Development 122, 1703-1709 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV4674

Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia

Takemitsu Furuchi[1], Kazue Masuko[1], Yoshitake Nishimune[2], Masuo Obinata[1] and Yasuhisa Matsui[1,*]

[1]Department of Cell Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980, Japan
[2]Research Institute of Microbial Diseases, Osaka University, Suita 565, Japan

*Author for correspondence

SUMMARY

During normal spermatogenesis, more than half of the germ cells undergo apoptosis, but the physiological significance and molecular mechanisms of this programmed cell death are largely unknown. Because Bcl-2 functions as a death repressor, we have investigated the effect of misexpressing Bcl-2 in spermatogonia in transgenic mice using the human *bcl-2* cDNA under the control of the human polypeptide chain elongation factor 1α (*EF-1α*) promoter. In the 2-week-old transgenic testes, exogenous Bcl-2 was expressed in spermatogonia and massive accumulation of spermatogonia was observed in seminiferous tubules by 4 weeks. At this time, only a few spermatocytes were apparent, and the accumulated cells degenerated, leading to vacuolization in some seminiferous tubules by 7 weeks. In older transgenic mice, abnormal accumulation of spermatogonia and degeneration of these germ cells was still observed, but some seminiferous tubules in which the level of Bcl-2 expression was reduced recovered normal spermatogenesis. These observations indicate that spermatogonial apoptosis is part of the normal program of mammalian spermatogenesis and is regulated by a pathway affected by Bcl-2.

Key word: transgenic mice, Bcl-2, apoptosis, spermatogenesis, spermatogonia

INTRODUCTION

Apoptosis or programmed cell death is an active process which controls cell numbers in a variety of tissues and is involved in morphogenesis during embryonic development and throughout adult life (Raff et al., 1993; Schwartzman and Cidlowski, 1993; Uker, 1991; Williams, 1991). Cell death also occurs spontaneously at various phases of germ cell development and appears to play a major role during spermatogenesis. (Allan et al., 1987; Bartke, 1995). However, the physiological significance and molecular mechanisms responsible for the process have remained obscure.

Although there is no consensus on the identity of spermatogonial stem cells, the A_s (isolated undifferentiated type A) spermatogonia are most widely thought to be the only population of stem cells (Russell et al., 1990; Meistrich and van Beek, 1993). When type A_s spermatogonia divide, they produce either two A_s cells or paired spermatogonia (A_{pr}) linked by an intercellular bridge, which results from incomplete cytokinesis. The production of type A_{pr} spermatogonia is the first step toward differentiation. Proliferation of type A_{pr} spermatogonia produces linked or aligned spermatogonia (A_{al}) in chains of 4-32 cells. They then give rise to several generations of differentiating spermatogonia, namely type A_1-A_4, intermediate (In) and type B spermatogonia. In the rodent, there is substantial death of spermatogonia of types A_2 and A_3, and lesser involvement of type A_4. Previous morphological studies have implicated apoptosis in spermatogonial death during spermatogenesis (Allan et al., 1987; Bartke, 1995). From quantitative studies, it has been shown that only 25% of the theoretically possible number of preleptotene spermatocytes is produced from the original population of A_1 spermatogonia (Allan et al., 1987). Death of selected spermatocytes and spermatids is also a regular feature of normal spermatogenesis and about 20% of germ cells degenerate between preleptotene primary spermatocytes and mature spermatids. Withdrawal of gonadotropins and/or testosterone enhances the germ cell degeneration and it seems that apoptosis of germ cells in the testis is under the control of FSH and testosterone (Allan et al., 1987; Bartke, 1995; Billig et al., 1995; Henriksen et al., 1995; Hikim et al., 1995; Kangasniemi et al., 1995).

The prototypic regulator of mammalian cell death is the protooncogene *bcl-2* (Korsmeyer, 1992a,b). Bcl-2 was originally cloned from the breakpoint of a t(14;18) translocation present in many human B cell lymphomas (Bakhashi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). This translocation results in the deregulated expression of the *bcl-2* gene as a result of its juxtaposition to the immunoglobulin heavy chain gene locus (Cleary et al., 1986; Graninger et al., 1987; Seto et al., 1988). The product of the *bcl-2* gene, which encodes a membrane-associated protein, prevents the normal course of cell death without altering proliferation in cultured cells (Hockenbery et al., 1990; Korsmeyer, 1992a,b; Reed, 1994). Transgenic mice expressing *bcl-2* in the lymphoid lineage have increased numbers of resting B cells (McDonnell et al., 1989,

1990; Strasser et al., 1990, 1991). However, expression in the T-cell lineage does not prevent clonal deletion of autoreactive T cells (Sentman et al., 1991; Strasser et al., 1991). Bcl-2 can also protect a proportion of neurons from cell death during development (Farlie et al., 1995; Martinou et al., 1994). These observations suggest that there are multiple death pathways, some of which are regulated by Bcl-2. Recently, several additional proteins homologous to Bcl-2 have been isolated and interaction among these proteins seems to be important for control of cell survival (Boise et al., 1993; Oltvai et al., 1993). One of these proteins, termed Bax, functions to counter the effects of Bcl-2 on cellular survival (Oltvai et al., 1993). The ratio of Bcl-2 to Bax proteins is the critical determinant of cell fate such that elevated Bcl-2 favors extended survival of cells, whereas increasing levels of Bax expression concomitantly accelerate cell death. A similar situation has also been described for products of the *bcl-x* gene (Boise et al., 1993). The *bcl-x* gene encodes two proteins: Bcl-x_L which promotes cell survival and Bcl-x_S, a splice variant of bcl-x_L, which antagonizes Bcl-2 function. However, it is not known how the interaction of these proteins regulates apoptosis in a physiological context.

