Apoptotic cell death and tissue remodelling during mouse mammary gland involution

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Summary

During post-lactational mammary gland involution, the bulk of mammary epithelium dies and is reabsorbed. This massive cell death and tissue restructuring was found to be accompanied by a specific pattern of gene expression. Northern blot analysis showed that weaning resulted in a dramatic drop in ODC, a gene involved in synthesis of a component of milk, and the nearly simultaneous induction of SGP-2, a gene associated with apoptotic cell death. These changes were followed by decreases in expression of milk protein genes to basal levels and expression of genes associated with regulation of cell proliferation and differentiation, p53, c-myc and TGF-β1. Subsequently, additional genes implicated in stress response, tissue remodelling, and apoptotic cell death were transiently expressed, expression peaking at about 6 days post-weaning. A non-random degradation of DNA yielding the oligonucleosomal length fragmentation pattern typical of apoptotic cell death (Wyllie, 1980; Wyllie et al., 1980) was detected in association with morphological changes and gene expression. The correlations between: (a) changes in morphology, (b) pattern of gene expression and (c) changes in DNA integrity suggest that complementary programs for cell death and tissue remodelling direct post-lactational mammary gland involution.

Abbreviations: ECM, extracellular matrix; hsp70, heat shock protein 70; LDH, lactate dehydrogenase; ODC, ornithine decarboxylase; SGP-2, sulfated glycoprotein-2; TIMP, tissue inhibitor of metalloproteinase; tPA, tissue plasminogen activator; tTG, tissue transglutaminase; TGF-β1, Transforming growth factor-β1; uPA, urokinase-type plasminogen activator; Wap, whey acidic protein.

Key words: apoptosis, cell death, DNA fragmentation, mammary epithelium, mammary involution, metalloproteinase, p53, SGP-2, TGF-β1.

Introduction

After completion of lactation, the mammary gland undergoes involution, regressing to a state resembling that of a virgin animal. This phase of mammary gland development is characterized by dramatic epithelial cell death and tissue remodelling (Ossowski et al., 1979; Pitelka, 1988) and has been described histologically and ultrastructurally as a process exhibiting morphological features consistent with an apoptotic cell death (Wellings and DeOme, 1963; Helminen and Ericsson, 1968a,b; Warburton et al., 1982; Walker et al., 1989). During involution, mammary gland mass and nucleic acid content also have been reported to decrease (Ota et al., 1962) but gene expression and nucleic acid integrity have not been characterized. We examined mammary gland involution for molecular evidence of apoptotic cell death and tissue remodelling.

In contrast to necrosis, which results from the perturbation of a physiological cell environment and causes severe cellular edema, loss of structure and random degradation of protein and nucleic acids, apoptotic processes typically involve cellular condensation and dehydration with maintenance of overall tissue structure and order (Wyllie, 1981). The first histological indication of mouse mammary gland regression is the breakdown of specialized cell junctions followed by collapse and dissolution of lobuloalveolar units, the secretory structures that form during lactogenic hormone-induced differentiation of mammary epithelium into a milk-producing tissue. This is accompanied by shedding of epithelial cells into the lumen of collapsing alveoli and alterations of the basement membrane that surrounds these epithelial structures (Wellings and DeOme, 1968; Martinez-Hernandez et al., 1976; Warburton, et al., 1982). Ultrastructural studies have detected compaction of nuclear chromatin and condensation of cell cytoplasm.
without apparent alteration of cytoplasmic organelles (Helminen and Ericsson, 1968a; Walker et al., 1989). Subsequently, membrane-bound vesicles containing nuclear fragments, cytoplasmic material and intact organelles bud from the cell. These vesicles are phagocytosed by adjacent epithelial cells and interstitial macrophages where they undergo autolysis (Helminen and Ericsson, 1968b; Walker et al., 1989). It is these findings which suggest that the morphological alterations observed during post-lactational regression of the mammary gland follow an orderly process consistent with apoptotic cell death.

