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Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis

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SUMMARY

Ductal morphogenesis in the rodent mammary gland is characterized by the rapid penetration of the stromal fat pad by the highly proliferative terminal endbud and subsequent formation of an arborized pattern of ducts. The role of apoptosis in ductal morphogenesis of the murine mammary gland and its potential regulatory mechanisms was investigated in this study. Significant apoptosis was observed in the body cells of the terminal endbud during the early stage of mammary ductal development. Apoptosis occurred predominately in defined zones of the terminal endbud; 14.5% of the cells within three cell layers of the lumen were undergoing apoptosis compared to 7.9% outside this boundary. Interestingly, DNA synthesis in the terminal endbud demonstrated a reciprocal pattern; 21.1% outside three cell layers and 13.8% within. Apoptosis was very low in the highly proliferative cap cell layer and in regions of active proliferation within the terminal endbud. In comparison to other stages of murine mammary gland development, the terminal endbud possesses the highest level of programmed cell death observed to date. These data suggest that apoptosis is an important mechanism in ductal morphogenesis. In *p53*-deficient mice, the level of apoptosis was reduced, but did not manifest a detectable change in ductal morphology, suggesting that p53-dependent apoptosis is not primarily involved in formation of the duct. Immunohistochemical examination of the expression of the apoptotic checkpoint proteins, Bcl-x, Bax and Bcl-2, demonstrated that they are expressed in the terminal endbud. Bcl-x and Bcl-2 expression is highest in the body cells and lowest in the non-apoptotic cap cells, implying that their expression is associated with increased apoptotic potential. Bax expression was distributed throughout the terminal endbud independent of the observed pattern of apoptosis. A functional role for Bcl-2 family members in regulating endbud apoptosis was demonstrated by the significantly reduced level of apoptosis observed in WAP-Bcl-2 transgenic mice. The pattern of apoptosis and ductal structure of endbuds in these mice was also disrupted. These data demonstrate that p53-independent apoptosis may play a critical role in the early development of the mammary gland.

Key words: apoptosis, p53, mammary gland, DNA synthesis, Bcl-2, Bcl-x, Bax, terminal endbud, WAP-Bcl-2, mouse

INTRODUCTION

Dramatic changes in the morphological organization and molecular expression patterns of the epithelial and stromal cells characterize the ductal and lobular-alveolar stages of postnatal development in the murine mammary gland (MMG). Ductal development is initiated in the virgin animal with the onset of puberty and decreases with the attainment of sexual maturity (Daniel and Silberstein, 1985; Knight and Peaker, 1982). The ductal pattern is created by the penetration of a highly proliferative and dynamic structure, the terminal endbud (TEB), through the stromal fat pad. The pattern of ducts generated during this stage of MMG development is a consequence of the restriction and organization of the proliferation of the TEB by systemic steroid hormones, locally acting growth factors and interaction with the stroma (Coleman et al., 1988; Topper and Freeman, 1980; Vonderhaar, 1987). Studies of the morphological characteristics and the dynamics of DNA synthesis of this early stage of ductal development have revealed little information about the molecular mechanisms that regulate the proliferation of the TEB epithelial cells and the formation of the ductal lumen.

The developing virgin rodent mammary gland contains several cell types that have been characterized by their morphology, differentiated function, ultrastructure (Williams and Daniel, 1983) and expression of cell surface markers such as cadherins (Daniel et al., 1995) and cytokeratins (Dulbecco et al., 1983). The TEB contains two histologically distinct cell types: the body cells and the cap cells. The cap cells are the outermost layer of the TEB and interact through a thin basal lamina with the surrounding stroma. Cap cells are thought to be the progenitors for the myoepithelial cells, which are characterized by their expression of myosin (Dulbecco et al., 1982, 1983; Williams and Daniel, 1983). The interior of the teardrop-

shaped TEB is filled with 6-10 layers of body cells. Body cells are characterized by the expression of specific cytokeratins (Dulbecco et al., 1986) and E-cadherins (Daniel et al., 1995). Proximal to the TEB is the neck region, which contains several layers of body cells and acts as the transition zone between the TEB and the subtending duct. Adjacent to the neck region is a simple duct with one to three layers of ductal epithelial cells surrounded by a single layer of attenuated myoepithelial cells. How the solid primordium of body cells in the TEB are organized to form the subtending duct and lumen has yet to be determined.

Cell death has been postulated to be an active participant in development for many years (Glucksmann, 1951; Saunders, 1966), but has only recently been demonstrated to be a common event in the development of several organs (Barres et al., 1992; Coles et al., 1993; Coucouvanis and Martin, 1995). Apoptosis is an active process of programmed cell death (PCD) with specific regulators and triggers. One of these regulatory molecules is p53. p53 functions as a guardian of the genome to prevent propagation of mutations to daughter cells. DNA damage induces p53 expression causing cell cycle arrest to effect either DNA repair or induce apoptosis. p53 expression is also associated with a change in the temporal appearance of apoptosis (Colombel et al., 1995). In addition, the members of the Bcl-2 family of proteins, Bcl-2, Bax, Bcl-x_S, Bcl-x_L, act together as a rheostat to regulate a cell's apoptotic potential. Bcl-x_S and Bcl-x_L are mRNA splice variants of *Bcl-x* correlated with induction or inhibition of apoptosis, respectively. Importantly, the relative levels of each protein isoform in a particular cell may dictate the progression to apoptosis (Chao et al., 1995; Middleton et al., 1996). However, previous analysis of a wide variety of tissues indicates that Bcl-x_S is only rarely present or present at relatively low levels compared to Bcl-x_L. The heterodimeric complexes that Bax forms with Bcl-2 regulate the apoptotic potential of various cells (Sedlak et al., 1995). It has been demonstrated that the absolute level of Bax is not the critical factor in the determination of apoptotic potential, but rather the ratio between Bax and Bcl-2 and the cell type in which they are expressed (Knudson et al., 1995).

In the mammary gland, apoptosis has been primarily examined during involution after lactation (Li et al., 1996b; Quarrie et al., 1995; Strange et al., 1992). A basic problem in understanding the development of branching organs like the mammary gland is dissecting the mechanisms that regulate the formation of the lumen of tubes and ducts from a solid primordium. Recently apoptosis has been implicated in the formation of the proamniotic cavity during rodent gastrulation (Coucouvanis and Martin, 1995). During the process of cavitation, apoptosis converts the solid primordium of the embryonic ectoderm into a hollow egg cylinder. While examining the effects of estrogen and progesterone on proliferation and apoptosis in the MMG, a significant apoptotic pattern was detected in the TEB of the MMG, prompting a more detailed study of the role of apoptosis in ductal development in the MMG.

