Development 115, 49-58 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

Apoptotic cell death and tissue remodelling during mouse mammary gland involution

ROBERT STRANGE[1,*,†], FENG LI[1], SUSANNE SAURER[1], ARNE BURKHARDT[2] and ROBERT R. FRIIS[1]

[1]*Laboratory for Clinical and Experimental Research, University of Bern, CH-3004 Bern, Switzerland*
[2]*Kreiskrankenhaus Reutlingen, D-7410 Reutlingen, Germany*

*Current address AMC Cancer Research Center, 1600 Pierce St , Denver, CO 80214, USA
†To whom correspondence should be addressed

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 (Ôssowski 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 (Ôta 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-bounded 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 morphology 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 shows the oligonucleosomal length fragmentation associated with apoptosis.

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 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 20×SSC. Nitrocellulose filters were hybridized for 16 hours at 42°C using [^{32}P]dCTP random-primed 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* (Chiocca 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, 4×SSC, 5×Denhardt'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 2×SSC with 0 2% SDS followed by 0.5×SSC with 0.2% SDS at 50°C before autoradiography.

Analysis of DNA 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, proteinase 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, TBE 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

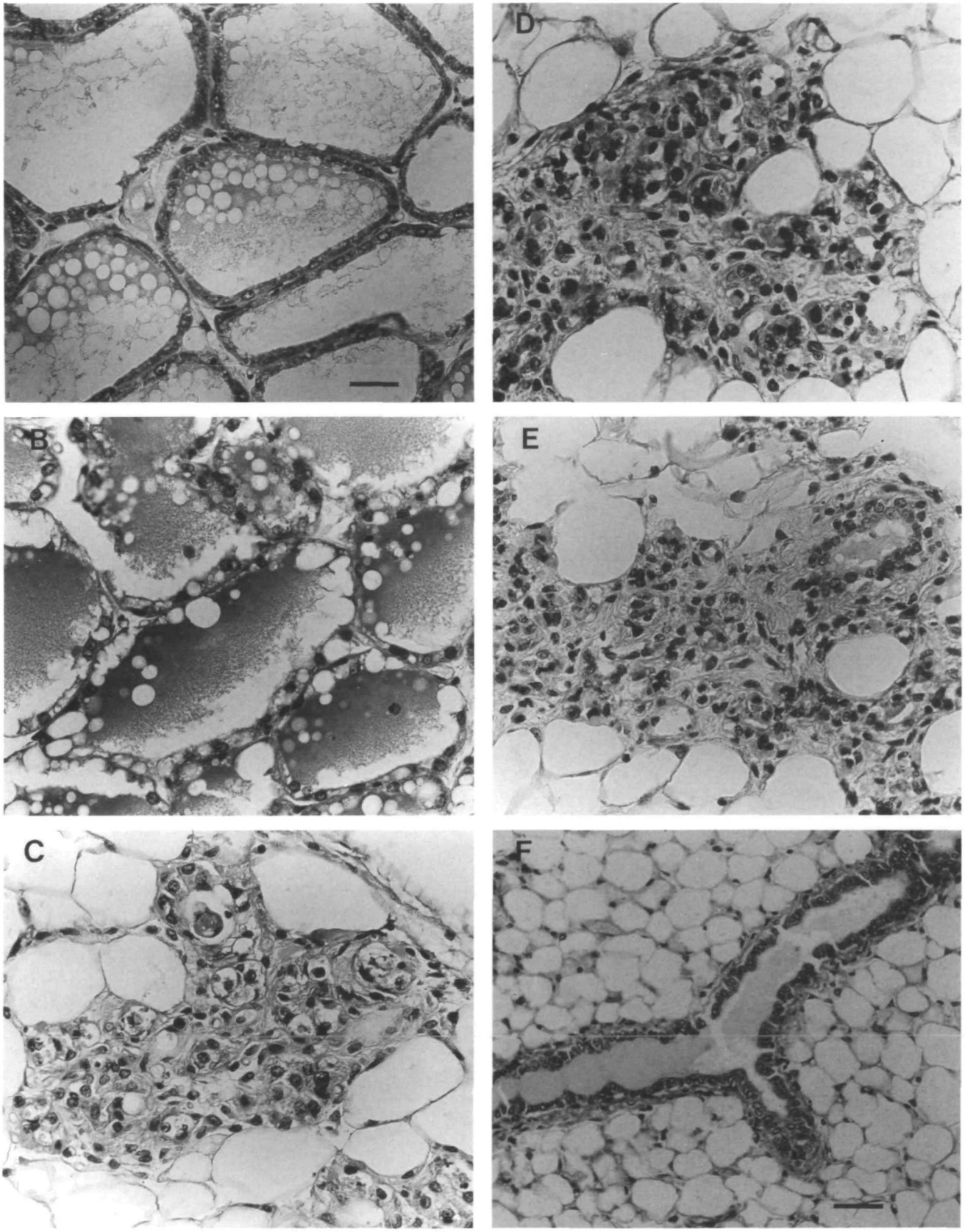

Fig. 1. Hematoxylin-eosin-stained paraffin sections of mouse mammary glands illustrate the morphological changes occurring during involution Sections from lactating mammary gland (A), mammary glands at 2 days (B), 4 days (C), 6 days (D) and 8 days post lactation (E), and a fully regressed mammary gland (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 remodelling process was not yet complete. In agreement with previous work (Ossowski 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.

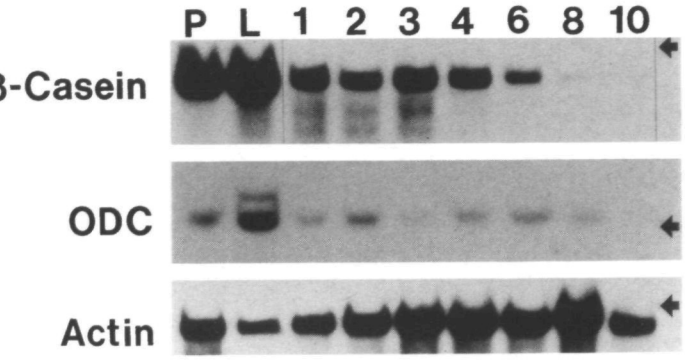

Fig. 3. Mammary epithelial differentiation. This figure shows Northern blot analysis of expression of *β-casein*, *ODC* and *γ-Actin* in mouse mammary glands during pregnancy (16-18 day), lactation (day 10) and days 1, 2, 3, 4, 6, 8 and 10 after weaning, (7.5 μg total RNA/lane). Arrows indicate migration of 18S rRNA.

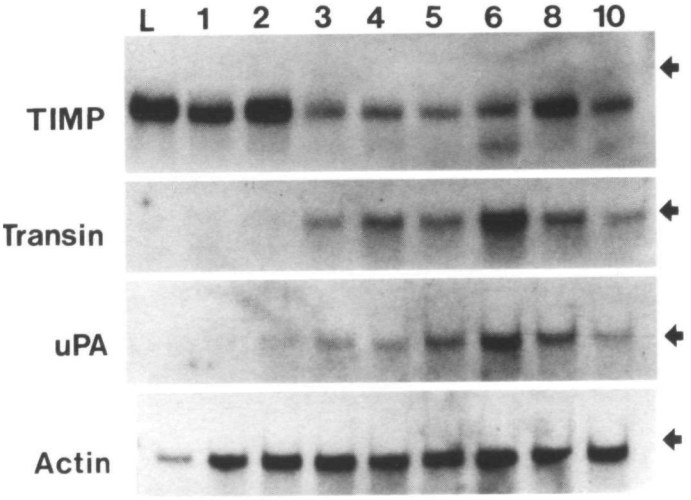

Fig. 4. Tissue remodelling enzymes. The figure shows Northern blot analysis of expression genes associated with tissue remodelling: *TIMP*, *uPA* and *stromelysin/transin* during lactation (day 10) and days 1, 2, 3, 4, 5, 6, 8 and 10 after weaning, (0.2 μg poly (A)+ RNA/lane). Arrows indicate migration of 18S rRNA.

