Gain of Bcl-2 Is More Potent Than Bax Loss in Regulating Mammary Epithelial Cell Survival in Vivo

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Abstract

The impact of gain of Bcl-2 function on mammary epithelial cell survival was compared with loss of Bax function during the two stages of mammary gland involution. Bcl-2 gain of function reduced apoptosis 50% during the first stage and increased cell survival 70% during the second stage. Complete loss of Bax reduced apoptosis by 20% during the first stage without second stage effect. Partial loss of Bax was ineffective but increased cell survival 2.4-fold when combined with Bcl-2 gain. Gain of Bcl-2 function is more potent than loss of Bax function in regulating mammary epithelial cell survival in vivo.

Introduction

Both gain of Bcl-2 function and loss of Bax function are associated with enhanced survival of breast cancer cells and resistance to apoptosis (1–4). This study exploited the two stages of mammary gland involution to measure the relative impact of gain of Bcl-2 function to partial and complete loss of Bax function in regulating mammary epithelial cell survival in vivo. Combinations of transgenic mice with varying expression levels of Bcl-2 and Bax in their mammary epithelial cells were used (5, 6). The relative bearing of specific factors on mammary epithelial cell survival and tissue structure in vivo can be assessed during mammary gland involution in genetically engineered mice (5, 7). Direct effects on local factor-induced apoptosis of mammary epithelial cells are measured during the first stage of involution (8, 9). Influences on proteinase-mediated remodeling of glandular architecture are evaluated at the end of the second stage of involution (10). Increased epithelial cell content at 10 days reflects enhanced epithelial cell survival during this stage. Defining the stage and impact of individual proteins on involution increases our understanding of this physiological process, provides insight into their role during breast cancer development, and serves as a model for understanding how epithelial cell survival is regulated during remodeling and development.

Materials and Methods

Generation of Five Mouse Models with Altered Expression Levels of Bcl-2 and Bax in Mammary Epithelial Cells. Mouse models with five different combinations of Bcl-2 and Bax expression levels in their mammary epithelial cells were generated by breeding C57bl/6 mice carrying a WAP1 human Bcl-2 (WAP-Bcl-2) transgene (5) to C57bl/6 SV129 mice carrying a germ line-targeted deletion of the Bax gene (6). The tissue-specific WAP promoter targets human Bcl-2 expression to mammary epithelial cells. The five different models generated include mice carrying: (a) a WAP-Bcl-2 transgene (WAP-Bcl-2 mice); (b) a WAP-Bcl-2 transgene in combination with one inactive Bax allele (WAP-Bcl-2/Bax+/− mice); (c) one inactive Bax allele (Bax−/−/− mice); (d) two inactive Bax alleles (Bax−/−/− mice); and (e) no transgene or inactive Bax allele (wild-type control mice). Wild-type mice were nontransgenic littermates of transgenic mice. Bax+/− mice were littermates of Bax−/− mice, WAP-Bcl-2 mice, and WAP-Bcl-2/Bax+/− mice.

Induction of Involution, Study Time Points, and Harvesting of Mammary Gland Tissue. First pregnancy was induced by breeding in 10 wild-type, 7 WAP-Bcl-2, 6 WAP-Bcl-2/Bax+/−, 9 Bax−/−, and 5 Bax−/− female mice. All dams delivered normally and lactated for 10 days before pup removal and induction of involution. Litter size ranged from 7 to 13. Four different time points were used to compare all mice: 10 days lactation to provide a uniform baseline, 48 and 72 h involution to examine the first stage of involution, and 10 days involution to examine the end of the second stage of involution. In addition, all mice were examined at 24 h involution and wild-type, Bax+/−, and Bax−/− mice were also examined at 4, 6, and 8 days involution to confirm that results from the 2- and 10-day time points accurately represented the entire process. Mammary tissue was removed by either biopsy (fourth mammary glands) or at the time of autopsy (second and third mammary glands) at the specified time points. Mammary gland tissue was divided, and samples were placed in either 10% neutral formalin for histological studies or snap-frozen in liquid nitrogen for RNA and protein studies. Each mouse was used for either two or three different time points. Examination of serial samples from individual mice was used to confirm there was no significant mouse-to-mouse variability in the parameters examined.

