The EMBO Journal Vol.16 No.9 pp.2262–2270, 1997

An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis

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Transgenic mice expressing high levels of the BclxL or Bcl2 proteins in the male germinal cells show a highly abnormal adult spermatogenesis accompanied by sterility. This appears to result from the prevention of an early and massive wave of apoptosis in the testis, which occurs among germinal cells during the first round of spermatogenesis. In contrast, sporadic apoptosis among spermatogonia, which occurs in normal adult testis, is not prevented in adult transgenic mice. The physiological early apoptotic wave in the testis is coincident, in timing and localization, with a temporary high expression of the apoptosis-promoting protein Bax, which disappears at sexual maturity. The critical role played by the intracellular balance, probably hormonally controlled, of the BclxL and Bax proteins (Bcl2 is apparently not expressed in normal mouse testis) in this early apoptotic wave is shown by the occurrence of a comparable testicular syndrome in mice defective in the *bax* gene. The apoptotic wave appears necessary for normal mature spermatogenesis to develop, probably because it maintains a critical cell number ratio between some germinal cell stages and Sertoli cells, whose normal functions and differentiation involve an elaborate network of communication.
Keywords: apoptosis/germinal cells/spermatogenesis/transgenic

Introduction

Mammalian spermatogenesis can be divided into two phases. The first round of spermatogenesis, which starts after birth, is characterized by the sequential appearance within seminiferous tubules of cells corresponding to each stage of germinal cell. The second phase is that of mature spermatogenesis, made up of the subsequent rounds of spermatogenesis which then occur permanently in adult animals, and which is, in contrast, characterized by the concomitant presence of all germinal cell stages, from stem cells and early spermatogonia to fully differentiated spermatozoa.

We report here that abnormal spermatogenesis accompanied by sterility occurs in transgenic mice expressing high levels of the Bclx long (BclxL) or Bcl2 proteins in their testicular germinal cells. This appears to result from the prevention by these apoptosis-inhibitory proteins, of a physiological early apoptotic wave, which occurs among germinal cells during the first round of spermatogenesis and which is necessary for the development of normal mature spermatogenesis. This apoptotic wave is concomitant with a temporary high expression of the apoptosis-promoting Bax protein in germinal cells, which is probably hormonally regulated. That a proper balance between cell death and survival-promoting proteins is critical to achieve this physiological apoptotic wave at an early stage of testicular germinal cell differentiation is substantiated by the finding of a comparable syndrome in mice defective in the *bax* gene (Knudson *et al.*, 1995). This early apoptotic wave of germ cells during establishment of spermatogenesis may be required to maintain a proper cell number ratio between maturing germ cells and Sertoli cells. An intricate relationship involving a constant modulation of activity and exchange of differentiation signals indeed exists between these two cell types (reviewed in Jegou, 1993) from the first stage of spermatogenesis, and its perturbation may extend to the life-long process of mature spermatogenesis.

Results

Male transgenic mice expressing Bcl2 or overexpressing BclxL in their germinal cells are sterile and display marked alterations in the late stages of spermatogenesis

Mice bearing a human *bcl2* or a human *bclx* transgene placed under the control of the promoter of the murine housekeeping phosphoglycerate kinase-1 (*pgk-1*) gene were generated. One male and four female founders bearing a *bcl2* transgene were obtained. The male (T41) was sterile and one of the females (T56) showed a lack of vaginal opening at sexual maturity. The ovaries of this founder female were, however, functional, since, when grafted onto ovariectomized normal female mice, they allowed transgenic progeny to be obtained. All transgenic female mice born from these transplanted ovaries had a closed vagina phenotype, and have been described elsewhere (Martinou *et al.*, 1994; Rodriguez *et al.*, 1997). All transgenic male mice from the T56 line were sterile, and the line was maintained by repeated ovary transplantation. One founder female died and the two others had apparently normal progeny, with fertile males, which did not express the transgene in their testis. One female and three male founder mice bearing the *bclxL* transgene were obtained. Two males were sterile (TX60 and TX85), and the description of their testicular abnormalities is included in the present report.

The sterile *bcl2* T41 transgenic male and males of the T56 line strongly expressed the human Bcl2 protein in their testis, as judged by Western blot analysis of testicular lysates (Figure 1b for T56); this protein was not detectable in lysates from the testes of the fertile males of the two