Biochemical analysis of various tissues and cells undergoing apoptotic cell death has suggested that this endogenous cell process requires: (1) de novo gene expression, (2) utilization of energy, (3) protein synthesis and (4) activation of an endonuclease that cleaves DNA into oligonucleosomal length fragments (Wyllie et al., 1980; Wyllie et al., 1984; Cohen and Duke, 1984; Compton and Cidlowski, 1987). Thus, apoptosis may be described as an active, physiological cell death process with a recognizable signature of morphological alterations and non-random nucleic acid degradation.

We examined involuting mouse mammary glands to ask: (1) if a specific pattern of gene expression can be detected in regressing mammary glands and (2) if there is molecular evidence for apoptotic cell death during this process. Histological and immunohistochemical analyses of mammary gland morphologic changes during post-weaning involution were correlated with analysis of DNA integrity and analysis of the expression of a panel of genes associated with mammary epithelial differentiation, tissue remodelling, apoptotic cell death and regulation of cell proliferation and differentiation. These studies revealed that during mammary gland involution there are recognizable patterns of gene expression that correlate with the morphological changes. In addition, genes associated with apoptotic cell death in other systems are expressed and DNA integrity is molecular evidence for apoptotic cell death.

### Materials and methods

#### Mice and induction of involution

Mid-pregnant, multiparous Swiss mice (BRL, Basel, Switzerland) were allowed to deliver their young and the litter sizes were normalized to eight pups. After full lactation was established (7-10 days of nursing), the young were removed. The mice were killed and mammary glands were removed at days 1, 2, 3, 4, 5, 6, 8 and 10 after weaning. RNA was prepared immediately from mammary glands, or the tissue was frozen in liquid nitrogen and stored at -70°C, or fixed for histological analysis.

#### Histology and immunohistochemistry

Tissues were fixed in 70% ethanol-acetic acid-formaldehyde (20:1:1), defatted in acetone, dehydrated through graded alcohols and paraffin embedded. Sections (4-6 μm) were cut and stained with hematoxylin and eosin. Myoepithelial cells were detected by a rabbit polyclonal anti-muscle actin and visualized by immunoperoxidase.

### Northern blot analysis

Total RNA was prepared from mouse mammary glands using the guanidinium thiocyanate extraction method described by Chomczynski and Sacchi (1987). RNA (5-10 μg total RNA/sample, or poly(A)-enriched RNA 200 ng/sample) was denatured with glyoxal and electrophoresed on a 1% agarose gel in phosphate buffer before blotting onto nitrocellulose in 20xSSC. Nitrocellulose filters were hybridized for 16 hours at 42°C using [32P]dCTP random-primerized probes for: β-casein (Hennighausen and Sippel, 1982); ODC (McConlogue et al., 1984); γ-actin (Ginzburg et al., 1980); transin/stomelysin (Matrisian et al., 1985a), urokinase (Belin et al., 1985); TIMP (Gewert et al., 1987); LDH (Matrisian et al., 1985b); hsp70 (Lowe and Moran, 1986); SGP-2 (Bandyk et al., 1990); tTG (Chocca et al., 1988); and TGF-β1 (Derynck et al., 1986), c-myc (Schoenenberger et al., 1988) and p53 (Bienz et al., 1984). The hybridization solution contained 50% formamide, 4xSSC, 5xDenhardt’s solution, 0.1% sodium pyrophosphate, 0.2% SDS and 30 μg/ml salmon sperm DNA. The filters were washed twice at 50°C for 30 minutes in 2xSSC with 0.2% SDS followed by 0.5xSSC with 0.2% SDS at 50°C before autoradiography.

