Targeted disruption of Bcl-2 $\alpha\beta$ in mice: Occurrence of gray hair, polycystic kidney disease, and lymphocytopenia

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ABSTRACT Mice carrying ablated coding regions of the *bcl-2 α* and *bcl-2 β* transcripts have been made. *bcl-2*^{-/-} mutants are smaller but viable, although about half of them die by 6 weeks of age. As shown earlier with somatic *bcl-2* gene-targeted mice, the number of lymphocytes markedly decreased within few weeks after birth while other hematopoietic lineages remained unaffected. Among lymphocytes, CD8⁺ T cells disappeared most quickly followed by CD4⁺ T cells, whereas B cells were least affected. *bcl-2*^{-/-} lymphocytes, however, could respond normally to various stimuli including anti-CD3, Con A, phorbol 12-myristate 13-acetate plus ionomycin, interleukin 2, lipopolysaccharide, and anti-IgM antibody. Abnormalities among nonlymphoid organs include smaller auricles, hair color turning gray at 4–5 weeks of age, and polycystic kidney disease-like change of renal tubules. These results suggest that Bcl-2 may be involved during morphogenesis where inductive interactions between epithelium and mesenchyme are important such as in the kidneys, hair follicles, and perichondrium of auricles. Surprisingly, the nervous system, intestines, and skin appear normal despite the fact that these organs show high levels of endogenous Bcl-2 expression in normal mice.

bcl-2 is a protooncogene initially found at the breakpoint of the t(14;18) chromosomal translocation associated in over 60% of human follicular lymphomas (1–3). The translocation juxtaposes the *bcl-2* gene with the immunoglobulin heavy-chain gene, resulting in the formation of *bcl-2*-immunoglobulin heavy-chain fusion gene (4–6). The deregulation of Bcl-2 may promote cell survival by preventing programmed cell death in many, but not all, types of cells (7–13). Endogenous Bcl-2 is normally expressed in mature T and B lymphocytes and in other long-lived cells such as neurons, stem cells in intestine and skin, immature progenitors of erythrocytes, myelocytes, thrombocytes, and hormonally regulated glandular epithelium (14). Specifically, fetal tissue studies suggest that Bcl-2 may be involved in kidney and hair follicle morphogenesis (15). Cells derived from transgenic mice bearing deregulated/overexpressed Bcl-2 display extended B- and T-cell survival (16–19). In addition, ectopic expression of deregulated Bcl-2 in immature thymocytes in transgenic mice results in the acquisition of resistance to apoptosis-inducing glucocorticoid, ionizing γ -irradiation, and anti-CD3 treatments. These transgenic mice studies suggested that Bcl-2 may play an important role in B-cell and T-cell differentiation as well as in the maintenance of B-cell memory (18–21). Human Bcl-2 rescues cells normally destined to die by programmed cell death in the roundworm, *Caenorhabditis elegans* (22). Moreover, the *ced9* gene in *C. elegans* appears to be structurally and functionally similar to the mammalian *bcl-2* gene (23–25). These findings implicate the general

importance of *bcl-2* and its homologues throughout phylogeny.

Recently, using somatic *bcl-2* knock-out mice, we demonstrated that Bcl-2 is necessary to maintain a stable lymphoid system but is not required for phenotypic lymphocyte maturation (26). The *bcl-2*^{-/-} lymphocytes develop normally until about 2 weeks postnatally. *bcl-2*^{-/-} mature T cells have a markedly shortened life span and increased sensitivity to glucocorticoid and γ -irradiation, which are characteristic of immature thymocytes. Thus, Bcl-2 plays an indispensable role *in vivo* in extending the life span of mature T cells and in protecting these cells from apoptotic cell death. Surprisingly, stimulation of the *bcl-2*^{-/-} mature T cells by anti-CD3 monoclonal antibody (mAb) rescued these cells from death. The number of mature T and B cells decreased with time, with almost complete disappearance by 6–8 weeks postnatally. In addition to the disappearance of mature cells, immature cells including thymocytes expressing low or medium levels of the T-cell receptor (TCR) and pre-B cells also disappeared with time, suggesting that the lymphoid precursor cells were depleted. To further characterize these lymphoid abnormalities and to elucidate the role of Bcl-2 in nonlymphoid cells, we have generated the germ-line-transmitted *bcl-2* knock-out mice.

MATERIALS AND METHODS

Establishment of *bcl-2* Germ-Line Mutant Mice. Vector constructions and generation of *bcl-2* mutant embryonic stem cells (E14) are described elsewhere (26). E14 clones containing one mutated *bcl-2* allele (*bcl-2*^{+/-}) were injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeras were mated with C57BL/6 females, and germ-line transmission of the embryonic stem cell gene background was judged by the coat color of the offsprings. Offsprings, homozygous or heterozygous for the mutation, were determined by Southern blot analysis of tail DNA as described (26).

