Successful lactation depends on a controlled process of cell proliferation and mammary gland growth during pregnancy and cell death during involution. The main type of cell death responsible for involution of bovine mammary gland is apoptosis, but programmed cell death type II (PCD II) – autophagic cell death is also observed. The regulation of mammary epithelial cells (MEC) apoptosis occurs at three levels, the molecular regulators of autophagy have not been identified yet. There are possible correlations between both processes, because cells sharing morphological features of apoptosis and autophagy were found in involuting mammary gland. Autophagy seems to be cellular defense against starvation, when it fails, a secondary response of apoptotic cell death is triggered.

Key words: mammary gland involution, apoptosis, autophagy

INTRODUCTION

The mammary gland is a complex organ, composed of secretory epithelium and diverse cells of the supportive stroma. This network of cells intricately communicates to control numerous cycles of mammary gland remodelling (1). Successful lactation depends on a controlled process of cell proliferation and mammary gland growth during pregnancy and cell death during involution. The development of the mammary gland is tightly regulated by a variety of hormones, both metabolic and reproductive (2). During pregnancy, estrogen and progesterone are critical for mammary epithelial cells (MEC) proliferation. Estrogen contributes
to ductal elongation and stimulation of prolactin release from the pituitary gland (3). Together with progesterone these hormones are strongly involved in the formation of alveolar structures and side branching (4, 5). After peak of lactation, the mammary gland undergoes gradual regression through the process of apoptotic cell death. Although cell death exceeds cell proliferation, considerable turnover of mammary cells occurs during lactation. During the course of a bovine lactation, the number of cells formed is approximately 50% of the number originally present in the mammary gland (6). During mammary gland involution the extracellular matrix (ECM) and the alveolar basement membrane are degraded. The alveoli lose their structural integrity and massive death of MEC is observed. (7, 8). The latter is responsible for the loss of cells during mammary involution after natural weaning or litter removal in rodents (9) and during drying off in ruminants (10). However, the most pronounced induction of MEC apoptosis is associated with cessation of milking at the beginning of the dry period (11).

In rodents, involution comprises two phases: early limited apoptosis of secretory cells followed by widespread apoptosis and tissue remodelling (12), which is regulated by local factors, not by systemic hormones (13, 14), whereas the second phase of involution is associated with a decline in circulating galactopoietic factors (15). It has been suggested that autocrine mechanism, triggering phase 1, is sensitive to the frequency and completeness of milk removal (16).

Mammary gland involution in dairy cows occurs at a slower rate than in rodents and it is defined as a regenerative involution, because involution of secretory tissue coincides in time with its renewal stimulated by pregnancy hormones (17). The environmental factors that orchestrate this process are poorly understood (18).

ROLE OF APOPTOSIS IN THE REMODELLING OF MAMMARY GLAND

It was reported that the withdrawal of hormones and growth factors initiates the programmed cell death of epithelium in mammary explants within 48 h, which was indicated by an increase in the intensity of nonrandom DNA degradation and apoptosis-associated genes (19, 20). The possible trigger of involution is loss of mechanic stimulation, and change in cell shape as the gland becomes engorged with milk and stretching of the mammary epithelial cells occurs (14, 18). Two systems may be affected by the altered cell shape. Firstly, tight junctions become leaky and pro-apoptotic factors may diffuse from the apical to the basolateral side of the mammary epithelial cells to induce involution and apoptosis (21). The second possibility is the altered interactions of the cell with the extracellular matrix, through focal adhesion complexes (22).

Involution occurs through apoptosis, Type 1 cell death, which is defined morphologically by condensation (pyknosis) and fragmentation (karyorhexis) of nucleus, without major ultrastructural changes of cytoplasmic organelles. It is
thought to involve the activation of caspases, highly specific proteinases that cleave a wide array of intracellular substrates (23), as well as a stereotyped pattern of mitochondrial alterations leading to the release of caspase activators and caspase-independent death effectors (24-26). Immunohistochemical labelling of CPP-32 (caspase-3) in goat mammary glands revealed a significant increase of this enzyme content, concurrent with a degree of mammary tissue involution, suggesting the involvement of caspase-dependent pathways in the execution of apoptosis in this tissue (27). In addition, the induction of caspase-3 from lactation peak to dry period was accompanied by a progressive loss of mammary epithelial cells and the increase in apoptotic cell number (27). Zarzynska et al. (28) also showed significant increase in CPP-32 expression in bovine mammary gland explants. Dallard et al. (29) in similar study revealed a significant increment in the percentage of stromal cells labelled with anti-active caspase-3 antibody at every observation period and of epithelial cells at 7 d and 14 d of involution.

