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Review

# Physiological apoptosis in hormone-dependent tissues: involvement of caspases

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## Abstract

**Physiological apoptosis in mammals is a type of programmed cell death, an important element in the developmental repertoire ensuring tissue homeostasis and proper disposal of cells that are no longer needed, such as milk-producing epithelial cells in the mammary gland after lactation, luteal cells in the post partum Corpus luteum or secretory cells in the prostate after castration. Although incompletely described, apoptosis in hormone-dependent tissues is apparently initiated and executed using common biochemical strategies. These include survival pathways governed by local and systemic factors and hormones, diverse regulatory pathways and caspase-dependent execution pathways. Using an antibody that recognizes processed effector caspases or a fluorogenic caspase substrate, we present for the first time evidence that caspases are activated in the mammary gland, in the prostate and in the ovary at the time when apoptosis occurs. Most likely phagocytosis of apoptotic cells by neighboring cells may represent an important step, since only a modest involvement of professional phagocytes is apparent. Here, we will summarize and discuss recent data and will attempt to draw a generalized picture of how physiological apoptosis may occur in these organs.**

**Keywords:** apoptosis; caspase; Corpus luteum; frizzled; mammary gland; programmed cell death

**Abbreviations:** bFGF, basic fibroblastic growth factor; CL, Corpus luteum; DDC, differential display coincidence gene; ECM, extracellular matrix; FIL, feedback inhibitor of lactation; FLIP, FLICE-like inhibitory protein; FSH, follicle stimulating hormone; gas-1, growth arrest specific gene 1; GH, growth hormone; hCG, human choriogonadotropin; IAP, inhibitor of apoptosis protein; IGF-1, Insulin-like growth factor-1; IGFBP, IGF-1 binding protein; LH, luteinizing hormone; Mn-SOD, manganese superoxide dismutase; PARP, poly(ADP-ribose) polymerase; SGP-2, sulfated glycoprotein 2; TIMP, tissue inhibitor of metalloproteinases; TNF-$\alpha$, tumor necrosis factor alpha; TRPM-2, testosterone repressed message 2; tTG, tissue transglutaminase; TUNEL, terminal transferase-mediated UTP nick end labeling; uPA, urokinase-type plasminogen activator

## Introduction

In multicellular organisms physiological cell death processes are used during organogenic and morphogenic processes in the embryos and to remove infected, defective or super-numerary cells in the adult.[1] Cell death in hormone dependent tissues is a well documented example of physiological apoptosis in mammals. In the female mammary gland and in the endometrium, epithelial cell proliferation is followed by epithelial cell death in a cyclical manner. Extensive germ cell loss occurs in the ovary where more than a million oocytes exist at birth, but only some hundred thousands form follicles and only a few hundred follicles ovulate during the reproductive life.[2] In addition to germ cell death, physiological cell death in the ovary occurs during follicular atresia and regression of the Corpus luteum (CL). In the reproductive organs of the male physiological cell death takes place in the testis and in the prostate after androgen ablation.

Here we will review hormonally-regulated cell death processes that are found in the mammary gland after lactation, in the CL during late pregnancy and after birth, and in the prostate after castration. We will first give a short overview of apoptosis as the best-known form of programmed cell death, then summarize apoptotic processes in the mammary gland, ovary and prostate, finally presenting a generalized model of physiological apoptosis in hormone-dependent tissues.

## Multiple facets of death processes

Programmed or developmentally determined cell death is always contrasted against the random, accidental death of necrosis. Originally described by pathologists on a morphological basis, apoptosis is a kind of programmed cell death characterized by cell shrinkage and chromatin condensation followed by the formation of membrane-bound nuclear and cellular fragments.[3] Most significantly, since in apoptosis the dying cells' membranes remain intact, an inflammatory response is avoided.[4–6] As a process, apoptosis exhibits recognizable phases:[7] the reversible induction or decision-

taking phase, the irreversible execution phase, and the cleanup phase in which the remains are disposed and recycled.

Caspases, aspartate-specific proteases with a catalytic cysteine in the active site,[8] are the primary executioners of many forms of apoptosis. They are usually constitutively expressed as inactive pro-caspases (zymogens). Activation of caspases involves hetero- or auto-proteolytic processing of pro-caspases usually at aspartic residues to give rise to active caspase fragments that join to form an active caspase. Caspases constitute a tightly regulated proteolytic network within the cell orchestrating the execution of apoptosis.[9–12] Most important for the understanding of how caspases regulate apoptosis is the identification of critical caspase substrates. To date over 50 potential substrates beside the caspases themselves have been implicated in various cell systems.[11,13] They include DNA repair enzymes, structural proteins and several protein kinases.[11]

Apoptosis can be induced by extracellular signals that trigger caspase activation (e.g. death receptor mediated apoptosis or T-cell mediated killing by granzyme/perforin), by the generation of intracellular signals that trigger caspase activation (e.g. DNA damage or damage to organelles), or by the removal of suppressing mechanisms that inhibit caspase activation or activity (e.g. loss of survival factors). Apoptosis is modulated or regulated at various levels. It turns out that almost always these regulatory processes target directly or indirectly the caspases. Numerous protein families have been identified that can inhibit caspase activation or caspase activity.[11] The anti-apoptotic members of the Bcl-2/Bax-family of proteins and caspase-like inhibitory proteins such as FLICE-like inhibitory proteins (FLIPs) interfere with signal transduction pathways leading to caspase activation.[14,15] Inhibitor of apoptosis proteins (IAPs) have been reported to inhibit apoptosis by directly binding to caspases.[16–19]