Morphological studies showed collapse of alveolar structures, death of epithelial cells and suggested that alterations of the basement membrane occurred during this time frame, 3-8 days post-weaning (Fig. 1). Expression of several proteases and an inhibitor of proteinase activity was examined for evidence of potential mediators for these morphological changes (Fig. 4; Li, Strange and Friis, unpublished data). Protease and protease inhibitor expression were inversely related. Expression of *TIMP*, a protease inhibitor, was maintained at a high level until three days post-weaning when it dropped significantly (Fig. 4). Proteases *uPA*, *tPA* (data not shown) and *transin/stromelysin* were not detected in lactating mammary gland but were expressed beginning about two days post-

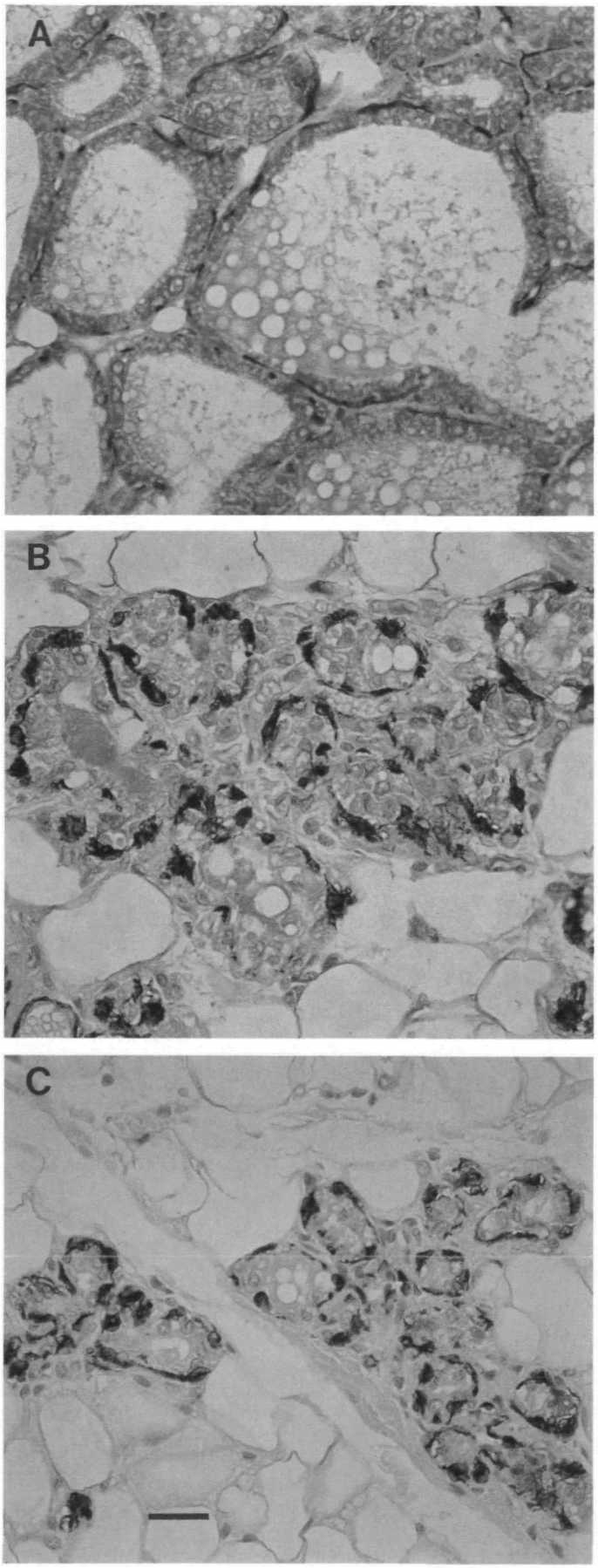

Fig. 2. Immunohistochemical analysis of paraffin sections of mammary glands to detect mammary myoepithelium. Sections from lactating mammary gland (A), day 4 post-weaning (B) and day 10 post-weaning (C) were stained with rabbit antisera to smooth muscle actin and detected by immunoperoxidase. Bar=20 μm.

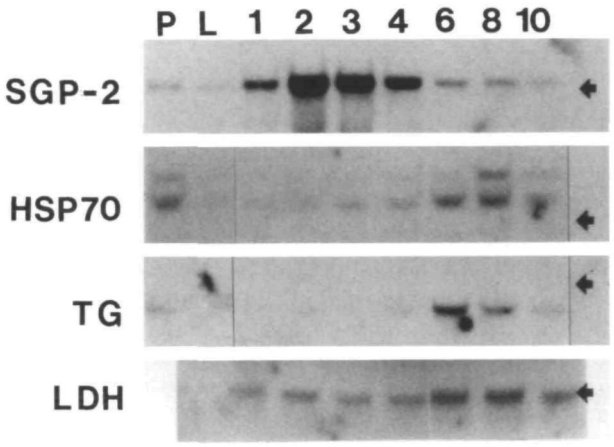

Fig. 5. Stress and cell death. Northern blot analysis of expression of genes associated with stress and cell death: *SGP-2*, *hsp70*, *tTG* and *LDH* during pregnancy (16-18 day), lactation (day 10) and days 1, 2, 3, 4, 6, 8 and 10 after weaning, (7.5 μg total RNA per lane) Arrows indicate migration of 18S rRNA, except for TG. Arrow to right of TG RNAs indicates migration of 28S rRNA.

