Developmental Cell
Article

BIM Regulates Apoptosis during Mammary Ductal Morphogenesis, and Its Absence Reveals Alternative Cell Death Mechanisms

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DOI 10.1016/j.devcel.2006.12.003

SUMMARY

The adult, virgin mammary gland is a highly organized tree-like structure formed by ducts with hollowed lumen. Although lumen formation during pubertal development appears to involve apoptosis, the molecular mechanisms that regulate this process are not known. Here, we demonstrate that disruption of the BH3-only proapoptotic factor *Bim* in mice prevents induction of apoptosis in and clearing of the lumen in terminal end buds during puberty. However, cells that fill the presumptive luminal space are eventually cleared from the adjacent ducts by a caspase-independent death process. Within the filled $Bim^{-/-}$ ducts, epithelial cells are deprived of matrix attachment and undergo squamous differentiation prior to clearing. Similarly, we also detect squamous differentiation in vitro when immortalized mammary epithelial cells are detached from the matrix. These data provide important mechanistic information on the processes involved in sculpting the mammary gland and demonstrate that BIM is a critical regulator of apoptosis in vivo.

INTRODUCTION

During embryogenesis and puberty, the mammary gland undergoes a morphogenetic program that leads to the development of a hollow ductal system terminating in alveolae. Mammary development during puberty is an excellent model by which to study the process of lumen formation in vivo. From 4 to 8 weeks after birth, the rudimentary mammary tree undergoes extensive expansion that results from proliferation and invasion at the leading front of the ductal outgrowths. The highly proliferative bulbous structures at the tips of these expanding outgrowths, referred to as terminal end buds (TEBs), develop in response to elevated levels of reproductive hormones. A luminal space forms behind TEBs during ductal expansion, and this process is hypothesized to require clearance of an inner cell population by apoptosis (Humphreys et al., 1996).

The TEB is composed of two main cell populations, distinguishable by their morphology and expression of specific markers: the cap cells and the body cells (Daniel et al., 1995). The cap cells are located in the outer layer that is in contact with the stroma and are considered to be progenitors of the myoepithelial cells. The body cells, organized with 6–10 cell layers within TEBs, are thought to be precursors for the luminal lineage. A rare scattered third population is occasionally observed in the TEB body, which expresses cap/myoepithelial cell markers and is hypothesized to comprise cap cells left behind during the extensive TEB outgrowth (Williams and Daniel, 1983).

It was previously shown that apoptosis is detected in nonproliferating body cells of TEBs (Humphreys et al., 1996). The spatial and temporal pattern of apoptosis suggested that caspase-mediated apoptosis maintains the lumen within the expanding duct. In addition, mosaic overexpression of *Bcl-2* was found to partially suppress body cell apoptosis and to disrupt TEB structure. However, lumen formation was not inhibited, and mature, virgin mammary glands developed normally in this transgenic model (Humphreys et al., 1996). Thus, the mosaic overexpression of *Bcl-2* precluded evaluation of the consequences of inhibition of apoptosis in this model. While apoptosis is detected in TEBs of the developing glands, the mechanisms underlying lumen formation and the mediators of this process are not known. Elucidation of this apoptotic program is critical not only for understanding mammary gland development, but also tumorigenesis, as repopulation of the luminal space with cancer cells is a hallmark of early breast tumors.

One potential mechanism for the regulation of luminal apoptosis has been proposed from studies utilizing a three-dimensional (3D) in vitro model of mammary acini. Human MCF-10A mammary epithelial cells (MECs) grown in 3D cultures form acini-like structures with a hollow lumen that clears by an apoptotic program. Because the inner acinar cells that lack attachment to the extracellular matrix (ECM) undergo apoptosis, it has been proposed that matrix deprivation, at least in part, contributes to the induction of apoptosis by a process analogous to anoikis.

This term refers to cell death programs caused by matrix detachment (Frisch and Francis, 1994). In the 3D model, caspase activation is regulated by induction of the proapoptotic factor BIM (Reginato et al., 2005). BIM is a BH3-only member of the proapoptotic Bcl-2 intracellular protein family, which also includes Bmf, Bik, Bad, Bid, Puma, Noxa, and Hrk (Huang and Strasser, 2000). During apoptosis, these proteins activate cytochrome *c* release and subsequent caspase activation by binding and inhibiting antiapoptotic Bcl-2 family member proteins or by triggering Bax oligomerization (Huang and Strasser, 2000). In the MCF-10A 3D model, *BIM* expression correlates temporally with lumen formation, and inhibition of *BIM* expression by small interfering RNA (siRNA) significantly delays apoptotic cell death of the central cell cluster (Reginato et al., 2005). Inhibition of *BIM* expression by siRNA also decreased apoptosis of MCF-10A cells during anoikis (Reginato et al., 2003). These data demonstrate that lumen formation in mammary acini and apoptosis during anoikis are dependent on the induction of BIM. However, whether BIM is a regulator of caspase activation and lumen formation in the mammary gland in vivo is not known. Furthermore, other mechanisms of anoikis in mouse MEC lines have been reported that apparently do not involve regulation by BIM (Wang et al., 2004). It is not known which of these in vitro models might be most representative of luminal death in the mammary gland in vivo.