Relevant to physiological apoptosis in hormone-dependent tissues is the fact that a loss of survival signals can induce apoptosis. Most likely caspase-inducing processes are constitutively active in some cell types which, therefore, have a constant tendency to undergo apoptosis unless prevented by specific survival-signaling events. Survival factors described to date include growth factors such as insulin, insulin-like growth factor-1 (IGF-1) and hormones like gonadotropins or steroids, as well as extracellular matrix elements.[20–24] It seems likely that Wnt : frizzled signaling functions also as survival pathway in some tissues.[25–27]

## Physiological apoptosis in hormone dependent tissues

Investigation of *in vivo* physiological apoptotic events in tissues is needed to establish the validity and generality of the known pathways. In the following section, three tissues exhibiting physiological, hormone-dependent apoptosis will be examined with special emphasis on the relevance of the known biochemical players. For clarity, the initial discussion will treat the induction and execution phases individually.

### Induction of apoptosis

*The mammary gland* Mammary gland development is characterized by proliferation of the epithelium during puberty and pregnancy and cyclically during each estrus, terminal differentiation of secretory epithelial cells and milk production during late pregnancy and lactation. Mammary epithelial cell proliferation and differentiation are under stringent hormonal control.[28,29] In the presence of insulin, prolactin, and hydrocortisone, cultured mammary explants remain in a lactating state.[30] After the lactation period the gland undergoes an extensive remodeling process that leads to the involution of the epithelial structure until a state is reached resembling that of a virgin gland.[28,31] The involution process is associated with changes in the pattern of gene expression, with apoptosis of about 80% of the epithelial cells and with the production and activity of extracellular matrix degrading proteases.[32–37]

All apoptotic signals are directly or indirectly a consequence of the loss of the suckling stimulus. Whereas hormonal changes and milk accumulation trigger early events during mammary gland involution, the activation of the ECM degrading proteases coincides with a later phase of involution that is characterized not only by epithelial cell apoptosis but also by a collapse of the alveolar structure and the subsequent tissue remodeling.[37] The following signals have been discussed as possible inducers of epithelial cell apoptosis during mammary gland involution: (1) changes in systemic hormone levels, (2) feedback regulation by factors in the milk and upregulation of locally acting factors, (3) the physical strain or stress of engorgement as milk accumulates, and (4) the loss of survival signals as basement membrane is degraded during tissue remodeling.

As a consequence of reduced suckling, oxytocin and ultimately prolactin levels fall. These systemic hormonal changes could induce apoptosis in milk-producing epithelial cells. In the mammary gland prolactin most likely signals via the prolactin receptor expressed on epithelial cells.[38,39] Li and co-workers, however, proposed that prolactin signaling is inhibited by local signals and not primarily by a decrease of systemic prolactin levels during early involution.[40] Local pro-apoptotic signals generated by the accumulation of milk are dominant over systemic hormone levels[40–42] and it remains controversial to what extent glucocorticoids can inhibit apoptosis during involution.[37,40,43]

Basically, effects of hormones on epithelial cell apoptosis were studied by experimentally decreasing or increasing their levels in the body of animals. Decreasing prolactin or growth hormone (GH) levels at lactation leads to epithelial cell death.[44] Elevating levels of prolactin, IGF-1 or hydrocortisone derivatives inhibited apoptosis and impaired expression of extracellular proteases.[28,43,45] It seems likely that the systemic downregulation of lactogenic hormone levels after weaning results in the loss of intracellular signals for survival of epithelial cells.[38,44]

Numerous experiments have shown local control of lactation and of apoptosis in the mammary gland. For example, a mammary gland undergoes involution following

'sealing', while in the same animal, milk demand can be compensated by increased production in unsealed glands.[40,41] Since reduced suckling in a gland results in accumulation of milk, regulation of milk production and local involution could be attributed to factors in milk acting themselves as 'feedback inhibitors of lactation' (FILs). The characterization of a FIL has been described[46,47] but it seems mainly to be responsible for regulating milk secretion.[42] Clearly local control is significant; apoptosis of mammary epithelial cells can occur independently of the systemic hormone levels. Accumulation of milk has engorgement as a consequence. The physical strain of engorged alveoli may cause tissue damage producing a stress response which triggers apoptosis.

In addition to induction of apoptosis by changes in the hormone levels or by accumulation of milk, it has been reported that expression of extracellular matrix-degrading proteases triggers apoptosis of mammary epithelial cells because the epithelial cells are thus deprived of contact with basement membrane.[20] Since ligation of certain integrins is a survival signal for epithelial cells,[48] this concept is plausible. Tissue remodeling does lead to degradation of extracellular matrix. Talhouk and co-workers demonstrated a transition in the ratio of extracellular protease inhibitors to proteases during involution.[33] The expression of tissue inhibitors of metalloproteinase genes (TIMPs) is downregulated around day 3 of involution with the concomitant upregulation of a number of metalloproteinases and serine proteinases such as stromelysin-1, stromelysin-3, gelatinase A and urokinase-type plasminogen activator (uPA).[28,32,33,37,49] Nonetheless, the fact that these tissue remodeling proteinases reach maximal levels after the onset of apoptosis speaks against the hypothesis that mammary involution is initiated by loss of basement membrane survival signals.[32,33,37]