In the present study, we generated transgenic mice expressing the human *bcl-2* gene in spermatogonia and have analyzed how Bcl-2 influences their development.

MATERIALS AND METHODS

Production of transgenic mice

The DNA fragment used for microinjection is shown in Fig. 1A. A 1.2 kb *Hin*dIII-*Eco*RI fragment containing human *EF-1α* promoter and a 0.7 kb *Xba*I-*Eco*RI fragment containing poly(A) adenylation signal from *G-CSF* cDNA were derived from pEF-BOS (Mizushima and Nagata, 1990). A 0.9 kb fragment of the *bcl-2* cDNA contains the entire coding sequence for the human *bcl-2* gene (Tsujimoto and Croce, 1985). Transgenic mice were produced by microinjection of a 2.8 kb *Cla*I-*Not*I fragment isolated from the plasmid into the pronuclei of fertilized mouse eggs (DBA/2 × C57BL/6 F_1) as described by Hogan et al. (1994). Integration of the transgene was detected by Southern blot hybridization with DNA obtained from tail biopsies using the 1.2 kb *Hin*dIII-*Eco*RI human *EF-1α* promoter fragment as a probe. All mice were mated with C57BL/6 mice.

RT-PCR analysis

Expression of the transgene was detected by RT-PCR analysis. Total RNA (5 μg) prepared by the method of acid guanidium thiocyanate phenol chloroform (Chomczynski and Sacchi, 1987) was reverse transcribed into cDNA using SuperScript RT (BRL SuperScript Preamplification System). The PCR was performed in 10 μl of solution containing PCR optimizer buffer F (Invitrogen), 10% dimethyl sulfoxide and AmpliTaq DNA polymerase. The cDNAs were amplified with specific primers for the exon1 region of the *EF-1α* promoter (5′-GGTTTGCCGCCAGAACACAG-3′) and for human *bcl-2* cDNA (5′-GGTTGACGCTCTCCACACAC-3′) for 30 cycles under the following conditions: 40 seconds at 94°C, 30 seconds at 65°C and 1 minute at 72°C. The amplified products were separated by 1.4% agarose gel electrophoresis. Southern blot was carried out at 42°C with random primed *bcl-2* cDNA probe (1× 10^9 cpm/μg), after which the membrane was washed three times with 2× SSC, 0.1% SDS at room temperature and then two times with 0.2× SSC, 0.1% SDS at 65°C. PCR of Hprt was performed as described previously (Nagamine et al., 1990).

Histology and immunocytochemistry

For hematoxylin and eosin staining and immunohistochemistry with anti-human Bcl-2 antibody, tissues were fixed in Bouin's fixative for 3 hours and routinely processed for paraffin embedding and sectioning (5 μm). For immunostaining with anti-human Bcl-2 antibody, sections were treated with 0.3% hydrogen peroxide in PBS for 20 minutes and washed with PBS. Sections were then treated with 5% skimmed milk (Difco) in PBS, followed by incubation with anti-human Bcl-2 polyclonal antibody (Pharmingen, 1:800 dilution) overnight at 4°C. Sections were washed 3 times with 0.02% Tween 20 in PBS and were treated with horseradish peroxidase complex conjugated to goat anti-rabbit IgG (Zymed, 1:500 dilution) for 30 minutes at room temperature, followed by 3 washings with 0.02% Tween 20 in PBS. Bound peroxidase was visualized with 0.01% hydrogen peroxide and 0.05% diaminobenzidine in PBS. Sections were counterstained with hematoxylin. Immunostaining with EE2 (Koshimizu et al., 1995) and BC7 (Koshimizu et al., 1993) was carried out as described.

In situ DNA 3′-end labeling

Tissues were fixed in 10% neutral buffered formalin and routinely processed for paraffin embedding and sectioning (5 μm). Sections were stained with an Apop Tag In Situ Detection Kit (Oncor) according to the instruction of the manufacturer.

RESULTS

Generation of transgenic mice expressing human bcl-2

We initially attempted to overexpress Bcl-2 in many tissues, including germ cells, and with this goal in mind we used the *EF-1α* promoter (Mizushima and Nagata, 1990; Uetsuki et al.,