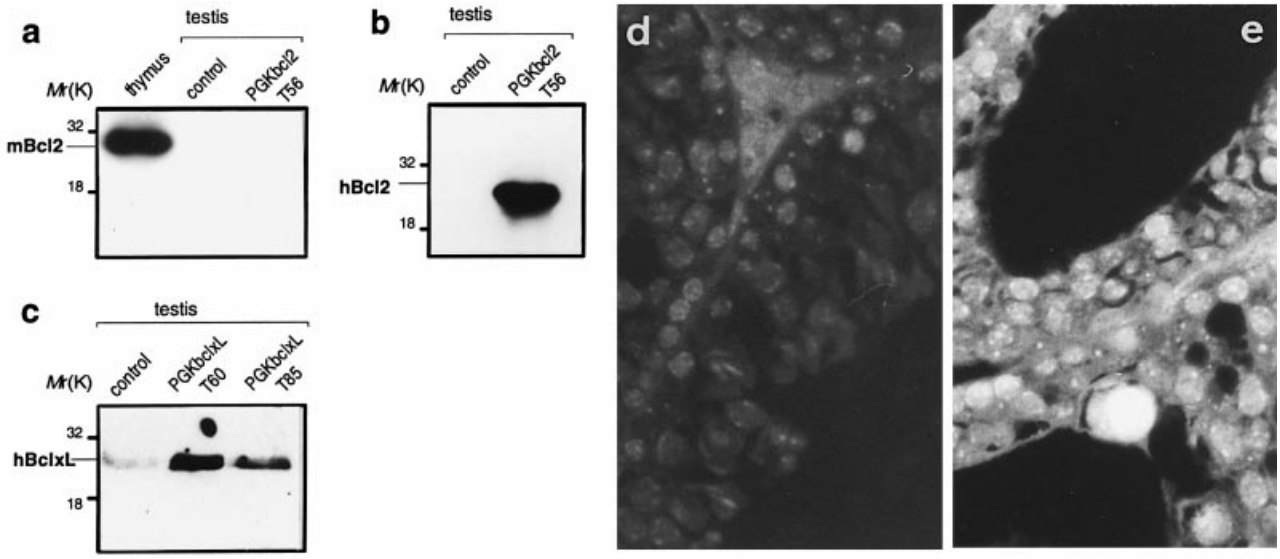

Fig. 1. Expression of the transgenic Bcl2 and BclxL proteins in the testes of transgenic mice. (a–c) Western blot analysis of testicular lysates of 3-month-old mice. (**a**) Murine Bcl2 is not detectable in lysates from control normal or T56 *bcl2* mice; the first lane, used as a control, shows the presence of mouse Bcl2 in the thymus lysate of a normal mouse. (**b**) Human Bcl2 is present in large amounts in the testicular lysate of a T56 mouse, but absent in the lysate of a control mouse. (**c**) BclxL, detected by an antibody which recognizes both mouse and human BclxL, is present in small amounts in the testis lysate of a normal adult mouse and in large amounts in the testis lysates of the two *PGKbclxL* transgenic mice TX60 and TX85. (d and e) Sections of the testis of a normal (**d**) and a transgenic T56 mouse (**e**) stained for the detection of human Bcl2: virtually all cells are stained in the transgenic testis, including a giant cell; strong expression of human Bcl2 was observed at all ages studied, including in 1-week-old mice.

other *bcl2* transgenic lines (not shown). The testis of normal or transgenic mice does not contain detectable mouse Bcl2 (Figure 1a). The human Bcl2 protein was detected by immunofluorescence on frozen sections in all cell types of the seminiferous tubules (Figure 1d and e). The two *bclxL* transgenic sterile males showed, on Western blots of the testicular lysates, a marked overexpression of the BclxL protein, compared with the testis of normal adult mice, in which BclxL is barely detectable (Figure 1c).

The testes of all the sterile adult transgenic males (T41, TX60 and TX85) and of all male progeny of the T56 line were in the normal scrotal position and appeared grossly normal, except that they were reduced in size and weight (~20% for T56 and T41, 50% for TX60 and 20% for TX85) compared with testes of non-transgenic adult mice. The seminiferous ducts were not obstructed. On histologic sections, the seminiferous tubules in all animals showed a range of abnormalities, varying in severity between tubules and between testes (Figure 2b–f), including: (i) the absence or severe depletion of spermatocytes and spermatids, with conserved spermatogonia and normal appearance and distribution of Sertoli and Leydig cells; (ii) complete cellular depletion of the tubules; (iii) the presence of multinucleated giant cells whose number and distribution ranged from sporadic (one giant cell per section of ~40 tubules) to high (more than one giant cell per tubule); the largest giant cells contained dozens of nuclei and their diameter reached one quarter of that of the tubule; and (iv) massively decreased numbers of spermatozoa compared with control animals (from 500-fold less to total absence) at the exit of the epididymis. In the T56 transgenic line, in which testes were examined at various ages, no relationship was found between the severity of the lesions and the age (see for instance in Figure 2b very severe lesions in a 10-week-old mouse, and in Figure 2d milder alterations in an 11-month-old mouse). Of special interest was the observation that the first major histologic abnormalities were observed in the T56 testes only from the age of 5 weeks, which is the time of sexual maturity (see below). Depletion of germ cells of the transgenic adult testes did not appear to result from a markedly decreased number of dividing spermatogonia since, after injection of bromodeoxyuridine (BrdU) to label S phase cells, the number of labeled spermatogonia observed in tissue sections was comparable with that found in normal mice (Figure 2h and i).

Expression of Bcl2 or overexpression of BclxL in male germinal cells make them more resistant to apoptotic-inducing conditions, in vitro and in vivo

Suspension of testicular cells, obtained from adult T56, TX60 or TX85 mice and from control mice of the same age, were cultured *in vitro* for 6 days in the presence of 10% fetal calf serum (FCS). The viability of transgenic cells was markedly increased (Figure 3a–c); the apoptotic nature of cell death in culture was assessed by propidium iodide staining of the nuclei and by internucleosomal DNA fragmentation on agarose gels (not shown). Cell suspensions from transgenic animals probably contained an increased percentage of somatic cells which may have contributed to the cells recovered alive at the end of the culture periods; since this accounted for ~80% of the cultured cells after 5 days, contrasting with the lack of cell survival in the cultures from control mice, it is clear that the transgene expression increased the survival of germ cells.

In vivo, moderate testicular hyperthermia (43°C) is known to inhibit spermatogenesis by inducing cell death in germ cells, the spermatocyte I population being the most sensitive to this form of injury (Allan *et al.*, 1987).