*The prostate* The prostate is a ductal network connected with the urethra. It functions as an exocrine organ to nourish and protect sperm cells subsequent to copulation. Main glandular structures are located at the distal end of the network. They are composed of secretory and non-secretory epithelial cells, smooth muscle cells, connective tissue, blood vessels and nerve cells. Prostate development and maintenance is strictly dependent on androgenic steroids which directly stimulate prostate epithelial cell proliferation and differentiation and protect cells from apoptosis.[50]

The rat ventral prostate is an established model for studying epithelial cell apoptosis.[50–55] Surgical castration induces regression of the gland and almost 90% of the cells are deleted by apoptosis within 2–3 weeks.[50,56] Induction of apoptosis seems to be entirely due to the lack of testosterone and derivatives thereof.[50] Interestingly, androgen ablation mediated induction of cell death has only been observed *in vivo* and the pathway by which androgens protect the prostate from involution remains largely unknown.[50]

Recent evidence indicates that Fas contributes to the induction of prostate epithelial cell death.[57] Bcl-2 is downregulated while Fas is upregulated following androgen ablation. Animals that harbor defects in Fas expression exhibit reduced apoptosis.[57]

Changes in the level of blood flow resulting in local hypoxia may also initiate epithelial cell apoptosis in the ventral prostate.[58,59] Following castration, blood flow in the prostate rapidly decreases, most likely due to endothelial cell apoptosis.[59] The resulting hypoxic conditions might be responsible for causing epithelial cell death.[50]

*The ovary* In the ovary massive apoptosis of germ cells occurs before birth to limit the number of follicles that can develop.[22] Oocytes mature within the follicle composed of granulosa cells and surrounding thecal cells. Granulosa cells support the growing oocyte. Of several hundred thousand follicles that are present at beginning of puberty only a few hundred develop to mature follicles and eventually ovulate. Survival of follicles is strictly dependent on gonadotropins such as follicle stimulating hormone (FSH) and luteinizing hormone (LH).[60] Follicles are constantly eliminated by a process termed follicle atresia that involves apoptosis of granulosa cells.[22,61] Germ cell survival is dependent on basic fibroblastic growth factors (bFGF) and stem cell factor and survival of follicles depends on FSH and LH. Consequently, experimentally decreasing levels of these factors result in massive germ cell apoptosis and atresia.[22,61,62] The Fas pathway has been implicated in contributing to follicular atresia since lpr mice devoid of an intact Fas pathway exhibit elevated numbers of secondary follicles.[63]

Another physiological process in which apoptosis has been implicated is regression of the Corpus luteum (CL).[64] CL is an organ within the ovary which differentiates from the ruptured follicle following ovulation. The main function of the CL is secretion of progesterone to maintain the lining of the uterus for implantation and support of a fertilized ovum.[65] Regression of the CL occurs at the end of each ovarian cycle or when it is no longer required for the maintenance of pregnancy.[66] While in the rat functional regression of the CL, evidenced by a significant decrease in progesterone secretion, begins at least 4 days prior to parturition,[67] the weight of the CL does not fall until after parturition.[68] Previous studies have found no evidence of vascular degeneration during natural regression of the pregnant rat CL.[68] Although previous studies have shown that the number of luteal cells in the rat CL remains constant,[68] recent studies have indicated that programmed cell death is occurring at day 20 of pregnancy.[69] Structural regression of luteal cells becomes apparent at day 1 post-partum and the weight of the CL declines over numerous estrous cycles following parturition.[70] Little is known about the mechanisms involved in structural regression. However, apoptotic cell death has been found to occur during luteolysis in many species. Apoptosis can be induced in CL of pregnant rats by treatment with a gonadotropin-releasing hormone agonist[71] and DNA fragmentation increases in luteal cells of rat CL following parturition.[71] Also, apoptotic cell death has been identified in CL of cycling rats treated with prolactin and during proestrous of the normal rat cycle.[72]

For the past several years the physiological signals which induce apoptosis in the CL have been explored. The initial starting point was the observation that luteal cells of rabbit CL underwent apoptosis at the time of regression.[73] To complement the *in vivo* analysis of luteal cell death

described above, an *in vitro* system employing individual CL collected from pseudopregnant rabbits and pregnant rats was developed and used to demonstrate a time-dependent onset of apoptosis in this tissue in serum-free organ culture.[73,74] Furthermore, using this model it was reported that human choriogonadotropin (hCG) blocks apoptosis in the rabbit and rat CL cultured *in vitro*.[73] However, the mechanisms by which this hormone, and other as yet unidentified endocrine factors, regulate apoptosis in the CL remain to be fully identified. Recent data indicate that the gonadotropin-mediated inhibition of apoptosis in luteal cells involves enhanced expression of the oxidative stress-response gene, Manganese super oxide dismutase (Mn-SOD), whose protein product may then function to rescue luteal cells directly from the damaging effect of reactive oxygen species and/or indirectly by acutely down-regulating expression of Bax.[75,76] Cytokines such as FasL and tumor necrosis factor-alpha (TNF-$\alpha$), and signaling molecules such as ceramide and sphingosine, appear to be of particular importance[74] and treatment of cultured granulosa or luteal cells with anti-Fas antibody induces apoptosis.[77]

## Execution of apoptosis

It is commonly believed that execution of apoptosis is brought about by the caspase family of proteins.[11] Although very obvious and clearly expected, almost no published evidence links caspase activation with mammary gland, prostate or ovarian CL involution *in vivo*. During mammary gland involution, induction of caspase-1 at the level of mRNA is the only known link so far between caspases and apoptosis of mammary epithelial cells.[20,37] Below we present data suggesting that indeed caspases do play a role for the apoptotic processes in these three tissues.