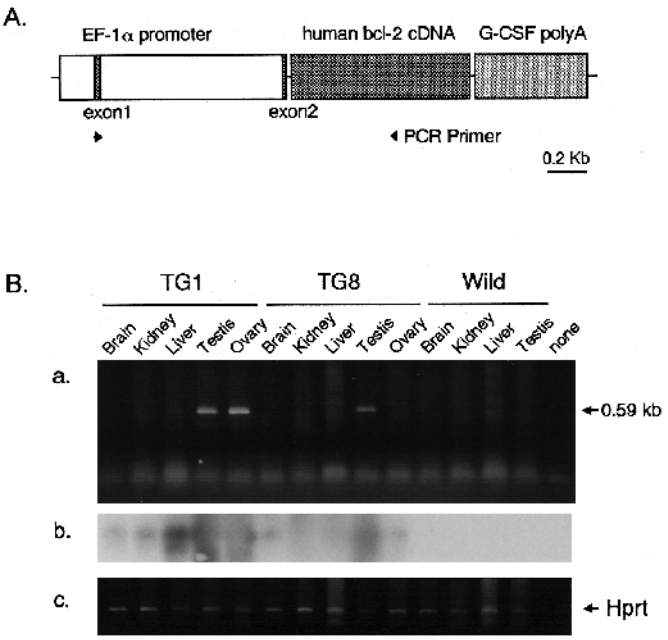

Fig. 1. Structure and expression of the *bcl-2* transgene. (A) *bcl-2* transgene construct used to generate transgenic mice. The construct contains the *EF-1α* promoter (1.2 kb) fused to the human *bcl-2* cDNA (0.95 kb) and the poly(A) adenylation signal from human granulocyte colony-stimulating factor (G-CSF) cDNA. The arrows indicate the primer used for RT-PCR. (B) Expression of human *bcl-2* in transgenic mice. (a) RT-PCR of total RNA from various tissues of a transgenic mouse (TG1 and TG8). The 0.59 kb band represents the RT-PCR products specific to the exogenous *bcl-2* gene. (b) Southern blot analysis of RT-PCR products with human *bcl-2* cDNA probe. (c) RT-PCR of hypoxanitine phosphoribosyltransferase (Hprt) used as an internal control.

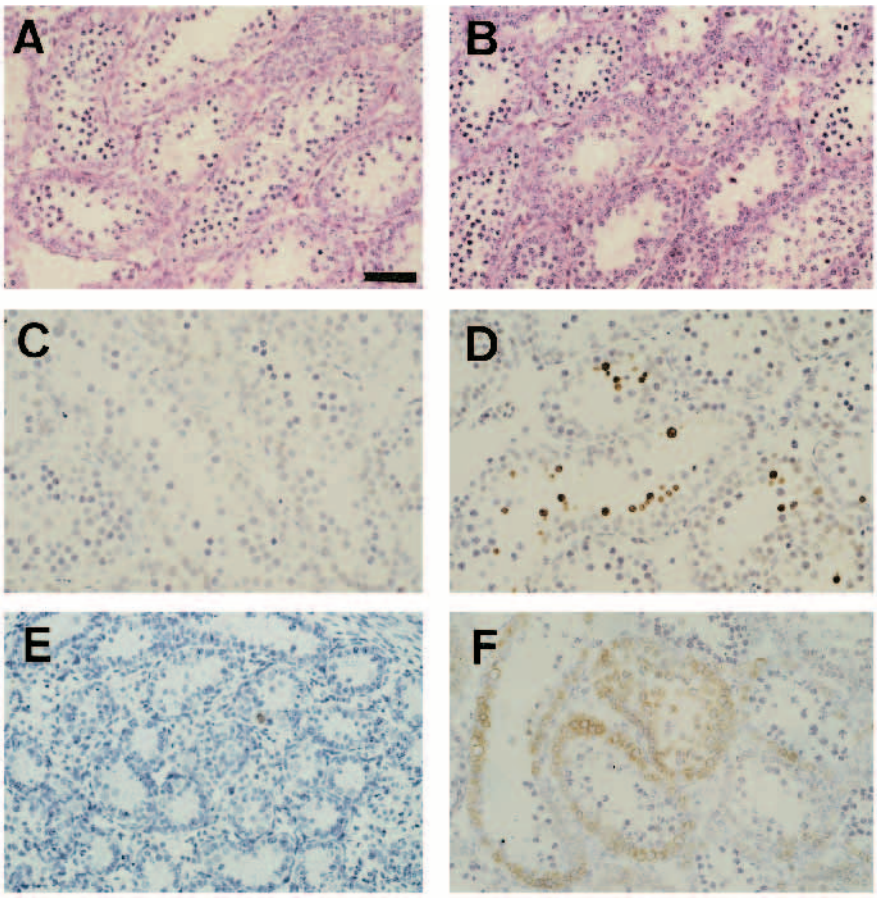

Fig. 2. Inhibition of germ cell death in 2-week-old *bcl-2* transgenic mice. (A,B) Sections of a testis from a 2-week-old transgenic mouse from line TG1 (A) and a normal littermate (B), stained with hematoxylin and eosin. (C,D) In situ 3′-end labeling of DNA by the TUNEL method using testis sections of a 2-week-old transgenic mouse from line TG1 (C) and a normal littermate (D). (E,F) Sections of testis from a newborn (E) and a 2-week-old (F) transgenic mouse from line TG1, stained with anti-human Bcl-2 antibody. Scale bar, 50 μm.

1989) for the transgenic construct (Fig. 1A). To our surprise, however, the transgene was preferentially expressed in gonads. As shown in Fig. 1B, high level expression of the transgene was detected in line TG1 and TG8 in the testes and/or ovary and at much lower levels in other tissues. A total of eleven different transgenic mice, eight females (TG1-TG8) and three males (TG9-TG11), were obtained. The copy number of the exogenous *bcl-2* gene varied in the range of 1-15 (TG1

