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 *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> 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 *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> ES cells (Table I). On the other hand, the contribution of *bcl-x*<sup>-/-</sup> ES cells to lymphoid organs such as spleen and thymus was significantly lower than that of *bcl-x*<sup>+/+</sup> or *bcl-x*<sup>+/-</sup> ES cells. This result is compatible with a previous report on the shortened life span of *bcl-x*<sup>-/-</sup> immature lymphocytes (21).

Two *bcl-x*<sup>-/-</sup> ES cell lines (clones 18 and 3a) were analyzed for the function of the *bcl-x* gene in hematopoiesis. Host blastocysts from the strain C57BL/6 are homozygous for the *Hbb*<sup>b</sup>  $\beta$ -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 *Hbb*<sup>d</sup> 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 *bcl-x*<sup>+/+</sup> or *bcl-x*<sup>+/-</sup> ES cells were used for chimera production, the contribution of the

**Table I.** Contribution of *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> ES Cells to Various Organs in Chimeric Mice

Organs	Genotype		
	+/+	+/-	-/-
	( <i>n</i> = 12)	( <i>n</i> = 3)	( <i>n</i> = 14)
Heart	62 $\pm$ 22	63 $\pm$ 15	52 $\pm$ 13
Kidney	60 $\pm$ 15	60 $\pm$ 27	55 $\pm$ 18
Muscle	99 $\pm$ 2	100 $\pm$ 0	99 $\pm$ 38
Thymus	38 $\pm$ 15	47 $\pm$ 21	21 $\pm$ 14*
Spleen	71 $\pm$ 9	63 $\pm$ 15	33 $\pm$ 21*

ES cells (GPI-1A) of *bcl-x*<sup>+/+</sup>, *bcl-x*<sup>+/-</sup>, and *bcl-x*<sup>-/-</sup> genotypes are microinjected into blastocysts of C57BL/6 mice (GPI-1B). Contribution of ES cells is examined by the enzyme activity of GPI-1 isozymes. Percentages of ES cell contribution are shown as mean  $\pm$  SD. Numbers of the examined chimeric mice are shown in parentheses. \**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 *bcl-x*<sup>-/-</sup> 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 *bcl-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 *bcl-x* to EryD production is cell autonomous, since the hematopoietic microenvironment in the chimeric animal could not complement the defective EryD production from *bcl-x*<sup>-/-</sup> 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 *bcl-x*<sup>-/-</sup> ES cells. One is the commitment failure of the multipotential hema-



**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 *Hbb*<sup>b</sup> haplotype (single) is specific for host blastocysts of strain C57BL/6, and the *Hbb*<sup>d</sup> 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 *bcl-x*<sup>+/+</sup> ES cells (lanes 3 and 4), *bcl-x*<sup>+/-</sup> ES cells (lanes 5 and 6), *bcl-x*<sup>-/-</sup> ES cells, clone 18 (lanes 7–9), and *bcl-x*<sup>-/-</sup> ES cells, clone 3a (lanes 10–12) were analyzed to examine the contribution of ES cells.

**Table II.** Differentiation of *bcl-x*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> ES Cells into Various Hematopoietic Lineages In Vitro

Colony type	Differentiation-induced ES cells		
	+/+	-/- 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 semi-solid 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  $\chi^2$  test. n, neutrophils; m, macrophages; E, erythroid cells; M, megakaryocytes.

topoietic progenitor cells into erythroid lineage cells, and the other is the proliferation and maturation failure of the committed erythroid cells. To analyze these possibilities, in vitro differentiation induction from *bcl-x*<sup>-/-</sup> and *bcl-x*<sup>+/+</sup> ES cells was carried out using OP9 stromal cells. The differentiation capacity of day 8 in vitro differentiation-induced 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*<sup>+/+</sup> or *bcl-x*<sup>-/-</sup> 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.

**Number and Viability of the In Vitro Differentiation-induced Erythroid Lineage Cells.** To further analyze the function of *bcl-x* in the production of erythroid lineage cells, in vitro differentiation induction into erythroid lineage cells from *bcl-x*<sup>-/-</sup> and *bcl-x*<sup>+/+</sup> ES cells was carried out using OP9 stromal cells in the presence of EPO. The number of hemoglobin-containing dianisidine-positive cells was counted between days 6 and 8 and between days 12 and 14. As previously reported, EryP and EryD appear in the former and latter periods, respectively (29). On day 6, the number of *bcl-x*<sup>-/-</sup> EryP was the same as *bcl-x*<sup>+/+</sup> EryP. However, on day 8, the difference between the number of *bcl-x*<sup>-/-</sup> EryP and *bcl-x*<sup>+/+</sup> EryP became pronounced. As shown in Table III, on day 8 the number of *bcl-x*<sup>-/-</sup> EryP was only ~10% that of *bcl-x*<sup>+/+</sup> EryP. Moreover, the number of *bcl-x*<sup>-/-</sup> EryP on day 8 was ~10% that of the day 7 *bcl-x*<sup>-/-</sup> EryP, suggesting that cell death occurred between these days. Similar results were obtained with EryD. There was no difference in the number of *bcl-x*<sup>-/-</sup> EryD and *bcl-x*<sup>+/+</sup> EryD on day 12. But, the difference became significant with maturation, and the number of EryD originating from *bcl-x*<sup>-/-</sup> ES cells was about one quarter that from *bcl-x*<sup>+/+</sup> ES cells on day 14.

