Apoptosis in mammary gland and cancer

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Abstract

Homeostasis in normal tissue is regulated by a balance between proliferative activity and cell loss by apoptosis. Apoptosis is a physiological mechanism of cell loss that depends on both pre-existing proteins and de novo protein synthesis, and the process of apoptosis is integral to normal mammary gland development and in many diseases, including breast cancer. The mammary gland is one of the few organ systems in mammals that completes its morphologic development postnatally during two discrete physiologic states, puberty and pregnancy. The susceptibility of the mammary gland to tumorigenesis is influenced by its normal development, particularly during stages of puberty and pregnancy that are characterized by marked alterations in breast cell proliferation and differentiation. Numerous epidemiologic studies have suggested that specific details in the development of the mammary gland play a critical role in breast cancer risk. Mammary gland development is characterized by dynamic changes in the expression profiles of Bcl-2 family members. The expression of Bcl-2 family proteins in breast cancer is also influenced by estradiol and by progestin. Since the ratio of proapoptotic to antiapoptotic proteins determines apoptosis or cell survival, hormone levels may have important implications in the therapeutic prevention of breast cancer.

Introduction

Breast cancer is the leading type of cancer in women and is the second leading cause (after lung cancer) of cancer death among women. In the United States in 1998, an estimated 178,700 new cases of breast cancer were expected to be diagnosed, and 43,500 women were expected to die of this disease. Approximately 2,000,000 women have been diagnosed with breast cancer (NCI statistics 1998). Breast cancer also occurs in men, although far more rarely than in women (approximately 1,600 cases in men were diagnosed in 1998); treatment for breast cancer in men is guided by our understanding of the disease in women.

Our limited understanding of the biology and developmental genetics of the normal mammary gland has been a barrier to progress in treating breast cancer. Much of the biological research in the recent past has focused on understanding the initiation and development of the disease. Data from the emerging models suggest that we need to better understand crucial signaling components in the normal mammary gland before any beneficial impact on the prevention and treatment of breast cancer can be expected.

In this review, we summarize some of the data from the last 10 years about the role, the expression, and the regulation of apoptosis regulatory proteins (Bcl-2 family proteins, caspases, Fas ligand, and transcription factors) in normal mammary gland development and carcinogenesis. Because of the nature and depth of the information available on this subject, we could only include representative studies, and we apologize to those whose work could not be covered. Extensive information is, however, available in earlier reviews (Carson & Ribeiro 1993, Williams & Smith 1993, Reed 1994, Steller 1995, Medina 1996, Baik et al. 1998, Benz 1998, Krajewski et al. 1999, Schorr et al. 1999).

Apoptosis

Homeostasis in normal tissue is regulated by a balance between proliferative activity and cell loss by apoptosis. Apoptosis is a physiological mechanism of cell loss that depends on both pre-existing proteins and de novo protein synthesis (Williams & Smith 1993, Steller 1995, Thompson 1995), and the process of apoptosis is integral to normal mammary gland development (Medina 1996). Apoptosis plays an important role during development, metamorphosis, and organ involution and in many diseases, including cancer (Steller 1995, Thompson 1995). Apoptosis is characterized by nuclear condensation and fragmentation and by degradation of DNA into oligonucleosome fragments (Thompson 1995).

The results of some studies suggest that cells derived from our human cancers have a decreased capacity to...
undergo apoptosis in response to various physiological stimuli (Carson & Ribeiro 1993, Thompson 1995). Thus, a defect in apoptosis may be involved in aberrant survival, the development of breast cancer or both, and deregulation of apoptosis may be involved in the development of breast cancer by the enhancement of cell survival and development of drug resistance by breast cancer cells.

Apoptosis is a highly regulated process. One important regulator of apoptosis is Bcl-2, a 26 kDa protein that protects cells against apoptosis in a variety of experimental systems (Reed 1994). Bcl-2 protein is primarily localized to the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membranes (Krajewski et al. 1993, Lithgow et al. 1994).

Apoptosis is also regulated by a number of genes, including Bcl-2 and related family members (such as Bcl-x, Bax, and Bad), which have significant structural homology with the Bcl-2 gene (Hockenbery et al. 1991, Korsmeyer 1992, Boise et al. 1993, Oltvai et al. 1993). The Bcl-x gene gives rise to two distinct mRNAs by differential splicing that encodes Bcl-xL and Bcl-xS proteins. Bcl-xL is related to Bcl-2 in inhibiting apoptosis. In contrast, Bcl-xS is a dominant negative inhibitor of both Bcl-2 and Bcl-xL. Different members of the Bcl-2 family have been shown to form homo- and heterodimers and it appears that the ratio of Bcl-2 to Bax, other family members, or both may play a regulatory role in apoptosis. It has been proposed that some of these interactions may be tissue specific (Sedlak et al. 1995).

