ADVANCES IN CELL AGING AND GERONTOLOGY

Programmed Cell Death Volume II

Role in Disease, Pathogenesis and Prevention

Mark P. Mattson, Steven Estus and V.M. Rangnekar
editors

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Volume II
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PATHOGENESIS AND PREVENTION

PREFACE

Many cancers are caused by cells not dying when they should. On the other hand, many other diseases, such as neurodegenerative disorders and diabetes, are caused by cells dying abnormally and not being replaced. Accordingly, therapeutic approaches to cancer involve drugs and other agents that selectively kill the cancer cells, whereas therapeutic approaches to neurodegenerative disorders involve drugs and other approaches that prevent neuronal death. *Programmed Cell Death, Volume II* considers in extensive detail the roles of apoptotic, biochemical and molecular cascades in the pathogenesis of a select group of major diseases. Two chapters focus on different types of cancer. Haim Werner, Y. Oh and C. T. Werner consider genetic and biochemical aspects of breast cancer, a major cause of death in women. Samuel R. Denmeade, Bertrand Tombal and John T. Issacs consider the roles of aging and altered hormonal status in prostate cancer, a major cause of death in men. Constantin Polychronakos considers the role of apoptosis in diabetes. Autoimmune attack on pancreatic beta cells plays a major role in the pathogenesis of diabetes. Increasing evidence suggests that the pancreatic beta cells die by apoptosis. Tomoko Hasunama and colleagues describe the evidence supporting the role for apoptosis in the pathogenesis of arthritis. Autoimmune attack on joint tissues underlies arthritis. Alterations in regulation of apoptosis play critical roles in the inflammatory response in arthritis. Accordingly, therapeutic approaches are being developed that target apoptotic signaling cascades and may prove effective in reducing the severity of symptoms in patients with arthritis.

AIDS (Acquired Immune Deficiency Syndrome) is caused by the HIV virus. Avindra Nath considers the roles of apoptosis in the pathogenesis of HIV infection, with a focus on neurodegenerative changes occurring in the brains of AIDS patients. The increased susceptibility of AIDS patients to certain forms of cancer further emphasizes the potential roles of altered apoptotic signaling in the pathogenic mechanism of HIV infection.

Stroke is a major cause of disability and death throughout the world. Matthias Endres, Lorenz Hirt and Michael A. Moskowitz consider the increasing evidence that nerve cells in the brain die by apoptosis following stroke. Studies of animal stroke models suggest that blocking key steps in the apoptotic cascade can reduce nerve cell damage and improve behavioral outcome.

Mark Mattson, Qing Guo, Wenzhuan Duan and Sic L. Chan critically examine the evidence supporting a role for programmed cell death in the degeneration of neurons in the brains of Alzheimer's and Parkinson's disease patients. Alzheimer's disease

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results in selective destruction of neurons involved in learning and memory processes, while Parkinson's disease selectively damages dopaminergic neurons in the substantia nigra that control body movements. Recent molecular, genetic and biochemical studies suggest roles for oxidative stress, perturbed calcium homeostasis and subsequent activation of apoptotic signaling cascades in the pathogenesis of Alzheimer's and Parkinson's diseases. Huntington's disease is a purely genetic disorder caused by polyglutamine expansion in the huntingtin protein. Vassilis Koliatsos and colleagues describe the evidence supporting a role for apoptosis in the degeneration of striatial neurons, which leads to severe motor dysfunction in Huntington's patients. The mechanisms whereby the polyglutamine expansions lead to altered activities of the huntingtin protein are being elucidated and linked to apoptosis. Progressive paralysis and ultimate death are the defining characteristics of amyotrophic lateral sclerosis (ALS). This disorder results from selective degeneration of spinal cord motor neurons. Ward Pedersen and colleagues describe the data linking increased oxidative stress to excitotoxic apoptosis in ALS. Finally, Christopher S. Adams and Walter E. Horton, Jr. present evidence supporting a role for apoptosis in bone diseases. As with most of the other diseases covered in this volume, bone diseases are age-related.