The percentage of viable cells was next examined, because apoptotic cell death of *bcl-x*<sup>-/-</sup> erythroid cells was suspected (Table IV). Here again, there were no significant differences between the day 7 EryP and the day 12 EryD, but the differences became significant thereafter. The percentages of viable cells mainly reflect viable erythroid cells, because the vast majority of the cells during differentiation induction belong to the erythroid lineage. More than 80%

**Table III.** Numbers of Dianisidine-positive Cells Induced from *bcl-x*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> ES Cells

Day of induction	Type of erythroid cells	Differentiation-induced ES 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‡

10<sup>5</sup> of the day 5 induced cells were cultured on the OP9 cell layer in the presence of EPO (2 U/ml). Data ( $\times 10^{-4}$  of primitive and  $\times 10^{-5}$  of definitive erythroid cells) are shown as mean  $\pm$  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.

**Table IV.** Percentages of Viable Cells during the Differentiation Induction from *bcl-x*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> ES Cells

Day of induction	Differentiation-induced ES cells		
	+/+	-/- 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<sup>5</sup> 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.

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*<sup>+/+</sup> ES cells were induced for differentiation. The percentage of viable *bcl-x*<sup>-/-</sup> EryP seems relatively high for the very low number of *bcl-x*<sup>-/-</sup> EryP (Table IV). This apparent discrepancy was probably due to the removal of dead *bcl-x*<sup>-/-</sup> EryP by adherent macrophages. Electron microscopic features at about day 8 of the differentiation induction showed macrophages with prominently phagocytosed dead EryP (data not shown).

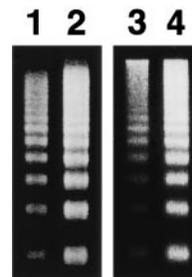
**Cell Death of In Vitro Differentiation-induced Erythroid Lineage Cells.** The morphological features and DNA fragmentation of the induced cells were analyzed to confirm that the decreased number and viability of *bcl-x*<sup>-/-</sup> cells were due to apoptosis. Immature EryP and EryD were purified by metrizamide density gradient separation on days 6 and 12 of the differentiation induction, respectively. At these times, no differences in number and viability were detectable between the *bcl-x*<sup>+/+</sup> and the *bcl-x*<sup>-/-</sup> erythroid cells as shown above. Using this purification method, >98% of the purified cells were erythroid lineage cells and their viability was 95–98%. These purified EryP and EryD were cultured on OP9 cells for 2 d in the presence of EPO, and the cells were harvested. Their morphological and molecular features were then examined. The *bcl-x*<sup>+/+</sup> EryP were viable and had mature morphology on day 8. In contrast, the *bcl-x*<sup>-/-</sup> EryP had fragmented nuclei with clumped chromatin, suggestive of apoptosis. On day 14, the vast majority of the cells were enucleated mature EryD when the *bcl-x*<sup>+/+</sup> ES cells were induced for differentiation, whereas enucleated EryD were rarely observed when the *bcl-x*<sup>-/-</sup> ES cells were induced. The hemoglobinized *bcl-x*<sup>-/-</sup> EryD were mainly nucleated erythroblasts. Thus, it was difficult to find viable, fully mature EryP and EryD on days 8 and 14 of the differentiation induction of *bcl-x*<sup>-/-</sup>

ES cells, respectively, although immature EryP and EryD were equally viable on days 6 and 12, respectively. Low molecular weight DNA was extracted from the cells, and agarose gel electrophoresis was carried out (Fig. 2). The nucleosomal DNA ladder, which is characteristic of apoptotic cells, was observed to be significantly more abundant in the *bcl-x*<sup>-/-</sup> erythroid lineage cells than in the *bcl-x*<sup>+/+</sup> erythroid lineage cells. These data clearly demonstrate that the *bcl-x*<sup>-/-</sup> erythroid lineage cells underwent apoptosis during the end stage of maturation.