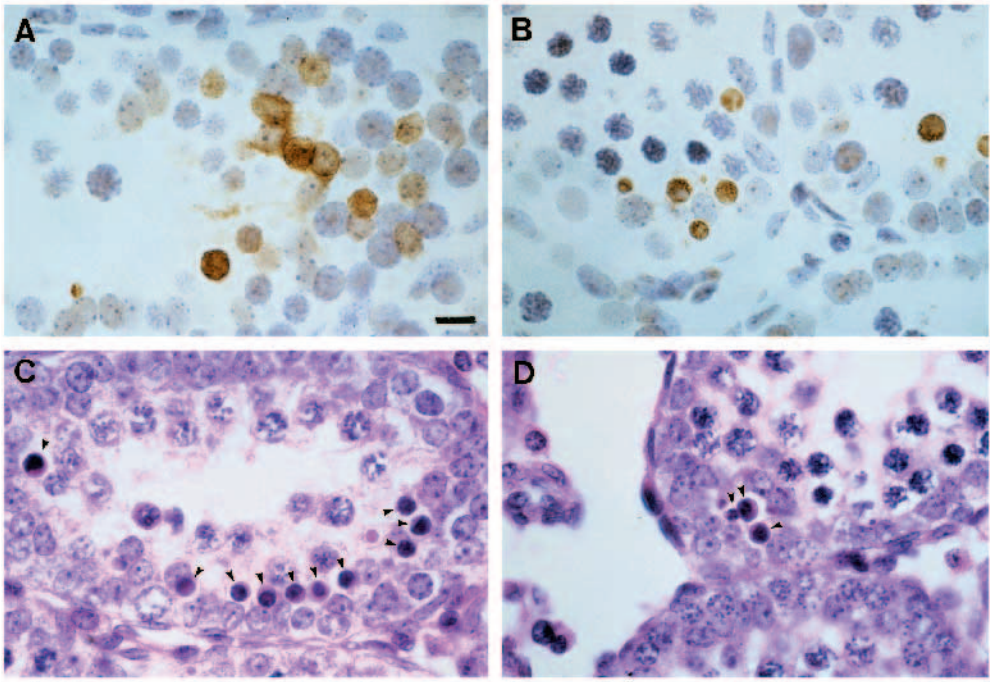

Fig. 3. Apoptosis of spermatogonia in 2-week-old wild-type mice. (A,B) High magnification photographs of testis sections from a 2-week-old normal littermate, stained by the TUNEL method. (C,D) High magnification photographs of testis sections from a 2-week-old normal littermate, stained with hematoxylin and eosin. Apoptotic cells showing dense, compacted nuclear chromatin (arrowhead). Scale bar, 10 μm.

contained >10 copies and TG8 contained 3-4 copies), as estimated by Southern blot hybridization (data not shown).

Sterility of transgenic mice

During the course of these experiments, it was noted that the male founders (TG9, 11) and male offspring from five lines (TG1, 2, 3, 5, 8) never bred. Fertility of the females from four lines (TG2, 3, 5, 6) also seemed to be reduced, but the male phenotype was more severe. None of the founders or their offspring showed gross abnormality other than reduced fertility, except line TG5. Two F_1 offspring of this line had webs between the 1st and 2nd toes on their hind limbs (unpublished result).

Histology of the male genital organs of transgenic mice

The male phenotype of lines TG1 and TG8 has been studied in detail and was identical. At birth, the testis was histologically normal and expression of exogenous Bcl-2 was observed only in a few cells (Fig. 2E). 2 weeks later, although testis histology still seemed to be normal (Fig. 2A,B), ectopic Bcl-2 expression was detected in basally located cells, many of which are spermatogonia and some spermatocytes (Fig. 2F). Expression of endogenous Bcl-2 was not detected in age-matched non-transgenic littermates (data not shown). At this age, degeneration of some germ cells was detected by the terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling (TUNEL) method (Gavriele et al., 1992) in normal mice (Figs 2D, 3). These TUNEL-positive cells, some of which seemed to be spermatogonia (Fig. 3A,B), also showed the typical morphological feature of apoptosis (arrowhead, Fig. 3C,D). However, much fewer positive cells were detected in the transgenic mice (Fig. 2C).

Testes of 4-week-old transgenic mice were found to be enlarged. The weight of the testes was about twice that of normal littermates (Table 1). Histological examination showed that numerous germ cells had accumulated in all seminiferous tubules (Fig. 4A). To identify these cells, we used germ cell stage specific monoclonal antibody EE2 (Koshimizu et al., 1995), specific for type A spermatogonia to zygotene spermatocytes, and BC7 (Koshimizu et al., 1993), specific for zygotene and early pachytene spermatocytes (Fig. 4F). Most of these cells were EE2 positive (Fig. 4H) and BC7 negative (Fig. 4E). From the staining specificity and morphological features of the cells, they were identified as spermatogonia. Although some spermatocytes were present near the center of some tubules and a part of them were stained by BC7 (Fig 4E), normal spermatogenesis was apparently inhibited. In contrast, the normal testis contained spermatids and spermatozoa (Fig. 4B). The expression level of exogenous Bcl-2 varied among the accumulated germ cells (Fig. 4G). At this time, a significant proportion of these germ cells in which Bcl-2 expression decreased tended to be positive for TUNEL staining (Fig. 4C), while only a few spermatocytes were stained in the normal testes (Fig. 4D).