REGULATION OF APOPTOSIS DURING MAMMARY GLAND REMODELLING

Motyl et al. (30) had proposed a scheme of MEC apoptosis regulation in bovine mammary gland, which occurs at three levels (Fig. 1). The first level comprises intercellular regulatory proteins, e.g. Bcl-2 family death promoters and inhibitors (e.g. Bax). The second level is represented by intramammary inducers of apoptosis, such as: FIL-feedback inhibitor of lactation (11) - synthesized and secreted in milk, and acting specifically through interaction with the apical surface of the mammary epithelial cell to reduce secretory efficiency by inhibiting the movement of proteins, insulin-like growth factor binding proteins (IGFBP₃) (31, 32), Fas ligand (33) and transforming growth factor β₁ (TGF-β₁) (34, 35). The expression and activity of these auto/paracrine inducers of apoptosis is controlled and modulated by the third level factors, e.g. systemic galactopoietic hormones, nutrition, reproductive status and milking management (frequency and efficiency) (36).

Studies on goat mammary glands revealed that Bax expression was the lowest at lactation peak, significantly increased in late lactation and remained elevated during involution (27). In addition, the increase in Bax expression during secretory tissue involution in sows has also been shown (37). Dallard et al. (29) in the study on bovine mammary glands ex vivo, have shown immunostaining for Bax in the alveolar epithelium and ducts, and a significant increase of stromal immunostaining was observed at 14 d and 21 d compared with 7 d of involution.

The insulin-like growth factor (IGF) system regulates numerous cellular functions including mitogenesis and survival. It plays a pivotal role in mammary tissue homeostasis, regulating cell proliferation and differentiation during lactogenesis (1). IGF-I is an endo/paracrine mediator of galactopoietic effects of growth hormone in the bovine mammary gland (38). In addition, IGFs protect cells from apoptosis under a wide variety of culture conditions including growth
factor withdrawal (39), i.e. suppress the apoptosis of murine primary mammary epithelial cells in culture (40) and bovine mammary gland in tissue culture and cells culture (20, 41, 42). Also Zarzynska et al. (28) reported in ex vivo studies, that in bovine mammary gland explants a high expression of α and β subunits of IGF-I receptor (IGF-IR) was found in early lactating mammary gland when the expression of pro-apoptotic TGF-β₁ was low. The protective effect of IGF-I against apoptosis is known to occur through the activation of phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt), followed by the phosphorylation-dependent inactivation of the pro-apoptotic protein Bad (43). It has been reported that both the quantity and the phosphorylation status of the IGF-IR and Akt decrease during involution of the mammary gland (44). Reducing the expression of IGFs in mammary tissue may, therefore, represent a mechanism for initiating apoptosis during involution (45).

The ability of IGF-I to regulate biological activity in mammary cells is regulated by six high-affinity binding proteins (IGFBP-1 through -6). mRNA for
all six IGFBPs has been detected in mammary tissue of several species and, similarly to IGF-I, their expression patterns and relative levels vary considerably between stages of development, which suggests specialized roles (38, 45, 46). Cultures of primary bovine mammary epithelial cells secrete IGFBP-2, -3, -4, -5, which by molecular weight analysis are identical to those present in bovine milk (47). An increase in IGFBP-5 protein concentration has been observed in rat milk 48 h after removal of the suckling young (48). The large concentrations of IGFBP-5 present in the mammary gland may act to neutralize the function of IGF-I as a survival factor for mammary epithelial cells and IGFBP-5 may therefore be instrumental in initiating the process of mammary gland involution (49). The over-expression of IGFBP-5 in the mammary gland can lead to increased expression of the pro-apoptotic molecules caspase-3 and plasmin, and to decreased expression of pro-survival molecules of the Bcl-2 family (32). It has been reported that of all the hormones related to mammary gland involution, only prolactin is able to repress IGFBP-5 synthesis (48). However, Sakamoto et al. (45) showed for the first time, that growth hormone (GH) also represses the expression of mRNA and protein of IGFBP-5 in bovine MECs.

