Apoptosis in the mammary gland and breast cancer

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Introduction

Apoptosis or programmed cell death (PCD) is an active, energy-dependent process of cell death which occurs during development, in response to certain physiologic stimuli, and secondary to cell injury and stress (reviewed by Wyllie et al. 1980, Arends & Wyllie 1991, Ellis et al. 1991, Wyllie 1992). This type of cell death occurs via controlled deletion of discrete cells within a tissue and plays a role in embryonic development and normal tissue homeostasis. It differs from necrotic cell death in that the cells eliminated by PCD die and are processed without initiation of an inflammatory response.

Kerr et al. (1972) introduced the term apoptosis to describe the characteristic morphologic changes seen during PCD. These changes involve initial disruption of cell-cell contacts, nuclear and cytoplasmic condensation, nuclear fragmentation, and packaging of cellular materials and organelles into membrane-bound vesicles termed apoptotic bodies. These apoptotic bodies are then shed, taken up via phagocytosis by other cells (including viable epithelial cells and macrophages surrounding the apoptotic cell), and degraded by lysosomal enzymes after fusion of the phagosomes with intracellular lysosomes.

The biochemical and genetic events involved in PCD are less well understood. Certain biochemical events and genetic pathways have been identified that are associated with the PCD pathway in multiple cell types including mammary epithelium. These include DNA fragmentation via endonuclease activation (reviewed by Compton 1992), protease activation and cleavage of intracellular proteins (reviewed by Patel et al. 1996), expression of bcl-2 family members (reviewed by Yin et al. 1994, Hockenbery 1995), tumor suppressor gene p53-directed events (Camman & Kastan 1995, reviewed by Levine 1997), proto-oncogene activation or dysregulation (Camman & Kastan 1995, Packham & Cleveland 1995), and activation of transmembrane receptor signaling pathways such as tumor necrosis factor and apo 1/has (Trauth et al. 1989, Itoh et al. 1991, Keane et al. 1996).

Activation of PCD plays a major role in virtually all phases of mammary gland development and remodeling. This process also occurs spontaneously in neoplastic breast tissue (Lipponen et al. 1994, Bodis et al. 1996), and the majority of chemotherapeutic agents used in breast cancer treatment trigger the PCD pathway (Dive & Hickman 1991, Kerr et al. 1994). Here we review the critical role of PCD in normal and malignant breast tissues and possible clinical implications.

Apoptosis in normal mammary gland biology and development

The normal mammary gland undergoes numerous hormonally regulated structural and functional changes during ductal morphogenesis, pregnancy, lactation and involution. PCD has been demonstrated to be the predominant mechanism for controlled cell deletion during this continuum (Ferguson & Anderson 1981a, Walker et al. 1989, Strange et al. 1992, Humphreys et al. 1996). Summarized below are studies in human tissues and cell lines as well as murine models which have enhanced our understanding of the PCD pathways in mammary epithelium. We recognize that most information on PCD in mammary epithelium is derived from murine models and there are inherent limitations to direct extrapolation of these data to humans. Nevertheless, these studies can be utilized as a foundation to help direct the research questions on the role of PCD in the human mammary gland.

The role of PCD in mammary gland development

Humphreys et al. (1996) evaluated the role of PCD during mammary gland ductal morphogenesis in the mouse. At puberty the ductal pattern is created via proliferation of terminal end buds (TEB) into the stromal fat pad. TEB are composed of two distinct cell populations, cap cells and body cells, which are the progenitors of the myoepithelium and epithelium of the mammary gland respectively. Significant numbers of apoptotic cells were noted in the body cells of the TEB during maximal ductal morphogenesis, which occurs at 4 to 7 weeks of age. They were localized predominantly in the body cells most proximal to the duct lumen, implicating a role for PCD in the organization of the mammary gland ductal structure.
The expression of members of the bcl-2 family during mammary gland development was also studied. Two bcl-2 gene family members, bax and bcl-xL, have been shown to have pro-apoptotic activity (Boise et al. 1993, Oltvai et al. 1993) whereas bcl-2 and bcl-xL have anti-apoptotic activity (reviewed by Korsmeyer 1992). Changes in levels of these proteins in mammary epithelium may play a critical role in mammary gland PCD.

Bcl-2 and bcl-x expression was prominent in the TEB body cells, especially those cells most proximal to the lumen. Examination of serial sections for apoptosis displayed no correlation between apoptotic cells and loss of bcl-2 or bcl-x expression. Bax expression was seen throughout the body and cap cells; and no correlation between bax expression and the pattern of apoptosis was seen. Since association of bcl-2 family proteins into homo- or heterodimers rather than their absolute level of expression appears to govern the response to an apoptotic signal (Boise et al. 1993, Oltvai et al. 1993, Yang et al. 1995), the lack of correlation observed between their expression and apoptosis does not eliminate a possible role for these proteins in mammary gland PCD. Indeed, the role of bcl-2 in mammary gland apoptosis was addressed more directly in transgenic mice. The level of apoptosis was found to be significantly lower in the TEB of whey acidic protein (wap)-bcl-2 transgenic mice than in controls and this was associated with a disruption in the structural organization of the TEB.