By 7 weeks of age, vacuolization of many seminiferous tubules was apparent (Fig. 5A). Most of the germ cells were deleted in these tubules, but a few spermatogonia could still be

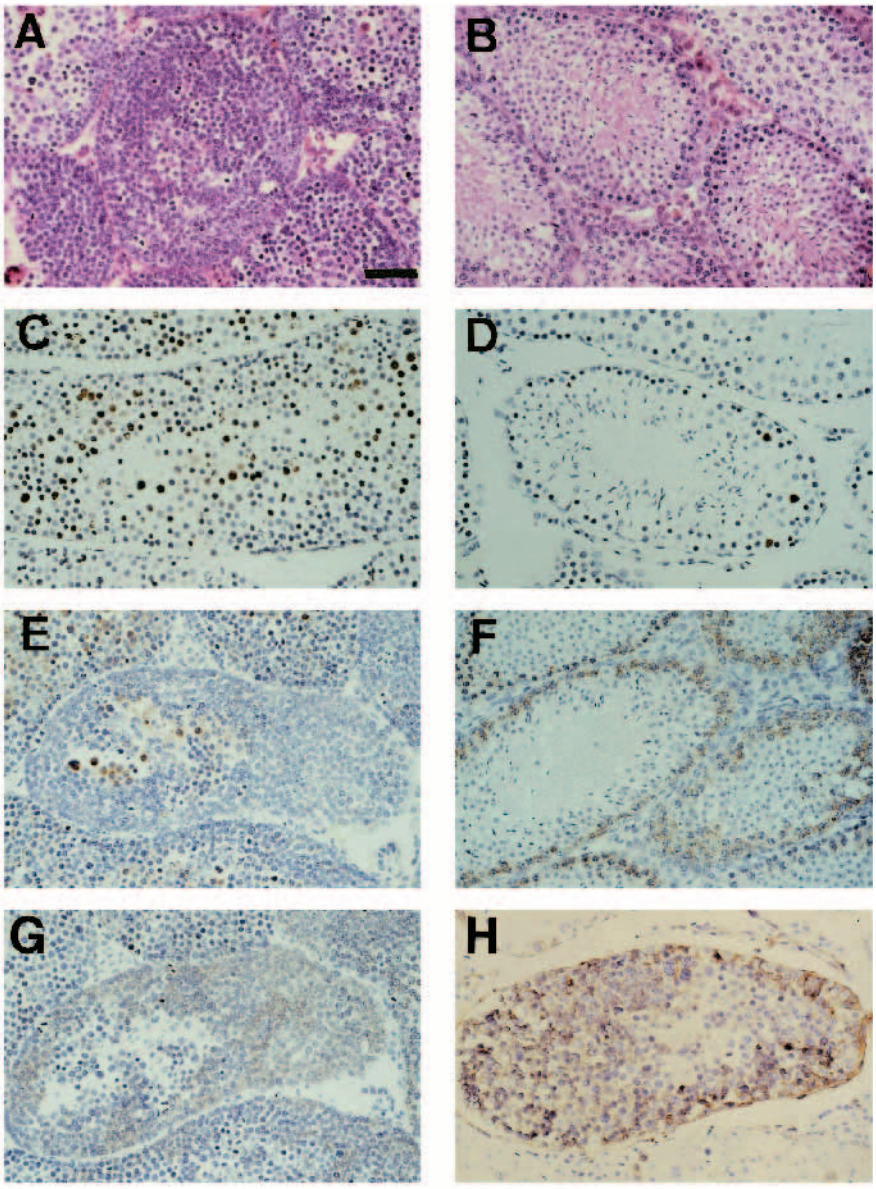

Fig. 4. Accumulation of spermatogonia in seminiferous tubules in 4-week-old *bcl-2* transgenic mice. (A,B) Sections of testis from a 4-week-old transgenic mouse from line TG1 (A) and a normal littermate (B), stained with hematoxylin and eosin. (C,D) In situ 3′-end labeling of DNA by the TUNEL method using testis sections from a 4-week-old transgenic mouse from line TG1 (C) and a normal littermate (D). (E,F) Sections of testis from a 4 week-old transgenic mouse from line TG1 (E) and a normal littermate (F), stained with monoclonal antibody (mAb) BC7, specific for zygotene and early pachytene spermatocytes. (G) Adjacent sections to those in E, stained with anti-human Bcl-2 antibody. (H) Sections of testis from a 7-week-old transgenic mouse from line TG1, stained with mAb EE2, specific for spermatogonia to zygotene spermatocytes. Scale bar, 50 μm.

Table 1. Weight of testes from transgenic mice and normal littermates*

Age (weeks)	Wild (mg)†	TG (mg)
4	46.1±1.6 (4)	81.8±3.1 (4)
7	100.2±2.5 (4)	36.7±1.3 (2)
12	112.6±2.5 (8)	28.5±1.0 (8)

*Testes from transgenic mice from lines TG1 and TG8 and normal littermates were dissected, and wet weight determined

†The mean±s.e.m. and the number of testes analyzed (in parentheses) is indicated.

Table 2. Weight of prostate and seminal vesicle from transgenic mice and normal littermates*

Organ	Wild (mg)†	TG (mg)
Prostate	20.9±2.2 (4)	22.7±3.4 (3)
Seminal vesicle	230.6±14.9 (3)	235.6±11.1 (3)

*Organs from transgenic mice from lines TG1 and TG8 and normal littermates were dissected, and wet weight determined.

†The mean±s.e.m. and the number of organs analyzed (in parentheses) is indicated.

detected. However, some tubules were still abnormally filled with undifferentiated and/or atypical germ cells, many of which were Bcl-2 positive (Fig. 5B). The weight of the transgenic testes had decreased to about one-third that of normal littermates (Table 1).

By 6 months of age, spermatogenesis had partially recovered and about one-third of the tubules contained elongated spermatids and even spermatozoa (Fig. 5C). Bcl-2 expression was not detected in these recovering tubules (arrow), while some tubules were still filled with Bcl-2-expressing spermatogonia and atypical germ cells (arrowhead, Fig. 5D). Recovering spermatogenesis was very rarely observed (one or two tubules per testis), even at 7 weeks of age (data not shown).