### Analysis of RNA integrity

Regressing mammary glands were frozen in liquid nitrogen and stored at -70°C. Tissue samples were minced rapidly and dissociated using a Dounce homogenizer in DNA extraction buffer (Tris-HCl 10 mM pH 7.5, NaCl 10 mM, EDTA 10 mM, protease K 0.2 mg/ml, 0.5% SDS). After two phenol/chloroform extractions, the DNA was ethanol precipitated and resuspended in TE pH 7.5. Samples (7.5 μg) were electrophoresed in 1.0% agarose gel DNA was visualized with ethidium bromide (0.5 μg/ml) and photographed.

### Results

#### Morphological changes during mammary gland involution

The lactating mammary gland (Fig. 1A) had well-defined lobuloalveolar structures containing secretory material. The nuclei were oval and pale suggesting active gene transcription. The alveolar structures consisted of a single layer of secretory epithelium with underlying myoepithelial cells and well-defined basement membrane. Very few adipocytes were seen in the lactating mammary gland. Two days post-weaning (Fig. 1B), secretory material was present in distended alveolar structures. Foci of collapsed alveoli were also observed. At four days post-weaning (Fig. 1C), no well-defined lobuloalveolar structure was apparent and both shrunken cells showing the nuclear changes characteristic of apoptosis and apoptotic bodies were seen throughout sections of mammary glands. This morphology was maintained through day six (Fig. 1D), when most epithelial cell nuclei were highly condensed. In addition, a significant incursion of cellular stromal elements was seen throughout the areas of densely packed epithelial cells. For example, in Fig. 1D, E adipocytes and other cellular stromal elements are seen...
Fig. 1. Hematoxylin-eosin-stained paraffin sections of mouse mammary glands illustrate the morphological changes occurring during involution. Sections from lactating mammary gland (A), mammary glands at 2 days (B), 4 days (C), 6 days (D) and 8 days post lactation (E), and a fully regressed mammary gland (F). Bar (Fig. 1A)=50 μm. Fig. 1F has different magnification, bar=100 μm.
in the midst of a mass epithelial cells. Few recognizable mammary acini were seen in sections of glands at 8-10 days post-weaning (Fig. 1E). Islands of epithelium were surrounded by adipocytes and interspersed by thickened stromal elements. In some cases, organized ductal remnants of the lactating mammary gland were present but resolution of the remodeling process was not yet complete. In agreement with previous work (Ossowsk et al., 1979), increased numbers of phagocytic cells were observed during involution but no massive infiltration of macrophages or granulocytes, such as seen associated with inflammation or necrosis, was observed. The fully regressed mammary gland, as seen in Fig. 1F, was largely ductal and reorganized for another cycle of lactation.

Myoepithelial organization was maintained during involution

Myoepithelial cells enclose the lobuloalveolar units in a basket-like structure. Paraffin sections of involuting mammary gland were stained by immunoperoxidase for expression of smooth muscle actin (Fig. 2), to show the myoepithelial cell organization. In the lactating mammary gland, myoepithelium was detected as thin extended brown-staining cells circumscribing the lobuloalveolar structures (Fig. 2A). As the process of mammary involution proceeded, the myoepithelium remained well-organized (Fig. 2B,C). Although collapsed, the remnants of alveoli could be seen within a defined ring of rounded myoepithelial cells. Thus, the myoepithelium remained organized and appeared to provide basic structure during mammary gland reorganization. In contrast, during this same time period, immunofluorescence studies (data not shown; Li, Strange and Friis, unpublished data) revealed basement membrane thickening and folding consistent with the disorganization reported in other morphological studies of mammary gland involution (Martin-Hernandez et al., 1979; Warburton et al., 1982).

Gene expression in regressing mouse mammary glands

Northern blot analysis was used to correlate changes in gene expression with observed changes in morphology. Specific genes were expressed in association with morphological changes in the mammary gland in a pattern that appears well-regulated. RNA extracted from involuting mammary glands was analyzed for expression of genes associated with: (1) mammary epithelial differentiation and metabolism: β-casein, ODC (Fig. 3) and Wap (data not shown); (2) tissue remodelling: TIMP, transin/stromelysin, uPA (Fig. 4) and tPA (data not shown); (3) stress and cell death: SGP-2, hsp70, tTG and LDH (Fig. 5) and (4) regulation of cell proliferation and differentiation: c-myc, p53 and TGF-β1 (Fig. 6).