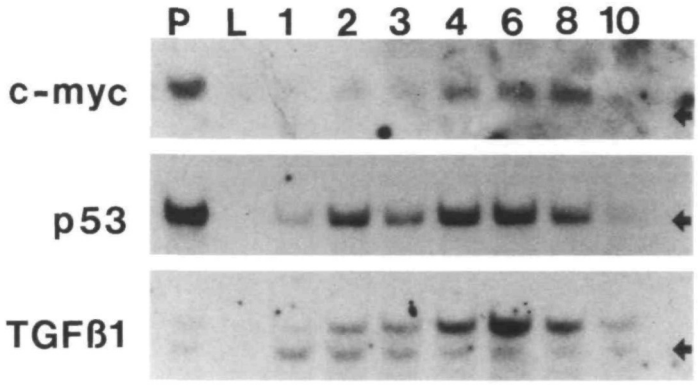

Fig. 6. Regulation of cell proliferation. Northern blot analysis of expression of genes implicated in regulation of cell proliferation growth and differentiation: *c-myc*, *p53* and *TGF-β1* during pregnancy (16-18 day), lactation (day 10) and days 1, 2, 3, 4, 6, 8 and 10 after weaning, (7.5 μg total RNA per lane).

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; Kyprianou 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 (Fig. 5). The expression of *tTG* also was not detected in lactating mammary gland but increased gradually to a peak at day 6 post-weaning before declining to an undetectable level at day 10 (Fig. 5).

Genes associated with the regulation of cell growth and differentiation also had definite patterns of expression during involution. *c-myc* and *p53* were expressed in pregnant mammary gland, downregulated to undetectable levels in lactating mammary gland and then were expressed again during involution (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). *TGF-β1* had a pattern of expression similar to *p53* during involution. It was detected at day 1 post-weaning and increased strongly during involution with a peak of expression at day 6 before declining to low levels by day 10.

Electrophoretic analysis of DNA isolated from involuting mammary glands

The non-random degradation of DNA into oligonucleosomal fragments is the most obvious feature for distinguishing between most apoptotic and necrotic death processes. This fragmentation is believed to be accomplished by activation of a specific, endogenous endonuclease (Wyllie, 1980; Cohen and Duke, 1984; Compton and Cidlowski, 1987). Electrophoretic analysis revealed DNA degradation consistent with activation of a specific endonuclease. DNA isolated from a control tissue (kidney), lactating mammary gland and mammary gland late in involution, was intact, high molecular DNA (Fig. 7A). In contrast, during the time that nuclear changes were seen and expression of *tTG* was detected, mammary gland DNA was degraded in a non-random pattern. Oligonucleosomal length DNA fragments were detected as early as day 1 post-weaning