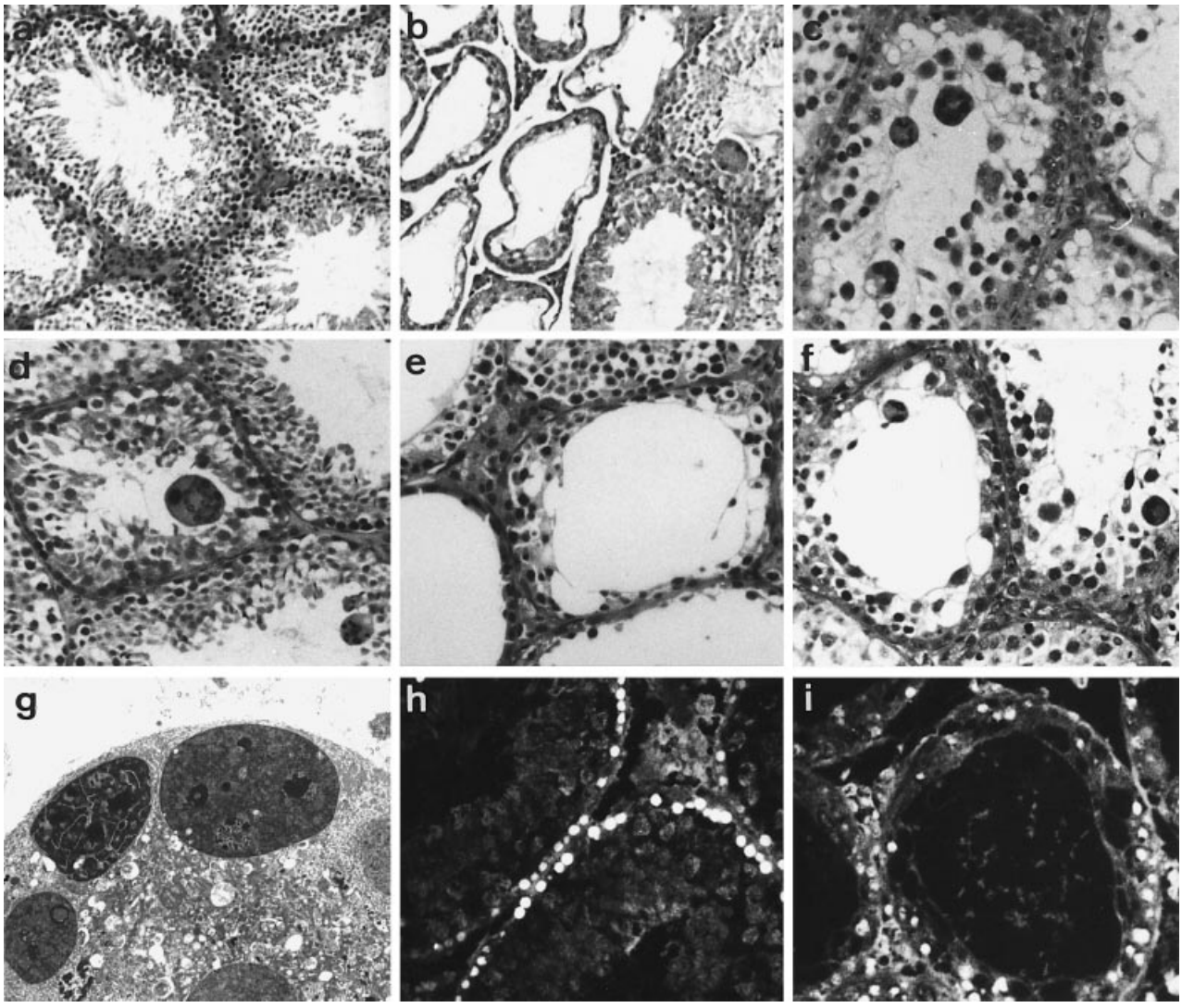

Fig. 2. (a–f) Histologic sections from various testes. (**a**) Ten-week-old normal mouse (100×). (**b**) Ten-week-old transgenic mouse of the T56 line: several seminiferous tubules are almost empty; a giant cell is seen on the right (100×). (**c**) Testis of the *PGKbcl2* T41 founder at 6 months: several giant cells are seen and few or no spermatids (200×). (**d**) Eleven-month-old transgenic mouse of the T56 line: note the presence of multinucleated giant cells in otherwise almost normal appearing tubules. (**e**) Testis of the *PGKbclxL* TX60 founder, removed at 3.5 months: note the severe depletion of the tubules (200×). (**f**) Testis of the *PGKbclxL* TX85 founder removed at 3.5 months: note the partial depletion and presence of giant cells (200×). (**g**) Electron micrograph of the numerous nuclei and dense bodies contained in a giant multinucleated cell. (h and i) BrdU-labeled mice: the stained cells indicate the existence of dividing spermatogonia in amounts not significantly different in control (**h**) and T56 mice (**i**).

The testes of control and transgenic mice of the T56 line were submitted *in vivo* to a hyperthermia of 43°C in a waterbath for 15 min, and 6 h later testis sections were analyzed by the TUNEL labeling technique (Gavrieli *et al.*, 1992) to detect apoptotic cells. In the testis of normal mice, apoptotic spermatocytes were numerous with some apoptotic spermatogonia; in contrast, apoptotic cells were much less frequent in the transgenic testis (Figure 3d and e). This observation confirmed the functional activity of Bcl2 expression in transgenic germ cells, leading to their protection from apoptosis-inducing conditions.

During normal adult spermatogenesis, apoptosis is a sporadic event, occurring mainly among spermatogonia; it is not significantly decreased in the testes of bcl2 or bclxL transgenic mice

Sporadic apoptotic cell death is known to occur in normal adult spermatogenesis, where it has been described to be largely restricted to spermatogonia (Allan *et al.*, 1992). Apoptotic cell death was evaluated by the TUNEL technique on sections of normal or transgenic testes. This confirmed that apoptosis can be observed in normal adult testes and occurs mainly in spermatogonia; unexpectedly, this phenomenon appeared not to be altered in the tubules of *bcl2* or *bclxL* transgenic animals (Figure 4c and d). When spermatogonial apoptosis was evaluated as a percentage of all spermatogonia observed (to take into account differences in cellular content between normal and transgenic tubules), the values observed (in percentage, controls, 0.21 ± 0.09; *bcl2*, 0.26 ± 0.09; *bclxL*, 0.39 ± 0.1; 25 000 cells counted in each case) again provided no evidence for a decrease in adult spermatogonial apoptosis in the transgenic mice. A few TUNEL-labeled spermatocytes were also observed in normal testes and were rarely detected in transgenic testes, but this probably resulted from the low number of these cells in the highly disorganized tubules. These observations indicate that the major