When taken together with the detailed coverage of molecular and biochemical mechanisms controlling apoptosis and signaling pathways that prevent apoptosis (which was presented in *Volume I of Programmed Cell Death),* this second volume of *Programmed Cell Death* provides critical disease-related data that will very likely lead to novel preventative and therapeutic approaches to a variety of diseases.

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APOPTOSIS IN BREAST CANCER

HAIM WERNER, YOUNGMAN OH and CHARLES T. ROBERTS, JR.

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Introduction

The mammary gland constitutes a unique model system in which to investigate the signals that are responsible for the initiation of the apoptotic process, as well as the signal transduction pathways involved. The mammary gland differs from most other organs in that it has the capacity to undergo multiple cycles of growth, differentiation, and involution. To accurately control cell number and tissue homeostasis at each developmental stage, including the lactation and post-lactation periods, mechanisms have evolved that efficiently regulate the finely tuned balance between cell death and proliferation. This chapter will focus on the molecular and cellular aspects of apoptosis in the mammary gland. In particular, we will discuss the role of apoptosis in physiologic situations such as development and involution of the gland, as well as

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the pathologic consequences of deregulation of the apoptotic machinery, including breast cancer development and progression.

Apoptosis in Ductal Morphogenesis

The human breast contains 10 to 12 main ducts that converge in the nipple, branching interlobular ducts, and terminal lobular units or alveoli (Petersen et al., 1998; Ronnov-Jessen et al., 1996). Three epithelial components can be identified in the functional mammary gland: the luminal epithelium, the alveolar epithelium, and the myoepithelium. The luminal epithelium forms an inner continuous layer that lines milk-collecting ducts. These cells are separated from basement membrane and from stromal extracellular matrix (ECM) by a more-or-less continuous outer layer of myoepithelial cells. The alveolar epithelium is responsible for milk production. It proliferates during pregnancy and, as will be discussed in the next section, dies by apoptosis after cessation of lactation. The alveolar epithelium interacts with myoepithelial processes that form a basket-like structure around alveoli. In addition, it interacts with the basement membrane that separates these epithelial components from the stroma.

Ductal development in the virgin rodent mammary gland is characterized by profound morphological changes in both the epithelial and stromal components of the gland (Daniel and Silberstein, 1985). Duct formation starts at the onset of puberty and results from the penetration of a highly proliferative structure, the terminal endbud (TEB), through the stromal fat pad. The TEB is comprised of two types of epithelial cells: an outer layer of cap cells and an inner mass of body cells. Cap cells interact through a thin basal lamina with the surrounding stroma and give rise to myoepithelial cells (Williams and Daniel, 1983). In addition, cap cells give rise to body cells that subsequently develop into luminal epithelial cells.

Using the TUNEL (Terminal deoxytidyl transferase UTP nick end labeling) technique on sections of the mammary gland obtained from virgin Balb/c mice, a significant level of apoptosis was detected in the body cells of the TEB. The vast majority of apoptotic cells were localized around the lumen of the newly forming ducts (Humphreys et al., 1996), suggesting that programmed cell death may be involved in ductal morphogenesis of the early mammary gland. The rate of apoptosis in the TEB during ductal development (11.3%) was higher than at any other stage of mammary gland development. Furthermore, apoptosis levels were higher than those reported in other organs, such as the kidney (3%) and optic nerve (0.25%) (Barres et al., 1992; Coles et al., 1993). In terms of the signals responsible for apoptosis induction in the TEB, it was clear that apoptosis was influenced by the position of the cell within the end bud, although the exact nature of this positional clue is still unclear.