To investigate the molecular mechanisms underlying apoptosis in mammary gland morphogenesis, we have examined the role of BIM-mediated cell death by utilizing $Bim^{-/-}$ mice (Bouillet et al., 2001). Our results indicate that *Bim* is expressed constitutively in the mammary epithelium from the earliest stage of embryonic development. MECs lacking *Bim* were deficient for apoptosis induction in TEBs and ducts at 5 weeks, and this was associated with an absence of lumen formation. Surprisingly, ducts in the $Bim^{-/-}$ mouse showed signs of caspase-independent death and squamous differentiation, and by 8 weeks the luminal spaces in these ducts had hollowed. These data demonstrate that BIM is required for caspase-dependent cell death in the mammary gland, and that in the absence of primary death mechanisms, such as apoptosis, alternative clearance mechanisms contribute to proper lumen formation in the mammary gland in vivo.

RESULTS

Bim Is Widely Expressed during Mammary Gland Development in the Mouse

Bim expression was analyzed during mouse mammary development by X-Gal whole-mount staining of heterozygous $Bim^{+/LacZ}$ embryos (Bouillet et al., 2001). *Bim* expression was detected in mammary buds that arise from the epidermal embryo flank at embryonic day 12.5 (E12.5) (Figure 1A). Thereafter, *Bim* expression was maintained in mammary epithelium throughout embryonic mammary gland development (Figures 1A–1C). *Bim* was also detected in the epithelial placode of primordial hair follicles at E14.5 (β-galactosidase activity), which, like the mammary buds, are appendages, derived from the ectoderm (Figure 1B). At E18.5, *Bim* expression was detected in the mammary gland as well as in the fat pad precursor (Figure 1C). After this period, *Bim* expression became restricted to the mammary epithelium (ducts and TEBs) during pubertal development (Figures 1D and 1E). Paraffin sections of the X-Gal-stained mammary glands at 5 weeks showed that *Bim* is expressed in both mammary luminal and myoepithelial cells (data not shown). A previous report had shown that *Bim* mRNA was detected in the mature gland by in situ hybridization (O'Reilly et al., 2000). Here, we show that BIM is also expressed at the protein level in mammary TEBs (Figures 1F and 1G) and ducts (Figure 1H). In a control, we detected no BIM expression in $Bim^{-/-}$ mammary tissue sections when the same antibody was used (data not shown).

Loss of *Bim* Triggers a Transient Luminal Filling in TEBs and Terminal Ducts during Puberty

To investigate whether BIM plays a role in postnatal mammary morphogenesis, whole-mount-stained mammary glands dissected from $Bim^{-/-}$ female mice were examined (Bouillet et al., 2001). No significant differences between wild-type and $Bim^{-/-}$ mammary glands were detected in glands from neonatal (1 week of age) or juvenile (3–4 weeks of age) periods of growth (see Figure S1 in the Supplemental Data available with this article online). In addition, the overall pattern of ductal branching and expansion in the fat pad was indistinguishable in glands from wild-type and $Bim^{-/-}$ mice throughout postnatal development (compare left panels of Figures 2A, 2D, and 2F to those of Figures 2B, 2E, and 2G; Figure S1). However, close examination of TEBs at 5 weeks indicated that while wild-type TEBs contained a well-defined lumen (Figure 2A), the structures from $Bim^{-/-}$ mice were filled (Figure 2B). Quantification indicated that 86.6% ± 9.3% of $Bim^{-/-}$ TEBs, versus 4.4% ± 1.3% ($p < 1E{-}04$) of wild-type TEBs, lacked a detectable lumen at 5 weeks (Figure 2C). Analysis of the distribution of terminal end size revealed that 12.4% ± 3% of the $Bim^{-/-}$ distal structures were greater than 50,000 μm^2 (terminal ends are designated as TEBs from the 30,000 μm^2 size range), compared to 5.5% ± 2.3% ($p < 0.01$) in the wild-type (Figure 2C). Thus, a significant percentage of the filled $Bim^{-/-}$ TEBs displayed an increase in size. By 6 weeks, the distal ducts in the $Bim^{-/-}$ mammary gland that were filled at 5 weeks began to show marked hollowing; indicating that the ductal-filling phenotype induced by loss of *Bim* was transient (Figure 2E). Finally, the $Bim^{-/-}$ ducts were completely hollowed at 8 weeks, and the whole mammary glands were indistinguishable from the wild-type glands (compare Figure 2F to Figure 2G).

Histological analysis of hematoxylin and eosin-stained serial sections from wild-type and $Bim^{-/-}$ mammary glands at 5–8 weeks (Figure 3 and data not shown) confirmed the results of whole-mount analysis described above (Figure 2). Although TEBs in wild-type mice at 5 weeks contained clearly discernable cap and body cells (Figure 3A), and the luminal and myoepithelial cell bilayer was organized in the ducts (Figure 3C), the luminal space

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