Analysis of apoptosis in the mammary gland of p53 null mice was utilized to evaluate the role of p53 in ductal morphogenesis. Whole mount analysis of p53 null and wild-type (wt) p53 mice demonstrated no difference in overall ductal development, TEB number, duct length or secondary branching. There was a decrease in the overall amount of apoptosis in the p53-deficient mice when compared with syngeneic wt p53 mice, but the lack of impact on ductal morphogenesis argues against an essential role for p53 in this setting. Together, these observations identify a role for PCD in mammary gland development during puberty and suggest a critical role for bcl-2 family members but not p53 in PCD during ductal morphogenesis.

PCD during mammary gland involution

Walker et al. (1989) evaluated the pathologic changes occurring in the rat and mouse mammary gland during post-lactational involution. In the mouse, apoptotic cell number increased more than 30-fold by 48 h after weaning, with a concomitant reduction in the volume of secretory epithelium. The secretory epithelium of the rat also comprised most of the breast parenchyma during lactation and this level was dramatically reduced by apoptosis after weaning. Both species also demonstrated regression of the mammary gland capillary beds after weaning via PCD of the endothelium. Thus, involution of the mammary gland after removal of lactogenic stimuli is achieved through reduction in both the glandular tissue and the vascular bed through PCD. Commitment to cell death after weaning is reversible for the first 24 to 48 h indicating that additional steps beyond the initial removal of the lactogenic stimulus are required for completion of involution.

Strange et al. (1992) characterized the biochemical and genetic changes of PCD in the involuting mouse mammary gland. Oligonucleosomal sized DNA fragments were apparent 24 h post-weaning, qualitatively increased through day 4, and were barely detectable by day 6. Expression of β-casein, wap, and ornithine decarboxylase was evident during lactation and decreased dramatically by 24 h post-weaning. Expression of the protease inhibitor gene, tissue inhibitor of metalloproteinase (TIMP), was inversely correlated with expression of proteases such as tissue plasminogen factor, urokinase-type plasminogen factor (uPA), transin, and stromelysin. Genes known to be expressed in association with cell stress such as heat shock protein 70 and lactate dehydrogenase, as well as PCD-associated gene products such as sulfated glycoprotein-2 (SGP-2), and tissue transglutaminase were also increased during involution in conjunction with the morphologic changes of apoptosis. c-myc, transforming growth factor (TGF)-β1 and p53 mRNA expression were easily detected in the pregnant state, undetectable during lactation, and reexpressed at detectable levels during involution.

Characterization of PCD in involuting mammary gland

In vitro and in vivo animal models of mammary gland involution have permitted the further delineation of key components of the PCD pathways in mammary gland. A brief review of pertinent studies follows.

The extracellular matrix and proteases

Several studies have examined the role of the extracellular matrix (ECM) in epithelial cell apoptosis (Talhouk et al. 1992, Frisch & Francis 1994, Boudreau et al. 1995, Lund et al. 1996, Pullan et al. 1996). ECM remodeling is minimal during lactation but increases dramatically from day 4 of involution when extensive tissue remodeling, high ECM degrading protease:protease inhibitor ratios, and collapse of the lobular/alveolar structure via apoptosis are seen (Lund et al. 1996). The importance of protease:inhibitor ratio in this process was demonstrated by studies where introduction of exogenous TIMP to the involuting mammary gland delayed onset of involution (Talhouk et al. 1992). Glucocorticoids, which have been shown to down-regulate matrix metalloproteinases and uPA, also resulted in a delay in mammary gland apoptosis,
ECM remodeling and regression in a dose-dependent manner in this model system. Studies in the immortalized, non-transformed CID-9 mouse mammary epithelial cell (mec) culture model have also defined a role of ECM in PCD. CID-9 cells demonstrated 10-20% apoptotic cells when grown on plastic dishes but minimal apoptosis when grown on ECM-coated plates (Boudreau et al. 1995). Induction of stromelysin-1, an ECM-degrading proteinase, increased apoptosis in the meccs grown on ECM and this was inhibited by TIMP. Interruption of ECM-cell signaling by β1-integrin antibodies also resulted in increased apoptosis in this model system. The role of induction of stromelysin-1 in vivo was also demonstrated by introduction of a wap-stromelysin-1 construct which was active during pregnancy and resulted in increased levels of apoptosis during mid to late pregnancy in the mouse mammary gland. Thus, ECM remodeling by proteinases and signaling through molecules such as β1-integrin are apparently important mediators of mammary gland PCD during involution.