Apoptosis plays an important role during the involution of the mammary gland. The 6-fold increase in the ratio of Bcl-xS to Bcl-xL during the first 2 days of involution suggests that apoptotic proteins play a role in predisposing mammary alveolar cells to cell death immediately after lactation.

**Mammary gland development**

The mammary gland is one of the few organ systems in mammals that completes its morphologic development postnatally during two discrete physiologic states, puberty and pregnancy. Thus the mammary gland is an excellent model for studying normal morphologic development and the early steps of tumor formation (Nandi 1959, Medina 1996).

Mammary gland growth and maturation consist of a series of highly ordered events involving interactions among several distinct cell types. These are regulated by complex interactions among many steroid hormones and growth factors. In 4-week-old mice, the mammary glands become increasingly sensitized to elevations in ovarian hormones, which signal the terminal end buds (club-shaped epithelial structures) to grow away from the nipple region to fill the fat pad. During this rapid but tightly regulated growth phase, an extensive network of epithelial ductal, tree-like branching develops. When the expanding mammary ductal mass reaches the limits of its fat pad, the terminal end bud structures are permanently replaced by mitotically quiescent terminal end ducts and alveolar buds (Fig. 1). At the onset of pregnancy, rapid epithelial-cell proliferation begins again, resulting in additional ductal branching and lobuloalveolar growth from the ductal skeleton. These alveoli are the functional units of milk production at lactation, the time at which the gland is fully differentiated. After lactation ceases, the gland undergoes massive restructuring and apoptosis, leading to involution and return of the primary ductal structures (Nandi 1959, Medina 1996, Daniels & Siberstein 1987).

In the normal mammary tissue in male mice, the mesenchyme condenses around the center of the mammary

**Figure 1** Schematic representation of various stages of mammary gland development.
bud at days 13–15 of gestation, and the cells of the cord die. Thus, a small cord of epithelial cells is detached from the skin and the mammary gland does not extend to the surface. No further development occurs. Apoptosis in the mammary cord of males has been shown to be induced by testosterone secretion, which targets the mesenchyme to destroy the epithelial cells (Durnberger & Kratochwil 1980).

**Mammary gland carcinogenesis**

The susceptibility of the mammary gland to tumorigenesis is influenced by its normal development, particularly during stages of puberty and pregnancy that are characterized by marked alterations in breast cell proliferation and differentiation. Numerous epidemiologic studies have suggested that specific details in the development of the mammary gland play a critical role in breast cancer risk (Kelsy & Gammon 1990).

Some of the risk factors for breast cancer include nulliparity or first full-term pregnancy after age 30, early menarche, late menopause, and a family history of breast cancer (Rondinelli et al. 1995). In contrast, early parity, late menarche, early menopause, and long periods of lactation provide protective effects against breast cancer (Russo & Russo 1980, 1982, Henderson et al. 1993). Therefore, systemic endocrine patterns and reproductive changes that occur in the human breast have important implications for breast cancer.

The observation that the same endocrine events control mammary development and influence breast cancer risk strengthens the hypothesis that mammary gland development and carcinogenesis are fundamentally linked. In addition, it is believed that the pluripotent cap cells play a key role in mammary gland carcinogenesis. For example, exposure of early-stage rat mammary glands to carcinogens resulted in significant mutations associated with malignant transformation and increased the susceptibility to tumor formation (Russo & Russo 1980, 1982). These hormonal risk factors provide clues for target-cell predisposition to breast cancer.

A balance between proliferation, differentiation, and death in the stem-cell population and throughout the cells of the mammary gland is critical for normal development. Perturbations in this balance can contribute to cancer development. Conditions that upregulate cell proliferation or downregulate apoptosis can allow the accumulation of mutations that contribute to the subsequent development of breast cancer. It is not clear, however, how normal mechanisms and signaling pathways controlling growth and apoptosis in the human breast act in the development of tumors, in the protection from tumor development, or in tumor dissemination.

Involution, an essential component of the mammary gland remodeling program, has been extensively characterized by gene products which are conditionally switched on or off during this phase of the mammary gland development. After weaning of the pups, the mammary gland involutes and proceeds through a rapid remodeling process that reduces the tissue to a state resembling the mature virgin gland. Some of the associated changes include the loss of secretory alveolar cells, the proteolytic degradation of the extracellular matrix, and the disappearance of lobuloalveolar structures. The majority of cells that die during involution undergo apoptosis (Guenet et al. 1994, Strange et al. 1995), being characterized by cellular condensation and nuclear fragmentation. They are eventually engulfed by macrophages. The transition from lactation to involution is accompanied by the proteolytic degradation of the supporting basement membrane and the systemic reduction in hormone levels (Strange et al. 1995, Lund et al. 1996). The rapid inactivation of milk-protein genes, mild ischemia, and increased intraluminal pressure are other phenotypic changes that could potentially participate in the induction of cell death and tissue remodeling (Richards & Benson 1971, Schmitt-Ney et al. 1992a,b).