Flow Cytometry Analysis. Single-cell suspensions were prepared from bone marrow, spleen, thymus, and lymph nodes. Lymphocytes (5 $\times$ 10⁵) were stained by a combination of anti-CD8-fluorescein isothiocyanate (FITC), anti-CD4-phycoerythrin (PE), and biotinylated anti- $\alpha\beta$ TCR, anti-B220-FITC, anti- $\alpha\beta$ TCR-PE, and/or biotinylated anti-IgM. All antibodies were purchased from PharMingen. Biotin conjugates were revealed by Red613-streptavidin (GIBCO/BRL). Dead cells were excluded by staining with propidium iodide (Boehringer Mannheim). All analysis was carried out using FACScan (Becton Dickinson).

Abbreviations: mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; PE, phycoerythrin; LPS, lipopolysaccharide; PKD, polycystic kidney disease; IoM, ionomycin; IL-2, interleukin 2; TCR, T-cell receptor.

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Complete Blood Count. Peripheral blood (0.2 ml) was analyzed by a Coulter counter (Coulter). Differential analysis was carried out by May-Giemsa staining of blood smears.

In Vitro Cell Survival Test. Lymph node cells were placed in culture for the indicated times. Culture conditions were identical to those in the previous study (26). Viable cells were counted and analyzed by FACScan to obtain profiles of CD8-FITC and CD4-PE staining. The cell number of each population was calculated as (total viable cell number) $\times$ (percentage of the population in viable cells). Percent survival was defined as [(cell number at the indicated times)/(cell number at time 0 hour)] $\times$ 100.

[³H]Thymidine Incorporation Assay. Spleen cells were prepared from 2-week-old mice. Cells (2×10^5) were placed in a V-bottomed 96-well plate (Costar) in RPMI-1640 containing 10% (vol/vol) fetal calf serum and $10 \mu\text{M}$ 2-mercaptoethanol. Anti-CD3 mAb (145-2C11; $1 \mu\text{g/ml}$), Con A ($5 \mu\text{g/ml}$), phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) plus ionomycin (IoM; 250 ng/ml), interleukin 2 (IL-2; 100 units/ml), lipopolysaccharide (LPS; $20 \mu\text{g/ml}$), or goat anti-mouse IgM F(ab')₂ ($10 \mu\text{g/ml}$) was added. Cells were cultured for 48 hr followed by a 12-hr pulse with $1 \mu\text{Ci}$ ($1 \text{ Ci} = 37 \text{ GBq}$) of [³H]thymidine per well.

Histological Analysis. Tissues were fixed in $1 \times$ Histochoice (Amresco, Euclid, OH) for 3–7 days according to manufacturer's protocol and routinely processed for paraffin embedding. Six-micrometer sections were stained with hematoxylin/eosin.

RESULTS

General Condition and Appearance of *bcl-2*^{-/-} Mice. Bcl-2-deficient mice were smaller and their body weight was 30–90% of control littermates. There was no gross abnormality in appearance except for a rounder nose and smaller ears, but $\approx 50\%$ died between 1 and 6 weeks after birth (Fig. 1). The hair color of mutant mice turned gray postnatally between 4 and 5 weeks (Fig. 1). Our preliminary studies show that melanocytes are present in the skin of the gray mice (data

FIG. 1. Appearance of the Bcl-2-deficient mouse. A representative Bcl-2-deficient mouse at six weeks of age (*bcl-2*^{-/-}; left mouse) is shown with its normal littermate (*bcl-2*^{+/+}; right mouse). Note that the mutant mouse has gray hair, smaller ears, and a rounder nose. Both mice were originally agouti. At 4–5 weeks of age, the hair color of the mutant mouse changed from agouti to gray.

not shown). Therefore, change of hair color may be due to a functional impairment of melanin synthesis or transport, rather than to actual cell death of melanocytes.

Lymphocyte Development and Function. As reported earlier for the somatic *bcl-2*^{-/-} mice, we observed (i) normal development of T and B cells in the young *bcl-2*^{-/-} mice, (ii) shortened *in vitro* life span of *bcl-2*^{-/-} mature T cells but normal life span of *bcl-2*^{-/-} B cells, (iii) increased sensitivity of mature T cells to glucocorticoid and γ -irradiation, (iv) inhibition of accelerated cell death by anti-CD3 mAb of *bcl-2*^{-/-} mature T cells, and (v) disappearance of *bcl-2*^{-/-} lymphocytes by 6 weeks postnatally.