When the patterns of apoptosis and DNA synthesis in TEBs were compared, it emerged that these two processes occurred in a reciprocal fashion; i.e. cells undergoing DNA synthesis were mainly found in zones where there was little apoptosis, or in adjacent areas. The proximity between zones of proliferation and apoptosis, however, was indicative of potential coordination of these two mechanisms during

ductal morphogenesis. In addition, no differences in ductal development patterns and TEB morphology were seen in mice in which the p53 tumor suppressor gene (described in more detail below) had been inactivated compared to control mice. Though there was a small reduction in the levels of apoptosis in p53-deficient animals (8.3% vs 11.5%), the absence of any major morphogenetic disruption suggested that p53 is not a key player in this developmental program.

Apoptosis During Involution of the Mammary Gland

Following the removal of the suckling stimulus at weaning, the mammary gland undergoes a process of lobular-alveolar remodeling termed involution. The process is characterized initially by milk accumulation within the alveolar lumen and by a reduction in the levels of systemic lactogenic hormones (Feng et al., 1995). Involution of the mammary gland goes through two distinct stages. During the first stage, alveolar cells undergo initial apoptosis, whereas, in the second stage, proteinases degrade the basement membrane and ECM. The initial stage is characterized by the induction of a number of apoptosis-associated genes, including the genes encoding sulfated glycoprotein-2 (SGP-2) and interleukin-1ß-converting enzyme (ICE, now termed caspase 1). The second phase is characterized by a dramatic increase in the activities of several proteolytic enzymes such as gelatinase A, stromelysin-1 and the urokinase type plasminogen activator (uPA), and downregulation of the tissue inhibitor of metalloproteinases-1 (TIMP-1) (Lund et al., 1996). The net result of these combined enzymatic activities is the obliteration of the lobular-alveolar structure of the mammary gland, with loss of its differentiated functions.

In the ovine mammary gland, apoptosis reaches a peak 4 days after weaning and continues at a slower rate thereafter, until attaining complete regression by 30 to 60 days post-weaning (Tatarczuch et al., 1997). As is the case with other species, the apoptotic cells were subsequently phagocytosed by alveolar epithelial cells and intraepithelial macrophages (Guenette et al., 1994). In the mouse, apoptosis was detected within 24 h of milk stasis, and augmented progressively for 4 days. Cell death was also detected before litter removal, suggesting that apoptosis is also, to a certain extent, a normal physiologic characteristic of the lactating tissue (Quarrie et al., 1995).

Hormonal Control of the Involution Process

Regression of the lactating mammary gland is induced by the decreasing levels of prolactin and glucocorticoid hormone associated with weaning (Kiess and Gallaher, 1998). The critical role of these hormones in the maintenance of lactation *in vivo* was illustrated by the observation that injection of high doses of hydrocortisone and prolactin inhibited mammary gland regression (Ossowski et al., 1979). Similarly, it was demonstrated that it is feasible to maintain mammary explants in a state of lactation by adding insulin, prolactin, and hydrocortisone to the culture medium (Topper et al., 1975). Removal of lactogenic hormones from mouse mammary glands

in whole organ culture resulted in a progressive regression of the lobuloalveolar structures, which was paralleled by a 4-fold increase in the frequency of apoptosis (Atwood et al., 1995).

The role of local as compared to systemic factors during the two stages of mammary gland involution were recently addressed using a number of experimental models that included sealing of the teats, mammary gland transplants that cannot release milk due to the absence of a teat connection, and inactivation of the oxytocin gene (Li et al., 1997). The results of this study clearly showed that local mammary-derived factors are sufficient for induction of apoptosis during the first (reversible) stage of involution. Systemic lactogenic hormones can prevent progression of the gland into the second (irreversible) stage of involution, but do not block apoptosis.

The process of involution is thus the result of an interplay between death and survival factors. The death signals are locally produced and act over a short range, whereas glucocorticoids and other hormones act as survival factors during the lactation period and the first stage of involution. The potential role of some of these local factors will be discussed in the following sections.

