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Process Which Can Be Prevented by the Overexpression of Bcl2

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In the mouse, opening of the vaginal cavity to the skin is a late event, occurring around the fifth week of life; it can be induced in sexually immature mice by β-estradiol injections. We have generated two lines of transgenic mice expressing the human Bcl2 protein in a variety of tissues. The vaginal cavity of the transgenic females remained permanently closed, a condition completely resistant to β-estradiol injections; this was accompanied by a considerable distension of the genital tract. Histologic studies of vaginal sections at the time of opening to the skin in normal mice showed, by the TUNEL method which detects nuclei with fragmented DNA characteristic of apoptosis, that this event coincides with extensive apoptosis in the lower part of the vaginal mucosa, a process prevented in the bcl2 transgenic mice, which express Bcl2 in suprabasal epithelial cells and in subepithelial cells of the vaginal mucosa. In contrast, two lines of mice bearing a Bcl2 transgene placed under the control of a K10 keratin promoter, whose expression is restricted to the suprabasal layers of the epidermis, had a normal phenotype. Eyelids' formation and opening of the external ear canals, which also occur after birth in the mouse, were not altered in any of these transgenic lines; histological study of eye and ear sections at the time of these events failed to detect apoptosis. In conclusion, the tissue remodeling required to complete maturation of the mouse female genital tract at the time of puberty is an hormonally triggered apoptosis-dependent process.

INTRODUCTION

Apoptosis, or programmed cell death, is probably a very common way to eliminate unnecessary or undesirable cells in the development of higher metazoa. It takes place even in organisms with a limited number of cells; in *Caenorhabditis elegans,* for instance, identification of the genes whose mutations prevent apoptosis and thus normal development has shed considerable light on the molecular basis of this process (Ellis and Horvitz, 1991; Hengartner and Horvitz, 1994; Yuan *et al.,* 1993). A family of proteins regulating programmed cell death has now been identified and is best known in the mouse, with inhibitors of death such as Bcl2 and $Bclx_L$, and promoters of death such as Bax, Bad, Bak and $Bclx_S$ (Oltvai and Korsmeyer, 1994; Farrow *et al.*, 1995; Yang *et al.,* 1995). Proteins directly involved in the execution of programmed cell death appear to belong to a class of cysteine proteases with a specificity of cleavage restricted to aspartic acid residues (Martin and Green, 1995). It has been proposed that programmed cell death represents the normal fate of all cells, from which they are rescued by the existence of survival factors which are represented by signals given to the cells by a large variety of molecules, such as hormones, growth factors, cytokines, and cell membrane receptors involved in intercellular contact or cell–extracellular matrix interaction (Raff, 1992). Programmed cell death appears to represent a physiological way to modulate normal tissue development, since, in contrast to cell damage inflicted to cells by outside agents, which results in cell necrosis, it is characterized by the absence of subsequent inflammatory reaction. Programmed cell death is also characterized by the very rapid elimination of the remnants of apoptotic cells (or ''apoptotic bodies'') through ingestion

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and final degradation by the surrounding surviving cells, without requiring the attraction of professional phagocytic cells such as macrophages (White, 1993).

Embryogenesis is characterized by numerous tissue remodelings, some of which are known to involve cell death (Saunders, 1966), such as interdigital tissue regression, palate fusion, or formation of the central nervous system (Raff *et al.*, 1993; Zakeri *et al.*, 1994; Mori *et al.*, 1994). Prevention of cell death by tissue overexpression of the Bcl2 protein in mice bearing a bcl2 transgene has been taken as an indication that this process results from apoptosis, and used to explore the importance of apoptosis in tissue remodeling before or after birth. This has been applied so far to the lymphoid (Sentman *et al.*, 1991; Strasser *et al.*, 1991) and nervous tissues (Martinou *et al.*, 1994). In the mouse, some definitive tissue remodelings, such as those leading to the opening of the eyes, the ears, and of the vaginal cavity, occur only after birth (Theiler, 1989). We describe here the apoptotic nature of the vaginal opening and its prevention in transgenic mice by the overexpression of the Bcl2 protein. In contrast, we could not find evidence for a comparable local cutaneous apoptotic process at the time of eye or ear openings, which were not altered in these transgenic mice.