Of the six IGFBPs, IGFBP-3 is the most abundant in the circulation and its regulation is principally linked to growth hormone (50). However, many tissues also produce IGFBP-3 where it is involved in local control of cellular growth by autocrine and paracrine mechanisms (51). IGFBP-3 (the major IGFBP in milk) in bovine milk increases during mid-lactation to late lactation (31) and during involution the highest expression of this protein in the bovine mammary gland is observed (31, 47). IGFBP-3 may inhibit action of IGF-I and its receptor via a mechanism independent on a physical interaction between IGF-I and IGFBP-3 (52, 53). Sakamoto et al. (45), reported that IGFBP-3 inhibited the phosphorylation of Akt mediated by IGF-I. Other studies using cultured primary mouse mammary epithelial cells indicate that both IGFBP-5 and IGFBP-3, can also inhibit IGF-I mediated phosphorylation of Akt (40). During mammary gland involution the effect of IGF-I is inhibited by IGFBP-3 also in ruminants (54).

TGF-β1 has a well-documented role in inhibition of MEC proliferation and modulating epithelial plasticity (55). TGF-β1 is also an apoptotic agent for murine MEC (56-58) and bovine MEC (34, 35, 42, 59). Execution of TGF-β1-induced apoptosis in bovine BME-UV1 cells occurs through the action of caspase-3 and m-calpain (34). Regulation of this process via mitochondrial pathway depends on interaction between a pro-apoptotic protein Bax and a voltage-dependent anion channel–1 (VDAC-1) on the outer mitochondrial membrane. Gajewska and Motyl (59) have reported that in the cascade of TGF-β1 apoptogenic signaling pathway Smads and AP1 complex of transcription factors are activated, followed by an up-regulation of IGFBP-3 and -4, decreased activity of PKB and activation of pro-apoptotic protein Bad.

It has been shown that expression of TGF-β1 and its receptors in MEC increases during involution of mammary gland in mouse (19), goat (27), sow (37)
and cow (28). There is only one paper showing an increase in TGF-β₁ expression in involuting bovine mammary gland at the transcript level (60). Our recent ex vivo study on bovine mammary gland explants (28) revealed that an increase in the extent of apoptosis measured by expression of CPP-32 and the 89 kDa fragment of poly(ADP-ribose) polymerase (PARP) (a product of caspase-3 activity) during the dry period was accompanied by highly significant increases in TGF-β₁ and its receptor’s (TGF-β-RII) expression. These results may indicate a casual relationship between expression of this cytokine and induction of apoptosis in vivo, as it has been reported earlier in cultures of bovine MEC exposed to exogenous TGF-β₁ (34, 35, 42, 59).

Our recent study on bovine MEC cells cultures revealed that expression of TGF-β₁ undergoes complex endocrine and auto/paracrine regulation by hormones of the somatotropic axis and sex steroids (41). Somatostatin, 17β-estradiol and progesterone (P4) enhance TGF-β₁ expression, whereas the main bovine galactopoietic hormones, GH and IGF-I suppress TGF-β₁ synthesis. TGF-β₁ expression may also be controlled by a local inhibitory action of epidermal growth factor (EGF) and through negative feedback of elevated extracellular TGF-β₁ concentration (41). Work of other researchers also indicates, that the GH axis/insulin-like growth factor system is one of the regulatory factors impinging upon MECs proliferation (61), mammary grand remodelling and functions (11, 62) and regulation of apoptosis (45).

Members of EGF subfamily contribute to epithelial proliferation during gestation and dry period, however EGF itself plays also a role in differentiation process during lactation (63) - it is needed for proliferation and for rendering cell response to lactogenic hormones and following differentiation, it also contributes to preventing apoptosis (64). Our previous studies revealed that IGF-I and EGF-dependent survival pathways are critical for suppression of apoptosis in the model of in vitro mammary gland involution (41, 42).

Sex steroids are another important regulators of bovine mammary grand remodelling and functions (65). The influence of those hormones on mammary gland involution is still scarce, however Schams et al. (66) showed that mRNA and protein levels of estrogen and progesterone receptors present clear regulatory changes, suggesting involvement of these receptors in bovine mammary gland involution.