**Bcl-2 family members in mammary gland apoptosis**

The Bcl-2 (B-cell leukemia/lymphoma 2) gene was first identified at the breakpoint of a chromosomal translocation t(14:18) in B- follicular lymphoma (Tsujimoto et al. 1985). It encodes a 26 kDa protein that protects cells against apoptosis. Overexpression of Bcl-2 has been shown to suppress the initiation of apoptosis in response to a number of stimuli, including anticancer drugs in a number of systems (Sentman et al. 1991, Hickman 1992, Dole et al. 1994, Teixeira et al. 1995). Furthermore, inhibition of Bcl-2 expression by antisense oligonucleotide (Teixeira et al. 1995), and dominant negative inhibitor Bcl-x<sub>s</sub> (Sumantran et al. 1995) has been shown to promote apoptosis and sensitize cells to chemotherapy-induced apoptosis.

Mammary gland development is characterized by dynamic changes in the expression profiles (both mRNA and protein) of Bcl-2 family members (Table 1). For example, Bax and Bcl-x are expressed evenly throughout mammary gland development. However, the levels of expression of Bak and Bad increase during late pregnancy and lactation, and these remain expressed at the time of maximal apoptotic involution. In contrast, Bcl-w (a new cell-death-suppressor member of Bcl2 family) was expressed in pregnancy and lactation but was downregulated at the onset of apoptosis. Bcl-2 is expressed in the mammary gland of the non-pregnant female and during early pregnancy but was undetectable in the lactating or early-involuting mammary gland. In contrast, the expression of Bcl-x<sub>s</sub>, Bcl-x<sub>l</sub>, and Bax continues through late pregnancy, is downregulated during lactation, and is upregulated with the start of involution.
### Table 1: Genes reportedly involved in mouse mammary gland involution.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Presumptive time of action</th>
<th>Identified aberrations during the mammary gland involution</th>
<th>References containing or citing evidence for roles in involution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bcl-2 family</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Bax</td>
<td>1st day</td>
<td>up</td>
<td>Heermeier et al. (1996)</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>first 2 days</td>
<td>up</td>
<td>Knudson et al. (1995)</td>
</tr>
<tr>
<td>Bak</td>
<td>1st stage</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td>Bad</td>
<td>1st stage</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td>Bcl-w</td>
<td>1st stage</td>
<td>down</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td></td>
<td>undetectable</td>
<td>Schorr et al. (1999)</td>
</tr>
<tr>
<td>Bfl-1</td>
<td></td>
<td>up</td>
<td>Jager et al. (1997)</td>
</tr>
<tr>
<td>Bcl-xS/xL</td>
<td>first 2 days</td>
<td>up</td>
<td>Heermeier et al. (1996)</td>
</tr>
<tr>
<td><strong>Mitochondrial genes</strong></td>
<td></td>
<td></td>
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<tr>
<td>Lactoferrin</td>
<td>days 1, 2 and 3</td>
<td>up</td>
<td>Lee et al. (1996)</td>
</tr>
<tr>
<td>Ferritin heavy chain</td>
<td>days 1, 2, 3 and 4</td>
<td>up</td>
<td></td>
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<tr>
<td>Cytochrome oxidase</td>
<td>days 4 and 7</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td>Subunit 1 and 2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cytochrome b</td>
<td>day 7</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td><strong>Transcription factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td></td>
<td>up</td>
<td>Chapman et al. (1999)</td>
</tr>
<tr>
<td>STAT5</td>
<td></td>
<td>down</td>
<td></td>
</tr>
<tr>
<td>STAT1</td>
<td></td>
<td>up, in absence of STAT3</td>
<td>Streuli et al. (1995)</td>
</tr>
<tr>
<td>p53</td>
<td>1st stage</td>
<td>no role</td>
<td>Li et al. (1996)</td>
</tr>
<tr>
<td>NF-1</td>
<td>2nd stage</td>
<td>up (repressor role)</td>
<td>Furlong et al. (1996)</td>
</tr>
<tr>
<td>NF-kB</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>STAT5A</td>
<td></td>
<td></td>
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<tr>
<td>ESX</td>
<td>prolonged</td>
<td>up</td>
<td>Clarkeston et al. (2000)</td>
</tr>
<tr>
<td>PKA</td>
<td>1st and 2nd</td>
<td>up</td>
<td>Humphreys &amp; Hennighausen (1999)</td>
</tr>
<tr>
<td>AP1</td>
<td>1st and 2nd</td>
<td>up</td>
<td>Marti et al. (1999)</td>
</tr>
<tr>
<td>JNK</td>
<td>1st and 2nd</td>
<td>up</td>
<td></td>
</tr>
<tr>
<td>Oct-1</td>
<td>days 1, 2, 3 and 4</td>
<td>down</td>
<td>Marti et al. (1999)</td>
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<tr>
<td><strong>Tyrosine phosphatases</strong></td>
<td></td>
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<tr>
<td>PTPs</td>
<td>prolonged</td>
<td>up</td>
<td>Aoki et al. (1999)</td>
</tr>
<tr>
<td>LAR</td>
<td></td>
<td></td>
<td>Schaapveld et al. (1997)</td>
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</table>