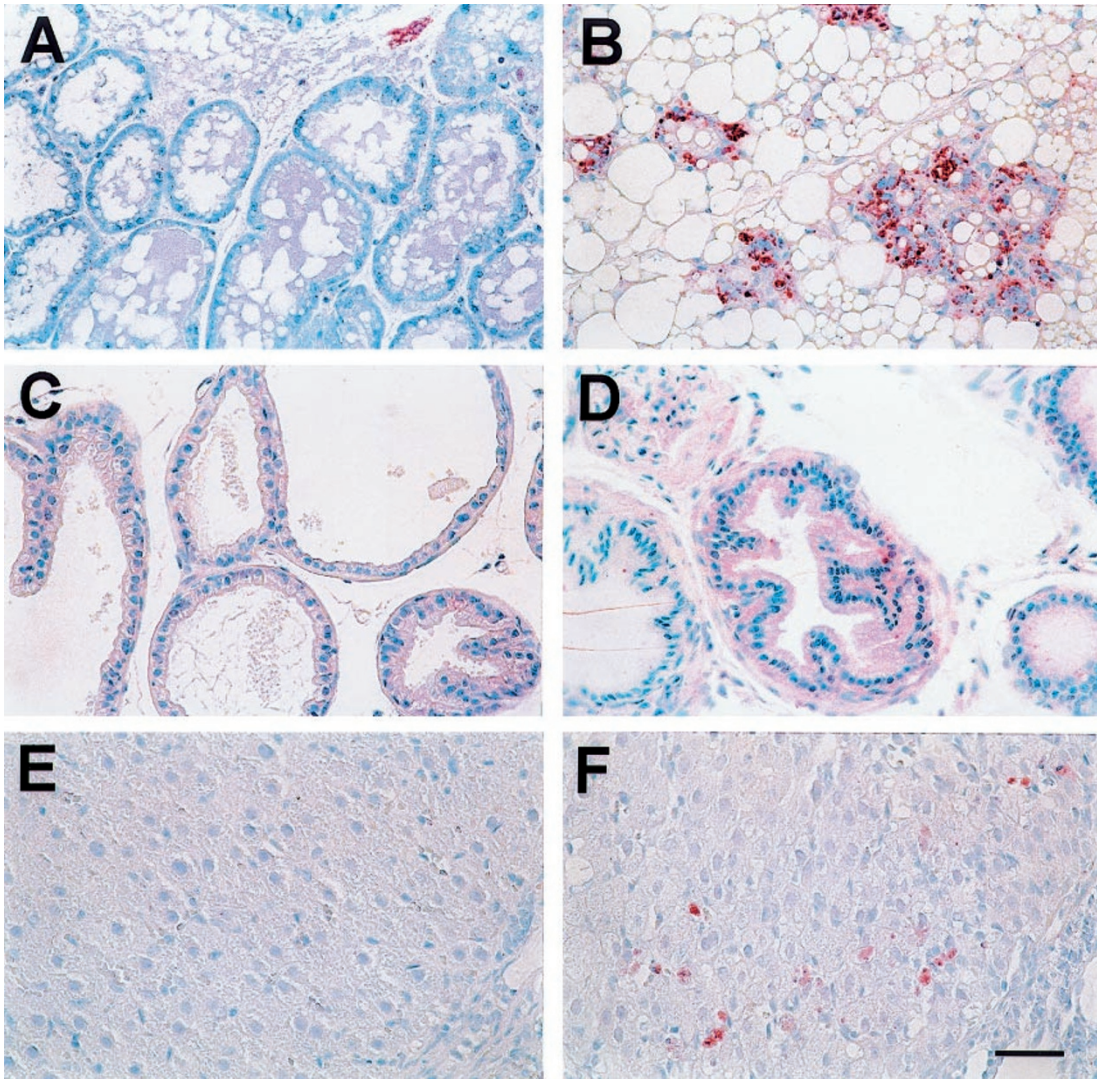


**Figure 1** Immunohistochemical analysis of active caspases using CM1 antibody. Shown are mammary glands at lactation (**A**) and 3 days of involution (**B**), normal prostate (**C**) and prostate 3 days after castration (**D**) and ovary at 16 d pregnancy (**E**) and 3 days after parturition (**F**). Paraffin embedded sections from paraformaldehyde fixed samples were incubated with CM1 antibody that recognizes active caspases.[78] Antigen-antibody complexes were detected with the EnVision System (DAKO, Glustrup, Denmark) using AEC as substrate. The bar represents 50 $\mu$m

*The mammary gland* Mouse mammary gland involution can be initiated any time at lactation by removing the pups. Analysis with the CM1 antibody that recognizes only processed caspases[78] revealed that caspase activation is readily observed during mammary gland involution. Positive staining of mammary epithelial cells is associated with epithelial cell apoptosis during mammary gland involution (Figure 1B). During lactation only few cells can be observed that stain positively for processed caspases (Figure 1A). It is not yet possible to clearly identify the caspases that are activated and recognized by the CM1 antibody in the mouse mammary tissue. However, it is likely that they belong to the DEVD cleaving subfamily such as caspase-3, caspase-7 and possibly other unknown caspases.[78] Figure 2 shows a DEVD-cleavage analysis[16,79] in cytoplasmic and nuclear extracts of mouse mammary tissue at lactation and at 1 day, 2 days, and 3 days of involution. Cytoplasmic and nuclear caspase activity was measured peaking at day 2 of involution. These results clearly confirm that execution of mammary epithelial cell apoptosis involves caspase processing and activity.