STATS and the Mammary Gland

The STAT (Signal Transducers and Activators of Transcription) proteins are a family of cytoplasmic transcriptional regulators activated by a number of cytokines and growth factors. Following activation, they form dimers and migrate to the nucleus where they bind to promoters containing a consensus 9-bp recognition element. Of relevance to mammary gland physiology, STAT5 is specifically activated by prolactin during lactation. STAT5, in turn, activates transcription of a number of milk protein genes (Burdon et al., 1994; Li and Rosen, 1995), including ß-lactoglobulin (Watson et al., 1991). Transcripts encoding STATs 1, 3, and 5 were found in the mammary gland (Philp et al., 1996). STAT4 mRNA, on the other hand, was detected only in undifferentiated mammary tissue but not in mammary cell lines. The levels of STAT5 mRNA increased during pregnancy, paralleling the rise in casein mRNA, whereas the levels of STAT1 and 3 did not change when compared to the virgin animal. STAT mRNAs decreased during lactation; however, significant levels of STAT5a and STAT5b proteins forming heterodimers and homodimers can be detected through pregnancy and lactation.

Finally, during involution of the mammary gland, STAT5 activity decreased, whereas STAT3 was specifically activated. It has been postulated that the decline in the levels of phosphorylated STAT5 results from the loss of systemic prolactin, and that this reduction is a key step in the subsequent involution of the gland. The reciprocal patterns of activation of STAT5 and STAT3 suggest that these factors have distinct roles in the growth and remodeling of this organ (Philp et al., 1996). Although preliminary, some reports have suggested that STATs may also be involved in other hormonal signaling systems that are relevant to mammary gland physiology and tumorigenesis, such as the insulin-like growth factor system described in a subsequent section.

The Bcl-2 Family

The Bcl-2 family of cytoplasmic proteins plays an important role in the process of apoptosis. Bcl-2 itself is a potent cell survival agent with significant antiapoptotic activity (Hockenberry et al., 1990; Oltvai et al., 1993). Members of this family can either promote (Bax, Bcl-X_S, Bad, Bak) or suppress (Bcl-2, Bcl-X_L) apoptosis in a number of cellular systems. These proteins function by homo- or hetero-dimerization through their BH (Bcl-2 homology) domains (Kroemer, 1997; Reed, 1995). For example, Bax is able to promote cell death via its capacity to heterodimerize with, and inactivate, Bcl-2. On the other hand, Bcl-2 and Bcl-X_L can heterodimerize and inactivate Bax and thus suppress apoptosis (Yang et al., 1995).

Deletion of the Bcl-2 gene by homologous recombination had no major effect on embryogenesis and on the development of the mammary gland (Veis et al., 1993). On the contrary, mice in which Bax was deleted exhibited enhanced cell death in their reproductive organs and abnormal mammary gland development (Heermeier et al., 1996; Knudson et al., 1995). The phenotypes of these "knock-out" animals indicate that Bcl-2 has no major role in mammary gland remodeling whereas, Bax may play a key role in remodeling of the gland during involution.

Western blot analysis of Bcl-2 and Bax proteins at various developmental stages showed that Bcl-2 was present in glands from resting, virgin, and lactating mice. The levels of Bcl-2 decreased two days after removal of the pups and were undetectable by the third day. The pattern of expression of Bax was diametrically opposed to that of Bcl-2. Bax was absent before and during pregnancy and increased at lactation and, especially, at involution periods. These patterns are therefore consistent with the pro-apoptotic and anti-apoptotic roles of Bax and Bcl-2, respectively (Merlo et al., 1997).

An additional member of the Bcl-2 family shown to be upregulated during involution was the pro-apoptotic gene product Bcl-X_S. Similar to Bax mRNA, the levels of Bcl-X mRNAs were low during lactation, increased within several hours after weaning, and remained high for 3 days (Heermeier et al., 1996). Furthermore, the ratio between the splice products of the Bcl-X gene, Bcl-X_S and Bcl-X_L, transiently increased within the first two days of involution. Given the pro-apoptotic role of Bcl-X_S, its specific increase may be of relevance for the involution process. The role of Bcl-2 family proteins in mammary carcinogenesis and their mode of action in apoptosis induction are discussed in a following section.