The ICE (interleukin-1β converting enzyme) family of proteases, also termed caspasas, have been shown to play a role in mammary gland PCD. ICE mRNA and protein expression was noted in the CID-9 cells growing on plastic but not on ECM-coated plates. Transfection of these cells with an expression vector containing Crm-A, a viral gene product known to inhibit the enzymatic activity of ICE, led to an 80% reduction in DNA fragmentation of CID-9 cells growing on plastic. Direct inhibition of this enzyme with the peptide BACMK also resulted in reduced DNA fragmentation. In vivo studies in the mouse also support a role for this protease in apoptosis during mammary gland involution. There was little expression of ICE mRNA in the lactating gland but increased expression was observed during involution.

Bcl-2 gene family

Using a transgenic SV40 large T antigen (Tag) mouse model, Heermeier and coworkers (1996) demonstrated that bax mRNA expression was low throughout lactation, induced within 6 h after weaning, and remained elevated for 72 h. Although the bax gene is known to be transcriptionally activated by p53, this increase in bax mRNA is via a p53-independent mechanism, since Tag is known to bind and inactivate endogenous wt p53. Bax protein was identified throughout lactation and involution, with increased levels noted at the terminal stages of mammary gland regression. Bcl-xL mRNA and protein were also detectable at low levels during lactation and increased after weaning. Bcl-xL mRNA levels were approximately 40-fold higher than bcl-xS during the resting state, pregnancy and lactation. However, during the first 48 h of involution, there was a transient six-fold increase in the ratio of pro-apoptotic bcl-xS to anti-apoptotic bcl-xL.

Jager and coworkers (1997) utilized a transgenic mouse model to show that bcl-2 overexpression during mammary gland involution resulted in inhibition of apoptosis. The mammary gland of the transgenic mice showed reduced apoptotic cell number on days 2 and 3 following weaning but no other histologic differences were noted between the two groups of mice. Evaluation of expression of multiple genes in the mammary gland in both groups of mice during involution (including SGP-2, p53, TGF-β1, TIMP, jun D, jun B, and β casein) did not show any significant differences. Thus these studies provide some support for a role for bcl-2 family members in mammary gland apoptosis during involution.

p53 and other transcriptional regulators

The role of p53 during pregnancy and involution has also been explored. Expression of Tag (as a means of suppressing p53 function) during pregnancy in mice (Li et al. 1996a) increased the number of apoptotic nuclei in the mammary gland from a basal level of <0.2% at days 18-19 of pregnancy in control animals to approximately 5% in the transgenic mice. The majority of the mammary epithelial cells in the transgenic animals expressed Tag, indicating that p53 inactivation was insufficient to induce apoptosis in the entire population. Bax mRNA and protein levels increased approximately five-fold over control. Bcl-x mRNA was increased approximately two-fold; both bcl-xS and bcl-xL expression increased but there was a relatively greater increase in pro-apoptotic bcl-xS compared with anti-apoptotic bcl-xL.

Li and coworkers (1996b) evaluated the role of p53 in murine mammary gland apoptosis during involution. They utilized two models, one in which the p53 was inactivated in mammary epithelium by Tag, beginning in pregnancy, and a second one where there was a defective germline p53. Whole-mount analysis of the mammary gland demonstrated no significant differences between the models during involution. Apoptotic cells were noted by day 1 after weaning and collapse of the lobular-alveolar structure was seen by day 3 in both control and p53-deficient mice. Increased expression of bax and bcl-x mRNA was seen within 1 day, with a decline back to levels similar to those in the lactating gland by day 10 in both groups of animals. Thus inactivation of p53 function did not have a major affect on PCD in the involuting mammary gland.

Merlo et al. (1995) utilized both human and murine immortalized, non-transformed mec lines to demonstrate p53-dependent and -independent pathways of apoptosis. These investigators showed that a murine mec line, HC11, containing mutant p53, did not undergo PCD after up to 12 h of exposure to mitomycin C, cisplatin, 5-fluorouracil
(5-FU), doxorubicin or vincristine. When a temperature-sensitive wt p53 was introduced into this cell line, the basal level of apoptosis was increased approximately two-fold; it increased another three-fold after treatment with mitomycin C but not the other chemotherapeutic agents. The MCF-10A human mec line which expresses wt p53 also demonstrated a three- to four-fold increase in the number of apoptotic nuclei in response to treatment with mitomycin C but not the other drugs. Two additional mecs with absent or inactivated p53 were also examined for drug-induced apoptosis with the same agents and no increase was observed after treatment. All four cell lines underwent PCD in response to serum deprivation at confluence, confirming a p53-independent PCD pathway in all four cell lines which was initiated by serum deprivation. However, the PCD response induced by treatment with mitomycin C in the same cell lines was p53-dependent, arguing for the presence of both p53-dependent and -independent pathways of apoptosis in these mec lines.