MATERIALS AND METHODS

Mice

A pgk–bcl2 construct was generated by insertion of a 900-bp cDNA containing the human bcl2 coding sequence excised with *Eco*RI from pB4 (Tsujimoto and Croce, 1986, gift of Dr. Y. Tsujimoto), into pEMBLpgkSV (gift of Melanie Price, Dept. of Pathology, Geneva) previously digested with *Eco*RI. A DNA fragment containing the mouse phosphoglycerate kinase 1 (PGK-1) promoter followed by the human bcl2 coding sequence, the intron and the polyadenylation signal of the SV40T gene, was excised by digesting the plasmid with *Sfi*I, isolated on agarose gel, and purified on NACS columns (Gibco BRL, Gaithersburg, MD). A K10-bcl2 transgene was generated by inserting the 900-bp hbcl2 fragment used for the pgk–bcl2 transgene into the *Eco*RI site of a plasmid previously generated by inserting a 1182-bp *Bam*HI/*Pvu*II fragment containing an intron and the polyadenylation signal of the rabbit β1-globin gene into a pBluescript SK$^-$ (Stratagene GMBH, Heidelberg) linearized with *Bam*HI and *Xho*I. This plasmid was linearized with *Not*I and blunted, and the K10 bovine promoter, excised from pUCK10 (kind gift of J. L. Jorcano, Madrid) (Bailleul *et al.*, 1990, Blessing *et al.*, 1989) with *Eco*RI and *Bgl*I and then blunted, was inserted. The resulting transgene was excised by *Bss*HII digestion. Transgenic mice were generated as described (Hogan *et al.*, 1986). The T56 PGK–bcl2 transgenic line was maintained by ovarian transplantation into (C57BL/6 × DBA2)F1 mice, whose own ovaries had been partially resected, since vaginal opening failed to occur and since transgenic males are sterile (Rodriguez *et al.*, 1997). The generation of transgenic lines of mice bearing a human bcl2 transgene placed under the control of a rat neuron-specific enolase (NSE) promoter has been described elsewhere (Martinou *et al.*, 1994). All transgenic mice were bred on a C57BL/6 background. Nontransgenic littermate or normal C57BL/6 mice were used as controls. All mice were purchased from IFFA CREDO (Lyon, France).

Histological Examination, in Situ DNA Labeling on Tissue Sections, and Immunohistochemistry

Tissues were fixed *in vivo* with 4% paraformaldehyde in PBS and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. For detection of DNA fragmentation characteristic of apoptotic nuclei, 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 μg/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 hr at 37°C. For immunohistochemistry, 5-μm deparaffinized sections were rehydrated in ethanol and treated with methanol–H_2O_2 5%. Slides were then rinsed in distilled water, plunged in 10 m*M* citrate buffer, pH 6, and heated three times for 5 min in a microwave oven. Slides were washed with distilled water and incubated for 15 min in phosphate-buffered saline with 5% BSA. An anti-human Bcl2 mouse monoclonal antibody (Dako A/S, Glostrup, Denmark) was added (1:20 dilution) for 1 hr at room temperature. After washing, an anti-mouse biotinylated antibody (Dako) was used at a 1:250 dilution for 30 min at room temperature. Biotin was revealed with a streptavidin–peroxidase complex at a 1:100 dilution (Dako).

Estradiol Injections

17β-Estradiol (Sigma Chemical Co. St. Louis, MO) was dissolved in isopropanol. Fifteen micrograms of 17β-estradiol diluted in flax oil was injected intraperitoneally into 12-day-old female mice, and the injections were repeated daily for 5 days.