Apoptosis and the Extracellular Matrix

The performance of most cellular functions by virtually every differentiated cell type depends on specific functional interactions with the ECM. In the mammary gland in particular, finely tuned interactions between epithelial cells and the surrounding ECM are crucial in order for the gland to progress through the various cycles of pregnancy, lactation, and involution (Roskelley et al., 1995).

Using normal mammary epithelial cells in culture, it has been demonstrated that adhesion and interaction with a basement membrane matrix protects them against cell death. In the absence of the correct matrix, cells die by apoptosis (Pullan et al., 1996). The implication of these observations is that the basement membrane has an important survival function during tissue morphogenesis (by eliminating cells that have migrated away from their micro environment) as well as during adult life (by maintaining tissue homeostasis). In addition to its survival role, signaling by the basement membrane is essential for the transcription of milk protein genes and for differentiation (Streuli, 1995).

The best-characterized interactions between the cell and the ECM are those mediated by the integrins, a family of heterodimeric transmembrane receptors that includes more than twenty different members. Integrins display characteristic affinities for specific ECM ligands, although many integrins can bind with high affinity more than one ligand (Hynes, 1992). The important role of integrins in survival of mammary cells was inferred from studies that demonstrated that blockage of ß1 integrin with a specific antibody resulted in a doubling in the rate of apoptosis (Pullan et al., 1996). The implication of these results, therefore, is that ligation of integrin receptors is obligatory to prevent a default apoptotic process.

Finally, there is an extensive remodeling of the ECM during involution, associated with loss of epithelial integrity and massive apoptosis. Among other processes that take place at this stage, laminin and type IV collagen are destroyed, whereas tenascin is deposited (Chammas et al., 1994). The ECM can be viewed, therefore, as an integrator of function that can positively influence gene expression (thus maintaining the differentiated state), or, on the other hand, regulate growth, apoptosis and the development of cancer.

Aberrant Apoptosis as a Common Theme in Breast Cancer Etiology

Abnormal regulation of the apoptotic process has been implicated in the origin and progression of a wide variety of diseases, as described elsewhere (Evan and Littlewood, 1998; Hetts, 1998) and in this volume. In the specific case of cancer, the classical view that prevailed for most of this century envisioned the disease as the result of excessive cellular proliferation. Accordingly, therapies were developed that were based upon the use of highly toxic chemicals or extremely energetic radiation that preferentially target DNA and cytoskeletal components of rapidly dividing cells. Proliferative models of cancer, however, cannot explain a number of clinical facts, including the observation that many slow-growing cancers are curable, whereas rapidly dividing tumors are often refractory to treatment (Fisher, 1994).

More recently, alternative models have been proposed, postulating that the accumulation of atypical cells in cancer is the result of insufficient apoptosis. The implication of this notion is that, in addition to its role in the etiology of cancer, a defect in the apoptotic program is likely to confer significant resistance to conventional antineoplastic protocols. In fact, mutations in a wide variety of pro-apoptotic genes are usually associated with poor prognosis in several tumor types (Hetts, 1998; Thompson, 1995).

The involvement of positive and negative regulators of apoptosis in the specific context of breast cancer will be discussed in the following sections.

The BRCA-1 and BRCA-2 Breast Cancer Susceptibility Genes

Although the great majority of breast cancer is sporadic in origin, approximately 5-10% have an inherited basis (Ellisen and Haber, 1998). The molecular foundation for many familial cases of breast cancer was elucidated by the cloning of the BRCA-1 (Miki et al., 1994) and BRCA-2 (Wooster et al., 1995) genes. BRCA-1 mutations are found in approximately 50% of familial breast cancers and BRCA-2 is mutated in a significant portion of the remainder. Recent studies suggest that the frequency of BRCA-1 mutations in familial breast cancer is actually underestimated (Puget et al., 1999), and BRCA-1 mutations have now been described in some non-familial cancers (Wilson et al., 1999). The BRCA-1 and BRCA-2 genes are unrelated, and the mutations described to date are scattered throughout the coding region of both genes. Exceptions are two sites of frameshift mutations in the BRCA-1 gene and one site in the BRCA-2 gene that occur at a high frequency in the Ashkenazi Jewish population (Ellisen and Haber, 1998).

