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Bcl-2 Gene Family and Related Proteins in Mammary Gland Involution and Breast Cancer

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The Bcl-2 gene family regulates tissue development and tissue homeostasis through the interplay of survival and death factors. Family members are characterized as either pro-apoptotic or anti-apoptotic, depending on cellular context. In addition to its anti-apoptotic effect, Bcl-2 also inhibits progression through the cell cycle. Functional interactions between family members as well as binding to other cellular proteins modulate their activities. Mammary gland tissue, similar to many other tissues, expresses a number of different Bcl-2 relatives including bcl-x, bax, bak, bad, bcl-w, bfl-1, bcl-2 as well as the bcl-2 binding protein Bag-1. Bcl-2 is expressed in the nonpregnant mammary gland and early pregnancy. In contrast, expression of bcl-x and bax continues through late pregnancy, is down-regulated during lactation, and up-regulated with the start of involution. Bak, bad, bcl-w, and bfl-1 are also up-regulated during involution. The specific roles of individual gene products are investigated using dominant gain of function and loss of function mice. Finally, different Bcl-2 family members are commonly over- or under-expressed in human breast cancers. Bcl-2 expression in human breast cancers has been associated with a good prognosis, while decreased Bax expression has been linked to poor clinical outcome. Understanding the role Bcl-2 family members play in regulating mammary epithelial cell survival is salient to both normal mammary gland physiology and the development of new therapeutic approaches to breast cancer.

KEY WORDS: Bcl-2 family; mammary gland; breast cancer; apoptosis; involution.

INTRODUCTION TO THE Bcl-2 FAMILY AND RELATED BINDING PROTEINS: REGULATORS OF CELL SURVIVAL

The Bcl-2 family of proteins are regulators of apoptosis (1). The *bcl-2* gene was originally identified in 1984 as a potential oncogene involved in pathogenesis of lymphoid cancers (2). Three types of important observations quickly followed. First, it was determined that Bcl-2 was not a typical transforming oncoprotein, but rather an anti-apoptotic cell survival protein (3,4). Second, Bcl-2 was identified as only one of a large family of proteins sharing similar structural motifs (5,6). Third, both anti-apoptotic cell survival proteins and pro-apoptotic cell death proteins were discovered within the Bcl-2 family (7).

Members of the Bcl-2 family share the presence of at least one of four different Bcl-2 homology (BH)[5] domains. As elucidated by site-directed mutagenesis,

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[5] Abbreviations: Bcl-2 homology domains, (BH); Simian virus 40 T antigen, (TAg); Whey Acidic protein, (WAP); estrogen receptor, (ER); progesterone receptor, (PR); Heat shock protein, (Hsp); dimethylbenz(a)anthracene, (DMBA).

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these highly conserved regions often serve as dimerization domains through which Bcl-2 family members homo- and hetero-dimerize (5,8,9). At the present time, more than fifteen different Bcl-2 family members have been reported in humans. These can be functionally classified as either anti-apoptotic or pro-apoptotic. Some of the more extensively studied members include the anti-apoptotic proteins Bcl-2 and Bcl-x_{Long} (Bcl-x_L), and the pro-apoptotic members Bax, Bak, Bad, Bcl-x_{Short} (Bcl-x_S), and Bid (10).

The mechanisms through which bcl-2 family members regulate apoptosis are only partly understood. Dimerization among different anti-apoptotic and pro-apoptotic Bcl-2 family members has been postulated to provide a mechanism for functional antagonism, acting as a rheostat for controlling the propensity of a cell to undergo apoptosis (11). Subcellular localization of the proteins may also be used to control their activity (12,13). The mitochondrial membrane appears to be a particularly important site of action (14) (Fig. 1). Several of the Bcl-2 members contain a pore-forming domain which could control the permeability of mitochondrial membranes (15). Disruption of mitochondrial membranes leads to release of cytochrome c, which can then activate downstream caspase family cell death proteases (16–18). A role for Bcl-2 downstream of mitochondria may also exist, particularly through interactions with Apaf-1/Ced 4-family proteins that directly control cytochrome c mediated activation of caspases (19). Bcl-2 family members may also regulate some mitochondria-independent pathways for apoptosis (1).