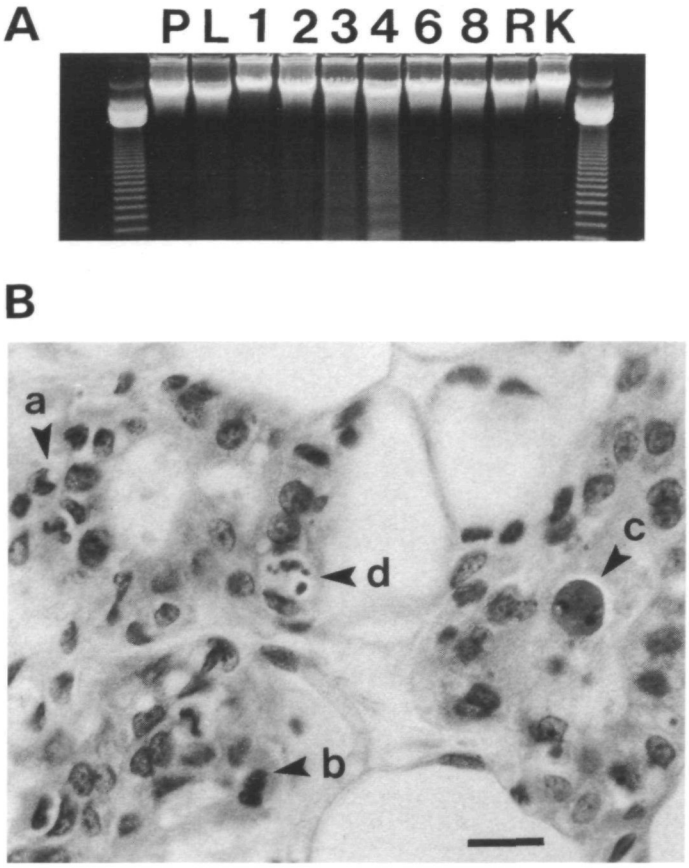

Fig. 7. (A) Electrophoretic analysis of DNA integrity during mammary gland involution. DNA extracted from mammary glands of pregnant (16-18 days; P), lactating (10 days, L), mammary glands post-weaning day 1 (1), day 2 (2), day 3 (3), day 4 (4), day 6 (6), day 8 (8), fully regressed mammary gland (R), 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

(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; Bandyk et al., 1990) and from a complex that is inhibitory for complement-mediated cytolysis (Kirzbaum 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, 1988). 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 *tTG* (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

Apototic 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; Fichett 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 tumorigenesis (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.

The authors thank Drs A.-C. Andres and A. Ziemiecki (Laboratory for Clinical and Experimental Research, Bern), Drs H. Thompson, P. Schedin and M. Singh (AMC Cancer Research Center, Denver) for critique of the manuscript and T. Tüscher for logistic support and assistance with manuscript preparation We also thank Dr R. Derynck for the TGF-β1 clone, Dr R. Buttyan for p1321 (SGP-2) and Dr P. J. A. Davies for mTg7.4 (mouse tissue transglutaminase). This work was supported by grants from the Swiss National Science Foundation, the Schweizerische Krebsliga, the Bernische Krebsliga and the Schweizerische Stiftung für klinische-experimentelle Tumorforschung.

References

Andres, A.-C., Bchini, O., Schubar, B., Dolder, B., LeMeur, M. and Gerlinger, P. (1991). H-*ras* induced transformation of mammary epithelium is favored by increased oncogene expression or by inhibition of mammary regression *Oncogene* **6**, 771-779

Bandyk, M. G., Sawczuk, I. S., Olsson, C. A., Katz, A. E. and Buttyan, R. (1990). Characterization of the products of a gene expressed during androgen-programmed cell death and their potential use as a marker of urogenital injury. *J Urol.* **143**, 407-412

Barcellos-Hoff, M. H., Aggeler, J., Ram, T. G. and Bissell, M. J. (1989). Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* **105**, 223-235

Battegay, E. J., Raines, E. W., Seifert, R. A., Bowen-Pope, D. F. and Ross, R. (1990) TGF-β induces bimodal proliferation of connective tissue via complex control of an autocrine PDGF loop *Cell* **63**, 515-524.

Belin, D., Vassalli, J.-D., Combépine, C., Godeau, F., Nagamine, Y., Reich, E., Kocher, H. P. and Duvosin, R. M. (1985) Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator *Br J Biochem* **148**, 225-232

Bienz, B., Zakut-Houri, R., Givol, D. and Oren, M. (1984) Analysis of the gene coding for the murine cellular tumour antigen p53. *EMBO J* **3**, 2179-2183

Buttyan, R., Olsson, C. A., Pintar, J., Chang, C., Bandyk, M., Ng, P.-Y. and Sawczuk, I. S. (1989) Induction of the *TRPM-2* gene in cells undergoing programmed death *Mol Cell Biol* **9**, 3473-3481

Buttyan, R., Zakeri, Z., Lockshin, R. and Wolgemuth, D. (1988). Cascade induction of c-*fos*, c-*myc*, and heat shock 70K transcripts during regression of the rat ventral prostate gland. *Mol. Endocrin* **2**, 650-657

Casey, G., Lo-Hsueh, M., Lopez, M. E., Vogelstein, B. and Stanbridge, E. J. (1991). Growth Suppression of human breast cancer cells by the introduction of a wild-type p53 *Oncogene* **6**, 1791-1797.