In the *bcl-2*^{-/-} mice, the number of peripheral blood lymphocytes was markedly decreased while the number of segmented neutrophils was comparable to that of *bcl-2*^{+/+} mice (Fig. 2). Other white blood cells, red blood cells, and platelets in peripheral blood were also within normal range (data not shown). Bone marrow cells appeared normal except for the decrease in the number of lymphocytes. Cells that are normally Bcl-2-positive such as promyelocytes, normoblasts, and megakaryocytes remain intact in the Bcl-2-deficient mice. The numbers of lymphocytes in thymus, spleen, and lymph nodes were also markedly decreased with age (Table 1). By 4 weeks of age, approximately 90–95% of lymphocytes had disappeared as observed earlier (Fig. 3) (26). The ratio of CD4⁻8⁺, CD4⁺8⁺, CD4⁺8⁻, and CD4⁻8⁻ thymocytes however remained unaffected despite this massive decrease of total thymocytes in *bcl-2*^{-/-} mice, suggesting possible precursor cell abnormality (Fig. 3). In contrast, the percentage of CD4⁻8⁺ cells was consistently lower in the periphery as reflected in the ratio of CD4⁻8⁺ to CD4⁺8⁻ lymph node T cells (less than 1:10 compared to 1:3 in control). To compare the life span of the CD4⁺8⁻ and CD4⁻8⁺ cells, lymph node T cells from *bcl-2*^{+/+} and *bcl-2*^{-/-} mice were placed in culture for the indicated times, and the numbers of viable remaining cells were counted (Fig. 4). *bcl-2*^{-/-} CD4⁻8⁺ T cells died more quickly than *bcl-2*^{-/-} CD4⁺8⁻ cells, possibly accounting for the accelerated decrease of CD4⁺8⁺ T cells in the periphery. In Bcl-2-deficient mice, the percentage of T-cell content in the spleen and peripheral blood was generally lower than in control, which is consistent with the more prominent decrease in the life span of T cells

FIG. 2. Decrease in the number of lymphocytes in peripheral blood of mutant mice. The number of lymphocytes (hatched bars) and segmented granulocytes (solid bars) in peripheral blood of control (*bcl-2*^{+/+}; left set of bars) and mutant (*bcl-2*^{-/-}; right set of bars) mice are shown.

Table 1. Percentage of cell number in lymphoid organs from Bcl-2-deficient mice

Age, week	Thymus	Spleen	Lymph node	Bone marrow
1	75	88	54	75
2	55	67	23	74
3	24	41	4.3	41
4	6.3	9.4	1.6	31
5	0.1	2.2	0.1	17

Cell numbers of thymus (both lobes), spleen, lymph nodes (cervical, axillary, brachial, and inguinal nodes), and bone marrow (one femur plus one tibia) in the *bcl-2*^{-/-} mice at the indicated age are shown as percentages compared to control littermates (*bcl-2*^{+/+}). The percentages are averages of three to five mice.

than of B cells (Fig. 3) (26). In bone marrow, B220⁺/surface immunoglobulin-positive mature B cells and B220⁺/surface immunoglobulin-negative pre-B cells were reduced by >80% compared to that in the *bcl-2*^{+/+} littermate (Fig. 3). Therefore, the lack of Bcl-2 seems to impair the early development of T and B lymphocytes.

We have earlier reported that the accelerated cell death of *bcl-2*^{-/-} mature T cells can be inhibited by anti-CD3 stimulation (26). To study the activation potential of *bcl-2*-negative lymphocytes, we used a variety of stimuli. *bcl-2*^{+/+}, *bcl-2*^{+/-}, and *bcl-2*^{-/-} cells responded comparably to anti-CD3, Con A, PMA plus IoM, IL-2, LPS, and anti-IgM stimuli (Fig. 5). We conclude that the *bcl-2*^{-/-} cells are not only phenotypically mature but are able to respond appropriately to activation signals.

Abnormality in Nonlymphoid Organs. Extensive histological examinations revealed that the heart, lung, liver, muscles, and bones appeared normal in the *bcl-2*^{-/-} mice (data not shown). Despite the fact that *bcl-2* is normally expressed highly in brain, intestines, skin, immature hematopoietic progenitors in bone marrow, or glandular epithelium, we could not find any morphological abnormality in these organs of *bcl-2*^{-/-} mice. However, the kidneys were smaller with a nonsmooth surface and were paler in color (Fig. 6A). Histological examination revealed decreased number of glomeruli and dilated renal tubules with hypertrophic epithelium

resulting in polycystic appearance of the kidney (Fig. 6B and C). Microscopic renal tubular dilatation could be detected in *bcl-2*^{-/-} mice as early as 1 week after birth. It is often accompanied with decrease in the number of glomeruli, interstitial hyperproliferation, and the disarray of cortex/medulla structure of the kidney. At 2–4 weeks old, macroscopic tubular dilatation became apparent.