Based on these studies in murine models in vivo, as well as murine and human mammary epithelium cell lines in vitro, mammary gland PCD appears p53-independent during pregnancy and involution but p53 may play an important role in PCD initiated by cytotoxic agents. These results underscore the observation that there are multiple pathways involved in PCD, and that different pathways may be active in different cell types or in response to different apoptotic signals. Elucidating the multiple PCD pathways active in normal tissues, including mammary epithelium, as well as breast cancers may aid us in developing more tumor-specific therapies and help us more rationally utilize p53 as a predictive factor in breast cancer treatment.

Other transcription factors such as AP-1 (activator protein-1) have also been examined as possible nuclear regulators of PCD. Marti et al. (1994) examined the temporal relationship between AP-1 activity and apoptosis during mammary gland involution in the mouse. AP-1 in the mouse mammary gland consisted primarily of c-fos and jun D with some jun B. c-fos and jun B mRNA was barely detectable during lactation but was easily appreciated by days 2 and 3 of involution while jun D expression was low during lactation and highly induced by day 2 after weaning. AP-1-DNA binding was undetectable during lactation, increased by day 1, and decreased by days 3 and 4 after weaning. These changes were seen in the mammary glands containing epithelium but not in cleared mammary gland fat pads devoid of epithelium, demonstrating epithelial cell specificity. The temporal relationship of these events to mammary gland PCD during involution suggests that AP-1 may be a nuclear regulator of this process.

Prolactin signaling during lactation has been shown to occur through the STAT (signal transducers and activators of transcription) family of transcription factors (Wakao et al. 1994, Liu et al. 1997). Phosphorylation of STAT proteins confers DNA binding activity and subsequent transcriptional activation of target genes. Li and coworkers (1997) demonstrated decreased phosphorylation (without changes in protein levels) of Stat 5a and 5b and increased phosphorylation of Stat 3 during the initial phase of involution in the mouse mammary gland. These changes coincided with increased bax expression and induction of alveolar PCD. What specific role, if any, these STAT transcription factors play in mammary gland PCD during involution remains to be elucidated.

**Protein tyrosine kinase receptors in mammary gland PCD**

Harris and coworkers (1995) noted that expression of a constitutively active form of c-neu (the rat homolog of c-erbB-2 or HER-2 oncogene, a receptor protein tyrosine kinase) in human mec in vitro resulted in induction of apoptosis in response to low serum whereas parental cells manifested only growth arrest. PCD so induced could be inhibited by glucocorticoids but apoptosis in this cell line could not be induced by treatment with growth factors or other non-glucocorticoid steroids. Of note, glucocorticoids can also inhibit apoptosis in the involuting mouse mammary gland in vivo (Ossowska et al. 1979).

As noted earlier, MCF-10A cells undergo apoptosis in response to mitomycin C but not other drugs tested (Merlo et al. 1995). When this cell line was engineered to overexpress the v-erbB-2 oncogene, induction of apoptosis occurred in response to cisplatin and 5-FU as well as mitomycin C. This study demonstrates that the chemotherapeutic drug-induced PCD response in mec can be altered by overexpression of a tyrosine kinase receptor proto-oncogene. Together, these studies imply a role for receptor protein tyrosine kinases in mammary epithelium PCD induced by serum deprivation and chemotherapeutic agents.

Systemic hormones clearly play a role in mammary gland PCD, but the microenvironment, including locally derived growth factors, also plays a role in this pathway. Neuenschwander and coworkers (1996) utilized transgene overexpression of insulin-like growth factor-I (IGF-I) or insulin-like growth factor-binding protein-3 (IGFBP-3) to evaluate the role of IGF-I signaling (through its protein tyrosine kinase receptor IGFR) in mouse mammary gland involution. Utilizing a Rap promoter, they demonstrated that expression of these proteins in the mammary epithelium occurred in mid-pregnancy, through lactation, and progressively declined during involution in the transgenic mouse. After weaning, control mice
demonstrated typical morphological changes characterized by extensive apoptosis and tissue remodeling while the mice transgenic for IGF-1 or IGFBP-3 showed incomplete involution with markedly reduced apoptosis and associated ductal hypertrophy. These studies support the possible role of local regulation of PCD through IGF-1 or IGFBP in mammary epithelium during involution. IGFBP proteins are known to regulate IGF activity and their level could modulate this mitogenic signaling pathway, thereby influencing the cell’s decision to undergo apoptosis. Regulation of the IGFBP gene by p53 is further evidence for a role of IGF in PCD (Buckbinder et al. 1995).