The activity of various Bcl-2 family members can be regulated either by phosphorylation and/or binding to other cellular proteins. Phosphorylation in turn may play a role in activation or inactivation of specific Bcl-2 family members. Phosphorylated Bad is bound to 14-3-3 protein and remains sequestered in the cytoplasm where it is unable to hetero-dimerize with Bcl-2 or Bcl-x_L and is thus prevented from exerting its pro-apoptotic function (20,21). Similarly, phosphorylation of Bcl-2 may interfere with its anti-apoptotic function under some circumstances (22). Binding of Bcl-2 to other cellular proteins can also regulate its activity. For example, Bag-1 originally was identified through its interaction with Bcl-2 (23), and may modulate the function of Bcl-2 through Hsp 70-family molecular chaperones (24). Bcl-x_L binds to APAF-1 and may act in a ternary complex with pro-caspase 9 to inhibit programmed cell death (25). Regulatory interactions with other cellular proteins are known (15).

Bcl-2 Protein and Cell Cycle Progression

At least one family member, Bcl-2, has regulatory functions which go beyond the inhibition of apoptosis, at least in some types of cells. In B and T lymphocytes, fibroblasts, and probably some epithelial cells, it can inhibit cell cycle progression; delaying entry into S phase and maintaining cells in G0 (26–29). In tissue culture cell lines the anti-proliferative activity of Bcl-2 is genetically separable from its anti-apoptotic function and maps to different regions of the protein (27,30–32).

More recently the effect of Bcl-2 on cell cycle progression during development of breast cancer *in vivo* was studied in two mouse models of breast cancer progression. The first study demonstrated that the addition of Bcl-2 to mammary epithelial cells expressing the viral oncoprotein Simian Virus 40 T Antigen (TAg) inhibited both oncoprotein induced cell cycle progression and apoptosis at very early stages of tumorigenesis (33). However during the course of tumorigenesis, the anti-apoptotic function and anti-proliferative function of Bcl-2 separated. The anti-apoptotic action was retained throughout carcinogenesis but the inhibitory effects on cell proliferation were lost as the tissues progressed through hyperplasia to cancer. In the second study, Bcl-2 mediated inhibition of cell cycle progression was demonstrated in a DMBA (dimethylbenz(a)anthracene) chemical carcinogenesis mouse model (34). In this model, the anti-proliferative effects dominated and led to a delay in tumor presentation. Interestingly, the rate of apoptosis was not significantly reduced in the DMBA induced tumors. The results in both the WAP-Tag mouse model and the DMBA mouse model substantiate that Bcl-2 can inhibit cell proliferation in mammary epithelial cells *in vivo*. Moreover, both models demonstrate that the anti-apoptotic and anti-proliferative effects of Bcl-2 are separable *in vivo* (Fig. 2). One can speculate that the disparate effects on tumorigenesis are due to the different types of oncogenic signaling pathways activated in the two models.

EXPRESSION OF Bcl-2 FAMILY MEMBERS AND RELATED BINDING PROTEINS IS REGULATED DURING MAMMARY GLAND DEVELOPMENT AND PHYSIOLOGY

Expression patterns of individual Bcl-2 family members in the mammary gland are regulated during ductal development as well as pregnancy, lactation and