This study demonstrates that significant apoptosis occurs in the body cells of the TEB. Apoptosis is most prevalent proximal to the lumen of the TEB and occurs in apoptosis-dense zones. This spatial restriction of apoptosis suggests a possible role for apoptosis in the maintenance of TEB structure and formation of the duct in the developing MMG. Examination of the apoptotic pattern of mammary glands from p53 knockout mice ($p53^{-/-}$) revealed that the level of apoptosis is reduced but that normal development of the TEBs and ducts, in these animals, is not detectably disrupted. Members of the apoptotic checkpoint pathway, Bax, Bcl-2 and Bcl-x, are distributed throughout the developing TEB and duct. The exclusion of Bcl-x and Bcl-2 from the cap cell layer and localization of Bcl-2 expression proximal to the lumen of the TEB implies Bcl-x and Bcl-2 may be associated with regulation of apoptotic potential in the body cells of the TEB. In support of this hypothesis, whey acidic protein (WAP)-Bcl-2 transgenic mice had reduced apoptosis and an altered ductal structure in the body cells, and inappropriately increased apoptosis in the neck of the TEB.

MATERIALS AND METHODS

Materials

Terminal deoxytidyl transferase (TdT) was obtained from Pharmacia (Milwaukee, WI). 16-dUTP-biotin and proteinase K were obtained from Boehringer Mannheim (Germany). Probe On Plus slides and Omniset tissue cassettes were from Fisher Scientific (Pittsburgh, PA). ABC reagent, goat serum and diaminobenzidine (DAB) were obtained from Vector Labs (Burlingame, CA). The cell proliferation kit for analysis of bromodeoxyuridine (BrdU) labeling was obtained from Amersham (Buckinghamshire, England). Mice were acquired from Charles River Labs (Wilmington, MA) or from a breeding colony at Baylor College of Medicine courtesy of Dr Daniel Medina. Aqueous mounting media and hematoxylin were obtained from Biomeda (Foster City, CA). All other chemicals were obtained from Sigma Chemical Company (St Louis, MO).

WAP-Bcl-2 mice

The WAP-Bcl-2 mice carry a transgene that consists of the human *Bcl-2* gene cDNA under the control of a 1.6 kb *Bgl*II/*Kpn*I fragment of the mouse WAP promoter. The mice have been bred into DBA/2 background. Details on the construction of the transgene and the complete phenotype of these mice is being published elsewhere (Jæger, R., Herzer, U., Schenkel, J. and Weiher, H., unpublished data). Mice used for this study were backcrossed twice into an FVB background.

Isolation of mammary glands

Age-matched 5- to 6-week-old virgin Balb/c mice (n=6) were injected with 20 μl of BrdU/gm body weight 2 hours before killing. The 4th (inguinal) and 3rd (thoracic) mammary glands were surgically removed, spread in Omniset tissue cassettes and immediately placed in Tellyesniczky's fixative for 5 hours. Glands were stored in 70% ethanol then paraffin embedded and sectioned or stained with hematoxylin for whole-mount analysis. Sections (5 μm) were mounted on Probe On Plus slides.

Whole-mount staining

The whole-gland staining was carried out essentially as described (Williams and Daniel, 1983) except that glands were stained for only 2 hours in hematoxylin.

TdT dUTP nick end labeling (TUNEL) analysis

TUNEL was performed as described (Gavrieli et al., 1992) with the following modifications. Proteinase K digestion was carried out at 2 μg/ml for 10 minutes at 24°C. Tissue sections were labeled with 1 unit of TdT and 1 nmol of 16-dUTP-biotin in 150 μl of TdT buffer. Labeled sections were incubated with ABC reagent following a standard manufacturer's protocol and then incubated with

diaminobenzidine-nickel substrate for 7-8 minutes. Tissue sections were counterstained with 0.1% (w/v) methyl green for one minute. Sections were then rinsed in distilled water, dehydrated through graded alcohols and xylene and mounted with Permount according to standard protocols.

Quantitation of apoptosis and cell proliferation

Images from TUNEL and BrdU analyzed TEBs were captured with a Sony 3CCD color video camera attached to a BX-50 Olympus microscope with Adobe Photoshop software. A manually drawn line on captured images defined a boundary 3 cell layers from the lumen. The percentage of apoptotic and BrdU-labeled cells was quantitated both inside (proximal) and outside (distal) this boundary. 30 TEBs from six mice were counted for apoptosis analysis representing a total of 10983 cells. 8 TEBs from three mice were counted for BrdU quantitation representing 3612 cells. 62 TEBs from eight mice were counted in the Bcl-2 analysis representing 18681 cells. 25 TEBs from eight mice were counted in the p53 analysis representing 4548 cells. Clusters of TUNEL-labeled cells that occurred within a single cell diameter were considered to be fragments of a central cell and counted as a single apoptotic cell.

Antibodies

The preparation and characterization of anti-peptide rabbit polyclonal antisera specific for the mouse Bcl-2, Bax and Bcl-x proteins have been described in detail previously (Krajewski et al., 1994a,b; Miyashita et al., 1994).

Analysis of $p53^{-/-}$ and WAP-Bcl-2 mice

The fourth (inguinal) and third (thoracic) mammary glands from $p53^{-/-}$ (n=5), $p53^{+/+}$ (n=3) (Donehower et al., 1992) and WAP-Bcl-2 (n=3), WAP-Bcl-$2^{+/+}$ mice (n=5) were collected and stained as whole mounts, as described, and then sectioned. Twelve glands from p53 genotypes and three glands from WAP-Bcl-2 genotypes were used to characterize the changes in ductal morphogenesis. The tissue sections were examined visually for changes in the number and size of ducts, the number of secondary branches, the extent of fat pad penetration and the size and number of TEBs. Eight glands from four mice of each p53 genotype and twelve glands from four mice of each WAP-Bcl-2 genotype were used for TUNEL analysis.