**Apoptosis in breast cancer**

**The study of PCD using breast cancer cell line models**


**Estrogens, anti-estrogens and PCD**

Kyprianou et al. (1991) first reported that regression of estrogen-dependent MCF-7 breast tumors in nude mice after estrogen withdrawal was associated with apoptosis. Decreased proliferation, increased apoptosis, oligonucleosomal DNA fragmentation, and increased expression of SGP-2 and TGF-β1 mRNA levels characterized this process. Subsequently, a number of investigators confirmed that estrogen deprivation or anti-estrogen treatment had similar effects on hormone-dependent breast cancer cell lines growing *in vitro* or *in vivo* (Warri et al. 1993, Perry et al. 1995, Wilson et al. 1995, Bursch et al. 1996, Chen et al. 1996, Otto et al. 1996, Cameron et al. 1997).

A functional role for TGF-β1 in this process is implicated by two studies. Perry et al. (1995) showed that treatment of estrogen receptor (ER)-positive MCF-7 or ER-negative MDA-MB-231 cells with 10 nM tamoxifen resulted in PCD characterized by intranucleosomal DNA fragmentation and enhanced TGF-β1 mRNA and protein expression. Tamoxifen-induced DNA fragmentation in both cell lines was inhibited by treatment with anti-TGF-β1 antibody. Similarly Chen et al. (1996) showed that treatment of MCF-7 cells with 1 mM tamoxifen induced PCD and increased TGF-β1 mRNA and activity. Addition of exogenous TGF-β1 mimicked tamoxifen’s ability to decrease proliferation and induce apoptosis, further suggesting that TGF-β1 may be an important mediator of tamoxifen-induced PCD in these breast cancer cells.

The importance of bcl-2 in hormone regulation of PCD has also been investigated. Several studies suggest that treatment of MCF-7 cells with 17β-estradiol leads to a dose- and time-dependent increase in bcl-2 mRNA, whereas bax and bcl-xL mRNA levels are unaffected (Teixeira et al. 1995, Wang & Phang 1995, Huang et al. 1997). No such effect is seen in ER-negative MDA-MB-231 cells. *In vitro* studies suggest that estrogen exposure diminishes the cytotoxic effect of chemotherapeutic agents like doxorubicin (Teixeira et al. 1995) or paclitaxel (Huang et al. 1997). Also, estrogen-deprived cells engineered to overexpress bcl-2 were more resistant to doxorubicin and expression of bcl-2 antisense increased doxorubicin sensitivity. Together, these *in vitro* studies support the concept that drug resistance in hormone-responsive breast cancer may be modulated by estrogen effects on bcl-2, an hypothesis which has not been addressed clinically.

**Peptide growth factors and their receptors**

The importance of peptide growth factor pathways in breast cancer cell growth and death is increasingly appreciated. The receptor tyrosine kinases epidermal growth factor receptor (EGFR) and c-erbB2 are overexpressed and/or amplified in 30% of human cancers including breast cancer (Hynes & Stern 1994). Overexpression of EGFR in breast cancer has been associated with a poor prognosis (Hynes & Stern 1994) and its level is inversely correlated with ER expression (Fitzpatrick et al. 1984, Davidson et al. 1987). Depending on the conditions, either ligand binding or antibodies directed against these two receptors may induce PCD. For example, EGF induced PCD in the ER-negative MDA-MB-468 cell line which overexpresses EGFR (Armstrong et al. 1994). Either ligand binding or antibodies directed against the extracellular portion of the receptor can inhibit proliferation and induce apoptosis in breast cancer cells that overexpress c-erbB-2 (McKenzie et al. 1989, Stancovski et al. 1991, Kita et al. 1996).
Dysregulation of protein tyrosine kinase signaling pathways because of receptor overexpression has also been shown to alter drug-induced PCD in breast cancer cells (Kumar et al. 1996, Dixit et al. 1997). Dixit and colleagues (1997) showed that antisense to EGFR abrogated apoptosis induced by cisplatin (but not doxorubicin, etoposide or paclitaxel) in MDA-MB-468 cells, possibly because of effects on DNA repair. Kumar et al. (1996) showed that overexpression of c-erbB-2 blocked tamoxifen-induced PCD in MCF-7 cells. This was associated with increased expression of anti-apoptotic bcl-2 and bclxL, while levels of pro-apoptotic bcl-xS and bax proteins were unaffected. These findings are consistent with preliminary clinical studies that have shown reduced response to hormonal therapy in women with ER-positive tumors which overexpress c-erbB-2 (Wright et al. 1992, Leitzel et al. 1995). Together, these studies demonstrate that receptor tyrosine kinase expression may modulate apoptotic pathways induced by endocrine manipulation or chemotherapy.