RESULTS

Vaginal Opening Does Not Occur in Bcl2 Transgenic Mice Showing Expression of the Bcl2 Transgenic Protein in the Vaginal Mucosa

Several mice bearing a pgk–bcl2 transgene (Fig. 1) were born normally and grew for a few weeks without any obvious phenotype. One of these mice exhibited a progressive swelling of the genital region, becoming prominent at about 3 months of age (Fig. 2a); it had no testes and no open vagina. At the opening of the peritoneal cavity, a massively distended female genital tract was observed (Fig. 2b). To ensure progeny to this mouse, its ovaries, whose gross appearance was normal, were transplanted into a normal (C57BL/6 × DBA2)F1 female mouse of the same age, whose own ovaries had been partially resected. All transgenic progeny born from the transplanted ovaries had a comparable phenotype, characterized by lack of vaginal opening in females, and sterility in males (resulting from testicular abnormalities (Rodriguez *et al.*, 1997). Thus, this pgk–bcl2 transgenic line, T56, could be maintained only by ovary transplantation. An identical phenotype was found in another transgenic line (T73 NSE–bcl2) in which the human bcl2 cDNA had been placed under the control of a NSE promoter (Forss *et al.*, 1990), which is, when used as a transgene, mainly expressed in neurons: lack of vaginal opening in females was accompanied by identical gross and histologic appearances of the female genital tract. Other NSE–bcl2 trans-

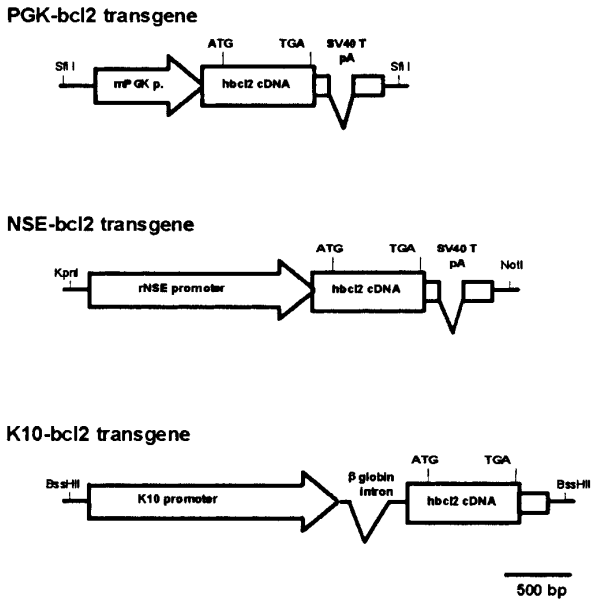

FIG. 1. PGK–bcl2, NSE–bcl2, and K10–bcl2 constructs. A mouse phosphoglycerate kinase 1, a rat NSE, and a bovine K10 promoter were used for the constructs; the complete coding sequence of a human bcl2 cDNA, followed by the intron and the polyadenylation site of the SV40 T gene were placed downstream for the PGK–bcl2 and NSE–bcl2 transgenes. A rabbit β-globin intron and polyadenylation signal were used for the K10–bcl2 transgene.

genic lines lacked this phenotype. Both the T56 pgk–bcl2 and T73 NSE–bcl2 transgenic lines expressed the human Bcl2 protein in the brain, and their brain and neuronal features have been reported elsewhere (Martinou *et al.*, 1994). Analysis of a number of other tissues of the pgk–bcl2 transgenic mice by Northern and Western blots showed that the human bcl2 mRNA and protein were expressed in the liver (Rodriguez *et al.*, 1996), testis (Rodriguez *et al.*, 1997), and at variable levels, sometimes very weak, in the kidneys, gut, spleen, and epidermis; all these tissues, with the exception of the testes and of the brain, appeared grossly and histologically normal, and no particular incidence of lymphomas was observed. Expression of the transgene in embryos was not explored. It should be noted that transgene expression might have been high, leading to lethality, in some embryos bearing a pgk–bcl2 transgene since the ratio of transgenic mice was markedly lower than expected after injection of oocyte with the pgk–bcl2 construct.