BRCA-1 was initially found to induce apoptosis in fibroblasts and human breast cancer cells in culture (Shao et al., 1996). A spate of recent studies have now shown that BRCA-1 and BRCA-2 have multiple modes of action, several of which may directly influence apoptosis. Both BRCA-1 and BRCA-2 proteins associate with the product of the rad51 gene to control DNA repair (Scully et al., 1997; Sharan et al., 1997). Inactivating mutations in either BRCA-1 or BRCA-2 would, therefore, be expected to lower the threshold for genetic instability due to DNA-damaging agents. BRCA-1 has also been shown to interact with E2F, cyclins and cyclin-dependent kinases in the nucleus (Wang et al., 1997); this may be one mechanism through which BRCA-1 may modulate cell proliferation. BRCA-1 and BRCA-2 have both been shown to exert transcriptional regulatory effects (Chapman and Verma, 1996; Milner et al., 1997), and Harkin et al. (1999) have recently shown that BRCA-1 can induce the expression the of GADD45 gene, triggering a JNK/SAPK-dependent, p53-independent apoptotic pathway.

A second mechanism through which BRCA-1 may directly influence apoptosis is through its interaction with p53 (Zhang et al., 1998c). This protein-protein association may partially explain the ability of BRCA-1 and p53 to cooperatively induce apoptosis in various cancer cell lines (Chai et al., 1999). Inherited and sporadic BRCA-1 and 2 mutations may, therefore, contribute to mammary carcinogenesis through both non-apoptotic and apoptotic mechanisms, the former involving increased sensitivity to DNA damage and aberrant cell cycle control, and the latter through defective induction of GADD45/JNK/SAPK-dependent and p53-dependent apoptosis.

The p53 Tumor Suppressor

As described in the previous section, one mechanism of BRCA action involves interactions with p53. The p53 gene product is a archetypal tumor suppressor that has been the subject of intense study since its discovery over 20 years ago (Agarwal et al., 1998; Levine,

1997; Soussi, 1995). The p53 protein is normally present at low levels in normal cells, but is rapidly induced through both transcriptional and post-transcriptional mechanisms in response to signals, as yet uncharacterized, resulting from DNA damage. Germ-line mutations in p53 are the basis of Li-Fraumeni syndrome (Li and Fraumeni, 1969), which predisposes to early appearance of several cancers, including breast cancer.

Inherited p53 mutations are rare in breast cancer families (Sidransky et al., 1992), but sporadic p53 mutations are found in 30-50% of non-familial breast cancer (Soussi, 1996). On the other hand, sporadic p53 mutations are frequent in familial breast cancer (Glebov et al., 1994), suggesting a possible synergy with BRCA mutations. The p53 protein consists of three major domains, an N-terminal transactivation domain, an internal DNA-binding domain, and a C-terminal regulatory domain. In contrast to the situation with the BRCA-1 and BRCA-2 genes, almost all sporadic and inherited p53 mutations are found in the sequence encoding the DNA-binding domain, with particular hot spots at codons 175, 248, and 273.

As is the case with BRCA-1, p53 has several modes of action, principally growth arrest and induction of apoptosis. The factors influencing which effect p53 will have in a given situation are still unclear, but are cell type-specific and probably depend upon the extent (repairability) of DNA damage. p53-dependent growth arrest requires the activation of p53 target genes (Pietenpol et al., 1994), which include $p21^{CIP/WAF}$ and insulin-like growth factor binding protein 3 (IGFBP-3; see below). Other regulatory targets of p53 directly influence apoptosis, such as Bcl-2 and Bax. Specifically, wild-type p53 can up-regulate Bax, which promotes apoptosis (Miyashita and Reed, 1995), whereas mutant p53 can down-regulate Bcl-2 (Haldar et al., 1994; Miyashita et al., 1994), thereby inhibiting apoptosis. Thus, in the context of apoptosis in mammary carcinoma, loss or alteration of p53 function would be expected to compromise both growth arrest and apoptosis, the latter effect potentially involving disrupted interactions with BRCA-1 and dysregulation of the Bax and Bcl-2 genes. A recent study has identified a proline-rich region in p53 as being required for induction of apoptosis but not for growth arrest (Sakamuro et al., 1997); the role of this domain in BRCA interaction or regulation of gene expression awaits further study.