Immunohistochemical analysis

Tissue sections collected and fixed as described, were deparaffinized and rehydrated according to standard protocols. The sections were heated in 0.01 M citrate pH 6.0 or 0.05 M acetate pH 5.5 for Bcl-2 detection, for 10 minutes at 90°C and allowed to cool for 15 minutes at 24°C. The sections were then rinsed in distilled water and endogenous peroxidases were quenched with 3% (v/v) H_2O_2/10% (v/v) methanol for 7 minutes in phosphate-buffered saline (PBS). After a 30 minute block with 10% (w/v) goat serum in 1× PBS, polyclonal rabbit primary antibody for Bax, Bcl-2 and Bcl-x was added at 1:500, 1:500 and 1:1000 (v/v) dilution, respectively, diluted in 10% goat serum/PBS. Slides were coverslipped and incubated in a humidified chamber for 60 minutes at 24°C. A PBS wash of 10 minutes preceded application

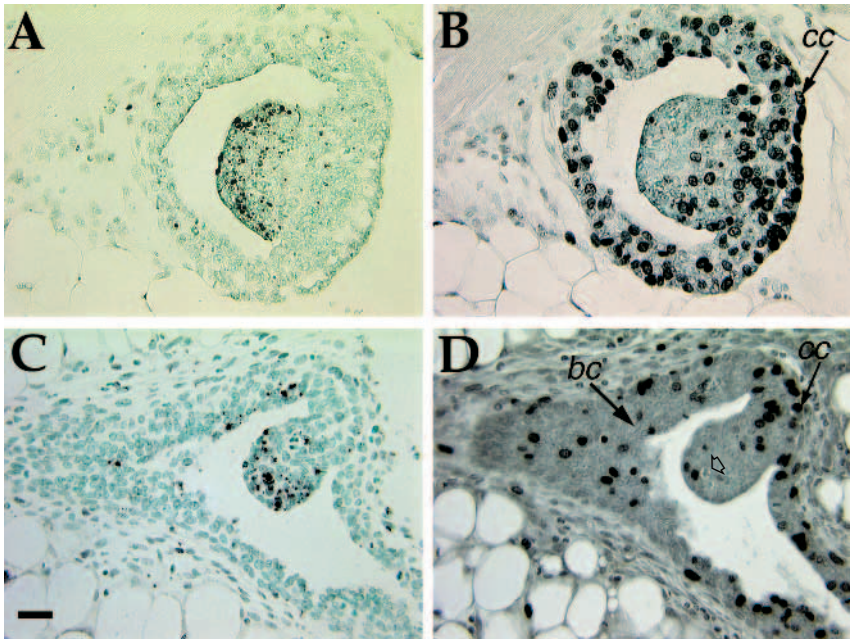

Fig. 1. Programmed cell death and DNA synthesis in the TEBs of 5-week-old virgin Balb/c mice. (A,C) TEBs analyzed by TUNEL; (B,D) serial sections analyzed by BrdU immunohistochemistry. cc defines the cap cell layer; bc defines body cells. The open arrow in D indicates an apoptotic cell adjacent to a cell undergoing DNA synthesis. All sections are counterstained with methyl green. Bar, 15 μm.

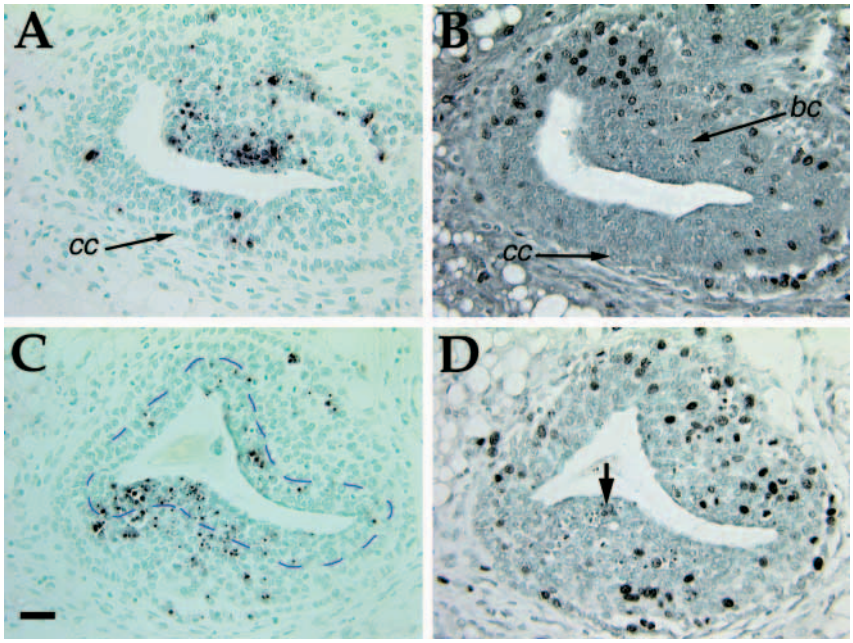

Fig. 2. Programmed cell death and DNA synthesis in the TEBs of 5 week old virgin Balb/c mice. (A,C) TEBs analyzed by TUNEL; (B,D) serial sections analyzed by BrdU immunohistochemistry. The dashed line in C defines the boundary used for quantitation of apoptosis. The arrow in D designates fragmented nuclei labeled with BrdU. cc defines the cap cell layer; bc defines body cells. All sections are counterstained with methyl green. Bar, 15 μm.

of biotin-conjugated goat anti-rabbit secondary antibody at 1:100 dilution in 10% goat serum/PBS for 45 minutes at 24°C. Biotin-avidin binding and detection was carried out according to manufacturer's protocol (Vector Labs). A 0.05% (v/v) Triton-X 100 wash for 1 minute was followed by detection with DAB for 7 minutes. Sections were counterstained with aqueous hematoxylin diluted 1:10, for 1 minute, mounted with aqueous mounting media and coverslipped. All immunohistochemical analyses included control sections without added primary or secondary antibodies.

RESULTS

Significant levels of apoptosis are present in the body cells of the terminal endbud

TEBs are most prominent and dynamic in the MMG during early ductal development (4-7 weeks of age). Using the TUNEL technique on sections of mammary glands isolated from 5 and 6 week old virgin Balb/c mice revealed significant levels of apoptosis in the body cells of the TEB (Figs 1A,C, 2A,C). Every TEB examined contained apoptotic cells. The majority of the apoptosis is conspicuously localized around the lumen of the newly forming duct. These highly apoptotic regions are primarily restricted to body cells that protrude into, or are proximal to, the ductal lumen (Figs 1A,C, 2A,C). In the densest apoptotic regions up to 60% of the body cells were undergoing apoptosis within a given section. This consistent and striking pattern suggested that apoptosis may be involved in the formation lumen of the duct formed by apoptosis of body cells most proximal to the lumen.

To test this hypothesis, the number of apoptotic cells within an arbitrary three cell layers of the lumen were quantitated (Fig. 2C, Table 1). A significantly greater percentage (14.5%) of the cells proximal to the lumen were undergoing apoptosis compared to those cells distal (7.9%) to this arbitrary division (P<0.001). Within an individual TEB, the level of apoptosis was as high as 26%. Only 0.5% of the cap cells in the TEB and 1.0% of the ductal epithelial cells and less than 0.01% of the myoepithelial cells in the subtending ducts were undergoing apoptosis (data not shown). The percentage of apoptotic cells detected in the ductal epithelial cells of the subtending ducts of the immature virgin is similar to the value observed in the mature virgin and proliferating pregnant gland (Table 2). These results were consistent between animals, among glands from the same animal and within endbuds from the same gland. No difference was observed in the apoptotic pattern between TEBs from the third and fourth mammary glands. DNA synthesis can precede a cell's entrance into apoptosis (Barres et al., 1992).