Chiang, H.-L., Terlecky, S. R., Plant, C. P. and Dice, D. F. (1989) A role for a 70-Kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* **246**, 382-385

Chiocca, E. A., Davies, J. A. and Stein, J. P. (1988) The molecular basis of retinoic acid action. *J Biol Chem* **263**, 11584-11589

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction *Anal Biochem* **162**, 156-159

Cohen, J. J. and Duke, R. C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death *J Immunol* **132**, 38-42

Compton, M. M. and Cidlowski, J. A. (1987). Identification of a glucocorticoid-induced nuclease in thymocytes· A potential "lysis gene" product. *J Biol Chem* **262**, 8288-8292.

Daniel, C. W., Silberstein, G. B., Van Horn, K., Strickland, P. and Robinson, S. (1989) TGF-β1-induced inhibition of mouse mammary ductal growth developmental specificity and characterization. *Dev Biol* **135**, 20-30.

Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B. and Goeddel, D. V. (1985) Human transforming growth factor β complementary DNA sequence and expression in normal and transformed cells *Nature* **316**, 701-705

Duvall, E., Wyllie, A. H. and Morris, R. G. (1985) Macrophage recognition of cells undergoing programmed cell death (apoptosis) *Immunology* **56**, 351-358

Fesus, L. and Thomazy, V. (1989) Apoptotic hepatocytes become insoluble in detergents and chaotropic agents as a result of transglutaminase action *FEBS Lett* **245**, 150-154.

Fesus, L., Thomazy, V. and Falus, A. (1987). Induction and activation

of tissue transglutaminase during programmed cell death. *FEBS Lett* **224**, 104-108

Fichett, J. E. and Hay, E. D. (1989). Medial edge epithelium transforms to mesenchyme after embryonic palate shelves fuse *Dev. Biol* **131**, 455-474

Fitzpatrick, D. R., Denhez, F., Kondaiah, P. and Akhurst, R. J. (1990) Differential expression of TGF beta isoforms in murine palatogenesis *Development* **109**, 585-595

Foulds, L. (1969) *Neoplastic Development.* New York. Academic Press

George, M. D., Vollberg, T. M., Floyd, E. E., Stein, J. P. and Jetten, A. M. (1990) Regulation of transglutaminase type II by Transforming Growth Factor-β1 in normal and transformed human epidermal keratinocytes *J Biol Chem* **265**, 11098-11104.

Gewert, D. R., Coulombe, B., Castellino, M., Skup, D. and Williams, B. R. G. (1987) Characterization and expression of a murine gene homologous to human EPA/TIMP A virus-induced gene in the mouse *EMBO J* **6**, 651-657

Ginzburg, I., De Baetelier, A., Walker, M. D., Behat, L., Lehrach, H., Rischauf, A. M. and Littauer, U. Z. (1980) Brain tubulin and actin cDNA sequences isolation of recombinant plasmids. *Nuc Acid Res* **8**, 3553-3565.

Gregory, C. D., Dive, C., Henderson, S., Smith, C. A., Williams, G. T., Gordon, J. and Rickinson, A. B. (1991) Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. *Nature* **349**, 612-614

Hassell, J. R. (1975) The development of rat palatal shelves *in vitro* An ultrastructural analysis of the inhibition of epithelial cell death and palate fusion by epidermal growth factor *Dev Biol* **45**, 90-102.

Helminen, H. J. and Ericsson, J. L. E. (1968a) Studies on mammary gland involution I On the ultrastructure of the lactating mammary gland. *J Ultrastruct Res* **25**, 193-213

Helminen, H. J. and Ericsson, J. L. E. (1968b) Studies on mammary gland involution II Ultrastructural evidence for auto- and heterophagocytic pathways for cytoplasmic degradation. *J Ultrastruct Res* **25**, 214-227

Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wand, F., Longnecker, R., Kieff, E. and Rickinson, A. B. (1991) Induction of *bcl-2* Expression by Epstein-Barr virus latent membrane protein 1 protects infected B cell from programmed cell death *Cell* **65**, 1107-1115

Hennighausen, L. G. and Sippel, A. E. (1982) Characterization and cloning of mRNAs specific for the lactating mammary gland *Eur J Biochem* **125**, 131-141

Hockenberry, D., Nuñez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death *Nature* **348**, 334-336

Hockenberry, D., Zutter, M., Hickey, W., Nahm, M. and Korsmeyer, S. J. (1991) Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death *Proc. Natl Acad Sci USA* **88**, 6961-6965

Hopwood, D. and Levison, D. A. (1976) Atrophy and apoptosis in the cyclical human endometrium *J Path* **119**, 159-166.