DISCUSSION

The germ-line *bcl-2*^{-/-} mice were unable to maintain the lymphoid system beyond a few weeks after birth, as reported earlier in somatic *bcl-2*^{-/-} mice. In addition, dramatic abnormalities in hair color, auricles, and kidney were found, whereas the rest of the body appeared remarkably normal.

Although every lymphocyte subpopulation appeared to decrease abnormally in *bcl-2*^{-/-} mice, the CD4⁺ T cells appeared most sensitive, and this was confirmed in our *in vitro* survival experiment. It has been reported earlier that in transgenic mice in which *bcl-2* was overexpressed among thymocytes, there was an increase in CD4⁺ T cells despite similar expression levels in the different thymocyte subpopulations in both normal and transgenic mice (27). Therefore, it appears that CD4⁺ T cells are more dependent on the presence of the Bcl-2 product and possibly explains why there are more CD4⁺ than CD4⁺ T cells in the normal periphery. Furthermore, it is possible that an alternative molecule such as Bcl-x (28) may be differentially involved in determining the life span of CD4⁺ T cells.

While ≈99% of *bcl-2*^{-/-} mature T cells died during *in vitro* culture by 48 hr in the absence of external stimuli, various T-cell stimuli including anti-CD3 mAb, Con A, PMA plus IoM, and IL-2 all gave rise to an increase of DNA synthesis, suggesting that the *bcl-2*^{-/-} T cells can be stimulated to grow as well as *bcl-2*^{+/+} or *bcl-2*^{+/-} T cells. Therefore, Bcl-2 is not necessary for the process of lymphocyte activation. The fact that growth signals suppress cell death in the absence of Bcl-2 implies that Bcl-2 is not necessary for cell survival during the presence of growth stimulation but is necessary only when growth stimulation is deprived. The *bcl-2*^{-/-} mature B cells responded normally to LPS and IgM-crosslinking. Given the

FIG. 3. Flow cytometric analysis of lymphocytes. Lymphocytes were prepared from thymus, lymph nodes, spleen, and bone marrow of a 4-week-old *bcl-2*^{+/+} littermate (Upper) or *bcl-2*^{-/-} mutant mouse (Lower). Antibodies used in this experiment (see *Materials and Methods*) are indicated. Cell numbers from the thymus (both lobes), lymph nodes (cervical, axillary, brachial, and inguinal), spleen, and bone marrow (one femur and one tibia) are shown below the fluorescence-activated cell sorting patterns. The data representing lymph nodes were gated on αβ TCR⁺ cells.

FIG. 4. *In vitro* cell survival experiment of CD4⁺8⁺ and CD4⁺8⁻ T cells. Lymph node cells from the mutant mouse (*bcl-2*^{-/-}; closed symbols) and its littermate (*bcl-2*^{+/+}; open symbols) were placed in culture for the indicated times. The definition of percent survival is given in *Materials and Methods*. The percent survival of CD4⁺8⁻ T cells (triangles) and CD4⁺8⁺ T cells (circles) is shown. Experiments were carried out in triplicate. The average values of triplicate samples (shown as symbols) and standard deviations (error bars) are shown.

fact that the *in vitro* life span of *bcl-2*^{-/-} B cells appeared normal (26), no abnormality in the mature B cells has been found in the *bcl-2* homozygous mutant mice. Although Bcl-2 is expressed in the follicular mantle and the light zone where plasma cells and memory B cells are selected and maintained (14), it is not clear whether Bcl-2 plays a significant role in mature B cells. The ability of *bcl-2*^{-/-} B cells to differentiate into plasma cells or memory B cells remains to be tested. However, initial experiments have detected serum immunoglobulins in the young Bcl-2-deficient mice, suggesting that Bcl-2 may be dispensable for the differentiation into plasma cells (unpublished results).