Table 1. Apoptosis and BrdU levels in the TEBs of 5-week-old virgin Balb/c mammary glands

	% BrdU ± s.e.m.	% Apoptosis ± s.e.m.
Proximal	13.8±4.2	14.5±1.1
Distal	21.1±2.5	7.9±0.9
Total %	19.7±3.6	11.3±0.8
Total labelled	619	1237
Total no. of cells	3612	10983

Table 2. Comparison of apoptosis levels with stage of murine mammary gland development

Stage	% Apoptosis	Reference
TEB	11.3	This study
Mature virgin, duct	0.4	Li, 1995; this study
17 day pregnant	0.2	Li, 1995; Li, 1996b
4 day involution	4.5	Quarrie, 1995
p53* transgenic	19.5	Li, 1995

This phenomena can be detected in the TEB as fragmented BrdU-labeled nuclei (Fig. 2D, arrow) suggesting that some apoptotic cells were very recently generated. The significant level of spatially restricted apoptosis that occurs in the body cells of the TEB suggests that this apoptosis may be involved in organizing TEB structure and possibly contribute to the formation of the lumen of the MMG duct.

Overall, 11.3% of the cells in the TEB were undergoing apoptosis. This represents a significant number of apoptotic cells within a single developing structure. To date, the TEB possesses the highest level of apoptosis detected during any stage of mammary gland development (Table 2). This observation reinforces the dynamic nature of the TEB and suggests that the mechanisms that regulate this apoptosis may be intimately involved in the development of this organ.

DNA synthesis has a reciprocal pattern to apoptosis in the TEB

Studies of DNA synthesis in the rodent mammary gland identify the cap cell layer and the neck region as the primary zones of proliferation in the TEB during ductal development. (Daniel et al., 1987; Dulbecco et al., 1982; Sapino et al., 1990). To understand the relationship between cell proliferation and apoptosis, the pattern of cellular proliferation in the TEB was examined by immunohistochemical analysis of mammary glands that had been injected with BrdU i.p., 2 hours prior to the mice being killed. DNA synthesis occurred at various locations throughout the TEB, but most significantly in the cap cells and body cells of the neck region (Fig. 1B,D).

Comparing the pattern of apoptosis and DNA synthesis in

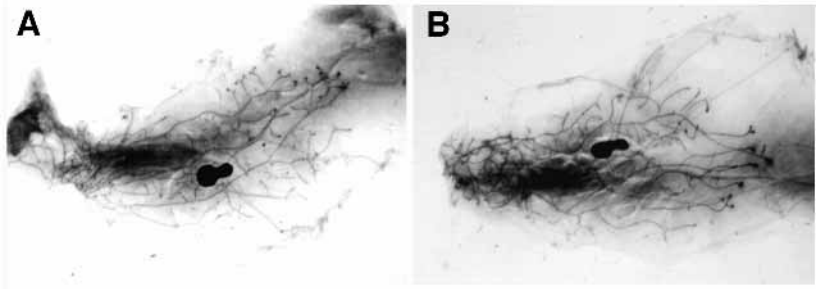

Fig. 3. The effect of *p53* deletion on the ductal development of the virgin mammary gland. Whole-mount hematoxylin-stained $p53^{-/-}$ (A) and wild-type (B) mammary glands. Bar 0.5 mm.

serial sections of TEBs revealed reciprocal zones of proliferation and apoptosis occurring within the same endbud and that they can exist in proximity within the TEB (Figs 1, 2). Cells undergoing DNA synthesis can occur in zones where there is little apoptosis, or adjacent to cells undergoing apoptosis (Fig. 1D arrow). Even though the majority of the apoptosis was around the lumen of the duct, BrdU labeling was not excluded from this region. 13.8% of the cells proximal to the lumen were labeled with BrdU. Distal to the arbitrary boundary, 21.1% of the cells were labeled with BrdU. This result represents a significant difference in the level of DNA synthesis depending on a cells proximity to the lumen of the TEB (P<0.02) (Table 1).

In the TEB, 17.9% of the cells were undergoing DNA synthesis. This level of BrdU labeling correlates with the previously reported levels of proliferation observed in the TEB of the mouse (Sapino et al., 1990). Compared to other organs undergoing extensive developmentally associated growth, the TEB of the mammary gland exhibits a high level of DNA synthesis (Barres et al., 1992; Coles et al., 1993).

Apoptosis in the TEB is not dependent on p53

To determine if the pattern of apoptosis observed in the TEB was dependent on the expression of p53, the TEBs of *p53*$^{-/-}$ mice were analyzed by TUNEL. If there was any p53 dependence then the pattern of apoptosis in the TEB and possibly ductal development in *p53*$^{-/-}$ mice should be disrupted. Comparison of whole-mount-stained mammary glands from *p53*$^{-/-}$ and wild-type mice revealed no difference in the ductal development pattern (Fig. 3). Several morphological characteristics were examined empirically including the overall ductal organization, number of TEBs, the length of ducts and number of secondary branches. These data support the observation that there are no gross developmental deficiencies in the mammary glands of *p53*$^{-/-}$ mice (Donehower et al., 1992). Microscopic examination of serial sectioned *p53*$^{-/-}$ TEBs showed no consistent changes in TEB morphology. A decrease in the level of apoptosis in *p53*$^{-/-}$ mice (8.3%) is detected when compared to syngeneic, age-matched control mice (11.5%, P<0.001) (Table 3) The majority of this decrease in apoptosis occurs in the cells that are distal to the lumen. However, the lack of a detectable effect on the development of ductal structures or TEBs in *p53*$^{-/-}$ mice suggests that p53 does not play an essential role in the development of the ductal structures or the formation of the lumen through apoptotic mechanisms.