Although to date no caspase substrates have been documented during mammary gland involution, it is very likely that similar substrates will be identified as have been reported for cells in culture. Many studies documented DNA fragmentation by *in situ* methods or by gel electrophoresis analysis after extraction of the DNA.[32,41,43] However, the DNases that are responsible for DNA cleavage during mammary gland involution remain to be identified.

Several laboratories demonstrated that intracellular regulators of caspases such as the pro-apoptotic Bax protein are upregulated during mammary gland involution.[40,80,81] Although the exact location of Bax in the gland remains to be identified, it is tempting to speculate that Bax contributes to the activation of caspases in epithelial cells. Induction of Bax does not depend on systemic hormonal control and local signals are sufficient to stimulate Bax expression.[40] Expression of the anti-apoptotic Bcl-2 protein does not correlate with survival of mammary epithelial cells.[21] However, overexpression of Bcl-2 in transgenic animals seems to inhibit involution.[22] Moreover, overexpression of Bcl-2 may enhance the development of mammary tumors by inhibiting mammary epithelial cell apoptosis.[82]

When analyzed morphologically, at least two types of epithelial cell death can be differentiated in the mammary gland, apoptosis and necrosis (Figure 3). It appears that phagocytosis of apoptotic cells is an early event in the mammary gland (C Vallan and R Jaggi, unpublished observations). Figure 3B,C shows phagocytosed cells undergoing apoptosis. In Figure 3B, the nucleus of the healthy cell can be easily differentiated from the apoptotic nuclei of the dying cell. Furthermore, the engulfed cell is completely rounded up indicating that it lacks any contact. It is presently unknown how dying cells are recognized and to what extent the phagocytosing cells contribute to the execution of apoptosis during involution. Numerous

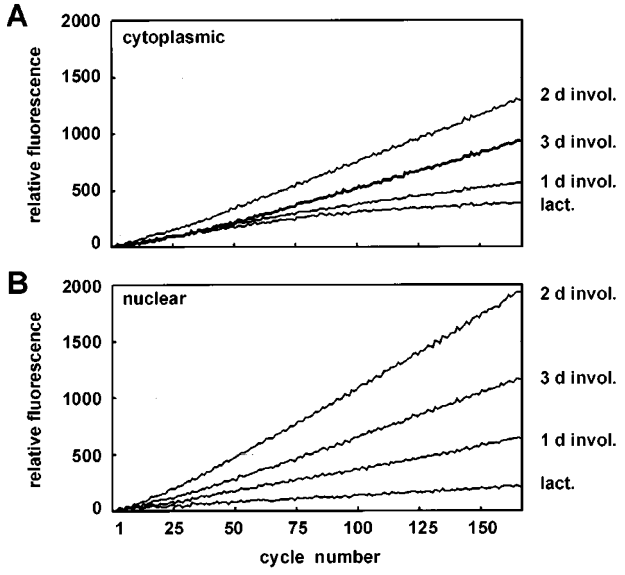


**Figure 2** Caspase cleavage activity of cytoplasmic (**A**) and nuclear extracts (**B**) prepared from mammary glands at lactation, and 1, 2 and 3 days of involution. Extracts were prepared from approximately 30 mg tissue by homogenization with a Polytron in hypotonic buffer as described.[34] Nuclei were pelleted by low spin centrifugation and nuclear proteins were extracted with hypertonic buffer.[34] Thirty $\mu$g protein in caspase buffer (Promega) were incubated in the presence of DEVD-amc and fluorescence at 460 nm was measured over a 50 min period (160 cycles of measurement) in a Fluorometer with exitation at 360 nm. Shown are arbitrary units of fluorescence

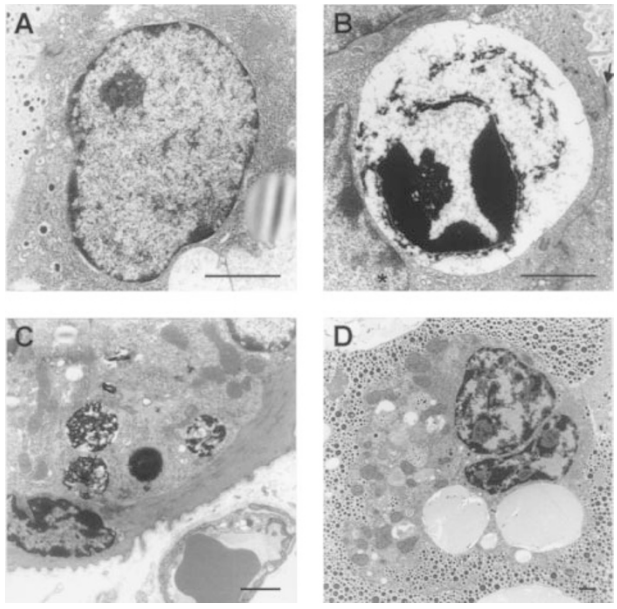


**Figure 3** Morphology of mammary epithelial cells in the transmission electron microscope at 3 days of involution. Mammary gland pieces were fixed in 50% Karnowsky solution, postfixed in 0.1 M Na-cacodylate buffered 1% OsO4 at pH 7.4, dehydrated and embedded in Epon 812. 80 nm thick sections were stained with lead citrate[126] and uranyl acetate.[127] (**A**) Represents a normal mammary epithelial cell. (**B**) Shows a phagocytosed cell with limited chromatin condensation at an early stage of cell death and (**C**) depicts an apoptotic cell at a late stage of cell death after fragmentation. (**D**) Represents a necrotic cell localized in the alveolar lumen. The arrow points to a tight junction and the bar represents 2 $\mu$m

epithelial cells that are shed into the alveolar lumen seem to undergo a primary or secondary necrosis (Figure 3D). Necrotic cells appear vacuolized with swollen mitochondria and they lack the highly condensed apoptotic chromatin.