ROLE OF AUTOPHAGY IN MAMMARY GLAND REMODELLING

So far most studies on mammary gland remodelling concerned only apoptosis as a way of MEC death in secretory tissue. Preliminary evidence indicates that type II PCD – autophagic cell death, is also observed in mammary epithelial cells. Autophagy is degradation and recycling process of cellular constituents (long-lived proteins and organelles) which plays a role in the bioenergetic management
of starvation (67). Although autophagy is a cell-survival response, morphological features of autophagy have also been observed in dying cells. Thus, it has been proposed that autophagy could also be a mechanism of death (autophagic cell death) (26). Type 2 cell death is characterized by an accumulation of autophagic vacuoles in the cytoplasm (68-70). Macroautophagy involves the sequestration of cytosol or cytoplasmic organelles within double membranes, thus creating autophagosomes (autophagic vacuoles). Autophagosomes subsequently fuse with endosomes and eventually with lysosomes, thereby creating autophagolysosomes or autolysosomes. In the lumen of these latter structures, lysosomal enzymes operating at low pH then catabolize the autophagic material (71, 72). The only reliable marker of autophagosome formation in mammalian cells is MAP1LC3/LC3 (microtubule-associate protein 1 light chain 3) (73). Autophagic cells are biochemically characterized by cleavage of LC3 and its punctate redistribution inside the cell. Another important marker of autophagy is Beclin1, which is required for vacuolar transport and autophagy (74). It forms a complex with class III PI3K (75), which participate in the early stages of autophagosome formation, promoting the nucleation of autophagic vesicles. This complex plays a key role in increasing the size of pre-autophagosomal membranes and in their biogenesis by recruiting proteins from the cytosol (67, 76).

Our recent studies revealed that similarly to apoptosis, the intensity of autophagy in bovine mammary gland is the highest in the dry period (28, 77). It was shown by the highest expression of Beclin1 and the highest number of cells with typical morphological features of autophagy (autophagosomes and autophagolysosomes).

However, the factors regulating autophagy in bovine MEC have not been identified yet. Zarzynska and coworkers (28) proposed that pro-apoptotic peptides (e.g. TGF-β1, IGFBPs), compromised activity of survival pathways (e.g. somatotropic pathway) and undernourishment (the reason is deficiency of galactopoietic factors) can be among the possible inducers of autophagy in drying off mammary tissue. The autophagic effect of TGF-β1 has been shown directly in vitro in bovine BME-UV1 cells culture (35) and indirectly by coincidence of the highest expression of TGF-β1, its receptor type II (TGF-βRII) and Beclin1 in mammary tissue explants from drying off cows (28). Suppression of somatotropic pathway, as another potential inductor of autophagy, was manifested by down-regulation of growth hormone receptor (GH-R) and α subunit of type I IGF-I receptor (IGF-IRα), and up-regulation of IGFBP-4 and −5 in bovine mammary glands during dry period (28). The compromised secretion of GH and IGF-I and expression of their receptors together with competition for nutrients with developing fetus at the end of lactation and drying off may create a transient undernourishment of bovine MEC. In this case, autophagy seems to be a natural cellular defense against starvation. However the direct effect of these peptides in the control of autophagy in bovine MEC has not been investigated yet. Apart from growth factors (IGF-I, EGF, TGF-β1), sex steroids are another group of bioactive compounds which may be involved in the regulation
of autophagy in bovine mammary gland in dry period (65, 66). It cannot be excluded that a high production of steroid hormones in pregnant dairy cows together with higher expression of ER\(\alpha\), ER\(\beta\) and P4 (65) may facilitate TGF-β1 expression (41), which in turn induces autophagy (35).