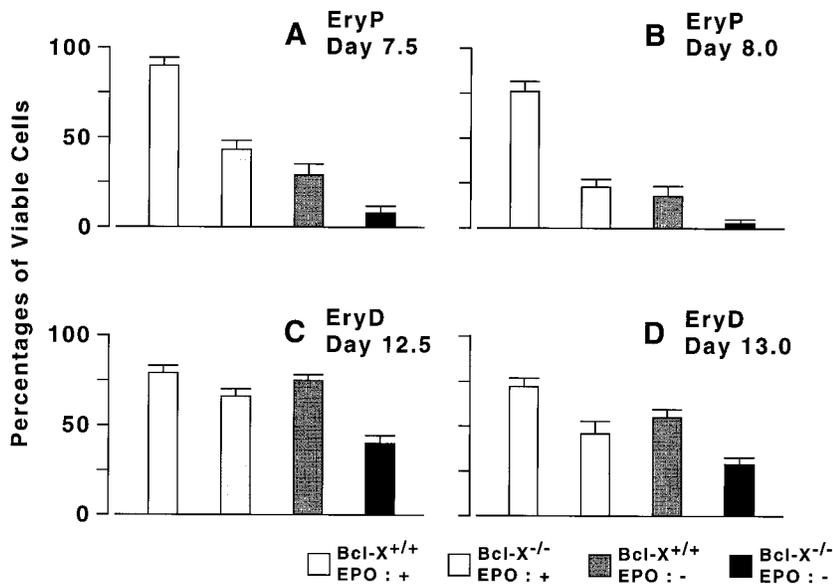
**Effects of Bcl-X and EPO on Apoptotic Cell Death.** EPO is required by immature erythroid lineage cells to prevent apoptosis. To analyze the roles of EPO and Bcl-X during the maturation of erythroid cells, an EPO deprivation experiment was performed. On days 6.5 and 11.5 of the differentiation induction, 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*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> 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*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> erythroid cells (*P* < 0.0005 by *t* test). Even in the absence of EPO, the differences in the viability were significant (*P* < 0.005 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.

## Discussion

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



**Figure 2.** Gel electrophoresis of low molecular weight DNA extracted from the cells after culture of purified day 6 differentiation-induced EryP and day 12 differentiation-induced EryD in the presence of EPO for 48 h. DNA extracted from the cells from the day 6 induced *bcl-x*<sup>+/+</sup> EryP (lane 1), the day 6 induced *bcl-x*<sup>-/-</sup> EryP (lane 2), the day 12 induced *bcl-x*<sup>+/+</sup> EryD (lane 3), and the day 12 induced *bcl-x*<sup>-/-</sup> EryD (lane 4).



**Figure 3.** Percentage of viable erythroid lineage cells in the presence or absence of EPO. Purified day 6.5 EryP (A and B) and purified day 11.5 EryP (C and D) derived from *bcl-x*<sup>+/+</sup> and *bcl-x*<sup>-/-</sup> (clone 18) ES cells were cultured in the presence or absence of EPO until the indicated day. The percentage of viable cells is shown as the mean  $\pm$  SD of six dishes. The data shown are representative of three independent experiments. *bcl-x*<sup>-/-</sup> (clone 3a) ES cells gave results similar to *bcl-x*<sup>-/-</sup> (clone 18) ES cells.

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  $\sim$ 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-x*<sup>-/-</sup> fetal liver and the absence of defects in the fetal liver of *bcl-2*<sup>-/-</sup> 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*<sup>-/-</sup> ES cells. There was no contribution by *bcl-x*<sup>-/-</sup> 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*<sup>-/-</sup> erythroid lineage cells was observed only at the end of maturation in both primitive and definitive erythropoiesis.

*bcl-x*-deficient 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*<sup>-/-</sup> liver compared with wild-type tissue. These data suggest that erythropoiesis in the fetal liver of *bcl-x*<sup>-/-</sup> mice is impaired because the vast majority of fetal liver hematopoietic cells at this gestational stage are erythroid lineage cells. The 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice was impaired to some extent. First, *bcl-x*<sup>-/-</sup> mice were paler than the control mice at day 12.5 of gestation

(21; our unpublished data). At this gestational stage, >95% of the erythrocytes are still EryP, although the relative percentage of EryP begins to decrease (42). Second, *bcl-x*<sup>-/-</sup> mice died at day 13 of gestation, which is earlier than the mutant mice lacking only definitive hematopoiesis by gene targeting of *c-myb* (43). The *c-myb* targeted mice were severely anemic by day 15; however, the mutant mice appeared normal at day 13 of gestation. On the other hand, EPO signal-deficient mice, which have a partial defect in primitive erythropoiesis and a complete defect in definitive erythropoiesis, die at day 13 of gestation, as early as *bcl-x*<sup>-/-</sup> mice (12, 13). Meanwhile, it is reasonable to consider that a similar time course of cell death of *bcl-x*<sup>-/-</sup> EryP and *bcl-x*<sup>-/-</sup> EryD would reflect a similar underlying molecular mechanism of cell death caused by the null mutation of *bcl-x*. The cause of cell death might be explained by the relationship between massive heme synthesis at the end of maturation of erythroid lineage cells and the antioxidant function of Bcl-X<sub>L</sub> (24).

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 *bcl-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 inseparable. 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 *bcl-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 *bcl-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.

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