Lack of vaginal opening in these transgenic mice persisted until their death, and was accompanied by genital swelling (Fig. 2a) resulting from a strongly distended genital tract (Fig. 2b), probably due to the accumulation of uterine and oviductal secretions and slough vaginal epithelial cells. Since vaginal opening is an hormone-dependent process, a lack of appropriate estrogen expression in ovaries at the time of sexual maturation might result in a closed vagina phenotype. To explore this possibility, β-estradiol was injected for 5 consecutive days into 6 control and 4 transgenic mice, starting on the 12th day of age. This treatment is known to induce precocious female sexual maturation, including vaginal opening on Day 17. While vaginal opening occurred at the expected time in all controls, all transgenic mice were resistant to this treatment (compare Figs. 2c and 2d). Immunohistochemical analysis of sections of these closed (transgenic mice) or open (normal mice) vaginas showed that, in the T73 NSE–bcl2 transgenic mice, although the NSE promoter is considered to be neuron-specific, the human Bcl2 protein was detectable within cells of the vaginal epithelium (to the exception of the basal layers) and also within some submucosal cells, probably, at least in part, fibroblasts (Fig. 2e). The localization of expression of the transgene in the T56 pgk–bcl2 animals was very similar and the expression was also poor in the basal layers of the vaginal epithelium (data not shown). No histological difference between the vaginas of control and pgk–bcl2 or NSE–bcl2 transgenic animals could be detected in immature animals not treated with hormones, thus excluding a possible effect of the transgene on the rate or the duration of proliferation of the cells during vaginal formation. The other lines generated with the two transgenes and which had normal vaginal opening did not express human Bcl2 in the vaginal mucosa. Specificity of the staining for human Bcl2 was ascertained by its absence in the vagina of normal mice (Fig. 2f). Attempts at detecting endogenous mouse Bcl2 protein around the time of vaginal opening did not show any conclusive staining in transgenic nor in normal mice.

Ear and eye openings occurred at the same time in transgenic mice and their negative littermates, i.e., within 3 days after birth for ears and on Days 12 to 14 for eye openings. Staining for human Bcl2 showed only a faint expression in skins of the T56 pgk–bcl2 transgenic mice in these locations and no detectable expression in the T72 NSE–bcl2 line (not shown).

Finally, two K10–bcl2 transgenic lines were also obtained. The keratin K10 promoter is active only in the suprabasal layer of the epidermis (Bailleul *et al.*, 1990). Expression of the transgene in the skin of these two lines was detected by RT–PCR. These lines had a normal phenotype, and vaginal opening was not delayed in comparison with nontransgenic female littermates. In order to evaluate the functionality of the transgene, one of these lines was used for crossing with a K10-myc transgenic line. Double transgenic mice, expressing both Myc and Bcl2 in the epidermal suprabasal layers, developed permanent skin papillomas after repeated topical exposure to phorbol myristic acetate, in contrast to single transgenic K10-myc mice (I.R. and P.V., unpublished experiments); this showed that expression of the K10–bcl2 transgene was effective. However, immunohistochemistry and RT–PCR analysis being insufficiently quantitative, it was not possible to compare in a precise manner the levels of Bcl2 epidermal expression between the pgk–bcl2 mice with a lack of vaginal opening and the K10–bcl2 mice with a normal phenotype (see discussion).