(Heermeier et al. 1996). Bak, Bad, Bcl-w, and Bfl-1 are also upregulated during involution (Schorr et al. 1999a). These results support a model in which mammary epithelial cells are primed for apoptosis during the transition from pregnancy to lactation by the de novo expression of the death-effectors Bak and Bad. It has been suggested that the anti-apoptotic Bcl-2 family proteins prevent the apoptotic function of Bak and Bad until involution, when the levels of Bcl-w decline (Metcalfe et al. 1999).

The Bcl-2 gene product represents the prototype of the antiapoptotic family of genes that regulate apoptosis (Hockenbery et al. 1990, Oltvai et al. 1993). Overexpression of Bcl-2 inhibits alveolar-cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice (Jager et al. 1997). Bcl-2 gain of function mutation reduces apoptosis 50% during the first stage of involution and increases cell survival 70% during the second stage of involution. Bcl-2 function has been shown to have a greater effect on the regulation of mammary epithelial-cell survival in vivo than the loss of Bax function (Schorr et al. 1999). Deletion of the Bcl-2 gene from the genome had no overt effects on embryogenesis and mammary gland development, indicating that Bcl-2 does not play a dominant role in mammary gland remodeling (Veis et al. 1993). On the other hand, mice lacking functional Bcl-xL died during embryogenesis, and massive cell death was observed in their nervous systems.

The expression of prosapoptotic Bcl-xL and Bax, unlike that of Bcl-2, has been shown to increase within the first day after weaning, coinciding with the onset of apoptosis (Heermeier et al. 1996). These events are associated with a decrease in the expression of milk proteins, a reduction in...
prolactin signal transduction through Stat5a and 5b, and the activation of STAT3 (Li et al. 1997).

The rise in the levels of Bax during the first day of involution does not depend on the presence or absence of p53. In one study, the Bax protein was detected after weaning in increasing numbers of cells, peaking at day 3 and decreasing thereafter. Bax-deficient mice displayed hyperplasia in their lymphoid system, showed enhanced cell death in their reproductive organs (Knudson et al. 1995) and exhibited aberrant mammary gland development (Shibata et al. 1999).

Bcl-x is another homolog of Bcl-2 and exists in two isoforms, Bcl-xL and Bcl-xS. The Bcl-xS splice variant of Bcl-x was shown to promote cell death, and Bcl-xL was shown to have a protective function. Low levels of Bcl-xS mRNA and protein, present during lactation even in the absence of detectable apoptosis, are followed by sharp increases during the first 2 days of involution. The ratio of Bcl-xS to Bcl-xL remained stable in the virgin, pregnant, and lactating gland. However, during the first 2 days of involution, Bcl-xS expression increased 6-fold more than Bcl-xL (Heermeier et al. 1996).

Mitochondrial genes in mammary gland apoptosis

By using partial sequence analysis, Lee and associates determined that expression of iron metabolism-related and mitochondrial genes was induced during mammary gland involution (Lee et al. 1996). The expression of: the lactoferrin gene was induced at involution days 1, 2, and 3; the ferritin heavy chain gene at involution days 1, 2, 3 and 4; the cytochrome oxidase subunit 1 and 2 genes at involution days 4 and 7; and the cytochrome b gene at involution day 7. These results imply that iron metabolism and mitochondrial function may be altered during mammary gland involution (Lee et al. 1996).

Transcription factors in mammary gland apoptosis


STAT3 is a well-studied transcription factor during mammary gland involution. Recent studies suggest that insulin-like growth factor 1 (IGF-I)-binding protein (IGFBP)-5 may be a downstream target of STAT3 during involution (Chapman et al. 1999). Since epithelial cells synthesize and secrete high levels of IGFBP-5, it has potential to induce apoptosis by sequestering IGF-I to casein micelles, thus preventing it from binding to its receptor. Stat5 activity, in contrast to that of STAT3, is downregulated at the start of involution, a phenomenon that appears to be required for transduction of survival signals from the extracellular matrix (Streuli et al. 1995). The physiologic significance of Stats during apoptosis was established by studies with STAT3-null mammary gland; these studies showed Stat1 activation and increases in the levels of p53 and p21, which may act as compensatory mechanisms for the eventual initiation of involution (Chapman et al. 1999). There were no marked differences in the regulation of Stat5, Bcl-xS, or Bax in the absence of STAT3.