APOPTOSIS-AUTOPHAGY CORRELATIONS IN MAMMARY GLAND

There are also possible correlations between apoptosis and autophagy in mammary gland. Zarzynska et al. (28) had found cells sharing typical morphological features of apoptosis (cell shrinkage and condensation of chromatin) and autophagy (autophagosomes and autophagic vacuoles) in involuting mammary gland. It is probable that when autophagy fails in its function of natural cellular defense against starvation, a secondary response of apoptotic cell death can be triggered. Autophagy may contribute to postponing the beginning of the apoptotic program which would be irreversible for the fate of the cell: if cells cannot be saved through autophagy, then apoptosis takes over. It is often reported that when autophagy is suppressed, apoptosis is initiated (67). We propose that apoptosis is the primary response of bovine MEC to the deprivation of bioactive compounds (i.e. fetal bovine serum (FBS) deficiency) in case of the subpopulations which are the most sensitive and ready to trigger apoptosis. Cells more resistant to undernourishment trigger autophagy as a defense mechanism, supplying indispensable amino acids and energy to maintain cellular homeostasis and survival. Ravikumar et al. (78) proposed the mechanism of damaged mitochondria sequestration. Lemasters et al. (79) suggested that autophagy may block apoptosis by preventing the release of pro-apoptotic mitochondrial factors to the cytoplasm due to the elimination of damaged mitochondria. These are not removed if autophagy is blocked and the mitochondria undergoing ΔΨ loss can prime apoptosis (67). During persisting starvation, mammary epithelial cells can trigger apoptosis despite autophagic defense. This could be due to the loss of mitochondrial membrane potential which could result from bioenergetic failure due to absence of nutrients and the impossibility of recruiting endogenous nutrients by means of autophagy-dependent catabolic reactions. In fact, in camptothecin-treated cells, autophagy delays apoptosis, while the inhibition of autophagy increases mitochondrial depolarization and apoptosis. The hypothesis is that the post-mitochondrial cascade is delayed by the disposal of damaged mitochondria into phagosomes. Other results suggest that mitochondrial functions (such as respiration) can block autophagic cell death, indicating that mitochondria need to be functional to allow the induction of autophagic cell death (80, 81). In the presence of these lysosomotrophic agents, autophagic vacuoles progressively accumulated in the cytoplasm of starving cells, thus producing a morphology that initially resembled autophagic cell death. With prolonged starvation and lysosomal inhibition, such cells acquired features of apoptosis including nuclear pyknosis and
karyorhexis (82). This result suggests a possible shift from type 1 cell death to type 2 (26). In addition, by preventing the turnover of long-lived proteins, autophagy inhibition could alter the equilibrium between pro-apoptotic and anti-apoptotic proteins which have different half-lives (82).

In general terms, it appears that similar stimuli can induce either apoptosis or autophagy (76, 83). In many other instances, autophagy and apoptosis develop in a mutually exclusive manner, perhaps as a result of variable thresholds for both processes, or as a result of a cellular ‘decision’ between the two responses that may be linked to a mutual inhibition of the two phenomena: the removal or functional inhibition of essential proteins from the apoptotic machinery, which can de-inhibit autophagy, or switch a cellular stress response from the apoptotic default pathway to a state of massively increased autophagy (Fig. 2). For example Beclin 1 BH3 domain was identified as a Bcl-2 interacting protein (84), thus a key apoptosis regulator also interacts physically with an autophagy regulator (76). A disruption of the interaction leads to increased autophagy (83). Beclin 1 also interacts with the other major anti-apoptotic Bcl family protein (Bcl-xL) and this interaction has been shown to regulate autophagy, so that not only Bcl-2/Bcl-xL inhibit apoptosis but also inhibit autophagy by binding with Beclin 1, and this interaction is important in the regulation of starvation-induced autophagy (85).

Fig. 2. The relationship between apoptosis and autophagy. There are findings of a mixed phenotype of apoptosis and autophagy at the single-cell level. The mutual inhibition of both processes could be regulated by Bcl-2, which inhibits autophagy by interacting with Beclin1 and could also inhibit apoptosis by blocking activation of Bax.
NEW PROCESS INVOLVED IN MAMMARY GLAND GLAND DIFFERENTIATION CYCLE

A new type of cell death was reported by Overholtzer et al. (86) in mammary epithelial cell lines in suspension: non-apoptotic cell elimination called “entosis”, that resembles cell cannibalism and the cell-in-cell phenotype, it is initiated by detachment of mammary epithelial cells from the ECM. This process requires the formation of adherens junctions in the absence of integrin signaling and force-driven invasion of one cell into another cell. Entosis is thus, not a phagocytic process but requires force generation from the actin/myosin cytoskeleton. Remarkably, viability of the internalized cells is sustained in the short term, and a fraction of the internalized cells can either extricate them or they can be expelled from the host cell (87). A key question is whether entosis is another mechanism to eliminate cells that detach from the ECM in vivo. This cell absorption may contribute to the elimination of ductal epithelial cells in normal development.

CONCLUSION

Apoptosis and autophagy are very important processes for involution, but probably future studies would find other types of cell death involved in remodelling of mammary gland.

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