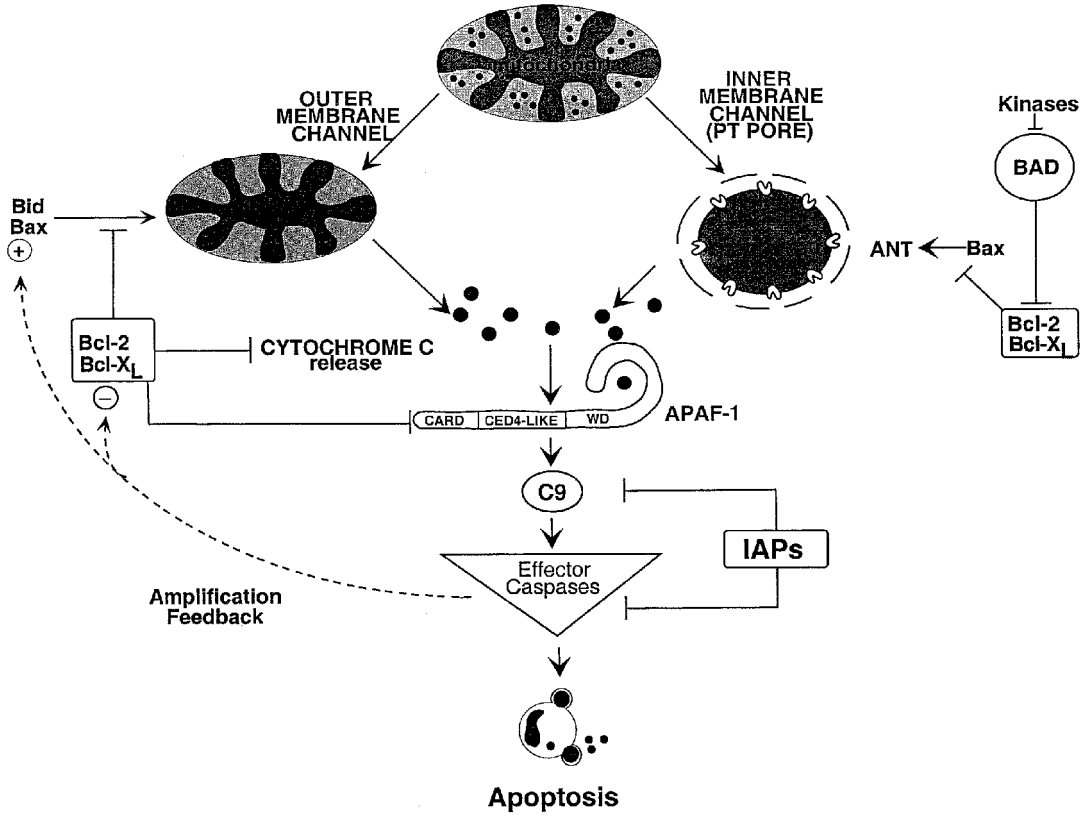

Fig. 1. Schematic diagram illustrating the relationships between different Bcl-2 family members, the mitochondria, and downstream activators of apoptosis. Changes in mitochondrial membrane permeability mediated by different Bcl-2 family members appear to be a key step in the execution of apoptosis. Whether or not apoptosis occurs is mediated by the balance of Bcl-2 family member survival factors as compared to Bcl-2 family member death factors. Bax and Bid are cell death factors which can increase mitochondrial membrane permeability and potentiate the release of cytochrome C, a downstream mediator of apoptosis. Bax acts on the permeability transition (PT) pore through an interaction with adenine nucleotide translocator (ANT). The cell survival factors Bcl-2 and Bcl-x_L can inhibit the actions of Bax and Bid on membrane permeability. The cell death protein BAD can block the cell survival activity of Bcl-2 and Bcl-x_L. Activity of BAD is controlled by kinases. APAF-1 is a downstream mediator of apoptosis which associates with caspase 9 (C9) in the cytoplasm. Activation of caspase 9 sets off a cascade of effector caspase activity resulting in the morphological appearance of apoptosis including nuclear fragmentation and membrane blebbing. Bcl-x_L can also block apoptosis by inhibiting the interaction between APAF-1 and Caspase 9. Effector caspases also promote apoptosis by feeding back through the system to promote Bax and Bid activity and block Bcl-2 and Bcl-x_L action. Under certain conditions, inhibitors of apoptosis (IAPs) can be present in the cytoplasm and abrogate caspase activity.

involution (35–37). In the mouse, Bcl-2 is expressed at detectable levels during early pregnancy (38). Expression is down regulated before late pregnancy and remains low during lactation and early involution. Expression increases during late involution (Fig. 3). In humans, the expression pattern of Bcl-2 throughout pregnancy, lactation, and involution is not well investigated. However, it is known that Bcl-2 is expressed in normal nonpregnant mammary epithelial cells as well as in a large percentage of breast cancers (39–41).