As anticipated, after weaning and withdrawal of lactogenic hormone stimulation, the expression of β-casein RNA (Fig. 3) and Wap (data not shown) were dramatically reduced. Nearly simultaneously, the level of ODC RNA, a gene strongly expressed in lactating mammary gland, dropped to barely detectable levels.
Fig. 2. Immunohistochemical analysis of paraffin sections of mammary glands to detect mammary myoepithelium. Sections from lactating mammary gland (A), day 4 post-weaning (B) and day 10 post-weaning (C) were stained with rabbit antisera to smooth muscle actin and detected by immunoperoxidase. Bar=20 μm.
weaning. This expression increased gradually with a peak at day 6 post-weaning before decreasing again (Fig. 4). The pattern of expression agreed well with the morphological changes detected by histological analysis and alterations in basement membrane suggested by immunofluorescence studies (data not shown, Li, Strange and Friis, unpublished data). Associated with increasing levels of proteases, TIMP expression increased again, with a smaller second transcript being detected at day 6 post-weaning before decreasing again to basal levels as protease expression also decreased (Fig. 4).

The observation of morphological changes typical of apoptotic cell death suggested that the expression of genes associated with cell stress or apoptotic cell death in other systems was likely to be found in involuting mammary glands. Expression of genes associated both with cell stress response in general and with apoptosis in particular was examined. LDH and hsp70, genes associated with stress response, were not detected in the lactating mammary gland but were detectable by one day post-weaning. hsp70 expression has been associated with lysosomal targeting of intracellular proteins (Chiang et al., 1989) and has been detected in prostate epithelium following hormone ablation (Buttyan et al., 1988). Expression of hsp70 increased to a peak at 8 days post-weaning (Fig. 5). LDH expression, a measure of anaerobic glycolysis, was present at an elevated level throughout the period of involution studied (Fig. 5). Expression of SGP-2 and tTG, genes specifically associated with apoptosis in other systems (Buttyan et al., 1989; Kypranou et al., 1990, 1991; Fesus et al., 1987; Piacentini et al., 1991a,b) was more pronounced. SGP-2 expression rapidly increased from undetectable levels in lactating mammary gland to strong expression by day 1 post-weaning, peaking at day 2 before decreasing to pre-involution levels by day 10. Increased c-myc expression was detected at day 1 post-weaning and peaked at days 4 and 6 before declining to low levels by day 10. Increased c-myc expression was detected later, between days 4 and 8, and was less strong than either TGF-β1 or p53. TGF-β1 was expressed at low levels in pregnant mammary gland but was not detected during lactation (Fig. 6). p53 expression was detected at day 1 post-weaning and peaked at days 4 and 6 before declining to low levels by day 10. Increased c-myc expression was detected later, between days 4 and 8, and was less strong than either TGF-β1 or p53. TGF-β1 was expressed at low levels in pregnant mammary gland but was not detected during lactation (Fig. 6). p53 expression was detected at day 1 post-weaning and peaked at days 4 and 6 before declining to low levels by day 10. Increased c-myc expression was detected later, between days 4 and 8, and was less strong than either TGF-β1 or p53. TGF-β1 was expressed at low levels in pregnant mammary gland but was not detected during lactation (Fig. 6). p53 expression was detected at day 1 post-weaning and peaked at days 4 and 6 before declining to low levels by day 10. Increased c-myc expression was detected later, between days 4 and 8, and was less strong than either TGF-β1 or p53. TGF-β1 was expressed at low levels in pregnant mammary gland but was not detected during lactation (Fig. 6).
Fig. 7. (A) Electrophoretic analysis of DNA integrity during mammary gland involution. DNA extracted from mammary glands of pregnant (16-18 days; P), lactating (10 days, L), mammary glands post-weaning day 1 (1), day 2 (2), day 3 (3), day 4 (4), day 6 (6), day 8 (8), fully regressed mammary gland (R), and normal kidney (K). The left and right outermost lanes contain a 123-bp DNA ladder (BRL) as relative molecular mass markers. Oligonucleosomal DNA fragments are detected at day 1 post-weaning and increase until day 4 post-weaning before declining to barely detectable levels by day 6 post-weaning. (B) Higher magnification photomicrograph of a mammary gland at four days post-weaning illustrating the morphological changes seen throughout the involuting mammary glands. Arrows indicate progressive features of apoptotic cell death, including: (a) a cluster of cells with abnormal, partially condensed nuclei, (b) a cell with condensed chromatin, (c) a cell with cytoplasmic condensation and nuclear fragmentation and (d) apoptotic bodies containing nuclear fragments. Bar=15 μm.