It is an intriguing coincidence that the lymphoid system and hair color in the Bcl-2-deficient mice initially appear normal but become abnormal only upon aging. Although the mechanism underlying the hair color change is not clear, our preliminary results suggest that it is not due to melanocyte cell death. Therefore, it is likely that synthesis or transfer of

FIG. 5. [³H]Thymidine incorporation upon various stimuli. Spleen cells from *bcl-2*^{+/+} (solid bars), *bcl-2*^{+/-} (hatched bars), and *bcl-2*^{-/-} (open bars) were placed in culture with the indicated stimuli and pulsed as described in *Materials and Methods*. Experiments were carried out in triplicate. The average values of triplicate samples and standard deviations (error bars) are shown.

melanin may be impaired by the absence of Bcl-2. Thus, the mechanisms for the disappearance of lymphocytes and change of hair color may be distinct. However, one explanation of the late onset of these abnormalities may be that some molecule may be accumulating with age in the absence of Bcl-2, resulting in toxicity to lymphocytes and disturbance of melanogenesis. Alternatively, certain developmentally regulated physiological changes such as changing steroid levels may be responsible for the delayed onset of the abnormal phenotype. Still yet, there may exist a functional isoform of Bcl-2 that is expressed early in development of mice and cease after birth. This scenario would be analogous to the switching of embryonic to adult forms of hemoglobin, actin, and myosin (29, 30).

Although the Bcl-2 expression in adult is restricted to tissues characterized by apoptotic cell death, it is expressed

FIG. 6. PKD-like change in Bcl-2-deficient mice. (A) Gross appearance of kidneys from *bcl-2*^{+/+} (Left), *bcl-2*^{+/-} (Middle), and *bcl-2*^{-/-} (Right) mice. Note that the kidney of the mutant mouse is smaller in size, paler, and has a rougher surface. (B and C) Histological analysis of kidneys from *bcl-2*^{+/+} (B) and *bcl-2*^{-/-} (C) mice. Proximal renal tubules were dilated, and the epithelium was hypertrophic in the Bcl-2-deficient mouse. In addition, the number of glomeruli was significantly decreased.

in a wide variety of fetal tissues (14, 15). It is of note that Bcl-2 is expressed in condensations of cells committed to the formation of more complex structures, such as hair follicles in the skin and developing proximal nephrons of the kidney characterized by inductive interactions between epithelium and mesenchyme (15), suggesting that Bcl-2 may be involved in aspects of morphogenesis. The renal abnormality suggests the critical role of Bcl-2 in the morphogenesis of the kidney. In addition, expression of Bcl-2 was noted in the condensation of mesenchymal cells that constituted the perichondrium of the cartilaginous masses of the auricle, possibly explaining why the auricles are smaller. It seems likely that Bcl-2 may also be important for the development of perichondrium of cartilage.

In humans, many types of congenital or acquired renal cystic diseases have been reported. Among them, polycystic kidney diseases (PKDs) are characterized as hereditary disorders with diffuse cystic changes involving both kidneys. They include two genetically distinct disorders, autosomal-recessive PKD and autosomal-dominant PKD (31). Since PKD-like disease does not take place in *bcl-2*^{+/-} mice, the PKD-like disease in *bcl-2*^{-/-} mice appears to be of the autosomal-recessive type. However, the kidneys are massively enlarged in both types of human PKD, whereas the kidney in the *bcl-2* mutant mice seems to be shrunken. In addition, autosomal-recessive PKD is characterized pathologically by dilated collecting tubes, whereas the most affected region in the kidneys of the *bcl-2*^{-/-} mice is proximal tubules. From these differences, it is likely that the pathogenesis of PKD-like disorder in the Bcl-2-deficient mice is distinct from that of autosomal-recessive PKD in humans. It is rather phenotypically similar to a certain type of the autosomal-recessive familial nephronophthisis medullary cystic disease, which is characterized by shrunken polycystic kidneys and pale color, although the pathogenesis of the disease has been unknown (32). The precise effect of the absence of Bcl-2 on morphogenesis of the kidney remains to be examined.

Our study using *bcl-2* homozygous mutant mice demonstrated that Bcl-2 is required not only for lymphocytes but also for other tissues such as the kidney, hair, and perichondrium. Other tissues characterized by long-lived cells such as neurons, stem cells in intestine and skin, and hematopoietic progenitors appeared normal in the mutant mice. Our *bcl-2* knock-out mice will provide a physiological experimental system to elucidate the function of Bcl-2 in the development, function and death of lymphocytes, as well as in the morphogenesis of certain tissues.

Note. After we submitted this paper, Veis *et al.* (33) reported the generation of Bcl-2 α -deficient mice. In their mice, Bcl-2 β , which lacks the membrane-spanning region, is preserved. In contrast, we disrupted most of 5' exon of the *bcl-2* gene, which encodes most of Bcl-2 α and Bcl-2 β . Although these mice are genetically different, they also reported hair color change, PKD, and early disappearance of lymphocytes. Therefore, it is suggested that the presence of Bcl-2 β may not be biologically significant.

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