In the mouse, we find that the pattern of bcl-2 expression during involution contrasts with that seen for at least six other Bcl-2 family members. Previous reports demonstrate expression of bax and bcl-x during late pregnancy (37,42). Here we show that the expression levels of bax, bcl-x, bcl-w, bfl-1, bak, and bad were relatively low during lactation. Expression of these genes was up regulated during involution whereas bcl-2 was not expressed (Fig. 3). Bax was the most highly up-regulated pro-apoptotic family member (37,42,43). Bcl-x, which has two splice variants Bcl-x_L and bcl-x_S was the most highly up-regulated family member. Bcl-x_L is a survival factor while bcl-x_S is a death inducer. During involution the relative levels

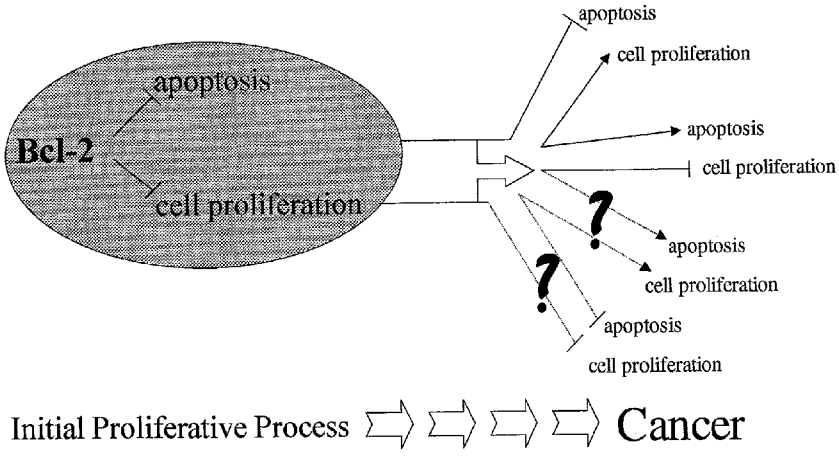

Fig. 2. Schematic diagram illustrating how Bcl-2 action can change during the course of tumorigenesis. Mouse studies demonstrated that the anti-apoptotic and anti-proliferative activities of Bcl-2 may separate during carcinogenesis. In the WAP-TAg model, anti-apoptotic activity was retained while anti-proliferative action was lost. In the DMBA chemical carcinogenesis model, Bcl-2 was anti-proliferative in the cancers but had no anti-apoptotic activity. Other models will need to be examined to determine if there are also examples of loss of both anti-apoptotic and anti-proliferative activities or retention of both actions in other cancers.

of bcl-x_S as compared to bcl-x_L increase (37,42,43). Expression levels of bcl-w, bfl-1, bcl-x, bak, bax, and bad all decreased by the end of involution. This is the time when bcl-2 expression increased. Analogous developmental studies have not been done in humans, but Bcl-x, Bax and Bad are expressed in human breast cancers (44–46).

Using immunohistochemistry, we followed the localization of Bcl-x, Bak, Bax and the Bcl-2 binding protein, Bag-1, in murine mammary tissue from lactation through involution (Fig. 4). All four proteins were expressed in mammary epithelial cells. Bcl-x, Bak, and Bax were localized primarily in the cytoplasm. Apoptotic mammary epithelial cells shed into the lumen appeared to express both Bak (Fig. 4F) and Bax (Fig. 4K). Significantly, intracellular localization of Bag-1 changed as the gland transitioned from lactation through involution. Bag-1 is a survival factor that interacts with Bcl-2, Raf-1, estrogen receptors (ER) and progesterone receptors (PR) as well as other proteins through its ability to bind Hsp-70 family chaperone (23,47,48). Different forms of the protein are expressed at varying levels and expression may be localized to the cytoplasm, the nucleus, or both. In the mouse mammary gland, Bag-1 exhibited nuclear localization during lactation and early involution. By ten days involution, nuclear localization was lost and the protein was present only in the cytoplasm (Fig. 4N–Q). This changing expression pattern could be indicating a role for Bag-1 in moderating mammary epithelial cell survival during lactation and involution.

ARE THERE SPECIFIC DEVELOPMENTAL AND PHYSIOLOGICAL ROLES FOR DIFFERENT Bcl-2 FAMILY MEMBERS?