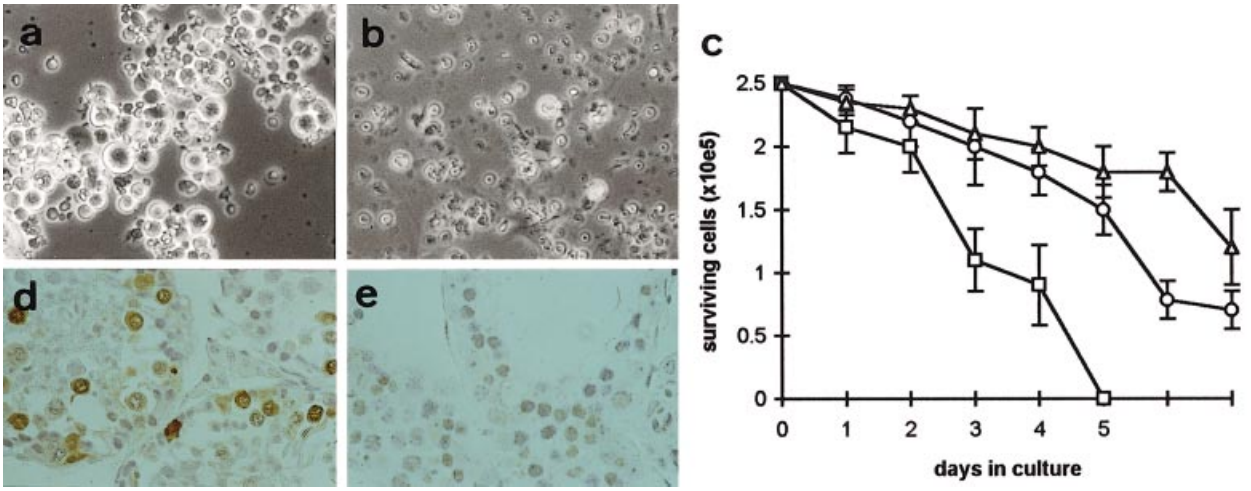

Fig. 3. Testis germinal cells of Bcl2 and BclxL mice are more resistant to apoptotic death *in vitro* (a–c) and to apoptotic-inducing themal injury *in vivo* (43°C for 15 min) (d and e). (**a** and **b**) Microscopic appearance of culture of testicular cells after 4 days: (a) T56 mouse; (b) normal mouse. (**c**) Plot of the survival (trypan blue staining) after 4 days of culture of testicular cells from normal □, T56 *bcl2* ○ and TX60 *bclxL* △ mice. One representative experiment is shown out of three for control and T56 cells. (**d** and **e**) TUNEL staining for the detection of apoptotic cells of sections of thermally injured testis from control (d) or T56 (e) adult mice. The testis of the normal mouse contains numerous apoptotic germ cells (compare with uninjured testis, Figure 4e), with little or no apoptosis detectable in the transgenic testis.

testicular alterations observed in the transgenic mice are not likely to result from the prevention, by the forced expression of a transgenic apoptosis-protecting protein, of a physiological apoptotic process occurring in the testis of sexually mature mice.

The first wave of spermatogenesis, culminating before sexual maturity, is accompanied by an outburst of focal apoptosis among germ cells; this early apoptotic wave, which takes place in cells expressing high amounts of the Bax protein, is markedly prevented in bcl2 transgenic mice

When testis sections of 3-week-old control or T56 mice were compared by the TUNEL technique (Figure 4a and b), the picture was completely different from that of sexually mature mice (compare Figure 4a and b with c and d). Numerous apoptotic cells and bodies were observed in normal mice (Figure 4a). These differences in size obviously represent different phases of the apoptotic process, in which germ cells phagocytosed by Sertoli cells fragment their nuclei while moving toward the basal lamina. Thus, while the precise nature and origin of the apoptotic cells and bodies was difficult to ascertain with precision, and although definite TUNEL-positive spermatocytes and spermatogonia were identified, spermatocyte degeneration may be the major origin of TUNEL-labeled cells and nuclear bodies seen during the first round of spermatogenesis. Transgenic testis, in contrast, showed a strikingly lower number of apoptotic foci (Figure 4b) and contained larger numbers of germ cells.

To explore more precisely the apoptotic phenomena normally occurring during the first wave of spermatogenesis, sections of testes of normal mice obtained every other day between birth and the 6th week were then analyzed by the TUNEL technique. While no apoptosis was observed during the first week, numerous apoptotic nuclei were observed for the 2nd to the 4th week, peaking around the 3rd week (Figure 4e). Staining of adjacent sections with antibodies to the Sertoli cell-specific GATA-1 protein (Yomogida *et al.*, 1994) showed all TUNEL-positive cells to be GATA negative (Figure 4f and g), establishing that this early apoptotic wave is restricted to the germ line.

To explore if this apoptotic wave is associated with modified levels of proteins involved in cell death or survival, Western blot analyses were performed on testicular lysates obtained every week between birth and sexual maturity (Figure 5a). The levels of the p53 and Bax proteins were strikingly elevated from birth until the 4th week, and hardly detectable in adult testis. Among other proteins known to be involved in cell death, Bad (Yang *et al.*, 1995) was more weakly expressed without change at maturity, Bak (Farrow *et al.*, 1995) was not detectable at any time (not shown) and BclxL was also expressed somewhat more in immature than in mature testes, but the difference was not as marked as for p53 and Bax. Bcl2 was not significantly expressed in testes at all ages (data not shown). This suggested that the increased expression of Bax, perhaps in association with that of p53, since the p53 protein may act as a *bax* promoter (Selvakumaran *et al.*, 1994; Miyashita and Reed, 1995), may be instrumental in the early apoptotic wave. By immunohistochemistry on testis sections of 3-week-old normal mice, a faint expression of Bax by germ cells of all stages was observed, being somewhat stronger in spermatogonia, and with very strong expression in focal groups of spermatocytes, with a frequency and distribution of highly expressing cells comparable with that observed for TUNEL-labeled cells at this age. [The specificity of the anti-Bax antibody was assessed by using an irrelevant rabbit polyclonal antibody as first antibody, and also by adding the peptide against which the anti-Bax antibody was raised (Krajewski *et al.*, 1994a) (data not shown)]. Serial sections were then performed to explore the possible