Detection of Local Tissue Apoptosis at the Time of Vaginal Opening

To evaluate whether vaginal opening was the result of cell death by apoptosis in the septum covering the lower

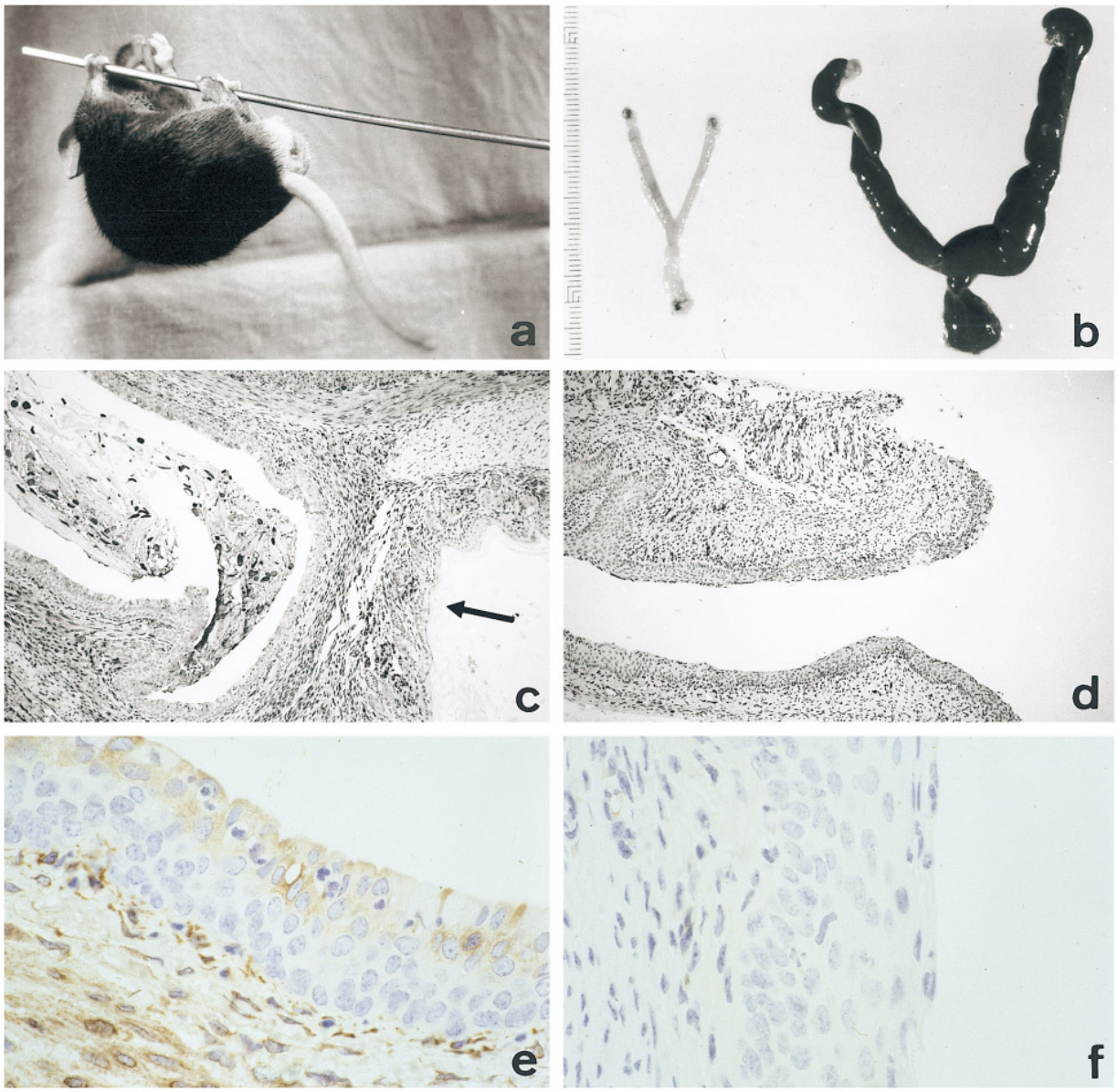

FIG. 2. (a) A 3-month-old pgk–bcl2 transgenic female mouse. Note the swelling of the genital region. (b) Female genital tract of a control mouse (left) and of a pgk–bcl2 transgenic female mouse, at 3.5 months of age. (c, d) Histologic sections of the lower extremity of the vagina in a 17-day-old T72 NSE–Bcl2 transgenic (c) and control (d) mouse, respectively, 5 days after 17β-estradiol injection. Opening of the vagina occurred in the control mouse (d) and failed to occur in the transgenic mouse (c), in which the arrow points to the region of the expected vaginal aperture. (e) Expression of the human Bcl2 protein in the transgenic T73 NSE–bcl2 female genital tract of a 17-day-old mouse treated with 17β-estradiol (see c), as detected by immunohistochemistry (brown color). Bcl2 is detected within the upper layers of the vaginal epidermis and in submucosal cells, probably fibroblasts. The localization of expression of the human Bcl2 protein is comparable in estradiol-treated and nontreated transgenic animals. (f) Control immunohistochemical staining for human Bcl2 in the genital tract with vaginal opening of a 17-day-old normal mouse treated with β-estradiol (see d). No specific staining is detected.

extremity of the vagina, serial sections of the genital tracts were performed on 4.5- to 7-week-old normal mice, and examined by the TUNEL method, which detects the high level of nuclear DNA fragmentation characteristic of apoptotic nuclei. No or very rare apoptotic cells were detected before vaginal opening. In contrast, large number of labeled cells were observed in the regressing tissue at the very time of vaginal opening, as shown in Fig. 3b in which an ''apoptotic plug,'' consisting in an aggregate of hundreds of apoptotic cells, is seen. Localization of these apoptotic cells was