Just like the mammary gland, many other tissues express more than one similarly acting Bcl-2 family member at the same time (45,49–52). Relatively few physiological and pathological roles have been definitively assigned to specific bcl-2 family members. Nevertheless, the combination of expression studies, germline loss of function, tissue specific loss of function, and transgenic dominant gain of function experiments provides some insight into the normal and abnormal functions of potentially redundant apoptotic regulators (Table I).

Germ-line loss of function experiments have identified tissues which require a particular family member for normal development. Murine phenotypes resulting from germ-line deletions of bcl-x, bcl-2, bax and bcl-w are reported. Loss of Bcl-x function is embryonic lethal at day 13 due to massive cell death in differentiating neurons and hemopoetic cells (53,54). In contrast, loss of Bcl-2 function yields viable mice with reduced numbers of lymphocytes, oocytes, and neurons, polycystic kidneys, hair hypopigmentation, and growth

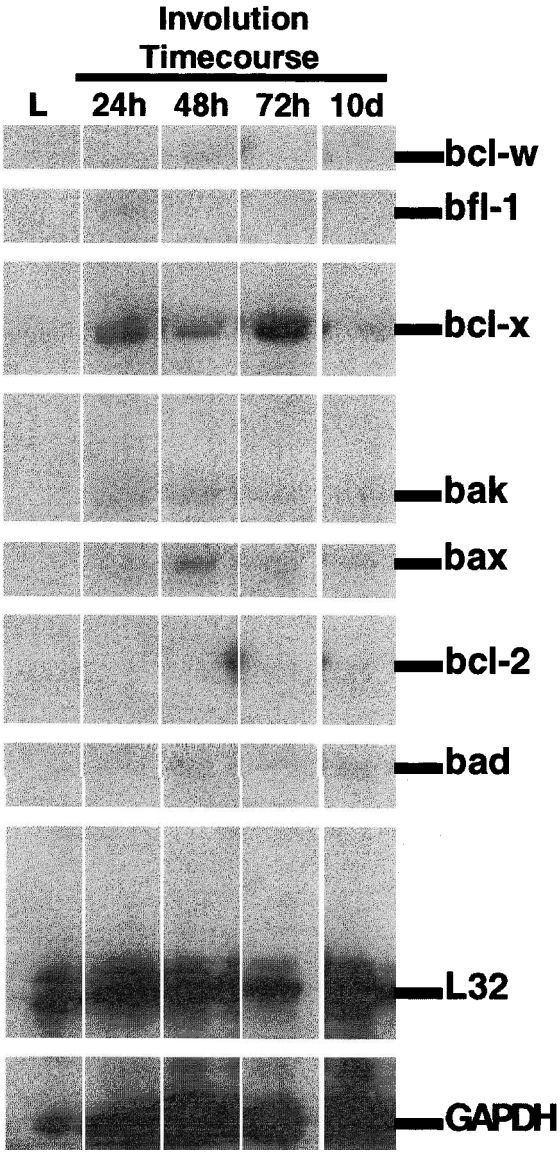

Fig. 3. Steady state RNA expression levels of bcl-2 family members bcl-w, bfl-1. bcl-x, bak, bax, bcl-2 and bad at ten days lactation and during the course of normal involution in C57bl/6 mice. RNase protection assay demonstrates that expression of bcl-w, bfl-1, bcl-x, bak, bax and bad is increased with the onset of the first stage of involution. Expression of bcl-w, bfl-1, bcl-x and bak rises first. Bax and bad RNA levels increase slightly later. Expression is down regulated by ten days involution. Bcl-x and bax are the most highly expressed family members. Significantly, in contrast to other family members, bcl-2 is not expressed during lactation or early involution but is expressed at ten days involution. Multi-probe RNase protection assays (Pharmingen, San Diego, California) were performed on 10 micrograms of RNA from each timepoint. L=Lactation day ten. Involution timecourse began when pups were removed at ten days lactation. 1d = 1 day after pup removal; 2d = 2 days after pup removal; 3d = 3 days after pup removal; 10d = 10 days after pup removal. Loading controls were L32 (ribosomal structural protein) and GAPDH (glyceraldehyde phosphate dehydrogenase).

retardation (55–60). Loss of Bax function results in viable mice with increased numbers of thymocytes, granulosa cells, and neurons (61–63). Male mice exhibit testicular atrophy (63). Loss of Bcl-w function in male mice produces testicular degeneration with disorganized seminiferous tubules and germ cell depletion (64,65).