and increased to a peak at day 4 before decreasing to barely detectable levels at six days post-weaning (Fig. 7A).

Higher magnification of a section of mammary gland 4 days after weaning illustrates nuclear changes observed during the time in which fragmentation was detected. Epithelial cells (Fig. 7B) exhibited nuclear condensation and fragmentation, cytoplasmic condensation and formation of apoptotic bodies typical of apoptosis. Similar cells were also seen within intact ductal structures suggesting that they were shed from collapsing alveoli upstream in a tributary to the larger duct.

Discussion

Initiation of mammary gland involution

During post-lactational mammary involution, the lactating breast which is composed of secretory epithelium regressed to a quiescent organ composed predominantly of fat cells surrounding a denuded mammary epithelial tree. The patterns of morphological change, concerted gene expression and non-random DNA degradation suggest that mammary gland involution is executed by specific and complementary programs for apoptotic cell death and tissue remodelling. The initiation and control of these processes are not well understood. An attractive hypothesis, based on the epithelial-mesenchymal interactions required for lactogenic differentiation (Levine and Stockdale, 1984; Barcellos-Hoff et al., 1989; Schoenenberger et al., 1990), is that destruction of the basement membrane induces mammary gland involution. However, sloughing of luminal epithelial cells, expression of genes associated with apoptotic cell death (e.g., SGP-2 and TGF-β1), nuclear changes and fragmentation of DNA presented here and described in other studies (Radnor, 1972; Martinez-Hernandez et al., 1976; Warburton et al., 1982; Walker et al., 1989), precede basement membrane changes. Thus, it appears likely that initiation of apoptosis precedes major alterations in the basement membrane. The rapid downregulation of genes whose expression depends upon lactogenic hormone stimulation and the early detection of fragmented DNA, presumably from apoptotic secretory epithelium, suggest that epithelial cell differentiation and the availability of lactogenic hormones are also important factors for initiation of cell death. In other systems, initiation of apoptosis appears to involve limitation of factors required for cell viability and often this is associated with a particular stage in differentiation (Buttyan et al., 1988, 1989; Kyprianou et al., 1990, 1991; Rennie et al., 1988; Williams et al., 1990). These observations are consistent with the hypothesis that the cell death observed during mammary gland involution results from a terminal differentiation event in which epithelial cells become committed to lactogenesis and dependent on lactogenic hormone stimulation for survival (Ossowski et al., 1979). The apoptotic death that follows loss of lactogenic hormone stimulation is consistent with this hypothesis. Loss of hormone stimulus is certainly the proximal signal for mammary gland involution. However, because mammary gland involution is reversible for a period of time (until between 1 and 2 days after weaning), the commitment to involution, particularly apoptotic cell death, must require additional signals.