Bcl-x, Bax and Bcl-2 expression are non-uniformly distributed in the TEB and reflect a combinatorial regulation of apoptosis

Since apoptosis in TEBs did not appear to be dependent on p53, the expression of other potential apoptosis-regulating molecules was examined. Using an antibody that recognizes

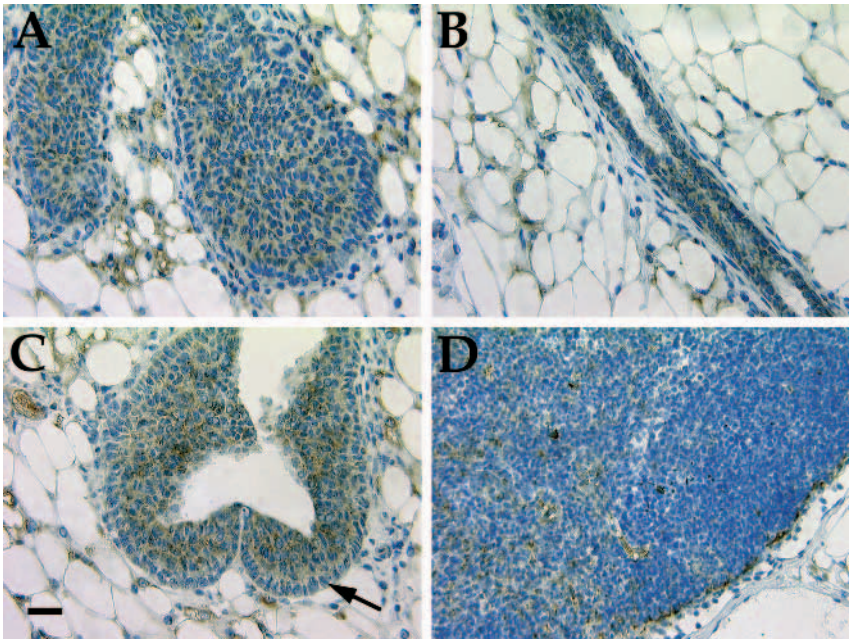

Fig. 4. Immunohistochemical detection of Bcl-x expression in the mammary gland of Balb/c mice. Bcl-x expression in the terminal endbud (A,C) and the duct (B). Arrow in C designates low Bcl-x expression the cap cell layer. The lymph node in D is a positive control for Bcl-x staining. Bar 15 μm.

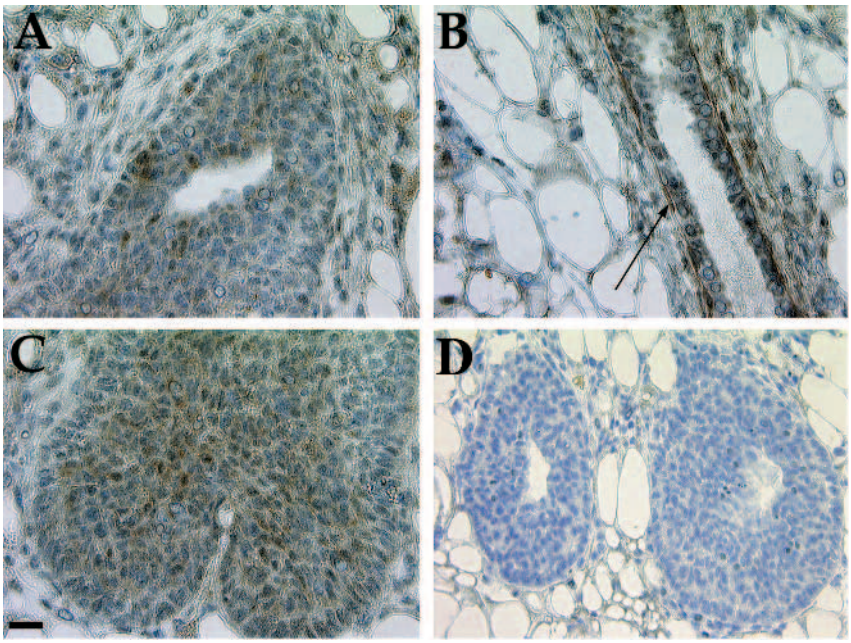

Fig. 5. Immunohistochemical detection of Bax expression in the mammary gland of Balb/c mice. Bax expression in the terminal endbud (A,C) and the duct (B). Arrow in B designates Bax expression in the myoepithelial cells. D is control terminal endbud with primary antibody excluded. Bar 15 μm.

both forms of the protein, Bcl-x expression was detected throughout the TEB in the body cells (Fig. 4A,C). Bcl-x is expressed in the ductal epithelial cells and weakly in the myoepithelial cells (Fig. 4B). In the TEB, expression was lowest in the cap cell layer (Fig. 4C arrow) and higher in the body cells. Body cells with the highest expression were located proximal to the lumen (Fig. 4A,C). The pattern of Bcl-x expression in specific body cells does not overlap with TUNEL labeling in serial sections. This lack of correlation is not surprising, since Bcl-x regulates the apoptotic checkpoint earlier than the moment when active DNA degradation occurs and can be detected by the TUNEL technique. Thus the pattern of expression of Bcl-x in the body cells and the low level expressed in the cap cell layer implies that Bcl-x may play a role in regulating the apoptotic pattern in the TEB.

Immunohistochemical examination of Bax expression in the TEB of 6-week-old Balb/c mice revealed expression of Bax throughout the TEB in both body and cap cells (Fig. 5A,C). High levels of Bax expression was detectable in both body and cap cells (Fig. 5A). Immunoreactive-Bax signal was apparent in the nucleus and cytoplasm. No overlap between the expression of Bax and the apoptotic pattern in serial sections was observed. Bax expression was also detected in the ductal epithelial cells (Fig. 5B). Interestingly, there was significant expression in the non-apoptotic myoepithelial cells (Fig. 5B arrow). This demonstrates that high levels of Bax expression do not necessarily correlate with induction of apoptosis in this cell type.

Bcl-2 is thought to repress apoptosis in most cells (Reed, 1994). Body cells in the TEB displayed significant Bcl-2 expression. Paradoxically, body cells most proximal to the lumen had high levels of Bcl-2 expression (Fig. 6A,B). There was reduced Bcl-2 expression in the cap cell layer (Fig. 6B). Examining serial sections of TUNEL-labeled TEBs, no direct correlation could made between non-apoptotic cells and the expression of Bcl-2. This pattern of Bcl-2 expression suggests that another Bcl-2 family member may be involved in repressing Bcl-2's anti-apoptotic effects. Bcl-2 is expressed in the ductal epithelial cells, but in contrast to Bax expression in myoepithelial cells, Bcl-2 expression was very low (Fig. 6C, arrow).