The p53 has an established role in apoptosis, but its participation in mammary gland involution remains unclear. In one study, the absence of p53 in p53 knockout mice had no apparent effect on involution (Li et al. 1996). These investigators examined the process of involution in the presence and absence of functional p53 in different mouse models: wild-type mice, transgenic mice that express simian virus 40 (SV40) T-antigen specifically in mammary tissue during pregnancy; and mice that carry non-functional p53 alleles in their germ line. The authors concluded that involution and remodeling, with the concomitant disappearance of the lobuloalveolar structures, proceeded normally in the absence of functional p53. In addition, the loss of p53 did not affect the expression of Bax or the ratio of RNAs encoding Bcl-xS to Bcl-xL (Li et al. 1996). However, investigators using p53−/− mice reported that there was a delay in mammary gland involution (Jerry et al. 1999). Thus, involution can be viewed as a biphasic phenomenon in which initial responses are sensitive to p53 whereas secondary responses are p53-independent (Jerry et al. 1999). A well-characterized p53 target is p21, an inhibitor of cyclin-dependent kinase, which may be required for the induction of apoptosis (Duttaroy et al. 1997). Similar results were obtained when a region of SV40 was expressed under the control of the whey acidic milk-protein promoter (WAP) in mammary gland epithelial cells (Tzeng et al. 1998). The authors reported that SV40T−/−-antigen synthesis causes premature mammary gland involution during late pregnancy by inducing apoptosis and leads to the development of mammary tumors after the first lactation period in both p53-positive (WAP-SV-T/t) and p53-negative double-transgenic animals (WAP-SV-T/t.p53−/−) (Tzeng et al. 1998).

The nuclear factor 1 (NF1) family of transcription factors has been shown to be critical for the tissue-specific transcription driving epithelial cell-specific milk-protein expression in the mammary gland, of a range of genes selectively expressed in terminal differentiation. (Marti et al. 1999). By using biochemical assays, Furlong and associates detected that induction of a 74 kDa NF1 protein was induced in mouse mammary gland during involution and not during...
lactation, as was the case for other NF1 proteins, of 110, 68, and 46 kDa (Furlong et al. 1996).

**Pathways upregulated during mammary gland involution**

Until recently, it was accepted that two series of events regulate distinct stages of mammary involution: the progressive gain of death signals and the loss of survival factors. An emerging view, however, suggests that mammary involution also involves the activation of specific survival factors. For example, a stage-specific pattern of NF-KB activation was observed in the mammary gland during postlactational involution, a period of extensive apoptosis of luminal epithelial cells (Clarkson et al. 2000). The results of that study suggested that NF-KB performs a homeostatic function during involution since its activity is maximally induced at the onset of the second phase of involution when proteases disrupt cell-extracellular matrix interactions. In another study, Stat5A activation in the mammary gland epithelium antagonized regression and involution-mediated apoptosis (Humphreys & Hennighausen 1999). The results of that study suggested that Stat5A acts as an antecedent, acting molecules that initiate the process of epithelial regression and reorganization during involution.

The epithelial restricted with serine box (ESX), a novel member of the erythroblast transformation specific domain (ETS) family of transcription factors is another molecule that has been shown to be maximally expressed during involuting mouse and rat mammary glands. The prolonged elevation of ESX levels in fully regressed mammary glands suggests that ESX expression can be induced by soluble growth factors and maximally upregulated in the epithelial cells destined to survive both the apoptotic and remodeling phases of glandular involution (Marti et al. 1999).

Nuclear activation of protein kinase A and transcription factor-activator protein 1 (AP1) also precede the irreversible phase of involution that is characterized by internucleosomal DNA fragmentation (Jaggi et al. 1996). Activation of AP1 and fragmentation of chromosomal DNA can be prevented by lactogenic hormone treatment in explant cultures derived from mammary tissue at lactation. The authors showed that the elevation in AP1 coincides with the epithelial expression of sulfated glycoprotein 2, a potential target gene of AP1 (Jaggi et al. 1996). In another study, Marti and associates described signal-transduction events, such as activation of protein kinase A, Jun-N-terminal kinase (JNK) and AP1 during the early and late phases of mammary gland involution, and suggested that this pathway plays a role in regulating and coordinating involution with an emphasis on the apoptotic process of involution (Marti et al. 1999). Elevated nuclear protein kinase A (PKA) activity was observed from postlactation day 1, paralleled by increasing levels of components of the AP1 complex. AP1 DNA binding activity was transiently induced, and the AP-1 complex was composed principally of cFos/JunD. In contrast, both the Oct-1 DNA binding activity and the Oct-1 protein were gradually reduced during the first 4 days of involution, although Oct-1 mRNA levels remained unchanged. Comparing nuclear extracts from normal mammary glands with nuclear extracts from glands which 3 weeks after birth had been cleared of all epithelial cells revealed that PKA activation, AP1 induction, and Oct-1 inactivation depend on the presence of the epithelial compartment. The increased Fos/Jun expression and the inactivation of Oct-1 could be the consequences of the increased PKA activity (Marti et al. 1994).