It is unlikely that this phenotype was caused by hormonal abnormality, because the weights of the prostate and the seminal vesicles of line TG1 and TG8 were normal (Table 2), suggesting that the androgen level was not altered in the transgenic mice.

DISCUSSION

We have generated transgenic mice which ectopically express the *bcl-2* transgene in spermatogonia. The *EF-1α*/human *bcl-2* fusion gene in these mice was strongly expressed in gonads, but only a faint signal was detected in other tissues. This is not due to the position of the transgene insertion, since at least two independent transgenic lines showed similar pattern of expression. Although the *EF-1α* gene is normally expressed in most tissues (Mizushima and Nagata, 1990; Uetsuki et al., 1989), and the regulatory region of the gene which we used for the transgene construct can direct strong expression in various cultured cells (Mizushima and Nagata, 1990), the truncated regulatory region apparently activates transcription preferentially in germ cells in a physiological context.

The most striking feature of the transgenic mice was adult testes with abnormal seminiferous tubules, some of which were filled with spermatogonia and others lacking most germ cells (Fig. 5A). Abnormal accumulation of spermatogonia was more apparent during the first wave of spermatogenesis. Although 2-week-old testes of the transgenic mice were seemingly normal, exogenous Bcl-2 was expressed in basally located germ cells, many of which are spermatogonia (Fig. 2F) and TUNEL-positive cells apparently decreased (Fig. 2C,D). However, there were a significant number of TUNEL-positive cells in normal testes and some of these cells were spermatogonia, judging from their morphology (Fig. 3). At 4 weeks of age, all tubules were abnormally filled with spermatogonia, most of which expressed exogenous Bcl-2 (Fig. 4G). These observa-

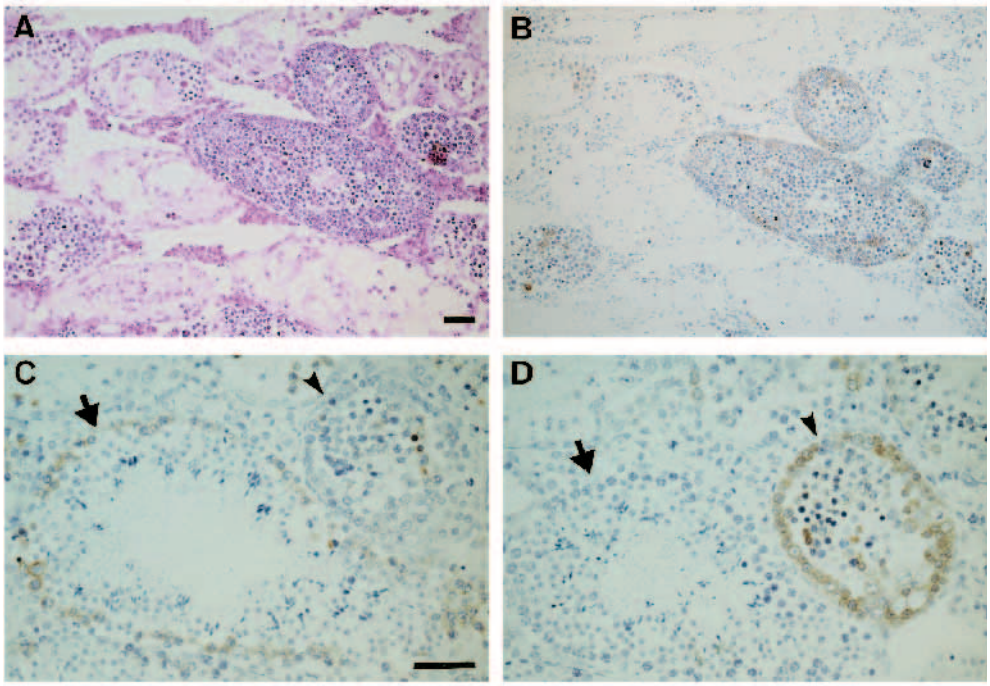

Fig. 5. Vacuolization of seminiferous tubules and recovery of spermatogenesis in 7-week-old and 6-month-old *bcl-2* transgenic mice. (A,B) Sections of testis from a 7 week-old transgenic mouse from line TG1, stained with hematoxylin and eosin (A) and anti-human Bcl-2 antibody (B). (C,D) Sections of testis from a 6-month-old transgenic mouse from line TG1, stained with mAb BC7 (C) and anti-human Bcl-2 antibody (D). Arrow, recovering tubule; arrowhead, tubule with Bcl-2-expressing germ cells. Scale bar, 50 μm.