Expression of death-associated genes

Candidate genes that regulate or execute apoptotic cell death have been identified in nematode development
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(Yuan and Horvitz, 1990). However, isolation of vertebrate homologs of these genes remains elusive. Expression of other genes has been associated with apoptotic cell death in vertebrates but their precise roles are not proven. For example, tTG has been detected in association with experimentally-induced apoptotic cell death (Fesus et al., 1987, 1989; Piacentini et al., 1991a,b). This enzyme has been suggested to play a protective role during apoptosis. By crosslinking and stabilizing membranes, tTG may prevent uncontrolled release of powerful lysosomal enzymes. In similar fashion, the potentially mutagenic release of fragmented DNA from apoptotic cells may be inhibited by tTG-mediated crosslinking of chromatin. Such insoluble protein crosslinks have been correlated with tTG expression and induction of apoptotic death (Fesus et al., 1989; Piacentini et al., 1991b). In addition, immunolocalization experiments have shown expression of tTG in cells undergoing apoptosis and apoptotic bodies (Piacentini et al., 1991a). tTG was expressed during mammary gland involution when morphological changes consistent with such crosslinking activity were detected.

The early and strong expression of SGP-2 during mammary gland involution suggests a more immediate role in programmed cell death. SGP-2 has been isolated from regressing rat prostate following castration-induced hormone ablation (Buttyan et al., 1989; Bandey et al., 1990) and from a complex that is inhibitory for complement-mediated cytolysis (Kizbauman et al., 1989; Jenne and Tschopp, 1989). In addition, expression of SGP-2 has been detected in the interdigital regions of cell death found during vertebrate limb development (Buttyan et al., 1989). The expression of SGP-2 detected here and in other studies (Rennie et al., 1988; Buttyan et al., 1989; Kyprianou et al., 1990, 1991) and its apparent cell surface location (Tung and Fritz, 1985) suggest that it may play a role in marking apoptotic cells for phagocytosis. This sort of targeting has been reported as important for macrophage recognition of apoptotic cells (Duvall et al., 1985; Savill et al., 1990). Another possible function of SGP-2 is suggested by studies of inhibition of complement-mediated cytolysis (Kirzbaum et al., 1989; Jenne and Tschopp, 1989). It is conceivable that such inhibition could be important to maintain orderly remodelling of the involuting mammary gland.

Tissue remodelling

The dramatic tissue remodelling observed during involution leaves little question about the role of protease expression. Plasminogen activator activity has been previously detected in involuting mammary glands (Ossowski et al., 1979) and expression has been detected during castration-induced regression of the rat prostate gland (Rennie et al., 1984). During these processes, the basement membrane encompassing epithelial structures must undergo significant changes as the bulk of epithelium undergoes cell death. Immunofluorescence studies of involuting mammary glands (data not shown; Li, Strange and Friis, unpublished data) detected alterations of the basement membrane consistent with ultrastructural (Martinez-Hernandez et al., 1979) and immunohistochemical (Warburton et al., 1982) studies. These morphological changes were observed when increased protease gene expression was detected. Protease expression was complemented by changes in expression of TIMP, an inhibitor of protease activity, suggesting that the activity of remodelling enzymes is under tight regulation. A similar pattern for expression of caseinases and gelatinases during mammary gland involution was recently reported (Talhouk et al., 1991). This study also found that some of the proteases detected were epithelial cell products; an observation consistent with the activation of an endogenous program for epithelial cell death. However, important questions remain to be investigated, including: (1) the identity of the cells expressing remodelling enzymes (are they the apoptotic epithelial cells or stromal cells?) and (2) the regulation of tissue remodelling.