Study of mammary gland function in the absence of Bcl-2, Bax, and Bcl-w is possible since all three loss of function models are viable. Published studies indicate that lactation is normal in mice who do not express any functional Bcl-2 but little information is available on involution in these mice. An examination of lactation and involution in the absence of Bax function was completed recently (66). The absence of Bax reduced the incidence of apoptosis during the first, locally stimulated, stage of involution. This suggested that Bax up-regulation during the first stage of involution is physiologically relevant. The lack of complete compensation from the other pro-apoptotic genes expressed, Bad and Bak, indicated that they are not completely functionally redundant. Rather, each family member expressed may play a specific role in moderating cell survival. When Bax was absent, increased cell death during the second proteinase mediated stage of involution compensated for the low rate of apoptosis during the first stage. This resulted in a normally remodeled tissue at day 10 involution. (see Fig. 5) There are possible effects on lactation. Most mice that did not express any Bax lactated normally. However, there was a higher percentage of lactation failure immediately following delivery in mice carrying homozygous deletions of the bax gene compared to either wild-type mice or mice carrying a heterozygous deletion of the bax gene (Li and Furth, unpublished observation.) Further study will be required to evaluate the significance of this observation, and to determine if it is secondary to direct effects on mammary development or indirect systemic actions.

Mammary specific deletion of bcl-x is being used currently to determine the role of this embryonic lethal gene in normal mammary gland development and involution (67) (Rucker, Wagner, and Hennighausen, personal communication). The same approach will be useful for evaluating the function of other embryonic lethal genes in the mammary gland.

Dominant gain of function experiments also provide information on relative roles of different family members in normal physiology and disease. In lymphoid tissue, overexpression of Bcl-2 led to follicular B-cell hyperplasia and progression to lymphoma

Fig. 4. Immunohistochemical localization of bcl-2 family member proteins bcl-x (A–D), bak (E–H), and Bax (J–M) as well as the bcl-2 binding protein Bag-1 (N-Q) at ten days lactation and during the course of involution in Sv129 mice. Bcl-x, Bak, Bax, and Bag-1 are expressed in mammary epithelial cells. Expression of Bcl-x, Bak, and Bax is localized predominantly to the cytoplasm. Nuclear localization of Bag-1 expression during lactation and the first stage is seen. Protein expression levels of Bcl-x and Bak appear highest at 1 day involution. Bax protein expression is increased though day 3 involution. Prominent nuclear localization of Bag-1 is observed from lactation through the first stage of involution. Involution timecourse began when pups were removed at ten days lactation. Thin arrows in Panel B point to apoptotic mammary epithelial cells shed into the lumen. Thicker arrows in Panels N, O and P point to cells exhibiting nuclear localization of Bag-1. (See 91, 92 for methodology).

(4). In contrast, in skin epidermis, Bcl-2 overexpression resulted in hyperplasia that did not progress to cancer unless the mice were exposed to chemical carcinogens (68). In the pancreas, bcl-x_L over-expression by itself did not produce either hyperplasia or cancer but did accelerate tumorigenesis when co-expressed with the TAg viral oncoprotein (69).

Dominant gain of Bcl-2 function in mammary epithelial cells did not induce either hyperplasia or cancer by itself (70). However, it did accelerate tumor presentation when the gene was co-expressed with either the c-myc oncogene or the TAg oncoprotein in mouse models of breast cancer (33,70). Tumor progression in both of these mouse models is marked by competition between cell proliferation and apoptosis and a reduced rate of apoptosis is likely one reason for the earlier tumor appearance. One can speculate that Bcl-2 overexpression may have also promoted earliest stages of cancer progression by increasing survival of cells containing genetic mutations. Significantly, in the DMBA mouse model, Bcl-2 expression delayed tumor appearance (34). As discussed earlier,

Table I. Effect of Loss of Function or Dominant Gain of Function of Bcl-2, Bcl-x, Bax, and Bcl-w in Mice