tions indicate that ectopically expressed Bcl-2 inhibited normal spermatogonial apoptosis, leading to accumulation of spermatogonia by 4 weeks. In normal spermatogenesis, a proportion of type A_2-A_4 spermatogonia undergo apoptosis (Allan et al., 1987), and death-survival balance may be controlled by Bcl-2 or other related molecules. Because Bcl-2 and a structurally related death repressor molecule, Bcl-x, are not expressed in spermatogonia (Hockenbery et al., 1990; Krajewski et al., 1994), other unidentified molecules of this family should be a physiological regulator for spermatogonial cell death. It has recently been reported that *bax*-deficient mice have a similar testicular abnormality as our *bcl-2* transgenic mice (Knudson et al., 1995). Bax has been identified as a Bcl-2 associated molecule which antagonizes Bcl-2 and accelerates apoptosis (Boise et al., 1993). In spermatogonia, Bax may associate with a Bcl-2 related molecule and together the two proteins determine cell survival or death. In testes of 4-week-old transgenic mice, some spermatocytes were found and they were often multinucleate (Fig. 4E). In contrast, numerous meiotic spermatocytes and spermatozoa were present in normal testis at this age (Fig. 4F). This result suggests that the differentiation of the surviving germ cells is prevented and even if the cells initiate the meiotic cycle, it may progress improperly. From these observations, we suggest that atypical spermatogonia, forced to survive by exogenous Bcl-2, may be at a disadvantage for further differentiation. Alternatively, Bcl-2 could also directly affect the progression of meiosis. In addition, the normal interaction between Sertoli cells and spermatogonia, which is known to be important for their growth and differentiation (Hecht, 1995; Russell and Griswold, 1993), may be disrupted in the transgenic mice, and this could lead to abnormal differentiation of spermatogonia. However, Sertoli cells themselves may be normal, because, as discussed below, spermatogenesis recovered in some seminiferous tubules in older mice due to decreased expression of the exogenous Bcl-2 in the germ cells. Moreover, androgen levels were not altered in the transgenic mice, and this suggests that Leydig cells function normally.

A significant propotion of the accumulated germ cells were stained by the TUNEL method and were degenerating (Fig. 4C). This may be why some seminiferous tubules lack most germ cells at 7 weeks of age. The germ cell death may be, at least in part, due to diminishing expression of exogenous Bcl-2 because a significant proportion of TUNEL-positive cells were found in the central region of the tubules where a lot of cells tend to lose the bcl-2 expression (Fig. 4C,G). In addition, defective spermatogonia and/or abnormal interaction between spermatogonia and Sertoli cells could be reasons for their failure to survive. After most of the germ cells were deleted, a few spermatogonia were still found that could have been renewing stem cells (Fig. 5A), and they may have filled the tubules again. However, each germ cell depleted tubule may start to accumulate spermatogonia at different times, i.e. spermatogonia in some tubules may remain resting and others initiate cycling immediately. This may result in the co-existence of filled and empty tubules in older mice.

We found that some seminiferous tubules, in which Bcl-2 positive germ cells were absent, recovered spermatogenesis at 6 months of age (Fig. 5C,D). The expression of the *bcl-2* transgene seemed to fluctuate in spermatogonia, and in the recovering tubules the Bcl-2 expression may be decreased in spermatogonia prior to the expansion, and these cells then could undergo normal apoptosis.

Our results show the consequence of ectopic expression of Bcl-2 in spermatogonia. They provide evidence that the renewal, differentiation and apoptosis of spermatogonia are closely correlated and that a pathway affected by Bcl-2 plays a major role in switching the developmental fates of spermatogonia. Several transgenic mice with sterile phenotypes have been reported (Al-Shawi et al., 1988; Hekman et al., 1988; Iwakura et al., 1988), but none of these showed the same abnormality as that shown here. Our transgenic mice thus provide a unique model for studying the choice between death and survival in spermatogonia.

We thank Dr Yoshihide Tsujimoto for the human *bcl-2* cDNA, Dr Shigekazu Nagata for the *EF-1α* promoter, Dr Brigid Hogan for critical reading and comments on the manuscript, and Dr Satoru Kanto for discussion. T. F. is a Research Fellow of the Japan Society for the Promotion of Science. This work was supported by the Ministry of Education, Science and Culture of Japan and the Princess Takamatsu Cancer Research Fund.

REFERENCES

Allan, D. J., Harmon, B. V. and Kerr, J. F. R. (1987). Cell death in spermatogenesis. In *Perspectives on Mammalian Cell Death* (ed. C. S. Potten), pp. 229-258. London: Oxford University Press.

Al-Shawi, R., Burke, J., Jones, C. T., Simons, J. P. and Bishop, J. O. (1988). A mup promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol. Cell. Biol.* **8**, 4821-4828.

Bakhashi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L. and Korsmeyer, S. J. (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* **41**, 889-906.

Bartke, A. (1995). Editorial: Apoptosis of male germ cells, a generalized or a cell type-specific phenomenon? *Endocrinology* **136**, 3-4.

Billig, H., Furuta, I., Rivier, C., Tapanainen, J., Parvinen, M. and Hsueh, A. J. W. (1995). Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. *Endocrinology* **136**, 5-12.

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

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156-159

Cleary, M. L. and Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. *Proc. Natl. Acad. Sci. USA* **82**, 7439-7443.

Cleary, M. L., Smith, S. D. and Sklar, J. (1986). Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* **47**, 19-28

Farlie, P. G., Dringen, R., Rees, S. M., Kannourakis, G. and Bernard, O. (1995). bcl-2 transgene expression can protect neurons against developmental and induced cell death. *Proc. Natl. Acad. Sci. USA* **92**, 4397-4401.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.

Graninger, W. B., Seto, M., Boutain, B., Goldman, P. and Korsmeyer, S. J. (1987). Expression of bcl-2 and bcl-2-Ig fusion transcripts in normal and neoplastic cells. *J. Clin. Invest.* **80**, 1512-1515.

Hecht, N. B. (1995). The making of a spermatozoon: a molecular perspective. *Dev. Gen.* **16**, 95-103

Hekman, A. C. P., Trapman, J., Mulder, A. H., van Gaalen, J. L. M. and

Zwarthoff, E. C. (1988). Interferon expression in the testes of transgenic mice leads to sterility. *J. Biol. Chem.* **263**, 12151-12155.