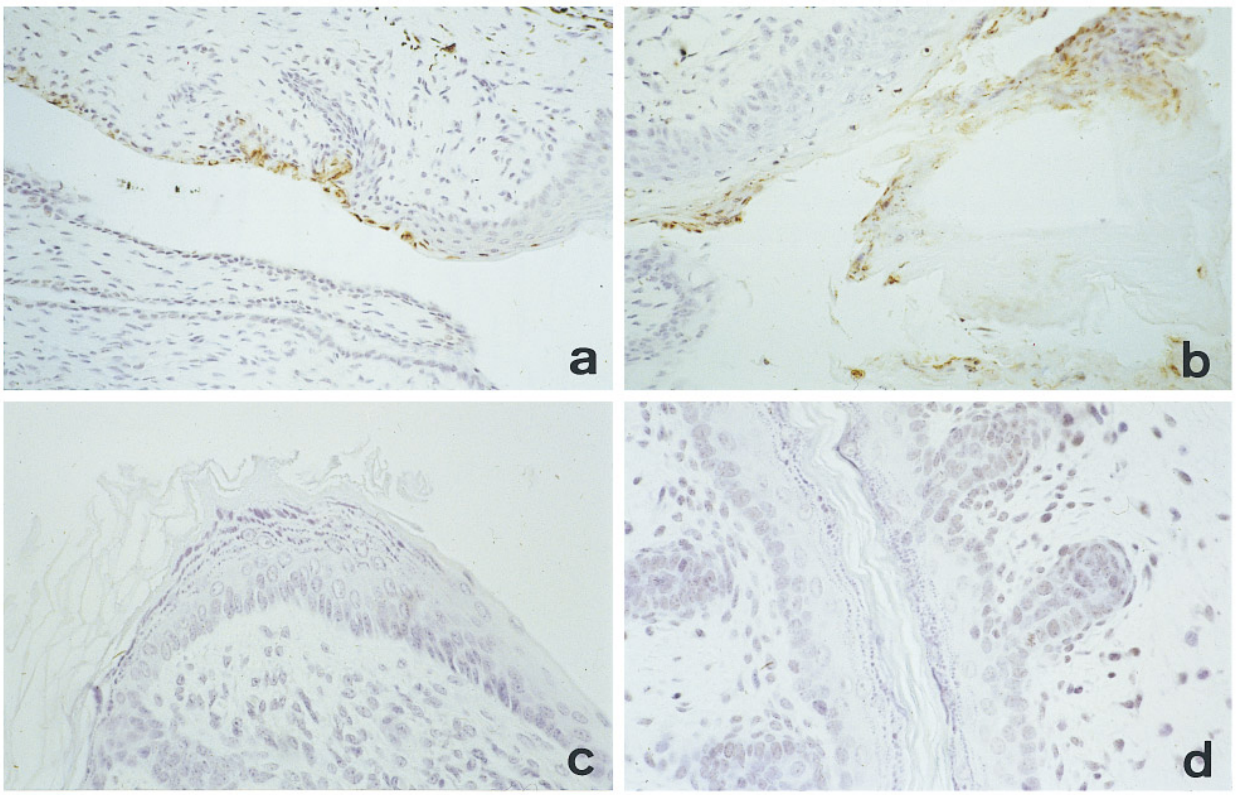

FIG. 3. Histologic sections of various tissues stained with the TUNEL method. Nuclei displaying DNA fragmentation are stained in brown. (a) Apoptotic cells 3 hr after vaginal opening in a 5-week-old mouse (200×). For a and b the inside of the genital tract is on the left. (b) Apoptotic ''plug'' during the vaginal opening of a 5-week-old female mouse (400×). (c) Histologic section at the level of lids formation in a 2-week-old C57BL/6 male mouse (800×). No labeled cells can be found. The picture represents the lower lid at the time of its separation with the upper lid. (d) Histologic section taken at the time of the opening of the external auditive canal in a 2-day-old C57BL/6 male mouse (800×). No labeled cells can be found.

topographically precisely limited to the area of vaginal opening; except for areas where the extent of apoptosis made identification of the origin of the apoptotic cells impossible, apoptosis of epithelial cells could be clearly identified, although it was probably not restricted to epithelium. This apoptotic process was not only massive but also restricted in time, since we were not able to observe it at its very beginning, i.e., with only sparse apoptotic cells, and since a few hours after vaginal opening only few cells labeled by TUNEL were detected (Fig. 3a), which had completely disappeared on the following day.

Serial sections of the genital tracts were also performed on 4.5- to 7-week-old pgk–bcl2 transgenic animals; no or very rare apoptotic cells could be observed in the sections, thus demonstrating the effect of Bcl2 expression on apoptosis.