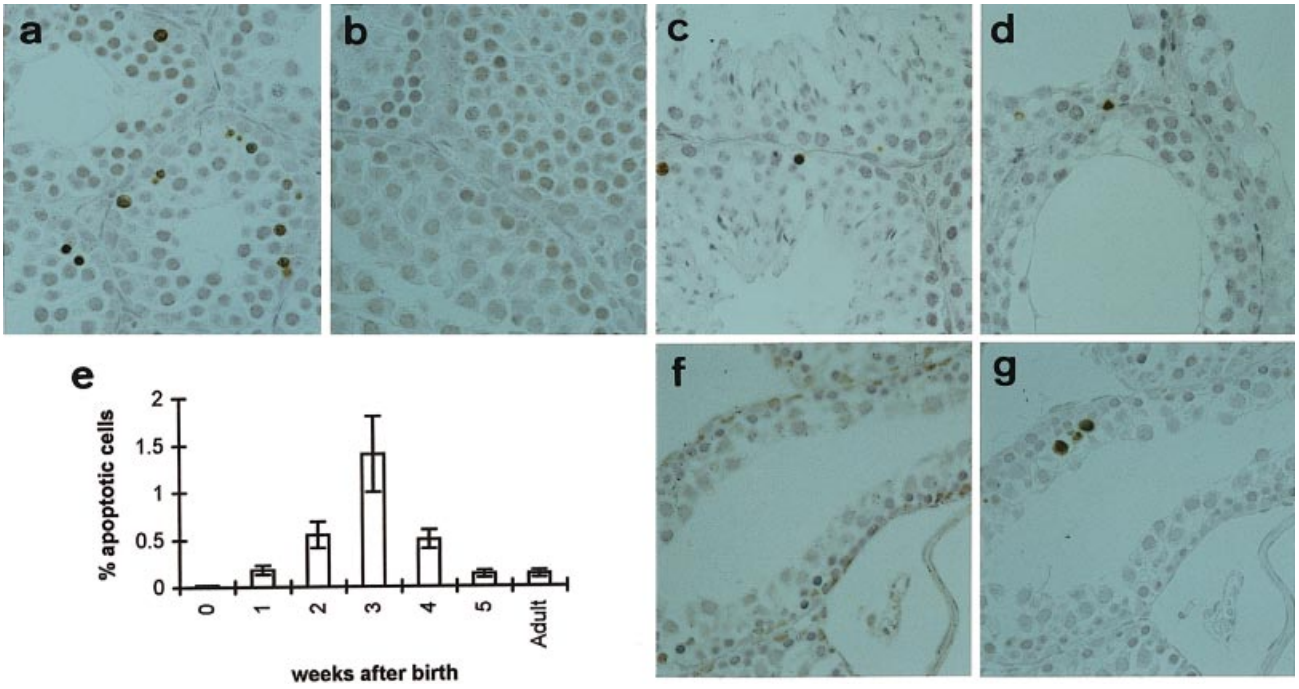

Fig. 4. (a and b) Extent of apoptosis in 3-week-old control (**a**) and T56 (**b**) animals. (c and d) Presence of apoptotic germinal cells detected by TUNEL in testis of adult control (**c**) and T56 (**d**) mice. (**e**) Plot indicating the percentage of TUNEL-positive germinal cells at various intervals after birth in normal mice (80 tubules scored per testis; three mice for each time point). Since TUNEL-positive cells were much rarer in transgenic testis, they were not studied at all comparable time points. At 3 weeks of age, the percentage of TUNEL-positive germ cells in T56 transgenic testis was 0.2 ± 0.06% (three animals analyzed, 80 tubules scored per testis). (f and g) GATA-1 (**f**) and TUNEL (**g**) staining of adjacent sections of the testis of a normal 3-week-old mouse. Sertoli cells (GATA positive) are restricted to the periphery of the tubules and are not TUNEL positive.

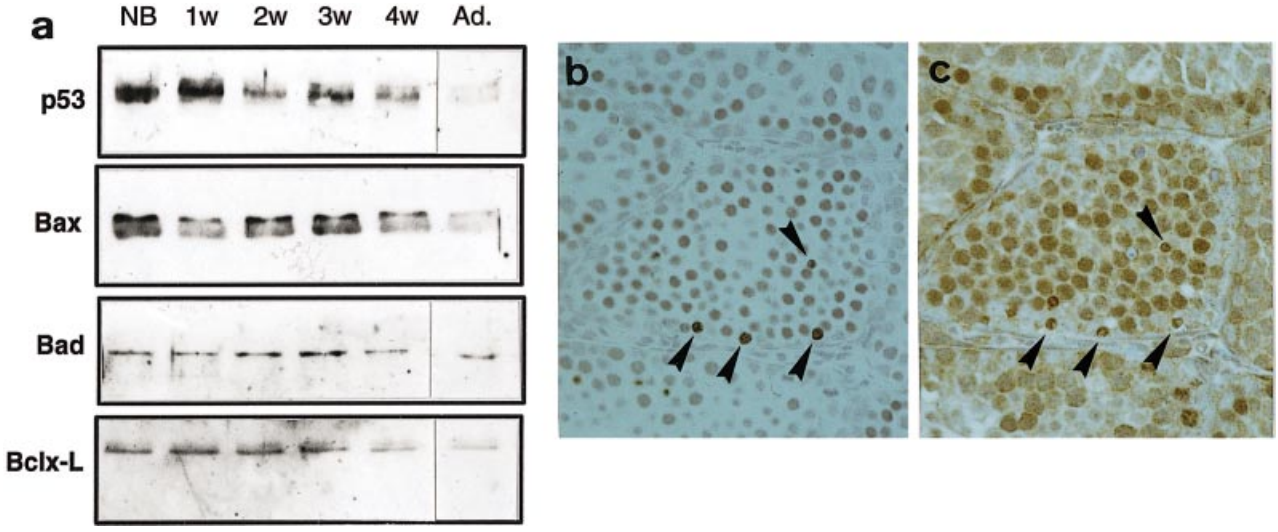

Fig. 5. (**a**) Western blot exploring the expression of proteins involved in cell death or survival during postnatal testicular maturation. Bax and p53 proteins are strongly expressed during the first round of spermatogenesis and almost disappear at sexual maturity. (b and c) Coincidence of strongly Bax-positive cells (**c**) and apoptotic cells (**b**) in adjacent sections of the testis of a 2.5-week-old animal.

coincidence of strongly Bax-positive cells and of apoptotic cells (Figure 5b and c), which showed that TUNEL-positive cells were often, but not always, high Bax expressors. Because of the overall coincidence of the areas with focal apoptosis and focal high Bax expression, it seems likely that the TUNEL-positive cells in which a large amount of Bax was not detected already had cytoplasmic alterations which were too extensive for an accurate detection of the Bax protein. In conclusion, the study of immature testes indicated the existence of an early apoptotic wave, coincident with an overexpression of Bax. Since this form of apoptosis was markedly prevented in immature testes expressing Bcl2, in contrast to the observations made on spermatogonial apoptosis in mature testis, it appears that overexpression of Bcl2 or of BclxL does alter normal mature spermatogenesis by interfering with the first wave of spermatogenesis rather than by acting at a later time.