Henriksen, K., Hakovirta, H. and Parvinen, M. (1995). Testosterone inhibits and induces apoptosis in rat seminiferous tubules in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethane sulfonate. *Endocrinology* **136**, 3285-3289.

Hikim, A. P. S., Wang, C., Leung, A., and Swerdloff, R. S. (1995). Involvement of apoptosis in adult rats after gonadotropin-releasing hormone antagonist treatment. *Endocrinology* **136**, 2770-2775.

Hockenbery, D. M., Nunez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* (London) **348**, 334-336.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Iwakura, Y., Asano, M., Nishimune, Y. and Kawade, Y. (1988). Male sterility of transgenic mice carrying exogenous mouse interferon-β gene under the control of the metallothionein enhancer-promoter. *EMBO J.* **7**, 3757-3762.

Kangasniemi, M., Wilson, G., Parchuri, N., Huhtaniemi, I. and Meistrich, M. L. (1995). Rapid protection of rat spermatogenic stem cells against procarbazine by treatment with a gonadotropin-releasing hormone antagonist (Nal-Glu) and an antiandrogen (flutamide). *Endocrinology* **136**, 2881-2888.

Knudson, C. M., Tung, K. S. K., Tourtellotte, W. G., Brown, G. A. J. and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270**, 96-99.

Koshimizu, U., Watanabe, D., Sawada, K. and Nishimune, Y. (1993). A novel stage-specific differentiation antigen is expressed on mouse testicular germ cells during early meiotic prophase. *Biol. Reprod.* **49**, 875-884.

Koshimizu, U., Nishioka, H., Watanabe, D., Dohmae, K. and Nishimune, Y. (1995). Characterization of a novel spermatogenic cell antigen specific for early stages of germ cells in mouse testis. *Mol. Reprod. Dev.* **40**, 221-227.

Korsmeyer, S. J. (1992a). Bcl-2: An antidote to programmed cell death. *Cancer Surveys* **15**, 105-118

Korsmeyer, S. J. (1992b). Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* **80**, 879-886.

Krajewski, S., Krajewska, M., Shabik, A., Wang, H.-G. Irie, S., Fong, L. and Reed, J. C. (1994). Immunohistochemical analysis of in vivo patterns of bcl-x expression. *Cancer Res.* **54**, 5501-5507.

Martinou, J.-C., Dubois-Dauphin, M., Staple, J. K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C. and Huarte, J. (1994). Overexpression of bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* **13**, 1017-1030.

McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P. and Korsmeyer, S. J. (1989). bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* **57**, 79-88.

McDonnell, T. J., Nunez, G., Platt, F. M., Hockenbery, D., London, L., McKearn, J. P. and Korsmeyer, S. J. (1990). Deregulated bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. *Mol. Cell. Biol.* **10**, 1901-1907.

Meistrich M. L. and van Beek M. E. A. B. (1993). Spermatogonoial stem cells. In *Cell and Molecular Biology of the Testis* (ed. Desjardins C. and Ewing L. L.), pp. 266-295. Oxford: Oxford University Press.

Mizushima, S. and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. *Nucl. Acids Res.* **18**, 5322.

Nagamine, C. M., Chan, K., Hake, L. E. and Lau, Y.-F. C. (1990). The two candidate testis-determining Y genes (zfy-1 and zfy-2) are differentially expressed in fetal and adult mouse tissues. *Genes Dev.* **4**, 63-74.

Oltvai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619.

Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. and Jacobson, M. D. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695-700.

Reed, J. C. (1994). Bcl-2 and the regulation of programmed cell death. *J. Cell. Biol.* **124**, 1-6

Russell, L. D., Ettlin, R. A., SinhaHikim, A. P. S., Clegg, E. D. (1990). *Histological and Histopathological Evaluation of the Testis.* Clearwater, FL: Cache River Press.

Russell, L. and Griswold, M. D. (1993). *The Sertoli Cell.* Clearwater, FL: Cache River Press.

Schwartzman, R. A. and Cidlowski, J. A. (1993). Apoptosis: the biochemistry and molecular bilology of programmed cell death. *Endocr. Rev.* **14**, 133-151.

Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O. and Korsmeyer, S. J. (1991). bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67**, 879-888.

Seto, M., Jaeger, U., Hockett, R. D., Graninger, W., Bennett, S., Goldman, P. and Korsmeyer, S. J. (1988). Alternative promoters and exons, somatic mutation and deregulation of the bcl-2-Ig fusion gene in lymphomas. *EMBO J.* **7**, 123-131.

Strasser, A., Harris, A. W., Vaux, D. L., Webb, E., Bath, M. L. and Cory, S. (1990). Abnormalities of the immune system induced by dysregulated bcl-2 expression in transgenic mice. *Curr. Top. Microbiol. Immunol.* **166**, 175-181.

Strasser, A., Harris, A. W. and Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67**, 889-899

Tsujimoto, Y. and Croce, C. (1985). Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc. Natl. Acad. Sci. USA.* **83**, 5214-5218.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. and Croce, C. (1985). The t(14;18) chromosome translocation involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* **229**, 1300-1303.

Uetsuki, T., Naito, A., Nagata, S. and Kaziro, Y. (1989). Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1a. *J. Biol. Chem.* **264**, 5791-5798.

Uker, P. S. (1991). Death by suicide: one way to go in mammalian cellular development? *The New Biologist* **3**, 103-109.

Williams, G. T. (1991). Programmed cell death: apoptosis and oncogenesis. *Cell* **65**, 1097-1098.

(Accepted 26 March 1996)