*The prostate* Morphologically, apoptosis of prostate epithelial cells is similar to mammary epithelial cell apoptosis. Apoptosis in the rat or mouse prostate can be initiated by castration. Dying prostate epithelial cells are either phagocytosed by neighboring cells or shed into the lumen where they may undergo secondary necrosis. In order to study the involvement of caspases, prostate tissue sections from animals before or 3 days after castration were stained as above for active caspases (Figure 1C,D). Distinct epithelial cells were detected after castration that express active caspases (Figure 1D). Only few cells with processed caspases are apparent in the involuting prostate indicating that apoptotic cells are either removed fast or that the number of apoptotic cells per given time point is low. Caspase-mediated events are likely to contribute to the morphological changes and to the fragmentation of the DNA into the 180 base pair ladder that is often observed during prostate regression. Induction of DNase activity is likely to be a secondary non-vital process as agents that inhibit DNA fragmentation do not inhibit prostate involution and epithelial cell apoptosis.[83]

Bcl-2 and Bax proteins have been described to be upregulated after castration.[84] Activation of the pro-apoptotic Bax protein occurs early and correlates well with the induction of prostate epithelial cell apoptosis. In contrast, induction of the apoptosis suppressor Bcl-2 takes place relatively late after castration and is expressed in surviving epithelial cells. It was proposed that induction of Bcl-2 might control the subsequent cessation of involution in the gland.[50,84]

*The ovary* At least three different apoptotic processes have been described in the ovary, oocyte apoptosis, follicular atresia and involution of the Corpus luteum (CL). Although presently not described in detail at the molecular level *in vivo*, caspase mediated events are likely to contribute to the morphological changes and to the fragmentation of the DNA into the 180 base pair ladder that is observed during atresia and luteolysis.[73,85–87] Bcl-2 and Bax may play a role in determining the number of oocytes, follicles and CL that survive in the ovary.[75,86,88,89] Transgenic overexpression of the Bcl-2 gene under the control of the inhibin-$\alpha$ promoter/enhancer results in an inhibition of follicular atresia and in Bcl-2 knockout animals the number of oocytes and primordial follicles are substantially reduced.[90,91] Novel members of the Bcl-2 family of proteins have been implicated in contributing to apoptosis in the ovary. Boo, an anti-apoptotic Bcl-2-like gene product and Diva and Bok, two pro-apoptotic proteins have been localized to the ovary and testis.[91–93] Their roles in regulating apoptosis in oocytes, secondary follicles or CL need to be determined.

In order to establish that caspases are activated in the ovary during CL degeneration, sections from CL before birth (day 16) and after birth (day 3 post-partum) were stained with the CM1 antibody (Figure 1E,F). Numerous cells express activated caspases 3 days post-partum in CL (Figure 3F) whereas almost no cell can be detected at 16 days of pregnancy exhibiting a positive staining for processed caspases (Figure 3E). These results clearly document for the first time that caspases are activated in the ovary *in vivo* during apoptotic processes.

Recently, a new family of broad-spectrum apoptosis suppressors has been identified, the inhibitor of apoptosis proteins (IAP).[19] The IAP family members demonstrate potent inhibition of apoptosis induced by a multitude of stimuli, suggesting that they regulate central and conserved components of the apoptotic signaling pathway. In addition, IAP family members physically associate with several key apoptotic signaling molecules, including TNF$\alpha$ receptors and specific caspases. It is possible that IAPs play a role in maintenance of the CL. A rat IAP was recently identified and cloned from CL cDNA which may increase our understanding of the apoptotic pathways and its suppression by IAP family members in the CL (AM Dharmarajan, unpublished observations).

## Apoptosis in hormone-dependent tissues: searching for regulated genes

The induction phase of apoptosis is cell-type specific and is influenced by the extracellular environment. Hormone-dependent tissues respond to hormonal changes or ablation, but the pathways leading to apoptosis are still undefined. How can we discover the proximal steps of induction? Several studies have been performed with the goal of identifying genes regulated in programmed cell death in different hormone dependent tissues, i.e. genes expressed at the onset of apoptosis in mammary gland, prostate or CL.[32,55,69,94,95] The initial studies of gene expression associated with programmed cell death in mammary involution showed a link with tissue remodeling.[32,49,94] Several extracellular proteases, including metalloproteinases, and inhibitors of metalloproteinases (TIMPs) have been investigated in the mammary gland (as described above in the section 'Induction of Apoptosis'). Because of the cyclical demand for mammary secretion, the tissue involutes rapidly and drastically: proteolytic activities degrade engorged milk proteins, basement membrane and other matrix elements simultaneously with the death and elimination of secretory epithelium.[32,33,37] Unfortunately, tissue remodeling in the mammary gland overlaps with and confuses any analysis of purely apoptotic events. Furthermore, several extracellular proteases, the cathepsins and matrilysins, have been described that are induced in the prostate after castration.[95–99] These enzymes seem to be involved in a tissue remodeling process that is similar to what has been described in the mammary gland.