**Tyrosine phosphatase in mammary gland development**

Recent studies have revealed that at the involution stage after weaning, most protein tyrosine phosphatases (PTPs) are upregulated, and their level of expression returned to almost virgin levels. Such upregulation was also induced by forced weaning in lactating mother mice. These results suggest that PTPs contribute to the development, involution, and remodeling of the mammary gland and may inhibit the expression levels of milk genes during lactation (Aoki et al. 1999). The results of another study showed that mammary glands of Leukocyte-common Antigen-Related Molecule (LAR) −/− females were incapable of delivering milk because of an impaired terminal differentiation of alveoli at late pregnancy (Schaapveld et al. 1997). As a result, the glands failed to switch to a lactational state and showed a rapid involution postpartum.

**Bcl-2 pathway in breast cancer cells and tumors**

Bcl-2 is an oncogene that contributes to malignancy by inhibiting apoptosis and thereby extending cell survival and is one of the most studied apoptotic genes in breast cancer (Table 2). Investigators analyzed apoptosis in untreated human ductal carcinoma in situ and adjacent invasive cancer and demonstrated that spontaneous apoptosis is lost concurrently with invasive transformation (Bodis et al. 1995). Breast epithelium is known to undergo periodic fluctuations in Bcl-2 levels and apoptosis during the menstrual cycle (Sabourin et al. 1994). The expression of Bcl-2 is known to fluctuate with the normal ovulatory cycle; its levels are modulated in a hormone-dependent manner within premenopausal breast ductal epithelial cells (Nathan et al. 1993, Sabourin et al. 1994). It has been demonstrated that the expression of Bcl-2 in human breast adenocarcinomas is significantly associated with hormone-receptor positivity and low histologic grade (Alsabe et al. 1996). Bcl-2 protein was detected in 80% of the breast
Table 2 Expression of Bcl-2 family genes in breast cancer cells and tumors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression status</th>
<th>Presumptive mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bag1</td>
<td>Over expressed in breast cancer cells and primary tumors</td>
<td>Regulation of Bcl-2 activity</td>
<td>Yang (1999)</td>
</tr>
<tr>
<td>Caspases</td>
<td>Increase in expression of caspases 3, 6, 8 and their inhibitors in invasive breast cancers Drugs NO, etoposide and flavopiridol activates caspases in cancer cells</td>
<td>Induction of apoptosis</td>
<td>Umansky et al. (2000) Krajewski et al. (1999)</td>
</tr>
<tr>
<td>FasL</td>
<td>Increase in expression of FasL in majority of breast tumors Fas positive tumors associated with metastatic lymph nodes and larger tumor size</td>
<td>Elimination of tumor infiltrating immune cells and facilitation of tissue destruction during invasion</td>
<td>Mottolese et al. (2000) Reimer et al. (2000)</td>
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cancers derived from women, and Bcl-2-positive patients had a better prognosis and survival rate than did Bcl-2-negative patients (reviewed in Krajewski et al. 1999). Bcl-2 and, to a lesser degree, Bax expression was inversely related to the distant metastases. A combined analysis of the ratio of Bcl-2 to Bax expression in relation to clinical outcome showed that the absence of both factors was more strongly associated with the development of distant metastases. These findings suggested that Bcl-2 and Bax apoptosis-related proteins could serve as good prognostic indicators in node-negative breast cancer patients, and their combined absence may imply a potential deregulation of the apoptotic process, which in turn could contribute to the biologic aggressiveness of the tumors (Kapranos et al. 1997). The suppression of p21WAF1 expression was proposed as one of the mechanisms by which Bcl-2 prevents apoptosis. A highly significant association was reported between reduced p21WAF1-protein expression and overexpression of Bcl-2 in tumors (Bukholm et al. 1997).

The expression of Bcl-2 family proteins in breast cancer is also influenced by estradiol and progestin (Kandouz et al. 1996). Since the ratio of proapoptotic to antiapoptotic proteins determines apoptosis or cell survival, hormone levels may have important implications in the therapeutic prevention of breast cancer (Krajewski et al. 1999). A strong, positive association was observed between Bcl-2 and estrogen receptor (ER) repression, and DNA aneuploidy. Eighty-five percent of Bcl-2-positive tumors were ER positive and 65% were aneuploid, while only 28% of Bcl-2-negative tumors were ER positive and 37% were aneuploid (Eissa et al. 1999). Estradiol markedly increased Bcl-2 protein content, in both short- and long-term treatments of MCF-7 cells, and anti-estrogens (4-hydroxytamoxifen and RU 58668) reversed this effect. Estrogen treatment had little effect on the Bax mRNA level. In addition, pretreatment with estradiol protected MCF-7 cells from apoptosis (Wang & Phang 1995). Estradiol regulation of Bcl-2 gene expression in breast cancer cells involves multiple enhancer elements, including Sp1 and CRE element, but does not require direct binding of the estrogen receptor with Bcl-2 promoter DNA (Dong et al. 1999). Anti-estrogens, such as tamoxifen, have been shown to induce apoptosis in breast cancer cells by downregulating Bcl-2. Tamoxifen did not, however, affect Bax, Bcl-xL, or p53 expression at the mRNA or protein levels (Zhang et al. 1997, 1999).