An interaction between another peptide growth factor, IGF-I (which interacts with the type 1 IGF receptor, also a tyrosine kinase), and drug-induced PCD pathways has also been demonstrated. IGF-I has been shown to be mitogenic and anti-apoptotic in mec and breast cancer cells (Neuenschwander et al. 1996, Dunn et al. 1997). Its activity is modulated by its binding to IGFBPs, and IGFBP-3 has been shown to promote apoptosis in vitro (Gill et al. 1997, Nickerson et al. 1997). A role for the IGF signal transduction pathway in anti-estrogen-mediated apoptosis is supported by two findings. First, the anti-estrogen, ICI 182 780, which inhibits cell growth and induces apoptosis in the MCF-7 cell line, also increases the secretion of IGFBP-3 and down-regulates the IGF receptor. Secondly, IGF-I or an analog can reduce or abolish ICI 182 780-induced apoptosis. IGF-I may also modulate PCD induced by chemotherapy. Dunn et al. (1997) showed that IGF-I inhibited PCD induced by serum deprivation in immortalized, non-transformed human HBL100 mec. Treatment of HBL100 cells with several anti-cancer agents including tamoxifen, methotrexate, 5-FU and camptothecin resulted in PCD; this effect was inhibited but not abolished by the addition of IGF-I, suggesting that this pathway also has the ability to partially overcome drug-induced PCD. These studies indicate that the IGF pathway is involved in breast cancer PCD induced by certain apoptotic signals, including anti-estrogens and chemotherapeutic agents, and changes in IGF-I or IGFBP-3 could modulate tumor cell sensitivity to these agents. This finding is not universal, however. For example, IGFBP-3 induces apoptosis of ER-negative Hs578t human breast cancer cells, but this effect appears to be independent of IGF-I and is instead mediated through the sphingomyelin/ceramide apoptotic pathway (Gill et al. 1997).

The bcl-2 gene family in human breast cancer cell models

Patterns of expression of bcl-2 family members may vary somewhat between normal and malignant mec. Bax expression is high in non-transformed mec relative to cancer cells, while bcl-2 and bcl-x are similar (Bargou et al. 1995). Bcl-xL is the predominant bcl-x species in normal and malignant breast cells. Numerous studies have attempted to correlate expression of bcl-2 family members with intrinsic sensitivity to apoptotic stimuli in these cell lines. Induction of PCD via serum deprivation or through the fas pathway was enhanced in the cell lines with relatively greater bax expression (Bargou et al. 1995). Bax was transfected into two breast cancer cell lines with relatively weak basal expression (compared with normal mec), and the resulting lines demonstrated increased sensitivity to serum-starvation-induced apoptosis and their growth in severe combined immunodeficiency mice was slowed (Bargou et al. 1996). Wagener et al. (1996) showed that overexpression of bax-α via an inducible tetracycline-dependent expression system in the same two cell lines enhanced their apoptotic response to epirubicin but not their rate of spontaneous apoptosis. Sakakura and coworkers (1996) showed that bax overexpression in MCF-7 cells resulted in increased sensitivity to ionizing radiation (IR), with resultant decreased cell survival and increased apoptosis. Since p53 is known to induce bax, this group measured p53 induction in the parental and bax-transfected MCF-7 cell lines and demonstrated a ten-fold induction of p53 by IR. In contrast, bax mRNA was increased only two-fold in the parental cell line at higher IR doses and there was no change in bax levels measurable in the transfected cell lines. Other cell types which show a high degree of bax induction secondary to p53 induction by IR are more sensitive to IR-induced apoptosis than the parental MCF-7 cells. The authors postulate that the inability of wt p53 to induce bax adequately in breast cancer may result in resistance to apoptotic stimuli such as IR.

Overexpression of the pro-apoptotic protein bcl-xS also modulates breast cancer cell sensitivity to apoptotic signals (Sumatran et al. 1995, Ealovega et al. 1996). MCF-7 cells transfected with bcl-xS show enhanced sensitivity to two chemotherapeutic agents, etoposide and paclitaxel. In addition, intratumoral injection of a replication-deficient adenoviral vector containing bcl-xS into MCF-7 tumors in nude mice led to partial tumor regression associated with apoptotic changes at the injection site seen histologically.
In contrast, Schott and coworkers (1995) demonstrated that bcl-xL protected breast cancer cells from p53-mediated apoptosis. High levels of the bcl-xL protein were seen in the breast cancer cell line T47D, which is known to have mutant p53. They utilized this cell line to evaluate the relationship between p53-mediated apoptosis and bcl-xL. When transfected with a temperature-sensitive p53, these cells were growth arrested but did not undergo apoptosis at the permissive temperature for wt p53. The hypothesis that increased levels of bcl-xL protected against p53-induced PCD was tested by utilizing a murine erythroleukemia cell line which does not contain bcl-2, bcl-xL, or p53. Transfection of the temperature-sensitive p53 resulted in induction of cell death at the permissive temperature while coexpression of p53 and bcl-xL resulted in growth arrest but not cell death. Whether this plays a role in breast cancer will need further study in breast cancer models and patient tissue samples.