Bcl-2 family member	Function	Germline deletion		Dominant gain	
		Developmental	Cancer progression	Developmental	Cancer progression
Bcl-2	Anti-apoptotic	Viable. Increased apoptosis of lymphocytes, decreased number of oocytes, postnatal degeneration of motoneurons, sensory and sympathetic neurons, polycystic kidneys, hypopigmentation of hair, growth retardation (55–60).		Lymphoid cells: Increased survival, polyclonal follicular lymphoproliferation (4, 11, 83). Neurons: Increased neuronal survival or decreased apoptosis (86–88) Mammary epithelial cells: Decreased apoptosis during involution (70).	Lymphoid cells: Lymphoma (4). Mammary epithelial cells: Accelerates progression of c-*myc* induced mammary cancer (70).
Bcl-x	Anti-apoptotic (bcl-x_L) and pro-apoptotic (bcl-x_S)	Embryonic lethal day 13 due to massive cell death in differentiating neurons and hemopoietic cells (53,54).		Lymphoid cells: increased survival (69,89,90)	Pancreatic acinar cells: Accelerates progression of TAg induced pancreatic cancer (69).
Bax	Pro-apoptotic	Viable. Thymocyte hyperplasia, excess granulosa cells, atrophic testes, increased survival of motoneurons and sympathetic neurons (61–63).	Retards progression of TAg induced choroid plexus cancer (84)	Retinal ganglion cells and hippocampal pyramidal cells: No change (85)	
Bcl-w	Anti-apoptotic	Viable. Testicular degeneration, disorganized seminiferous tubules, severely depleted germ cells (64,65).			

in this model inhibitory influences on cell proliferation dominate and there is little effect on apoptosis in the tumors. The divergent results in the different mouse models are likely due to the specific signaling pathways activated during carcinogenesis and may offer clues to understanding phenotypic variability in human cancer.

During mammary gland involution, gain of Bcl-2 function decreased the incidence of apoptosis during the first stage (70). Interestingly, gain of Bcl-2 function was much more effective in promoting mammary epithelial cell survival than loss of Bax function when the two were compared directly (66). In contrast to loss of Bax which had no effect on mammary epithelial cell survival during the second stage of involution, gain of Bcl-2 function increased the number of epithelial cells remaining at the end of the second stage, consistent with its more pronounced effect on mammary epithelial cell survival than loss of Bax. Bcl-2 is also able to inhibit apoptosis of mammary epithelial cells induced by several different stimuli. In separate apoptosis triggered by expression of the SV40 TAg as well as p53 during late pregnancy was reduced (33,34).

FUNCTIONAL IMPLICATIONS OF Bcl-2 FAMILY MEMBERS AND RELATED BINDING PROTEINS IN PROGRESSION OF HUMAN BREAST CANCERS AND RESPONSE TO THERAPY

Bcl-2, Bax, Bcl-x, Bak, and Bag-1 are expressed in human breast cancers (39,40,44,51). Expression of bcl-2 is sensitive to estrogen (71,72) and its presence in most breast cancers is highly correlated with estrogen receptor positivity (40). The association of Bcl-2 with a good prognosis in breast cancer has been proposed to be linked to the ability of estrogen receptor antagonists