Coordination of cell death and tissue remodelling

The expression of TGF-β1 detected early in mammary involution was provocative as a potential modulator of both apoptotic cell death and expression of tissue remodelling enzymes. In vivo studies of hormone-dependent epithelial tumor lines in nude mice have detected increased expression of TGF-β1 in association with hormone ablation-induced apoptotic cell death (Kyprianou et al., 1990, 1991). In vitro experiments also have demonstrated that TGF-β1 is able both to inhibit epithelial cell proliferation (Yamanishi et al., 1990) and to induce apoptotic cell death of epithelial cells (Martikianen et al., 1990; Rotello et al., 1991). In the mammary gland, TGF-β1 treatment of developing endbuds by slow release implants resulted in the cessation of DNA synthesis in proliferating endbud epithelium, development of a stromal hyperplasia and endbud involution with appearance of masses of epithelial cells, some of which were autophagic, within the lumen of the endbud (Silberstein et al., 1990; Daniel et al., 1989). TGF-β1 also has been reported to modulate expression of iTG (George et al., 1990), extracellular matrix-degrading proteases (Keski-Oja et al., 1988; Laiho and Keski-Oja, 1989; Kerr et al., 1990), and to regulate connective tissue proliferation (Battegay et al., 1990). Such observations are consistent with a role for TGF-β1 in both the induction of apoptotic cell death and the modulation of tissue remodelling during mammary gland involution. Thus, it is particularly significant that strong expression of TGF-β1 is detected during mammary gland involution in which epithelial cell death and tissue remodelling play such important roles. The source of the TGF-β1 in involuting mammary glands remains to be determined and this work is in progress.

Apoptosis and initiation of mammary neoplasia

Apoptotic cell death is believed to play a significant role in normal development and maintenance of tissue homeostasis, e.g. embryonic morphogenesis, endome-
trial cycling, neuronal cell deletion and immune system cell selection (Wyllie et al., 1980; Hassell, 1975; Fichet and Hay, 1989; Fitzpatrick et al., 1990; Hopwood and Levison, 1976; Oppenheim et al., 1988; Rotello et al., 1991; Tomei and Cope, 1991). In these settings, apoptosis is a physiological means for limiting cell number, removing unneeded and aged cells, or eliminating potentially detrimental classes of cells. These functions raise the question of what would result from the failure or misregulation of apoptosis. Recent studies of the BCL2 gene may provide some insight. BCL2 is a mitochondrial gene expressed in long-lived populations of glandular epithelium in which hormones or growth factors regulate hyperplasia and involution, such as breast epithelium (Hockenberry et al., 1991). Misregulated BCL2 expression in B-cells has been shown to block apoptotic cell death resulting in prolonged cell survival that contributes to tumorigensis (Hockenberry et al., 1990; Henderson et al., 1991; Gregory et al., 1991). In the mouse mammary gland, prolonged cell survival is also a characteristic of a premalignant lesion associated with increased tumor risk called a hyperplastic alveolar nodule (HAN). Such lesions morphologically resemble mid-pregnant mammary epithelium yet fail to fully regress following pregnancy and lactation (Foulds, 1969; Medina, 1978; Morris and Cardiff, 1987). Furthermore, the inability of mammary epithelium to regress has been shown to increase tumor frequency and to decrease tumor latency (Andres et al., 1991). Thus, mutation of a gene instrumental in the apoptotic death of mammary epithelium would be a likely factor contributing to initiation of neoplasia. Indirect evidence for this hypothesis is provided by studies of the Ha-ras oncogene, an oncogene associated with inhibition of apoptotic cell death (Wyllie et al., 1987). Introduction of a Ha-ras oncogene into normal mammary epithelial cells resulted in mammary dysplasias (Strange et al., 1989) which had characteristics of HANs. Like HANs, these premalignant dysplasias were not proliferative but did not regress following hormone ablation (Strange et al., 1989, unpublished observations). The strong expression of p53, a tumor suppressor gene that is important for cell cycle control (Levine et al., 1991) during mammary gland involution is particularly interesting in this context. Mutation of this gene is the most frequent defect found in tumors, including breast tumors (Levine et al., 1991). Introduction of a functional p53 is also reported to induce apoptosis (Yonish-Rouach et al., 1991) and inhibit growth of breast tumor cells (Casey et al., 1991). Such findings are consistent with the hypothesis that a defect in apoptotic cell death can be an early event in neoplastic development of the mammary gland.

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