Changes in bcl-2 may also modulate sensitivity to PCD in breast cancer cells. Treatment of MCF-7 cells with sodium butyrate down-regulated bcl-2 mRNA and protein expression and led to PCD (Mandal & Kumar 1996). Overexpression of bcl-2 decreased MCF-7 cell sensitivity to sodium butyrate-induced apoptosis. Low doses of sodium butyrate which did not induce apoptosis increased MCF-7 cell sensitivity to doxorubicin-induced apoptosis. These studies demonstrated that modulation of levels of bcl-2 by agents such as sodium butyrate may be useful clinically to increase breast cancer cell sensitivity to chemotherapy.

Finally, other changes in bcl-2 family proteins may alter their activity. Treatment of cells in vitro (including breast cancer cells) with chemotherapeutic drugs which interfere with cell microtubules by either stabilization (i.e. taxanes) or destabilization (i.e. vinca alkaloids) is associated with bcl-2 phosphorylation (Haldar et al. 1995, 1997, Blagosklonny et al. 1996). Phosphorylation of bcl-2 inhibits its anti-apoptotic activity, perhaps through interference with its binding to the pro-apoptotic protein, bax (Haldar et al. 1995). These studies demonstrate that post-translational modification of bcl-2 by phosphorylation as well as its absolute cellular level may play a role in tumor cell sensitivity to chemotherapy. Thus, modulation of bcl-2 activity through phosphorylation could be conceivably utilized to design chemotherapeutic regimens which maximize tumor cell response.

**Apoptosis in human mammary epithelium**

Several investigators have provided evidence that apoptotic pathways are also operative in normal human mammary tissues. Ferguson & Anderson (1981a) noted that the classical morphologic changes of apoptotic cell death were present in the parenchyma of normal breast epithelium. Rare apoptotic cells were scattered throughout the duct and ductules of the breast. Phagocytosis of apoptotic bodies by neighboring cells, including breast epithelium, myoepithelium and macrophages, was observed.

These investigators also examined the relationship between mitosis and apoptosis during the menstrual cycle (Ferguson & Anderson 1981b). There was a cyclical variation in the number of mitotic and apoptotic cells, with peak mitotic activity occurring at day 25 and peak apoptotic levels seen at day 28 of the menstrual cycle. Apoptotic and mitotic cells were distributed evenly throughout the lobules examined rather than clustered within specific lobules. These observations suggest that apoptosis is the primary mechanism for deletion in the normal human mammary gland and occurs in response to cyclical fluctuations in hormones.

Several members of the bcl-2 family including bcl-2, bax, and bcl-xL are expressed by normal mammary epithelial cells in culture and breast tissue (Hockenbery et al. 1991, Leek et al. 1994, Sabourin et al. 1994, Bargou et al. 1995, Ferrieres et al. 1997). Sabourin et al. (1994) examined the expression of bcl-2 in normal breast tissue through the menstrual cycle. Enhanced staining was noted in the follicular phase with a decrease in number of stained cells and intensity of staining during the luteal phase. This study argues for hormonal regulation of bcl-2 in normal breast epithelium.

**Evaluation of apoptosis in breast cancer**

Apoptosis has been noted to occur spontaneously in cancer tissue including breast carcinomas (Kerr et al. 1972, Lipponen et al. 1994, Bodis et al. 1996). Studies have attempted to correlate the amount of apoptosis within the malignant epithelial cells with known histologic and prognostic factors in breast cancer (Lipponen et al. 1994, Sierra et al. 1996). In addition, molecular markers associated with PCD such as p53, p21 and bcl-2 gene family members have been evaluated in breast cancer (Gee et al. 1994, Leek et al. 1994, Elledge et al. 1995, Hurlimann et al. 1995, Sierra et al. 1996, Silvestrini et al. 1996, Bukholm et al. 1997a,b, Kapranos et al. 1997, Keen et al. 1997, Krajewski et al. 1997). A particular focus has been on their ability to help define prognosis (prognostic factor) or predict response to a particular type of therapy (predictive factor). Finally, since the majority of chemotherapeutic agents work through induction of PCD (Dive & Hickman 1991, Kerr et al. 1994), studies have also attempted to address the possibility that defects in the PCD pathway may be associated with malignancy as well as how PCD might be manipulated as a therapeutic target.
Lipponen et al. (1994) evaluated the apoptotic index in 288 invasive breast cancers. A high apoptotic index was correlated with increased tumor grade, aneuploidy, high S-phase fraction, high mitotic index, ER and progesterone receptor (PR) negativity, mutant p53, tumor necrosis, and increased lymphocytic infiltration. In multivariate analysis, only increased mitotic index correlated with a high apoptotic index. Although increased apoptotic index was significantly associated with decreased survival in both lymph node positive and negative patients, it had no independent prognostic value. Bodis and coworkers (1996) also found a positive correlation between apoptosis and cell proliferation in ductal carcinoma in situ. These studies suggest that correlation between apoptosis and proliferation may be important in breast cancer. A strong apoptotic signal and tumor cell proliferation may be associated with accumulation of molecular derangements within the cells. Escape of tumor cells which become resistant to apoptosis could help explain tumor progression and decreased survival in patients.