Jenne, D. E. and Tschopp, J. (1989). Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma· Identity to sulfated glycoprotein 2, a constituent of rat testis fluid *Proc Natl Acad Sci USA* **86**, 7123-7127.

Kerr, K. D., Miller, D. B. and Matrisian, L. M. (1990). TGF-β1 inhibition of transin/stromelysin gene is mediated through a **fos** binding sequence. *Cell* **61**, 267-278.

Keski-Oja, J., Blasi, F., Leof, E. and Moses, H. L. (1988). Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cell by Transforming Growth Factor-β *J Cell Biol* **106**, 451-459.

Kirzbaum, L., Sharpe, L. A., Murphy, B., D'Apice, A. J. F., Classon, B., Hudson, P. and Walker, I. A. (1989) Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40· a link between the complement and reproductive systems *EMBO J* **8**, 711-718

Kyprianou, N., English, H. F., Davidson, N. E. and Isaacs, J. T. (1991). Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res.* **51**, 162-166.

Kyprianou, N., English, H. F. and Isaacs, J. T. (1990). Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. *Cancer Res* **50**, 3748-3753

Laiho, M. and Keski-Oja, J. (1989) Growth factors in the regulation of pericellular proteolysis A review *Cancer Res* **49**, 2533-2553

Levine, A. J., Momand, J. and Finlay, C. A. (1991) The p53 tumour suppressor gene *Nature* **351**, 453-456.

Levine, J. F. and Stockdale, F. E. (1984). Cell-cell interactions promote mammary epithelial cell differentiation *J Cell Biol* **100**, 1415-1422

Lowe, D. G. and Moran, L. A. (1986) Molecular cloning and analysis of DNA complementary to three mouse M_r=68,000 heat shock protein mRNAs. *J Biol Chem* **261**, 2102-2112

Martikianen, P., Kyprianou, N. and Issacs, J. T. (1990) Effect of Transforming Growth Factor-β_1 on proliferation and death of rat prostatic cells *Endocrinology* **127**, 2963-2968

Martinez-Hernandez, A., Fink, L. M. and Pierce, G. B. (1976). Removal of basement membrane in the involuting breast *Lab Invest* **34**, 455-462

Matrisian, L. M., Glaichenhaus, N., Gesnel, M.-C. and Breathnach, R. (1985a) Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. *EMBO J* **4**, 1435-1440

Matrisian, L. M., Rautmann, B., Magun, B. E. and Breathnach, R. (1985b) Epidermal growth factor or serum stimulation of rat fibroblasts induces an elevation in mRNA level s for lactate dehydrogenase and other glycolytic enzymes. *Nuc Acid Res* **13**, 711-726

McConlogue, L., Gupta, M., Wu, L. and Coffino, P. (1984). Molecular cloning and expression of the mouse ornithine decarboxylase gene *Proc Natl Acad Sci. USA* **81**, 540-544

Medina, D. (1978) Preneoplasia in breast cancer In *Breast Cancer* Vol. 2 (ed W.L. McGuire) pp 47-107. Philadelphia Plenum.

Morris, D. W. and Cardiff, R. D. (1987) Multistep model of mouse mammary tumor development. *Adv. Viral Oncology* **7**, 123-140

Oppenheim, W., Haverkamp, L. J., Prevette, D., McManaman, J. L. and Appel, S. H. (1988). Reduction of naturally occurring motoneuron death in vivo by a target-derived neurotrophic factor. *Science* **240**, 919-922

Ossowski, L., Biegel, D. and Reich, E. (1979) Mammary plasminogen activator. Correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue *Cell* **16**, 929-940

Ôta, K., Yokoyoma, A. and Shinde, Y. (1962). Effects of administration of oxytocin and prolactin on nucleic acids and phosphoproteins contents of mammary glands in lactating rats. *Nature* **195**, 77-78

Piacentini, M., Autori, F., Dini, L., Farrace, M. G., Ghibelli, L., Piredda, L. and Fesus, L. (1991a) "Tissue" transglutaminase is specifically expressed in neonatal rat liver cells undergoing apoptosis upon epidermal growth factor-stimulation *Cell and Tissue Res* **263**, 227-235.

Piacentini, M., Fesus, L., Farrace, M. G., Ghibelli, L., Pireddal, L. and Melino, G. (1991) The expression of "tissue" transglutaminase in two human cancer cell lines is related with the programmed cell death (apoptosis). *Eur J Cell Biol* **54**, 246-254.