A number of mechanisms by which Bcl-2 exerts its positive growth-supporting effects and its anti-apoptotic function have been proposed. It has been shown that Bcl-2 plays a role in the regulation of cell division. High proliferation rates and high tumor grade (Bloom – Richardson grading scheme) have been shown to be strongly associated with the absence of Bcl-2 expression. The loss
of Bcl-2 expression was strongly correlated with increased apoptotic and necrotic cell death, high proliferation rates and high tumor grade (van Slooten et al. 1998). Other investigators showed that Bcl-2 overexpression prolongs cell-cycle progression by increasing doubling time (Knowlton et al. 1998). Decreased tumor proliferation may account for the association of Bcl-2 expression with a favorable outcome in breast cancer, even though Bcl-2 may mediate chemoresistance in some patients (Knowlton et al. 1998).

Bcl-2 has also been shown to work in combination with hypoxia to modulate vascular endothelial growth factor (VEGF) expression and the in vivo angiogenic response in doxorubicin-resistant MCF7 cells (Biroccio et al. 2000). Bcl-2 protein also contributes to oncogenesis; in cooperation with known oncogenes such as c-myc, ras, or viral genes, Bcl-2 can transform and immortalize cells. In animal models, Bcl-2-overexpressing clones induce a significantly higher number of experimental and spontaneous lung metastases than do the control transfectant clone (Del Bufalo et al. 1997).

Because overexpression of human epidermal growth factor receptor-2 (HER-2) in MCF-7 cells results in upregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL, deregulation of anti-apoptotic Bcl-2 and Bcl-xL may be associated with the enhanced survival of HER-2–overexpressing and ER-positive breast cancer cells (Kumar et al. 1996). In some breast cancer cells the overexpression of heregulin, a ligand of HER-3 and HER-4 receptors, has been shown to induce apoptosis, and heregulin overexpression has been shown to downregulate expression of Bcl-2 (Weinstein et al. 1998). Overexpression of Bcl-2 in the human breast cancer cell line (MCF-7ADR) enhances the cell line’s tumorigenicity and metastatic potential by inducing metastasis-associated properties such as increased secretion of the matrix metalloproteinase-9 (MMP-9) and enhances NF-B-dependent transcriptional activity (Ricca et al. 2000). Basic fibroblast growth factor (bFGF, FGF-2), a classical transforming factor and angiogenic factor in breast tumors, downregulates Bcl-2 expression, and promotes apoptosis in MCF-7 breast cancer cells (Wang et al. 1998, Maloof et al. 1999).

Bag-1 is an anti-apoptotic protein that binds to and enhances the anti-apoptotic activity of Bcl-2 (Takayama et al. 1995). Like Bcl-2, Bag-1 has been shown to interact with a number of signaling proteins and receptors, including Raf1 kinase (Wang et al. 1996), platelet-derived growth factor receptor, hepatocyte growth factor receptor (Bardelli et al. 1996), and nuclear hormone receptors (Prat & Toft 1997). Bag-1 mRNA and protein are overexpressed in human breast cancer cell lines. In one study, the expression of two isoforms of Bag-1, p46 and p33, was also much higher in breast primary tumors (Yang et al. 1999). Bag-1 expression has been detected in normal breast epithelial cells and most ER-positive tumors, but was undetectable or weakly expressed in ER-negative tumors. A correlation was also observed between ER and Bag-1 in breast cancer-derived cell lines (Brimmel et al. 1999). Immunolocalization studies with an antibody that recognizes all three isoforms of Bag-1 revealed that Bag-1 is expressed in the cytosol and nuclei of normal mammary epithelial cells but was found predominantly in the cytosol in invasive carcinomas (Turner et al. 1997). Bag-1 was shown to regulate retinoid activities through its interactions with retinoic acid receptor (RAR). The overexpression of Bag-1 in MCF-7 and ZR 75–1 breast cancer cells reduced the ability of the all transretinoid acid to inhibit cell growth and induce apoptosis (Liu et al. 1998).