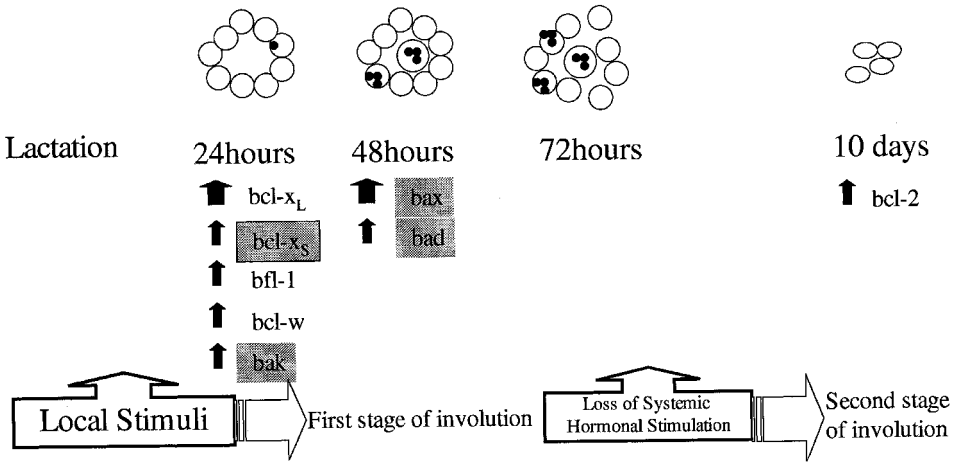

Fig. 5. Schematic diagram illustrating how bcl-2 family members may regulate the rate of cell death during mammary gland involution. Both death (in shaded boxes) and survival inducing family members are expressed during the first stage of involution. Expression of the bcl-x_L survival factor may preserve the gland during the early phase of "reversible involution". Apoptosis becomes more frequent as expression of the death inducing factors bax and bad rise at 48 hours involution. Expression of bcl-2 in mammary epithelial cells during the second stage of involution which ends at day 10 may help protect cells from death during proteinase mediated tissue remodeling. The first stage of involution is induced by local factors and lasts approximately 72 hours. Onset of the second stage of involution is triggered by loss of systemic lactogenic hormone stimulation, begins around 72–92 hours after suckling is discontinued, and is typified by tissue proteinase mediated tissue remodeling. Alveolar structures composed of mammary epithelial cells are diagrammed at 24, 48, and 72 hours involution. Cells undergoing apoptosis are indicated by the presence of small black circles which represent degenerating apoptotic nuclei. Alveolar structures are gone at 10 days involution. Bcl-2 family member survival factors include bcl-x_L, bfl-1, bcl-w, and bcl-2. Bcl-2 family member death factors include bcl-x_S, bax, and bad.

such as tamoxifen to down-regulate its expression and thereby promote apoptosis of the malignant cell (73,74). However, one can speculate that the actions of Bcl-2 on cell cycle progression also may be directly associated with good prognosis (34). Additional experimental work will hopefully reveal the molecular pathways involved.

Alterations in the relative expression levels of individual bcl-2 family members appear to influence breast cancer progression (75,76). Decreased expression of bax was correlated with poor clinical outcome in some circumstances (39,77). Increased levels of Bax or Bcl-x_S were observed to sensitize breast cancer cells to chemotherapy (78). Gene transfection experiments in breast cancer cell lines have provided evidence that those elevations in Bax protein increase the incidence of apoptosis (79–81) and delay tumor progression (82). However, studies focusing on a single family member may not reveal phenotypes due to interactions between family members and more studies are needed in which expression levels of several different family members are examined or varied simultaneously. Finally, the question of how different expression levels of Bcl-2 family members affect breast cancer by promoting either survival or death of those first mutated cells has not been conclusively investigated.

CONCLUSIONS

Bcl-2 family members are important regulators of apoptosis. Several bcl-2 family members and related proteins are expressed in mammary epithelial cells and expression levels are regulated during pregnancy, lactation, and involution. Expression levels of several family members are altered in breast cancer cells and the relative amounts of survival and death factors have been related to responses to chemotherapy and overall prognosis.

Information derived from studying mammary epithelial cell survival during normal involution may be translatable to the treatment of breast cancer. As key regulators of cell survival, bcl-2 family members and the signaling pathways they govern represent attractive

therapeutic targets. Investigation of the specific roles of individual family members in cancer progression and response to therapy likely will be useful for focusing efforts on the most critical family members. Increased understanding of their transcriptional regulation, intracellular control, and mechanisms of action of Bcl-2 family proteins hopefully will accelerate development of novel therapies targeted against this gene family of cell survival regulating genes. Agricultural scientists have a direct interest in promoting mammary epithelial cell survival during lactation. Extension of effective lactation in dairy cows has direct financial benefits for the modern farmer. It would be interesting to know if manipulation of the expression levels of different Bcl-2 family members could extend lactation by promoting survival of milk producing mammary epithelial cells under different conditions. But the key to translating observations from basic science studies to the clinic or the barn will be a thorough understanding of their significance. Model systems may be used to demonstrate potential roles and elucidate mechanisms but they also can provide over-simplified explanations of complicated physiological problems or disease processes. A combination of careful in vivo studies in mammary cell systems and animal models coupled with meticulous clinical and agricultural investigations will provide the best basis for realizing potential applications.

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