WAP-Bcl-2 mice have a reduced level and altered distribution of apoptosis in the TEB and disruption in the formation of ductal structures

To demonstrate a functional role for Bcl-2 family members in TEB-apoptosis, the level of apoptosis was examined in transgenic mice where Bcl-2 expression was targeted to the mammary gland using the regulatory sequences from the mouse WAP gene. Overexpression of Bcl-2 has been

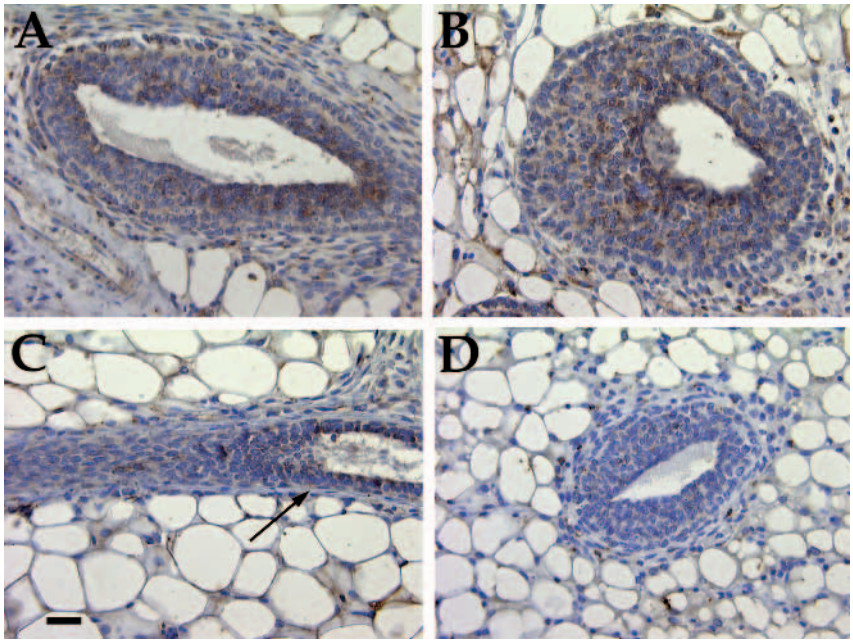

Fig. 6. Immunohistochemical detection of Bcl-2 expression in the mammary gland of Balb/c mice. Bcl-2 expression in the terminal endbud (A,B) and duct (C). Arrow in C indicates myoepithelial cells with no Bcl-2 staining. D shows staining in the TEB after competing with Bcl-2 control peptide. Bar 15 μm.

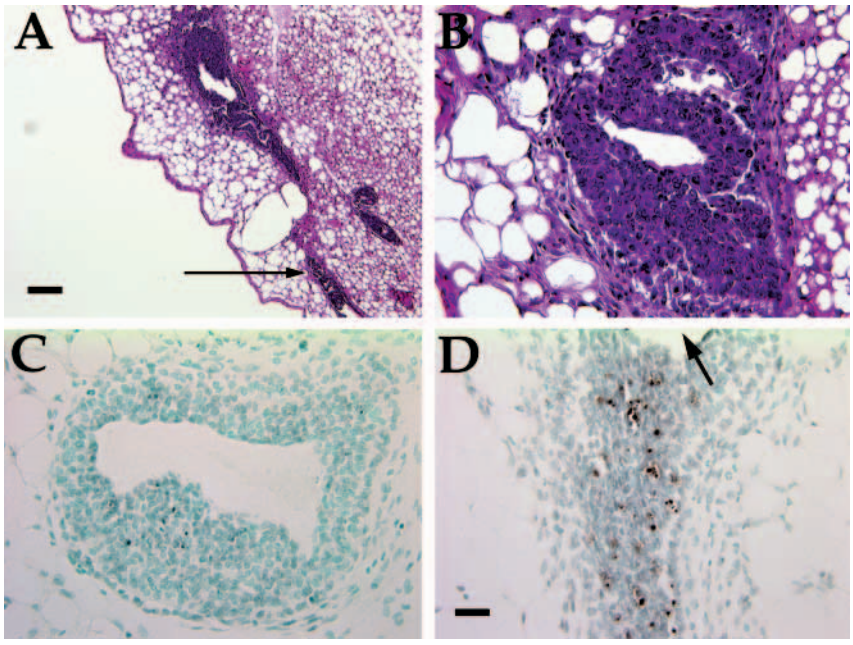

Fig. 7. Hematoxylin and eosin staining of TEBs from WAP-Bcl-2 mice showing a disruption in the organization of the body cells (A). Arrow in A shows cells in the duct. B shows convoluted layers of epithelial cells in the TEB. Bar in A, 100 μm. TUNEL analysis of WAP-Bcl-2 mammary glands revealing lowered levels of apoptosis in the TEB (C) and increased levels in the neck region (D). Arrow in D indicates the location of the lumen of the TEB. Bar in B-D, 15 μm.

Table 3. Percentage apoptosis in the TEBs of $p53^{-/-}$ mice is reduced compared to $p53^{+/+}$ mice

	Proximal	Distal	Total
$p53^{-/-}$	11.7±0.99*	4.2±0.63	8.3±0.71
$p53^{+/+}$	13.4±1.14	8.3±0.83	11.5±0.57

*Mean ± s.e.m.

demonstrated to block p53-mediated apoptosis in vitro (Garcia et al., 1992; Strasser et al., 1991) and in vivo (Vaux et al., 1988). Apoptosis was significantly lowered in WAP-Bcl-2 TEBs (6.3%) when compared to wild-type controls (10.7%, $P<0.001$, Fig. 8). The persistent pattern of apoptosis observed in the TEBs of wild-type mammary glands was noticeably absent in transgenic glands (Compare Fig. 1A to Fig. 7C). In some large transgenic TEBs, there were no apoptotic cells present (data not shown). Additionally, the localization of apoptosis in the TEB was disrupted. A dramatic increase in the level apoptosis was observed in the neck of some TEBs (Fig. 7D). There was also an effect on the structural organization of the TEB. In some TEBs, the body cells were present as highly convoluted layers of epithelial cells. These layers of cells were found in the central cavity of the TEB and in the neck region (Fig. 7A,B). Unattached cells could be found in the newly formed duct (Fig. 7A, arrow). Cells were observed some distance from the TEB into areas that were clearly ductal (data not shown). In addition the cellular organization of the cap cells was disrupted and were often difficult to identify. These data demonstrate that WAP-driven Bcl-2 expression has disruptive effects on the pattern and level of apoptosis and cellular organization in the TEB. These results support the hypothesis that apoptosis is involved in ductal morphogenesis and that Bcl-2 family members play a role in regulating this mechanism.