### Bax in breast cancer cells and tumors

An analysis of Bax expression in human breast cancer specimens revealed the localization of Bax in normal mammary epithelium. Bax-alpha, a splice variant of Bax that promotes apoptosis, is expressed in high amounts in normal cell lines and breast tissue but is expressed only weakly or not at all in cancer cell lines and malignant tissue (Bargou et al. 1995). The results of some studies have shown a correlation between the loss of Bax expression and shorter overall patient survival and faster tumor progression (Krajewski et al. 1997, Kapranos et al. 1997). Despite the ability of p53 to bind directly to the Bax gene promoter, the regulation of Bax in human breast cancers does not correlate with p53 status, implying that regulation of this pro-apoptotic gene in these tumours is complex and probably influenced by several factors (Krajewski et al. 1997). The expression of the pro-apoptotic protein Bax did not correlate with either Bcl-2 expression or the frequency of apoptotic cells (van Slooten et al. 1998). Increased degradation of the Bax protein via the ubiquitin proteosomal pathway was reported as one mechanism by which cancer cells regulate the expression of Bax (Li & Dou 2000). Inactivating mutations in the Bax gene in colon cancers have been reported (Rampino et al. 1997); however until now there was no evidence that these mutations also exist in breast cancers.

Overexpression of Bax also sensitizes breast cancer MCF-7 cells to cisplatin and etoposide (Sakakura et al. 1997) and to radiation-induced apoptosis (Sakakura et al. 1996). Using the tetracycline-inducible system, the expression of Bax-alpha was shown to have no effect on the viability of breast cancer cells; however, the expression of Bax-alpha strongly increased the chemosensitivity of breast cancer cells to epirubicin and this sensitization is due to increased apoptosis (Wagener et al. 1996). The upregulation of Bax and p21WAF1 were proposed as the molecular mechanisms by which genistein (a soy isoflavone) induces apoptosis in the breast cancer cells (Li et al. 1999). Using a transgenic mouse model system Shibata et al. (1999) demonstrated a role for Bax in inhibiting mammary cancer progression.
Caspases in breast cancer cells and tumors

Caspases are the principal intracellular effectors of apoptosis. In a study comparing the intensity of immunostaining in normal mammary epithelium with that of invasive carcinoma, caspase-3 immunointensity was generally higher in invasive cancers (Zapata et al. 1998). In another study, increases in the expression of caspase-3, -6, and -8 were associated in parallel with apoptosis and histologic aggressiveness of the breast lesions (Vakkala et al. 1999). Since tumorigenesis is associated with a resistance to apoptosis, enhanced levels of caspases in tumor cells suggest that caspases may have different functions in tumor cells or may be inactive because of overexpression of inhibitors (Krajewski et al. 1999). Survivin, an inhibitor of apoptosis protein (IAP) has been shown to be expressed in a number of breast cancer cell lines. Survivin binds caspase-3 and -7 and thus may have a role in inhibiting caspase activity and cell death in cells exposed to diverse apoptotic stimuli (Tamm et al. 1998). Nitric oxide, which induces apoptosis in breast cancer cell lines induces activation of caspase-1, -3, and -6, mitochondrial damage but does not require the CD95/CD95L pathway (Umansky et al. 2000). Flavopiridol, a drug currently used in cancer therapy, inhibits growth and induces apoptosis in a number of breast cancer cell lines. One mechanism by which flavopiridol induces apoptosis is activation of caspases, specifically caspase-3 (Motwani et al. 1999). The activation of caspase-3 was also implicated as a mechanism through which topoisomerase inhibitor etoposide induces apoptosis in breast cancer cell line MCF-7 (Benjamin et al. 1998).

Fas in breast cancer cells and tumors

The Fas ligand FasL, and its receptor Fas (APO-1 or CD95) are members of the tumor necrosis family. Interaction of FasL with its receptor Fas initiates signaling, leading to initiation of an apoptotic pathway. Non-transformed mammary epithelial cell lines express high levels of Fas mRNA and protein, but several breast cancer cell lines express low levels of Fas (Keane et al. 1996). In that study, the level of FasL protein in the majority of breast carcinomas was higher than that in normal breast tissue or benign breast disease. A significant association was observed between FasL and the presence of metastatic lymph nodes and larger tumor size but Fas expression correlated with node-negative status and smaller tumor size. Patients with Fas-positive tumors exhibited longer disease-free survival than did those with Fas-negative carcinoma. (Mottolese et al. 2000). Increased expression of FasL was speculated to confer an advantage on breast cancer cells, possibly by eliminating tumor-infiltrating immune cells, by facilitating tissue destruction during invasion, or both (Gutierrez et al. 1999, Mullauer et al. 2000). High levels of FasL mRNA expression in breast carcinomas seems to be positively correlated with histologic grading. A ratio of FasL to Fas mRNA transcripts greater than one was found to be significantly associated with shorter disease-free survival and increased mortality (Reimer et al. 2000). Vitamin E succinate inhibited growth and induced apoptosis in estrogen receptor-negative human breast cancer cells; the pathway implicated was Fas-mediated apoptosis (Turley et al. 1997).

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