Bcl-2 in Breast Cancer

As mentioned above, one of the targets of the p53 gene product is Bcl-2. The Bcl-2 gene was first identified as a translocation partner in a B-cell lymphoma (Tsujimoto et al., 1985; Vaux et al., 1988) and, as described in a previous section, is a member of a large family of proteins that can homo- and heterodimerize and exhibit anti-apoptotic (Bcl-2, Bcl-X_L) or pro-apoptotic (Bax, Bad, Bak, Bcl-X_S) effects (Kroemer, 1997; Reed, 1995). Bcl-2 expression has been evaluated as a diagnostic marker for breast carcinoma staging, based upon the observation that non-invasive, early-stage breast carcinomas tend to exhibit high levels of Bcl-2, whereas invasive or metastatic tumors have lower levels of Bcl-2 expression (Zhang et al., 1998b). The levels of Bcl-2 seen in early-stage breast cancer may be similar to those in normal mammary epithelium, so that the major change in Bcl-2 expression may be its down-regulation in aggressive tumors. This loss of antiapoptotic Bcl-2 would be in accord with the increased apoptosis

and high cell turnover characteristics of invasive tumors, and it is tempting to speculate that the inverse relationship between p53 and Bcl-2 noted in a large sample of breast carcinomas is the result of the down-regulation of Bcl-2 by mutant p53 (since assessment of p53 status does not typically distinguish wild-type p53 from missense mutants of ...p53). In this scenario, Bcl-2-positive tumors would be more likely to retain wild-type p53, and this may explain their more favorable diagnosis (Zhang et al., 1998a).

While Bcl-2 family members have been recognized as mitochondrial membrane proteins for some time (Hockenberry et al., 1990), their mechanism of action in initiating apoptosis has only recently been elucidated. Bcl-2 has been shown to effect the release of cytochrome *c* from the mitochondrial intermembrane compartment (Kluck et al., 1997). Using reconstituted synthetic membranes, Shimizu et al. (1999) have now demonstrated that Bcl-2 family members regulate the voltage-dependent anion channel (VDAC) to modulate cytochrome *c* release, which is one component on the activation of the caspase cascade. This terminal phase in apoptosis is discussed in the following section.

The Caspase Family

The existence of a family of intracellular proteases that are major effectors of apoptosis was first suggested by the finding that the mammalian interleukin-1ß-converting enzyme (ICE) shared sequence homology with the ced-3 gene of *C. elegans*, which was involved in developmentally regulated apoptosis (Yuan et al., 1993). Since that report, more than 11 members of the family have been characterized (Cohen, 1997; Cryns and Yuan, 1998; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998) and designated caspase-1 (ICE) through caspase-11 (thus clarifying a bewildering assortment of previous names for these proteins). The caspase term reflects the cysteine protease nature of these proteins and the fact that they cleave after aspartate residues in specific amino acid contexts. Caspases are synthesized as proenzymes that can be cleaved autocatalytically and by other caspases into two cleavage fragments that heterodimerize. Subsequent association of two heterodimers produces the active caspase heterotetramer. The various caspase family members can, to some extent, be divided into initiator and executioner categories, depending upon where they function in a particular apoptotic cascade. The specific substrates and relationships between caspases that can autoactivate and activate other caspases are still being worked out.