Pitelka, D. R. (1988) The Mammary Gland, In *Cell and Tissue Biology*, (ed L Weiss), pp 877-898, Baltimore Urban and Schwarzenberg

Rennie, P. S., Bouffard, R., Bruchovsky, N. and Cheng, H. (1984). Increased activity of plasminogen activators during involution of the rat ventral prostate. *Biochem J* **221**, 171-178.

Rennie, P. S., Bruchovsky, N., Buttyan, R., Benson, M. and Cheng, H. (1988). Gene expression during the early phases of regression of the androgen-dependent Shionogi mouse mammary carcinoma. *Cancer Res* **48**, 6309-6312.

Rotello, R. J., Liebermann, R. C., Purchio, A. F. and Gerschenson, L. E. (1991) Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β1 in cultured uterine epithelial cells. *Proc Natl Acad Sci. USA* **88**, 3412-3415.

Savill, J., Dransfield, I., Hogg, N. and Haslett, C. (1990) Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature* **343**, 170-173

Schoenenberger, C.-A., Zuk, A., Groner, B., Jones, W. and Andres, A.-C. (1990) Induction of the endogenous whey acidic protein and a c-myc transgene in primary murine mammary organoids *Dev Biol* **139**, 327-337

Silberstein, G., Strickland, P., Coleman, S. and Daniel, C. W. (1990) Epithelium-dependent extracellular matrix synthesis in Transforming Growth Factor-β1-inhibited mouse mammary gland *J. Cell Biol* **110**, 2209-2219

Strange, R., Aguilar-Cordova, E. C., Young, L. J. T., Billy, H. T., Dandekar, S. and Cardiff, R. D. (1989). Harvey-*ras* mediated neoplastic development in the mouse mammary gland. *Oncogene* **6**, 309-315.

Talhouk, R., Chin, J. R., Unemori, E. N., Werb, Z. and Bissell, M. J. (1991). Proteinases of the mammary gland: developmental regulation *in vivo* and vectorial secretion in culture *Development* **112**, 439-449

Tomei, L. D. and Cope, F. O. (1991). *Apoptosis the molecular basis of cell death.* Cold Spring Harbor: Cold Spring Harbor Lab. Press

Tung, P. S. and Fritz, I. B. (1985). Immunolocalization of clusterin in the ram testis, rat testis and excurrent ducts *Biol Reprod* **33**, 177-186

Walker, N. I., Bennett, R. E. and Kerr, J. F. R. (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats *Am J Anat* **185**, 19-32

Warburton, M. J., Mitchell, D., Ormerod, E. J. and Rudland, P. (1982). Distribution of myoepithelial cells and basement membrane proteins in the resting, pregnant, lactating and involuting rat mammary gland. *J Histochem Cytochem* **30**, 667-676.

Wellings, S. R. and DeOme, K. B. (1963) Electron microscopy of milk secretion in the mammary gland of the C3H mouse III. Cytomorphology of the involuting gland *JNCI* **30**, 241-261.

Williams, G. T., Smith, C. A., Spooncer, E., Dexter, T. M. and Taylor, D. R. (1990) Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**, 76-79

Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555-556

Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980) Cell death. The significance of apoptosis *Int Rev Cyt* **68**, 251-306

Wyllie, A. H., Morris, R. G., Smith, A. L. and Dunlop, D. (1984) Chromatin cleavage in apoptosis Association with condensed chromatin morphology and dependence on macromolecular synthesis *J. Pathol* **142**, 67-77

Wyllie, A. H., Rose, K. A., Morris, R. G., Steel, C. M., Foster, E. and Spandidos, D. A. (1987). Rodent fibroblast tumours expression human *myc* and *ras* genes growth, metastasis and endogenous oncogene expression. *Br J Cancer* **56**, 251-259

Yamanishi, H., Nonomura, N., Tanaka, A., Yasui, T., Nishizawa, Y., Matsumoto, K. and Sato, B. (1990) Roles of Transforming Growth Factor β in inhibition of androgen-induced growth of Shionogi carcinoma cells in serum-free medium *Cancer Res* **50**, 6179-6183.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimichi, A. and Oren, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6 *Nature* **352**, 345-347

Yuan, J. and Horvitz, H. R. (1990). The *Caenorhabditis elegans* genes *ced-3* and *ced-4* act cell autonomously to cause programmed cell death *Dev Biol.* **138**, 33-41

(*Accepted 21 January 1992*)