Histological Analyses, in Situ Detection of Apoptosis, Quantification of Apoptosis, and Tissue Remodeling. Mammary gland specimens were fixed in 10% neutral formalin and embedded in paraffin using routine methods. Five-μm sections were used for either H&E staining or in situ detection of apoptotic cells. H&E sections were used to establish that all mice exhibited equivalent mammary gland development at 10 days lactation, to quantitate the number of mammary epithelial cells shed into the lumen at 2 and 3 days involution, and to quantitate the relative area covered by epithelial cells as compared to fat cells at 10 days involution. Quantiﬁcation of luminal cells was performed by counting the total number of epithelial cells shed into the lumen in 10 contiguous high-power (×40) ﬁelds. Quantiﬁcation of the relative areas covered by epithelial cells versus fat cells were calculated using grids on 10 contiguous high-power (×40) ﬁelds and then expressed as relative percentages. In situ detection of apoptotic mammary epithelial cells was performed by the ApopTag Detection Kit (Oncor, Gaithersburg, MD), as described previously (7–9). The apoptotic index was calculated after counting the number of digoxigenin-UTP-labeled cells within a population of 1000 cells.

RNA Preparation and RNase Protection Assays. Total RNA was isolated from frozen mammary gland tissue using acid guanidium, phenol, and chloroform as described (11) and quantitated on a spectrophotometer (model DU 640; Beckman Instruments, Fullerton, CA). Ten-µg aliquots were used for...
Fig. 1. Histology of normal mammary gland involution and expression of Bcl-2 family members in the five different mouse models. 

a. H&E-stained sections of mammary gland during normal involution in C57bl/6/SV129 mice. At 10 days lactation, normal histology is characterized by open alveolar structures that are composed of mammary epithelial cells and contain milk secretions. The first stage of involution begins with withdrawal of the pups. At 24 h involution, apoptotic cells appear in the alveolar lumens (arrows). At 48 h involution, the number of apoptotic cells within the lumens increases. Around 72 h involution, the alveolar structures begin to collapse and the second stage of involution begins. At 10 days involution, the second stage of involution is complete and the gland is remodeled to near its prepregnant state. Most of the gland is composed of fat pad. Mammary epithelial ductal structures are embedded within the fat pad. 

b. Time course of Bcl-2 family member expression during mammary gland involution in wild-type C57bl/6/SV129 mice using a multiprobe RNase protection assay. bfl-1, bcl-x, bak, bax, and bad RNA are expressed during the first stage of involution with a fall in expression levels by day 10 involution. Bcl-2 expression is not detectable through day 3 of involution but increases to detectable levels by day 10. 

c. Involution timecourse total RNA combined from A20, S49.1, and J774A.1 cell lines used as a positive control (PharMingen, San Diego, CA); L32, 18S rRNA used as a loading control. 

d. WAP-bcl-2 transgenic mice express high levels of human bcl-2 RNA throughout lactation and early involution. Levels drop by day 10. L10, lactation day 10; Lanes B, WAP-bcl-2 transgenic mouse; Lanes WT, wild-type nontransgenic mouse. 

e. Mice carrying one inactive Bax allele (Bax+/− mice) express reduced amounts of Bax protein at all time points during mammary gland involution. Spleen (S) was used as a positive control in Lane 1. Mammary tissue from wild-type (WT) control mice was analyzed in Lanes 2, 4, 6, and 8. Mammary tissue from mice carrying one inactive Bax allele (+/−) were analyzed in Lanes 3, 5, 7, and 9. 