A particular caspase cascade initiated by the Bcl-2 family described in the previous section involves Bcl-X_L, cytochrome c, activation of caspases-9 and 3, and DNA fragmentation. In this pathway, Bcl-X_L, which can elicit release of cytochrome *c* from mitochondria through modulation of VDAC activity (Kluck et al., 1997; Shimizu et al., 1999), can also form a ternary complex with caspase-9 and APAF-1 (Pan et al., 1998). APAF-1 also binds cytochrome c, which, along with dATP, is required for proteolytic activation of caspase-3. Thus, Bcl-X_L-induced cytochrome *c* release and the subsequent formation of a Bcl-X_L/APAF-1/cytochrome c/caspase-9 complex would result in the dATP-dependent activation of caspase-9, which then proteolytically activates caspase-3. One substrate of caspase-3 is ICAD, an inhibitor of CAD (Enari et al., 1998), previously shown to be identical to the DFF DNA fragmentation factor (Liu et al., 1997).

Activated caspase-3 then inactivates an inhibitor of DFF, leading to fragmentation of genomic DNA, one of the hallmarks of apoptosis. Other substrates of executioner caspases such as caspase-3 include PARP, lamins that are integral components of the nuclear membrane, and the Rb protein (Allen et al., 1998). It is possible, therefore, to envision a pathway leading from BRCA-1, through p53, Bcl-2 family proteins, and caspases to the final events of apoptosis. It is important to bear in mind that this would represent only one of a multitude of possible apoptotic pathways whose dysregulation could contribute to mammary tumorigenesis.

Growth Factors

A variety of growth factors regulate growth or differentiation of breast cancer cells by modulating mitogenesis and/or apoptotic processes in an endocrine, paracrine or autocrine manner. These growth factors include insulin-like growth factors (IGFs), transforming growth factor-ß (TGF-ß), and platelet-derived growth factor (PDGF). These growth factors interact with specific cell-surface receptors to induce signals that inhibit the apoptotic process by either up-regulating negative modulators of apoptosis such as Bcl-2 (Nass, 1996; Singleton, 1996; Staiger, 1998) and Bag (Bardelli et al., 1996) or down-regulating positive modulators of apoptosis such as Bax (Nass, 1996; Wang, 1998a) and Bad (Kulik, 1998). During cancer progression, or as a result of tumorigenesis, breast cancer cells overexpress receptors such as IGF receptors (Surmacz, 1998), EGF receptors and erb2/Her2 receptors (Kumar, 1996) that mediate anti-apoptotic as well as mitogenic functions.

Other growth factors, such as transforming growth factor-ß (TGF-ß) (Yu, 1997), tumor necrosis factor-α (TNF-α) (Rozen, 1998), interleukin-4 (IL-4)(Chiu et al., 1996; Gooch et al., 1998), heregulin (Lupu, 1996) and basic fibroblast growth factor (bFGF) (Wang, 1998b) induce apoptosis by regulating positive or negative modulators of apoptosis in breast cancer cells. Recent studies have revealed that heregulin induces apoptosis in human breast cancer cells via downregulation of Bcl-2 through its interaction with members of the EGF receptor tyrosine kinase family (Weinstein et al., 1998). Furthermore, IL-4 appears to inhibit breast cancer cell growth through the induction of apoptosis; this pro-apoptotic effect is reversed by the addition of IGF-I (Gooch et al., 1998).

The IGF System

The IGF system is composed of ligands (insulin, IGF-I, and IGF-II), receptors (insulin, type 1 and type 2 IGF receptors) and a family of binding proteins (IGFBPs 1-6). The IGFs, formerly known as somatomedins, are structurally related to insulin, sharing approximately 50% amino acid homology. The IGFs have been recognized as major regulators of mammary epithelial cell and breast cancer cell growth (Oh, 1998). The mitogenic actions of the IGFs are mediated largely through the type 1 IGF receptor, which, like the insulin receptor, is a heterotetrameric, membrane-spanning tyrosine kinase. The importance of the IGF system in breast cancer was demonstrated by recent clinical studies showing that serum IGF-I levels are increased in breast