In order to search more selectively for genes associated with apoptosis, Guenette and co-workers[95,100] compared mammary gland and prostate involution, searching for specific, apoptosis-associated gene expression. Another approach called differential display coincidence analysis was developed by Bielke *et al*.[55] to discover common denominators expressed in association with programmed

cell death in the mammary gland, prostate and ovarian CL.[55,69]

Table 1 presents a catalog of genes and gene products that are induced in their expression or activity during apoptosis. These genes are candidates as players in regulatory pathways leading to apoptosis. Since for purposes of this discussion, expression in involuting mammary, prostatic and/or luteal tissues is a prerequisite, products of these genes can be considered viable candidates for functional involvement in apoptosis. For convenience, candidates are ordered according to extracellular, cytoplasmic, or nuclear localization or site of action. Prominent among the extracellular candidates is sulfated glycoprotein-2 (SGP-2), initially described as testosterone repressed prostate mRNA (TRPM-2).[101,102] Subsequent studies showed SGP-2 to be associated with mammary gland involution.[32] Although, SGP-2 has proven to be a very good *in vivo* marker for apoptosis, its functional role remains unknown.

Induction of apoptosis in the mammary gland, in the prostate and in the ovarian Corpus luteum involves a local upregulation of IGF-1 binding proteins (IGFBPs) which may sequester and inactivate the survival factor IGF-1 thereby facilitating apoptosis.[44,95,103–107]

The differential display coincidence analysis identified five genes, of which two, DDC-3 and DDC-4 are likely to play roles in regulating early events in apoptosis.[55] DDC-3 proved to be *gas-1*, a growth arrest gene previously described in connection with serum-deprivation of fibroblasts in culture.[108] It is expressed in mammary gland, prostate and CL involution, and scarcely otherwise *in vivo*. Del Sal and co-workers[108] showed that *gas-1* is upregulated in quiescent cells, and that its effect, if ectopically expressed, is dominant over oncogenes like *ras* or *src*. *Gas-1* is dependent for its function on cellular p53.[109] That *gas-1* should be so specifically upregulated in all three hormone-dependent apoptosis models is surprising and raises the question, why cells preparing for apoptosis should need to arrest within the cell cycle. In spite of intensive study, so far no functional role for *gas-1* in epithelial apoptosis has emerged.

DDC-4 proved to be a secreted form of *frizzled*.[27] The transmembranal forms of *frizzled* have recently attracted attention as receptors for *Wnt* ligands, whereas the secreted forms compete for the ligand : receptor signaling.[110] The history of the *Wnt* genes is closely tied to the history of the mouse mammary tumor virus, because the first transforming gene found to be activated by viral integration[111] proved to be identical with *Drosphila* Wingless and the mammalian *Wnt-1*. Furthermore, studies over the past 8 years have shown that developmental events in the mammary gland are regulated in a complex way by *Wnt* ligand expression.[25,112] The biochemical pathway by which *Wnt* : *frizzled* signaling stabilizes free $\beta$-catenin levels intracellularly and leads to transcription factor activation is becoming increasingly well understood.[113] DDC-4 presumably plays a role by interdicting a survival pathway by which specific *Wnt* : *Frizzled* signaling is interrupted. Somewhat similar secreted *frizzled* forms which play a role in apoptosis have also been reported by Melkonyan *et al*.[26]

Transgenic mice overexpressing DDC-4 under the control of the mouse mammary tumor virus long terminal repeat have been prepared in our laboratory. Figure 4 illustrates the alveolar morphology observed in the expressing line 721 at lactation. Plaques or masses of alveolar epithelial cells express DDC-4 at high levels according to *in situ* hybridization, while locally, the TUNEL assay for DNA fragmentation indicates a high level of apoptosis in these

**Table 1** Induction of gene expression and protein activity during involution. Depicted are a selection of genes and gene products that have been shown to be induced during involution in hormone dependent tissues

| | | Level of induction | | |
|---|---|---|---|---|
| **Gene-protein** | **Organ** | **Expression** | **Activity** | **Reference** |
| Extracellular/surface | | | | |
| TRPM/SGP-2 | M, P | + | | 32,102,103 |
| MMPs | M | + | + | 32,33,49,99 |
| TIMP | M | + | | 33,94 |
| DDC-3 | M,P,O | + | | 55,69,108,109 |
| DDC-4 | M,P,O | + | | 27,55,69 |
| IGFBP-5 | M,P,O | + | | 95,104–106 |
| Cytoplasmic | | | | |
| PKA | M | | + | 34 |
| JNK | M | | + | 36 |
| tTG | M,P | + | | 32,53,115 |
| caspase-1 | M | + | | 20 |
| caspase-3 | M,P,O | + | + | 114, this paper |
| caspase-9 | M | | + | A Marti and R Jaggi (unpublished) |
| Bax | M,P,O | + | | 40,80,81,84,86 |
| Bok | O | + | | 85 |
| Nuclear | | | | |
| STAT3 | M | | + | 38,40 |
| NF-1 (74 kDa form) | M | | + | 121 |
| AP-1 (Fos/Jun) | M,P | + | + | 34,122 |
| myc | M | + | | 32 |
| p53 | M | + | | 32,125 |

The gene products are subgrouped according to their localization (extracellular, cytoplasmic or nuclear). Induction is shown at the level of gene expression or protein activity in the mammary gland (M), prostate (P) or ovary (O)

regions. The expression is not uniform across the sections; a degeneration is apparent in the gland at the time of lactation, but milk production is sufficient that small litters (average of four animals) can be suckled. These results suggest that high levels of DDC-4 expression may induce apoptosis and imply this gene product as a pro-apoptotic protein that may locally contribute to the initiation of apoptosis not only in the mammary gland but also in the prostate and the ovary.