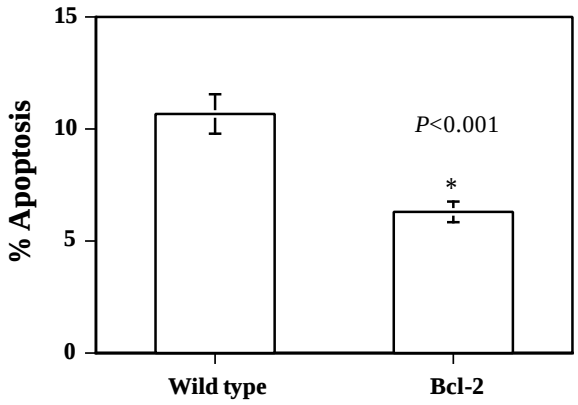

Fig. 8 Quantitation of the reduction of apoptosis levels in WAP-Bcl-2 TEBs by TUNEL analysis. A significant difference is observed between WAP-Bcl-2 (n=3) and wild-type control (n=5) mice ($P<0.001$). Values represent percentage of total cells labeled positive for apoptosis in transgenic and wild-type TEBs.

DISCUSSION

Apoptosis in the TEB is a critical mechanism of ductal morphogenesis

This study reveals that the TEBs of the MMG exhibit a dramatic pattern and level of apoptosis in the body cells. These apoptotic body cells are often restricted to an area projecting into the presumptive lumenal space. This reproducible pattern of apoptosis suggests that programmed cell death deletes specific body cells and may be a critical mechanism in the ductal morphogenesis of the early mammary gland.

The very high levels of apoptosis (11.3%) detected in the TEB during normal ductal development of the MMG are higher than the published levels for any other stage of mammary gland development. During involution in the mammary gland, when there is dramatic tissue reorganization, the maximal level of apoptosis observed is approximately 4% (Quarrie et al., 1995) In SV40 T-antigen transgenic pregnant mammary glands, approximately 5% apoptosis was detected (Li et al., 1996a). Only in the mammary glands of transgenic mice overexpressing p53 have levels of apoptosis (20%) been detected that exceed those observed in the TEB (Li et al., 1995) (Table 2). The level of apoptosis in most quiescent tissues is usually 0.1-1%. Apoptosis in the TEB exceeds that detected during development in other organs like the kidney, 3% (Coles et al., 1993) and optic nerve, 0.25% (Barres et al., 1992). The clearance rate of apoptotic cells in these structures is rapid and consequently it is estimated that 50% of the cells undergo apoptosis. Thus, there are examples of other organs that utilize large scale apoptosis as a mechanism of development.

How is this pattern of apoptosis in the TEB generated? Given the striking pattern observed in some TEBs, apoptosis is clearly influenced by a cell's position within the TEB. This suggests that there is an apoptotic signal that is regulated by positional cues from the TEB and its environment. Interestingly, for TEBs with large, well-defined lumens, apoptotic body cells are distributed evenly around the lumen (data not shown). Only in TEBs with large numbers of body cells protruding into the presumptive lumenal space is the apoptosis narrowly restricted. Examination of serial sections of TEBs indicate that the zones of apoptosis penetrate several cell layers (data not shown). These observations imply it is not just the distance from the external cap cell layer that induces apoptosis in a particular cell, but that there is another presently undefined mechanism influenced by a cell's position within the overall structure of the TEB.

A precedent for the derivation of an apoptotic signal originating from outside the zone of apoptosis has been provided by studies from Coucouvanis and Martin (1995). These authors demonstrated that the external group of endodermal cells sends a signal causing apoptosis in the centrally located ectodermal cells during rodent gastrulation. Attachment to the basal lamina protects the internal ectodermal cells from this apoptotic signal. There is striking similarity between this system and the TEB, and it is tempting to speculate that this same mechanism of apoptosis induction may be occurring in the MMG. Attachment to the basal lamina by the cap cells may provide a protective effect as indicated by their relatively low apoptotic rate. It is unknown whether the cap cells or the stromal cells are performing the same role as the endodermal cells in the rodent embryo and are, in fact, the source of the apoptotic signal.

This model of developmentally associated apoptosis implies that structural organization; i.e., attachment to the basal lamina, can protect cells from apoptosis. Attachment to the basal

lamina has been demonstrated to regulate the induction of apoptosis and integrins may play a role in the regulation of this response (Coucouvanis and Martin, 1995; Frisch and Francis, 1994; Ruoslahti and Reed, 1994; Zhang et al., 1995). Interaction between mammary epithelial cells, in vitro and in vivo, and the extracellular matrix can regulate their apoptotic potential (Boudreau et al., 1995; Pullan et al., 1996). Specifically, antibodies against β-1 integrin or overexpression of the matrix metalloproteinase, stromelysin-1, can induce apoptosis of mammary epithelial cells. Overexpression of stromelysin-1 can also alter the developmental pattern of transgenic MMGs (Sympson et al., 1994; Witty et al., 1995). Increased expression of several proteinases correlates with an increase in apoptosis during mammary gland involution (Lund et al., 1996). Thus, the signal for apoptosis may originate from an interaction between the cap cells and the extracellular matrix surrounding the TEB. The characteristics of the stroma immediately surrounding the TEB are different from the stroma not involved with the TEB and the thickness and molecular components of the basal lamina change with respect to position on the TEB (Daniel and Silberstein, 1985; Williams and Daniel, 1983). In this way, positional cues could be transmitted to the cap cells. Thus, it is conceivable that the interaction between the ECM and the cap cells may be involved in the generation of the apoptotic pattern in the course of regulating the development of the TEB.

Apoptosis in the TEB is independent of p53 and may depend on combinatorial regulation by several Bcl-2 family members

Given the presence of apoptosis during ductal morphogenesis, the expression of several proteins that have demonstrated roles in regulating the apoptotic pathway, p53, Bax, Bcl-x_{s+L} and Bcl-2, was examined immunohistochemically. p53 can cause both cell cycle arrest and apoptosis in response to DNA damage (Shaw et al., 1992; Yonish et al., 1993; Zhan et al., 1993), but is apparently not involved in apoptosis induced by growth factor withdrawal and cell density in mammary epithelial cells in vitro (Merlo et al., 1995). Whether p53 is involved in developmentally associated apoptosis in vivo is controversial. *p53* gene expression increases with the onset of involution (Strange et al., 1992). However, *p53*$^{-/-}$ mice showed no overt differences in mammary gland ductal or lobular-alveolar development when compared to syngeneic wild-type mice (Donehower et al., 1992; Li et al., 1996b) and this study. The level of apoptosis in SV40 T-antigen transgenic mice is increased abnormally during pregnancy preventing attainment of a complete lactogenic phenotype (Li et al., 1996a). Since p53 is sequestered by T-antigen in these mice, the increased apoptosis probably occurs by a p53-independent mechanism. There is some evidence to suggest that p53 may delay the onset of apoptosis. In *p53*$^{-/-}$ mice, prostate involution occurred normally (Berges et al., 1993), but Colombel et al. (1995) demonstrated that prostate gland involution was delayed in the absence of p53. These data suggest the role of p53 in the mammary gland may be of secondary importance in defining the developmental pattern of apoptosis as suggested by Li et al. (1996b). This study supports the conclusion that p53 is not primarily involved in the regulation of the developmentally associated apoptotic pattern in the TEB.