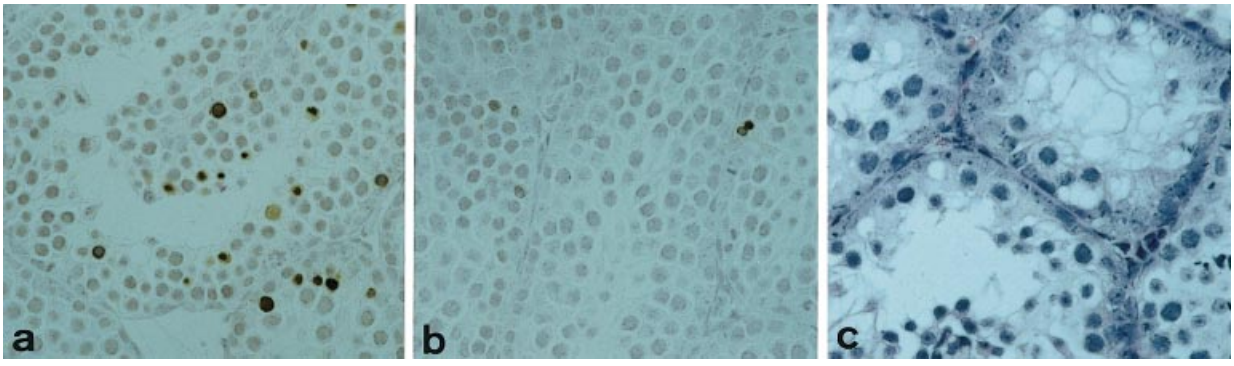

Fig. 6. Prevention of germ cell apoptosis by testosterone during the first round of spermatogenesis. TUNEL staining of the testis of 3-week-old control (**a**) and testosterone-treated (**b**) animals. (**c**) Testicular alterations at puberty after testosterone treatment.

Testosterone injections partially inhibit the early germ cell apoptotic wave and lead to altered spermatogenesis

Recently, it has also been observed in the rat that sexually immature males show an increased level of apoptosis in the testis, appearing 1 week after birth and disappearing at puberty, and primarily affecting spermatocytes (Billig *et al.*, 1995). This phenomenon is under hormonal, in particular gonadotropin, control, since it is considerably increased after hypophysectomy or treatment with a long-acting gonadotropin-releasing hormone (GnRH) antagonist, and largely reversed by injection of follicle-stimulating hormone (FSH), and to a lesser extent by injection of testosterone. To explore whether the early apoptotic wave described here could be modulated by hormonal treatment, perhaps through a change in expression of apoptosis-related proteins, we injected 1-week-old male mice with testosterone every other day for 2 weeks (long-acting FSH was not available to us). This induced a decrease in apoptosis in 3-week-old animals (Figure 6a and b) and some decrease in frequency of cells with an especially high Bax content (although, on Western blots, the high Bax content characteristic of the immature testis was not significantly decreased; not shown). At sexual maturity (6 weeks), the testes of the testosterone-treated mice showed marked tubular abnormalities (Figure 6c) and a very low content of spermatozoa. Although partial, these experiments confirm that the early apoptotic wave observed in the immature testis is under sex hormone control, and that the low rate of apoptosis resulting from a temporary hormonal change is probably responsible for the alterations in spermatogenesis observed at the age of sexual maturity.

Discussion

Several lines of evidence indicate that the syndrome of defective adult spermatogenesis and testicular atrophy observed in the transgenic mice described in the present report results from the overexpression of the apoptosis-protecting BclxL or Bcl2 proteins in germ cells. (i) A damaging effect of the transgene on the host genome can be ruled out since, for both *PGKbcl2* and *PGKbclxL* transgenes, two independent integration sites were involved. (ii) A deleterious use of the transgenic PGK promoter, resulting from competition for transcription factors with the endogenous promoter required for the synthesis of PGK-1, a housekeeping protein, is unlikely because of our observation of comparable but less severe lesions in mice of a *NSEbcl2* transgenic line. These mice, bearing the same human *bcl2* cDNA transgene but placed under the control of another promoter, not widely used in testis, express a lesser amount of Bcl2 in their testes than do *PGKbcl2* mice (Martinou *et al.*, 1994; data not shown). (iii) Expression of the *bcl2* and *bclxL* transgenes in other sites (since their expression was not limited to the testis: Martinou *et al.*, 1994; Rodriguez *et al.*, 1996; I.Rodriguez and P.Vassalli, submitted) may have resulted in a sex hormone imbalance favoring testicular degeneration. This appears unlikely, since the transgenic males had a sexually normal behavior and since the sizes of their prostate and seminal vesicles, which are also under hormonal control, were not significantly different from that of control mice. (iv) Not only were the two transgenic proteins strongly expressed in the testis (and, in the case of Bcl2, its germ line localization was ascertained by immunohistochemistry), but increased resistance of the germ cells to apoptosis-inducing conditions, *in vivo* and *in vitro*, showed that these proteins were functional. (v) Finally, the major, if not the only, disturbance responsible for the defective adult spermatogenesis appears to be the failure of the germ cells to undergo apoptosis at a critical period, which is precisely what can be expected from an overexpression of these proteins.

Although by itself only circumstantial, this last line of evidence becomes more compelling when associated with the observation that the testicular syndrome of these transgenic mice is closely comparable, if not identical, to that found in male mice defective in the *bax* gene (Knudson *et al.*, 1995). The Bcl2 and BclxL proteins are known to dimerize with the Bax protein, and it has been shown that the balance between the expression of these apoptosis-protecting and apoptosis-inducing proteins is critical for cell survival or death (Korsmeyer, 1995). This is consistent with the finding that genetically directed disturbances of this balance in the testis, by transgenic expression of Bcl2 or overexpression of BclxL, or by disruption of the *bax* gene, lead to the same consequences, and suggests that prevention of the physiological process of apoptosis essential to the normal maturation of the testis is at the center of the testicular syndrome observed. This notion receives additional support from the direct relationships (in time and localization) observed *in situ* between a high expression of Bax in the germ cells and their apoptosis (Figure 5b and c). Thus, the salient notion emerging from the various

observations and correlations made in the present work is that of a peculiar early apoptotic wave, occurring in the testicular germ line and which is essential for the development of normal adult spermatogenesis. This leads to the discussion of the following questions: when is this physiologically critical apoptotic wave involving male germ cells taking place? How is it regulated? How can a defect in a temporary process result in permanent impairment of spermatogenesis? What is the function of this apoptotic wave?