Serial sections of the ears and eyes were performed at the time of ear septum and eyelids openings, respectively. In contrast to what was observed for the vaginal tract, no significant presence of cells labeled by the TUNEL technique was detected. (Figs. 3c and 3d).

DISCUSSION

The detection, at the time of vaginal opening, of extensive apoptosis in the lower end of the vagina and in the septum closing the vaginal cavity, combined with the failure of two distinct bcl2 transgenic mouse lines expressing the transgene in this very location to display vaginal opening, even after hormonal induction, provides compelling evidence that this postnatal tissue remodeling event rests upon an hormonally triggered process of apoptosis. This process appears to be very restricted not only in its site, but also in its duration, and has probably virtually ceased a few hours after vaginal opening. It affects mostly the suprabasal layers of the vaginal epithelium, but also some submucosal cells, probably fibroblasts. Prevention of vaginal opening in the pgk–bcl2 transgenic mice might result from the expression of Bcl2 in both subepithelial and epithelial cells (Fig. 2e). The K10–bcl2 transgenic mice, in which use of the keratin K10 promoter restricted transgene expression to the suprabasal layers of the epidermis, were phenotypically normal. Thus, it appears that the initiating apoptotic events in

vaginal opening may not be those involving the epithelium, or at least are not restricted to the epithelium. However, since a precise comparison of Bcl2 expression levels in suprabasal epithelial cells between the different bcl2 transgenic lines was not possible, the exact place of epithelial cell apoptosis in the chain of events leading to vaginal opening remains to be determined.