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of a normal involution, local factors induced apoptosis of mammary epithelial cells with shedding of apoptotic cells into the alveolar lumen (Fig. 1a, see cells indicated by arrows at 24 and 48 h involution). Loss of systemic hormonal stimulation initiated the second proteinase-mediated tissue remodeling stage of involution. This began 72 h after suckling ended and returned the gland to a near-virgin state. At the end of the second stage (10 days involution), the gland was composed primarily of fat pad with scattered mammary epithelial cell structures (Fig. 1a). Multiple Bcl-2 family members were expressed during mammary gland involution (Fig. 1b). Bax was the most highly up-regulated Bcl-2-related death factor. Murine bcl-2 is not expressed during early involution, but expression increases to detectable levels by day 10 (Fig. 1c). Levels of Bcl-2 and Bax were varied both individually and coincidentally to measure interactions between gain of Bcl-2 and loss of Bax. WAP-bcl-2 transgenic mice expressed high levels of human Bcl-2 through the onset of the second stage, with a drop by day 10 (Fig. 1c). Bax protein expression levels were reduced 50–80% in mice carrying only one active Bax allele (Fig. 1d). Bax expression was undetectable in mice carrying two inactive Bax alleles (Fig. 1e). Expression levels of other Bcl-2 family members were not altered by either gain of Bcl-2 or loss of Bax (Fig. 1f).

Mammary Gland Development and Lactation in the Four Experimental Mouse Models. All four experimental mouse models exhibited normal lactation. Histology of the glands at 10 days lactation was indistinguishable from that of the wild-type control gland (Fig. 2).

Bcl-2 Gain of Function Was More Effective Than Bax Loss of Function in Reducing Apoptosis during the First Stage of Involution. Gain of Bcl-2 function was more effective than loss of Bax function in reducing the number of apoptotic cells shed into the lumen (Fig. 3a; Table 1). An in situ assay was used to detect apoptotic cells present in both the alveoli and alveolar lumens. The calculated apoptotic index was reduced >50% in WAP-bcl-2/+ mice and dropped only 20% in Bax−/− mice (Fig. 3b; Table 2). Partial loss of Bax function did not reduce the number of apoptotic cells detected in the lumen or the apoptotic index.

Bcl-2 Gain of Function Increased Mammary Epithelial Cell Survival during the Second Stage of Involu- tion but Loss of Bax Function Had No Effect. Cumulative effects on mammary epithelial cell survival during the second stage of involution were measured by comparing the percentage of area covered by epithelial cells at day 10 involution (Fig. 4). In wild-type mice, epithelial cell structures covered ~25% of gland area at day 10 (Fig. 1a; Table 3). Fat cells comprised the remaining 75%. The combination of gain of Bcl-2 function and partial loss of Bax function had the most marked effect on epithelial cell area. In WAP-bcl-2/Bax−/+ mice, mammary epithelial cells covered >60% of the tissue area at day 10, a 2.4-fold increase over the area covered in wild-type mice. Forty-four % of the area was covered by epithelial cells in WAP-bcl-2 mice. There was no increase in the area covered by epithelial cells in mice with either complete or partial loss of Bax. Bcl-2 gain of function affected not only the first apoptotic stage of involution but the second remodeling stage as well. Partial loss of Bax function enhanced the effect of Bcl-2 on survival of mammary epithelial cells during the second stage.

densitometry analysis was used to quantify protein expression levels in wild-type as compared to Bax−/− mice. The bands from Bax+/− mice were 20% of the intensity of wild-type at 24 h, 54% of that at 48 h, 31% of that at 72 h, and 21% of that at 10 days involution. Coomassie blue staining was performed to verify equal protein loading of all samples. e, mice carrying two inactive Bax alleles (Bax−/− mice) express no intact Bax protein during involution. S, spleen positive control. f, RNA expression levels bfl-1, bcl-x, bak, and bad were not significantly altered by either gain of Bcl-2 or loss of Bax. Mammary tissue from a mouse carrying the WAP-Bcl-2 transgene and one inactive Bax allele (B/+−/−) at 48 h involution was analyzed in Lane 1. Mammary tissue from a wild-type non-transgenic mice mouse (WT) at 48 h involution was analyzed in Lane 2. Analysis of mice carrying only one inactive Bax allele or two inactive Bax alleles and analysis at other time points during involution demonstrated the same findings.
Apoptotic index at 48–72 h involution in the five different mouse models is shown below. The total number of luminal apoptotic cells within their alveolar lumens. WAP- Bcl-2/Bax+/- mice as compared with wild-type control mice. The apoptotic index of WAP- bcl-2 bcl-2 mice is significantly less than that measured for wild-type mice. Bax+/- mice have a slightly higher apoptotic index than either WAP- bcl-2 or WAP- bcl-2/Bax+/- mice.