p53 in human breast cancer

Aberrant p53 expression has been widely studied as a prognostic and predictive factor in invasive breast cancer. In aggregate, these studies suggest that mutant p53 (as detected by immunohistochemistry, single strand conformation polymorphism, and/or sequencing) is noted in 20 to 50% of primary breast cancers (Osborne et al. 1991, Dornagala et al. 1993, Gretarsdottir et al. 1996, Lizard-Nacol et al. 1997). Most studies also showed that the presence of mutant p53 is correlated with other poor prognostic factors such as negative ER, high S-phase fraction, and high tumor grade. It is not certain if mutant p53 is an independent predictor of recurrence or survival. However, Silvestrini et al. (1996) addressed whether p53 status is a predictor of distant failure in women with node-negative breast cancer. In 1400 patients treated only with locoregional therapy until relapse (with a median follow-up of 10 years), mutant p53 was associated with negative receptors, age greater than 65 years, tumors greater than 2 cm, overall relapse and development of distant metastatic disease. Using multivariate analysis, mutant p53 correlated with overall relapse and survival as did tumor size, proliferative index, receptor negativity, and patient age.

Because experimental studies suggest a function for wt p53 in drug sensitivity (Lowe et al. 1993, 1994), the role of p53 as a predictor for response to anti-neoplastic therapy has also been evaluated. Two small studies of preoperative chemotherapy (with doxorubicin alone or a doxorubicin containing combination) in aggregate show that patients whose tumors expressed mutant p53 were more likely to manifest progressive disease during chemotherapy (Aas et al. 1996, Lizard-Nacol et al. 1997). However, two other studies failed to demonstrate any relationship between p53 status and response to preoperative chemotherapy (Linn et al. 1996, Makris et al. 1997). In addition, a large study evaluating p53 status and clinical outcome after adjuvant cyclophosphamide, methotrexate, 5-FU, and prednisone chemotherapy for node-negative breast cancer failed to confirm any relationship between p53 expression and clinical outcome (Elledge et al. 1995). Thus the importance of p53 status as a predictive factor is uncertain.

Bcl-2 and bax expression in breast cancers


Surprisingly, the relationship between lymph node status and bcl-2 expression is uncertain. Several studies have failed to link the two (Leek et al. 1994, Hurlimann et al. 1995, Bukholm et al. 1997b). However, a study by Sierra et al. (1995), utilizing tumor samples from women with tumors less than 2 cm, demonstrated a significant correlation between bcl-2 expression and lymph node involvement. Whether bcl-2 and/or bax expression is an independent prognostic factor remains to be seen. Kapranos and coworkers (1997) noted that the absence of bcl-2 or bax expression in node-negative breast cancers was associated with subsequent development of metastatic disease. Absence of both bax and bcl-2 expression was an even stronger predictor of distant relapse.

Three studies have examined the link between bcl-2 expression and response to endocrine therapy (Gee et al. 1994, Elledge et al. 1997, Keen et al. 1997). Together they suggest that bcl-2 expression is associated with enhanced response to endocrine therapy. Patients whose tumors co-expressed ER and bcl-2 derived the greatest benefit from hormonal therapy (Gee et al. 1994). This may be explained in part by the laboratory studies suggesting that bcl-2 (like PR) is an estrogen-regulated protein. More work will be required to elucidate a role for bcl-2 family members as prediction factors for response to systemic therapy.
Conclusions

Apoptosis is the primary mechanism of cell death in the normal mammary gland at all phases including ductal morphogenesis, pregnancy, lactation and involution. This pathway has been retained in breast cancer cells, and it is likely that aberrations in this pathway are central to tumorigenesis, tumor progression, and response to therapy. Multiple apoptotic signals have been identified which initiate the PCD pathway in normal mec and breast cancer cells, and a variety of mediators and pathways have been identified to play a role in PCD in various models of breast cancer. Biochemical and molecular markers identified in the PCD pathway in breast cancer cells may prove useful as prognosticators as well as predictors of tumor sensitivity to chemoendocrine treatments. With further elucidation, the biochemical and molecular pathways of PCD in mammary epithelium and breast cancer will broaden our understanding of mammary gland biology and the development of cancer in this tissue.

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