The induction of numerous cytoplasmic gene products has been reported during apoptosis in the mammary gland, the prostate and the ovary (Table 1). Guenette *et al*.[100] reported that steady-state levels of expression for Poly(ADP-ribose) polymerase (PARP) were increased. Key events seem to be induction of several kinases (e.g. protein kinase A, Jun kinase),[34,36] induction of Bcl-2 family members such as Bax[40,80,81,84] or Bok[85] and induction and activation of several caspases[20,114] (Figure 1).


**Figure 4** DDC-4 expression under the control of the mouse mammary tumor virus long terminal repeat. (**A**) *In situ* hybridization illustrates DDC-4 expression in the mammary gland of line 721 animals 2 days after parturition. Note that expression is not uniform; luminal epithelial cells are variably positive, while endothelial cells, fibroblasts and adipocytes are negative. Positive cells are clustered, and co-localize with abnormal alevolar plaques or cell masses. (**B**) *In situ* hybridization for DDC-4 with non-expressor transgenic line 722 reveals no detectable expression of DDC-4 at 2 days of lactation. The morphology of the gland is normal. (**C**) An *in vitro* terminal transferase reaction (TUNEL) is illustrated to show DNA fragmentation in nuclei of an alveolar cell mass from the lactating line 721 mammary gland. The bar corresponds to 100 $\mu$m

Expression of tissue transglutaminase is a general feature of apoptosis.[32,100,115,116] Tissue transglutaminase (tTG) may assist in stabilizing cellular structures during apoptosis by crosslinking cytoskeletal and other proteins,[117] but more complicated explanations for the role tTG can also be adduced, based on its G protein-like function.[118]

Nuclear proteins are obvious candidates for fulfilling requirements for regulation of gene expression in connection with apoptosis. Numerous studies have demonstrated that mammary and prostate involution is associated with induction of transcription factors such as Fos, Jun, Myc, p53 and NF-1.[32,34,35,119–121] For only a few of these gene products, however, has a direct regulatory role in epithelial cell apoptosis or caspase activation been shown. c-Fos seems to play an essential role for the initiation of apoptosis in prostate epithelial cells after hormone ablation and animals that lack the c-*fos* gene fail to undergo castration mediated epithelial cell apoptosis in the prostate.[122]

In animals that lack p53, prostate involution was shown to proceed with a temporal delay.[123] Whereas Li and co-workers[124] proposed that mammary gland involution proceeds through a p53-independent pathway, Jerry and co-workers[125] reported that even 5 days after forced weaning, a 60% greater alevolar area remained in p53 deficient animals as compared to wild-type animals, suggesting that p53 does play a role in mammary epithelial cell apoptosis during involution.

**Table 2** A generalized model of apoptosis in hormone dependent tissues. The scheme summarized the common denominators of apoptosis during involution of the mammary gland, the prostate and the ovary

| Gland | Initiation | Regulation | Execution | | Tissue remodeling | | Disposal |
|---|---|---|---|---|---|---|---|
| Mammary | hormonal changes FIL engorgement stress on intergrins hypoxia? | milk componements, Fos/AP-1, cAMP/PKA? ⟶ ⊤ glucocorticoids prolactin IGF-1, Bcl-2 | caspases other proteases DNases | → | rapid, drastic Metalloprot. (e.g. stromelysin) SGP-2 | → | phagocytosis by neighboring cells luminal shedding |
| Prostate | hormone ablation hypoxia? | AP-1, c-Myc FAS p53, TGF-β, Bax hypoxia? ⟶ ⊤ androgen IGF-1, Bcl-2 | caspases DNase | → | modest cathepsin B and D matrilysin SGP-2/TRPM C-CAM Par-4 | → | luminal shedding phagocytosis |
| Ovary | hormones hypoxia? | androgens Fas, TNF-α Bax, Diva, Bok ⟶ ⊤ FSH, LH, estrogen IGF-1, Bcl-2, Boo | caspases DNase | → | slow, minimal SGP-2 others? | → | persistance |

The phases of apoptosis are separated into an initiation phase and an execution phase that are both subject of complex regulation and a tissue remodeling/disposal phase

Table 2 presents an overview of apoptosis in the three hormone-dependent tissues we have discussed. While the induction phase is vague and unsharp, probably because different cell types respond to such a variety of stimuli, the execution phase exhibits many common features. Seemingly shared by all these physiological apoptotic processes is caspase activation. Variability among responses by different cell types is apparent again in the modulating influences such as those imposed by the Bcl-2/Bax family, the IAPs and others. Results presented in this review now support the view that caspases are universal effectors in apoptosis. For the first time caspase activation has been demonstrated *in situ* in the mammary gland, in the prostate and in the CL in close association with the apoptotic process. Perhaps the most important feature of apoptotic cells in order to avoid inflammation within the organism is their ability to remain intact until clearance by phagocytosis. In fulfiling this criterion tissue transglutaminase (tTG) may be of decisive importance.[118] Much work still needs to be done in order to define in more detail common features among cell types from tissues which can be induced to enter apoptosis. The physiological *in vivo* models with masses of cells entering apoptosis synchronously will offer good material for study.

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