### Table 1 Apoptotic index during the first stage of involution

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean percentage cells undergoing apoptosis between 48 and 72 h</th>
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<tbody>
<tr>
<td>WAP-Bcl-2</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>WAP-bcl-2/Bax+/-</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Bax+/-</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>Bax+/-</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.4 ± 0.3</td>
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</table>

### Table 2 Luminal apoptotic cells during the first stage of involution

Quantitation of the number of apoptotic cells within alveolar lumens at 48–72 h involution in the five different mouse models is shown below. The total number of luminal apoptotic cells from 10 contiguous high power (×40) fields was calculated for each mouse analyzed. Bax+/- and wild-type mice exhibit almost the same number of apoptotic cells within their alveolar lumens. WAP-Bcl-2, WAP-bcl-2/Bax+/-, and Bax+/- mice demonstrate statistically significantly fewer luminal apoptotic cells.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean no. of cells at 48–72 h involution</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAP-Bcl-2</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>WAP-bcl-2/Bax+/-</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td>Bax+/-</td>
<td>4.2 ± 1.1</td>
</tr>
<tr>
<td>Bax+/-</td>
<td>35.0 ± 5.7</td>
</tr>
<tr>
<td>Wild-type</td>
<td>31.0 ± 6.6</td>
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*Values ± SE.

#### Discussion

These results provide new information on the respective contributions of Bcl-2 and Bax to the regulation of apoptosis in mammary epithelial cells *in vivo*. The experiments demonstrate directly that gain of Bcl-2 function is significantly more potent than loss of Bax function in reducing apoptosis of mammary epithelial cells *in vivo*. This supports previous observations in lymphocytes, which demonstrated that Bcl-2 and Bax functioned independently (12), but extends these findings to epithelial cells *in vivo*. Moreover, the study clearly demonstrates that gain of Bcl-2 function is not biologically equivalent to loss of Bax function in normal mammary epithelial cells.

The ratio of Bcl-2 to Bax has been studied as a prognostic indicator in cancer (13). Elevated Bcl-2:Bax ratios in breast cancer cells are correlated with decreased rates of cell death in response to apoptotic stimuli *in vitro* (1, 2, 4). The enhanced mammary epithelial cell survival observed during tissue proteinase-mediated tissue remodeling when Bcl-2 expression was increased in conjunction with reduced Bax expression provides experimental support for the significance of Bcl-2:Bax ratios.

Like normal mammary gland, many breast cancers express multiple different Bcl-2 family members (14). Many of these genes have redundant functions *in vitro*. Nevertheless, these results demonstrate that they do not have redundant functions *in vivo*. Loss of Bax function was not fully compensated by expression of the other death-inducing Bcl-2 family members during the first stage of involution. Although the survival-inducing Bcl-x gene was highly expressed...
serve to modulate the apoptotic rate. It may be one factor responsible for maintaining some mammalian epithelial cell survival during the first reversible stage of involution (8, 9).

In conclusion, gain of Bcl-2 function was much more potent than loss of Bax function in promoting mammalian epithelial cell survival in vivo. Nevertheless, alterations in either Bcl-2 and Bax levels were able to modulate the rate of mammalian epithelial cell apoptosis. Loss of Bax function in the mammary gland was not fully compensated by the presence of other death-inducing family members. Expression of these other family members was not altered by either gain of Bcl-2 function or loss of Bax function.

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References


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