Since the most conspicuous testicular lesions occurred only in adults, normal and transgenic adult testes were compared first for the extent of *in situ* apoptosis. It was unexpected to find that, in both cases, this process occurs at about the same levels, being confined mainly to spermatogonia; it was only when transgenic germ cells were submitted to non-physiological apoptosis-inducing conditions, *in vitro* and *in vivo*, that their increased resistance to apoptosis became apparent. Apoptosis is a well-described and common event in the adult rodent testis, where it is restricted mainly to early spermatogonia (Allan *et al.*, 1992). It thus appears that this normal process corresponds to a form of apoptosis which is not sensitive to the presence of Bcl2, BclxL or Bax, since we found this last protein virtually absent in adult testis, as is Bcl2, while the amount of BclxL is low. Since the number of dividing spermatogonia was roughly comparable in normal and depleted adult testis, this suggested that abnormalities of spermatogenesis leading to subsequent severe seminiferous tubule depletion were occurring at an earlier stage, i.e. during the first round of spermatogenesis, which lasts in mice from the second to the fifth week of age; this is the developmental process leading to testicular maturity. Sequential analysis of the first round of spermatogenesis for the presence of apoptotic cells led to the detection of a striking early wave of apoptosis, culminating in the third week and probably affecting spermatocytes more than spermatogonia, often with a focal distribution. If the average disappearance time of an apoptotic nucleus once it has become detectable is ~3–4 h, which is probably an overestimation (Surh and Sprent, 1994), it can be calculated that at least 80% of the germ cells are eliminated during the first round of spermatogenesis. This early apoptotic wave was absent in transgenic mice, the testes of which contained more germ cells than did normal immature testes. The testis contained high levels of the BclxL, Bax and p53 proteins (Bcl2 appears never to be expressed at significant levels in mouse germ cells) which were detectable from birth and decreased abruptly or disappeared after the 4th week. As mentioned above, there was a strong local and temporal coincidence between the level of Bax expression and the occurrence of apoptosis. Apoptosis during the first round of spermatogenesis thus differs from apoptosis observed in mature testis by the following features: (i) it occurs as a single wave; (ii) it is not restricted to spermatogonia and may, on the contrary, affect mainly spermatocytes; and (iii) it is sensitive to, and probably dependent on, variations in the BclxL–Bax balance. It is the disturbance of the first apoptotic wave rather than that of the adult testicular apoptosis which appears to be critical for the normal development and function of the adult testis.

How is this apoptotic wave regulated by external stimuli? It has been observed recently that immature testes in the rat are extremely sensitive to gonadotropin depletion, which markedly enhances apoptosis in germ cells, while the gonadotropins, FSH and, to a lesser extent, testosterone (Billig *et al.*, 1993; Tapainen *et al.*, 1993), exert an opposite effect. Sertoli cells are known to bear high amounts of FSH receptors and to release, under FSH stimulation, a number of germ cell-stimulating products (reviewed in Jegou, 1993). Testosterone, mainly released by Leydig cells, may act at some specific stages of spermatogenesis, where androgen receptors have been detected (Bremner *et al.*, 1994). These observations parallel those made in immature rat ovary, where apoptosis of the granulosa cells of the ovarian follicles is inhibited by gonadotropin treatment; this is accompanied by a decrease in Bax and a maintenance of *bclxL* mRNA levels (Tilly *et al.*, 1995). Thus the early apoptotic wave in the testis may be due to the expression of gonadotropin levels lower than those present in adults, acting in part by insufficient repression of *bax* transcription in germ cells. Our observation that an early cycle of testosterone injection decreases the early apoptotic wave (with only a moderate effect on Bax levels, however) and results in marked alterations of spermatogenesis after sexual maturity (Figure 6c) partially substantiates this possibility. In conclusion, the early apoptotic wave during testis maturation appears to be regulated, as are many other apoptotic processes, by the levels of relevant hormones (and of growth factors released through their action), which remain too low to allow survival of all germ cells.

How could inhibition of a temporary phenomenon, the early wave of testicular apoptosis, have such a long-lasting effect, preventing normal spermatogenesis from being restored? Sertoli cells control germ cell differentiation. Their number does not appear to be modified during the early apoptotic wave, which spares them (Figure 4f), nor in the altered adult transgenic testes, at least in non-empty tubules. However, far from representing a homogeneous cell population subject mainly to external hormonal influences, Sertoli cells display an extremely elaborate network of communication with the germ cells; this network is essential for the regulation of the seminiferous epithelial cycle. For instance, in the normal adult testis, Sertoli cell morphology and function is critically influenced by the presence of late spermatids. It has been proposed that, at each step of spermatogenesis, the Sertoli cell activity is directed by the most advanced generation of germ cells within the seminiferous epithelium (for review, see Jegou, 1993). Since normal spermatogenesis, probably the most complex differentiation pathway permanently at work in the organism, requires the permanent fine tuning of a very elaborate cross-talk between Sertoli and germ cells, it may not be so surprising that disruption of this network, by prevention of the physiological process of germ cell decrease at a critical time during development, has the capacity to impair spermatogenesis permanently.

What is the function of the early apoptotic wave in the testis? The ratio of the different stages of germ cells to Sertoli cells remains relatively constant in mammalian spermatogenesis, and, as just discussed, control of this ratio is probably a critical requirement during testis differentiation. It has been proposed that a supra-optimal production of spermatogonia may, even in adult testes, require

subsequent death of part of these cells to keep an optimal ratio (reviewed in Allan, 1987), and this may be the role of the permanent apoptotic processes in early spermatogonia observed in the adult, whose regulation, however, is different from that of the first apoptotic wave, as discussed above. Maintenance of an adequate ratio between germ cells and Sertoli cells may be such an essential process, during both development and adult life, that it may be the main, if not only, function of the early apoptotic wave. However, germ cells are extremely sensitive to DNA damage, which is especially incompatible with their ultimate function. It may thus be asked whether the early apoptotic wave may not also be triggered in part by DNA alterations, such as would occur in cells unable to achieve correct DNA rearrangements during the chromosomal crossing over of the pachytene phase of the first meiotic division; this would result in early elimination during development of defective germ cells and of their progenitors arising from a common spermatogonium (because of the unique syncytial nature of this system). The p53 protein, whose accumulation is induced by DNA damage and which participates in apoptosis induction, is present in strikingly large amounts in immature testes, and decreases to very low levels in adult germ cells (Figure 5a). This protein is a positive regulator of *bax* gene expression (Selvakumaran *et al.*, 1994; Miyashita *et al*., 1994; Miyashita and Reed, 1995). It has been shown that p53 is expressed mainly in pachytene spermatocytes; however, p53 is also abundant in newborn testis (Figure 5a), well before the occurrence of DNA rearrangements, and, while p53 –/– mice show some testicular abnormalities including occasional giant cells, these alterations appear to be minor and these mice are not sterile. Possible links between DNA alterations and the early apoptotic wave in the testis thus remain at present very uncertain.