Bcl-2 is a proto-oncogene that has demonstrated anti-apoptotic effects (Garcia et al., 1992; Reed et al., 1990; Strasser et al., 1991; Vaux et al., 1988). Bax, Bcl-x_L, Bcl-x_s, Bad and Bag can form heterodimeric protein complexes with Bcl-2 and act as apoptotic checkpoint regulators (Boise et al., 1993; Oltvai et al., 1993; Takayama et al., 1995; Yang et al., 1995). Expression of these proteins has been correlated with induction and repression of apoptosis, dependent upon the cell type and expression levels of the competing heterodimer partners. The expression of these proteins during the initial phase of ductal development in the mammary gland has not been characterized to date.

The pattern of Bcl-x expression in the TEB suggested that it may be involved in the regulation of the apoptotic pattern. This is based on the two observations; the cap cell layer has very low Bcl-x staining and Bcl-x staining is correlated with proximity to the ductal lumen. Although the antibody employed in this study recognizes the Bcl-x_s and Bcl-x_L forms of the Bcl-x protein, there may be a role for Bcl-x in the appearance of the apoptotic pattern. Li et al. (1996b) have reported that an increase in the level of both forms of Bcl-x mRNA occurs at the onset of involution when a majority of the apoptosis occurs in MMG development. However, the relative increase in the level of Bcl-x_s mRNA may be responsible for the increased apoptosis. The ratio between the two splice forms regulate the apoptotic potential of the particular cell, and the largest increase in Bcl-x_s expression occurs during mammary gland involution when there is significant apoptosis. In addition, Bcl-x_s overexpression in transfected mammary epithelial cells resulted in an increase in apoptosis (Heermeier et al., 1996). Thus, Bcl-x expression detected in the body cells may be due to an increase in the amount of Bcl-x_s which may, in turn, cause increased apoptosis of the body cells. In the absence of antibody reagents that are capable of unambiguously distinguishing between the Bcl-x_s and Bcl-x_L proteins, however, this idea remains speculative.

The presence of Bax in the TEB, and the lack of correlation between its expression and the apoptotic pattern in the TEB, do not exclude Bax from being involved in the induction of apoptosis. It does suggest, however, that other members of the Bcl-2 family may be required to induce PCD in this structure. Interestingly the high level of Bax in the myoepithelial cells and their characteristically low level of apoptosis shows that expression of Bax does not induce apoptosis in these cells. This may be another example where apoptosis induction is dependent on the ratio of Bcl-2 family members within a cell. Alternatively, studies of Bax knockout mice have suggested that Bax may paradoxically promote cell survival in some cellular contexts (Knudson et al., 1995).

The expression of Bcl-2 in the TEB is similar to that of Bcl-x; i.e. low expression in the cap cells with increased expression in the body cells proximal to the lumen. Bcl-2 was originally characterized as capable of preventing apoptosis and its expression pattern is the inverse of what is expected given the apoptotic pattern in the TEB. This suggests that Bcl-2 expression alone does not regulate TEB-apoptosis. A recent study of the role of matrix attachment and Bax expression in promoting apoptosis in alveolar cells demonstrated that Bcl-2 is expressed in ductal, but not in alveolar, cells. These authors suggested that as survival factors, Bcl-2 is dominant in the ductal cell while matrix attachment is dominant in the alveolar cell (Pullan et al., 1996). This supports the theory that Bcl-2

may be a dominant molecule in regulating apoptosis in the ductal epithelial cells and their progenitors in the TEB. The patterns of expression of these three apoptosis-regulating proteins in the TEB imply that no single protein is responsible for regulating apoptosis in the TEB. Thus, although there may be a correlation between the expression of Bcl-2, and possibly Bcl-x, and the apoptotic pattern in the TEB, it is most probably the ratios of these and other Bcl-2 family proteins that decides a cell's fate, as has been suggested previously (Sedlak et al., 1995).

The pattern of apoptosis and ductal development of WAP-Bcl-2 mammary glands is disrupted

In order to establish a functional correlation between the expression of these apoptosis-regulating molecules, the pattern of apoptosis and ductal development was studied in WAP-Bcl-2 transgenic mice. In TEBs of WAP-Bcl-2 mice, there was a significant decrease in the level of apoptosis even though there is presumably an effect of incomplete penetrance and estrous cycle-dependent expression on the WAP transgene. Some transgenic TEBs lacked apoptotic cells altogether. In addition to the decrease in the body cells, an increase in apoptosis was observed in the neck region where there is normally little apoptosis. This new localization of apoptosis suggests that there is a possible compensatory mechanism for the lack of normally occuring apoptosis in the TEB. In some transgenic TEBs there was a disruption in the cellular organization of the TEB, which was manifested as convoluted layers of body cells in the TEB and neck region. This morphological disruption may have resulted from an increased number of cells present in the TEB forcing a loss of structural integrity. Despite the observed increase in apoptosis in the neck of transgenic TEBs there were still unattached cells present in ductal regions well past the neck. Often these unattached cells were located quite far from the TEB suggesting that compensatory mechanisms could not completely account for the lack of apoptosis in the TEB. These data demonstrate that disruption of the pattern and level of apoptosis in the TEB by altering the balance of apoptosis-regulating molecules can lead to alterations in the ductal development of the mammary gland. Presumably, this occurred by alteration of the balance between Bcl-2 and its co-regulatory molecules.

The proximity and reciprocity of pattern and levels of DNA synthesis and PCD in the TEB reflect a potential coordination between mechanisms of cell proliferation and cell death during mammary gland morphogenesis. TEBs, the most proliferative structure in the mammary gland, are at their highest density in early ductal development (Russo and Russo, 1978b). This maximal proliferation and presence of large numbers of TEBs in the young virgin is correlated with an increased sensitivity to carcinogens (Anderson and Beattie, 1992; Russo and Russo, 1978a). Thus, the high level of apoptosis in the TEB could provide another mechanism sensitive to transformation. It will be interesting to test whether disruptions in the apoptotic mechanism will increase the TEB's sensitivity to transformation.

In conclusion, these studies suggest that the TEB is a dynamic structure regulated by a balance of proliferation and apoptosis. Significant apoptosis occurring in the primordium of body cells may be a critical mechanism of ductal morphogenesis in the MMG.

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