It should be emphasized that there is no established relationship between the level of expression of a bcl2 transgene in a given tissue and its effect on the development of this tissue or even its resistance to apoptosis. Thus, in the pgk–bcl2 mice, the highest expression of Bcl2 was observed in the brain and the testes (which both showed abnormal development: Martinou *et al.*, 1994, Rodriguez *et al.*, 1997), and in the liver (which is structurally normal: Rodriguez *et al.*, 1996), while Bcl2 expression at the vaginal area of opening was apparently weaker, as judged by immunohistochemistry. Furthermore, we have observed that in some tissues considered to undergo permanent and extensive apoptosis in relation with cell renewal, such as the small intestine, even a very high expression of transgenic Bcl2 in enterocytes (obtained in mice bearing a bcl2 transgene placed under the control of the intestinal fatty acid-binding protein promoter: Sweetser *et al.*, 1988) does not lead to tissue changes, nor protects enterocytes *in vivo* or *in vitro* against apoptotic stimuli (I.R. and P.V., unpublished observations). It can be concluded that if a developmental event shows both involvement of apoptosis and prevention by forced expression of Bcl2, even weakly, this association shows conclusively that this event is mediated by an apoptotic process: this is the case for vaginal opening in mouse; on the other hand, failure to modify a development process by an apparently high forced expression of Bcl2 does not rule out a critical role for apoptosis in this process.

Two other forms of tissue remodeling resulting from a change in sexual hormones levels and accompanied by a local process of apoptosis may occur in adult life: prostate involution after castration (Barnejee *et al.*, 1995), a nonphysiologic process, and involution of the mammary gland after the lactating period (Strange *et al.*, 1992). In these instances, however, whether the forced overexpression of a ''survival'' gene, such as bcl2, which can be only obtained by a transgene whose local expression is independent from hormonal control, prevents these tissue changes has not been explored. In the transgenic mice used in the present experiments, the effect of possible overexpression of the transgene in lactating mammary glands could not be explored, since pregnancy was impossible.

The intracellular mechanisms leading to the local process of apoptosis of the vaginal mucosa remain to be determined. It is unlikely that they involve a sudden drop in Bcl2 level, since local expression of endogenous Bcl2 appears to be very weak in this tissue, and since vaginal opening occurs normally in bcl2 −/− mutant mice (Veis *et al.*, 1993). An increased expression of the Bax protein, a molecule present in many cell types and whose overexpression may promote cell death (Oltvai *et al.*, 1995), is also unlikely to be involved, since bax −/− mutant mice have no defect in vaginal opening (Knudson *et al.*, 1995). A drop in the BclxL protein, whose antiapoptotic property is very comparable to that of Bcl2 but is more indispensable in fetal life, since bclx −/− embryos die *in utero* (Motoyama *et al.*, 1995), or an increased expression of the Bclxs protein, remain distinct possibilities as a mechanism leading to this localized apoptosis. Detection of local variations at the time of vaginal opening in the mRNA and protein levels of BclxL or Bclxs and also of Bak, Bax, and Bad, all involved in the control of programmed cell death, is presently under study. It must be indeed realized that the intracellular levels of at least some of these apoptosis-related proteins has been shown in some conditions to be regulated by external signals received by the cells, among which sexual hormones (Tilly *et al.*, 1995).

Two other remodeling events involving mucosae also occur after birth in the mouse, i.e., opening of the ear conducts and formation of the palpebral slits. They were not altered in these bcl2 transgenic mice, but this may have resulted from insufficient expression of the transgene in these locations. However, the failure to detect any evidence of apoptosis by the TUNEL technique on tissue sections obtained from the ear and eye regions at the time of occurrence of epidermis remodeling suggests that massive cell death is not involved in these events, or that cell elimination occurs through mechanisms distinct from these identified under the name of apoptosis.

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