Finally, the continuous formation of multinucleated giant cells in the transgenic adult testes probably simply reflects a process of abnormal and incomplete germ cell differentiation favored by the syncytial nature of differentiating germ cells; multinucleated giant cells are observed within seminiferous tubules after a variety of testicular injuries, including moderate hyperthermia and transient ischemia (Allan *et al.*, 1987).

While this report was in the process of being written, a paper appeared (Furuchi *et al.*, 1996) describing comparable abnormalities in the testis of mice bearing a *bcl2* transgene.

Materials and methods

Generation of mice

PGKbcl2 transgenic animals bore a transgene containing a human cDNA containing the complete coding sequence of *bcl2* which was described previously (Martinou *et al.*, 1994). A cDNA encoding human BclxL was excised from pcDNAbclxL by *Xba*I and *Not*I digestion, blunted and inserted into pPGKbcl2, in which the human *bcl2* cDNA had been removed by *Sma*I and *Eco*RV digestion. The resulting construct was called pPGKbclxL. The transgene used for oocyte injections was excised by digesting pPGKbclxL with *Sfi*I, isolated on agarose gel and purified on a NACS column (Gibco BRL, Gaithersburg, MD). Transgenic animals were generated as described (Hogan *et al.*, 1988) using (C57BL/6×DBA2)F1 mice. Founder transgenic mice, identified by Southern blotting, were then mated with C57BL/6 mice. C57BL/6 and (C57BL/6×DBA2)F1 were purchased from IFFA CREDO (Lyon, France).

In vivo hyperthermia

Mice were anesthetized with tribromoethylene and the testes were immersed in a 43°C water bath for 15 min. Testes were removed 6 h later.

In vitro germ cell culture

Testes were homogenized with a hand glass homogenizer and debris were removed by filtration through a nylon membrane. Cells were then washed twice with RPMI medium and plated at a density of 2.5×10^5 cells/ml in RPMI medium containing 10% FCS. Cell viability was assessed by trypan blue exclusion. Triplicate cultures were counted and averaged for each time point.

Histologic examination, immunohistochemistry and TUNEL staining

Tissues were fixed *in vivo* with 4% paraformaldehyde in phosphate-buffered saline (PBS), paraffin-embedded and 5 μm sections were stained with hematoxylin and eosin. For electron microscopy, organs were fixed by *in vivo* perfusion with 2% glutaraldehyde, 1% sucrose in PBS. Immunohistochemistry was performed on 5 μm cryosections of optimal cutting temperature (OCT)-embedded or on 4% paraformaldehyde-fixed paraffin-embedded tissue samples. We used antibodies against human Bcl2 (Dako A/S, Glostrup, Denmark), anti-mouse Bax and anti-mouse GATA-1 (GATA-N6 and anti-Bax, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). BrdU labeling was performed with a Boehringer Mannheim kit following the manufacturer's instructions, 2 h after BrdU injection. For TUNEL staining, testes were fixed in 4% paraformaldehyde and paraffin sections were prepared by standard procedures. A modified protocol of the TUNEL technique (Gavrieli *et al.*, 1992) was used. Sections were deparaffinized in xylol and treated for 15 min with 3 mg/ml proteinase K, followed by 5 min in 2% H_2O_2, and the tailing reaction was then carried out in the TdT buffer [0.5% bovine serum albumin (BSA)] for 1 h at 37°C. Sections were counterstained with hematoxylin and eosin. Counts of TUNEL-positive cells were made on testis sections of three animals of each age analyzed, and 80 tubules of each testis were scored.

Northern and Western blot analysis

Proteins were extracted from minced testis fragments for 15 min at 4°C with RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing aprotinin and phenylmethylsulfonyl fluoride. After centrifugation at 15 000 *g*, the amount of protein in the supernatants was quantitated by the Bradford method. Tissue lysates (30 μg/lane) were submitted to 10% polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes, vacant sites were blocked with 5% non-fat milk in TBS-T [TBS (20 mM Tris–HCl, 500 mM NaCl, pH 7.5) and 0.1% Tween-20] and the washed membranes were incubated for 1 h at 20°C with either a mouse anti-human Bcl2 monoclonal antibody (Bcl2 100, kindly provided by D.Mason, John Radcliffe Hospital, Oxford, UK) at a 1:200 dilution, a hamster anti-mouse Bcl2 monoclonal antibody (3F11, Pharmingen, San Diego, CA) at a 1:1000 dilution or a mouse anti-p53 monoclonal antibody (DO1, Santa Cruz Biotech. Inc.) at a 100 ng/ml concentration. Rabbit anti-mouse Bax (13686E, Pharmingen) was used as described in Krajewski *et al.* (1994a), and rabbit anti-Bclx was produced and used as described in Krajewski *et al.* (1994b). Polyclonal rabbit anti-Bak and anti-Bad antisera were generated by injecting rabbits following standard procedures with the peptides YSEFQTMLQHLQPT and GRELRRMSDE respectively, coupled to keyhole limpet hemocyanin, and used at a 1:1000 dilution. Peroxidase-conjugated $F(ab)_2$ goat anti-hamster IgG (Jackson Immuno-Research Labs Inc., West Grove, PA), goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotech. Inc.) were used for 1 h as secondary antibodies at a concentration of 400–800 ng/ml. Membranes were washed with TBS-T, incubated in enhanced chemiluminescent (ECL) detection reagents (Amersham International, Amersham, Bucks, UK) for 1 min at room temperature, and exposed to X-Omat films (Eastman Kodak Co., Rochester, NY).

Testosterone injections

One-week-old male mice were injected subcutaneously every 2 days with 5 mg of testosterone in ricini oil (Testoviron Depot, Schering AG Pharma, Germany).

Acknowledgements

We thank Mr Christian Vesin and Ms Mireille Redard for technical assistance, Ms J.Ntah for secretarial work, Mr E.Denkinger and Mr

J.-C.Rumbeli for photographic work, and Dr D.Mason for the gift of anti-Bcl2 antibodies. This work was supported by a grant from the Swiss National Foundation (31-37516.93)

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Received on November 7, 1996; revised on December 20, 1996