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Regulation of Apoptosis and Functional Activity in Bovine Mammary Acini

Sang Hoon Kim*

Department of Biology, College of Liberal Arts and Sciences, Kyunghee University, Seoul 130-701, Korea

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Programmed cell death, apoptosis, is a mechanism to maintain tissue homeostasis. Although the apoptotic process in rodent mammary tissues has been known to occur at the onset of involution, little is known about programmed cell death in the bovine tissues. Therefore, the purpose of this study was to investigate the molecular and cellular basis of apoptotic process in bovine mammary cells. Mammary tissues were obtained at different lactational and involutional stages. By apoptosis in situ end-labeling assay, apoptotic cells were found around the acinar cell lining in regressing bovine mammary tissues. The apoptosis-related genes bcl-2 and bax were detected throughout involution by Northern blotting assay. The level of bax mRNA was dominantly expressed during involution. On the other hand, the bcl-2 RNA transcripts were constantly expressed by 14 of post-lactation and declined thereafter. The expression of the testosterone-repressed prostate message-2 (TRPM-2) RNA transcripts, a marker for tissue remodeling, was increased as involution progressed. TNF α, were induced the DNA fragmentation and enhanced the expression of bax mRNA. In addition, milk protein secretion and amino acid uptake were decreased in mammary acinar culture treated with TNF α. These results indicate that bovine mammary cells undergo apoptotic process after the cessation of milking and that TNF α may trigger apoptosis in lactating bovine mammary acini.

The bovine mammary cells have a dynamic function in production of milk during lactation. After the cessation of milking, the bovine mammary tissue enters a transition phase to undergo a regression process. The cessation of milking increases intramammary pressure, which may induce the involutionary process in bovine mammary gland (Helminen and Ericson, 1968). During this period, composition of the mammary tissue is changed. Large vacuoles, containing residual lipid and protein, begin to form in involuting mammary tissue. Decreased synthesis and secretion in involuted bovine mammary tissue are consistent with histological changes such as higher percentages of stromal area accompanied by lower percentages of lumina (Holst et al., 1987).

Programmed cell death (PCD), apoptosis, is a type of cell death which is important for the maintenance of homeostasis of many tissues, and appears to be the mechanism responsible for the cell loss during physiological regression of a variety of tissues (Tenniswood et al., 1992; Juengel et al., 1993). Studies have reported apoptotic process as the mechanism of lactation turnover in rodent mammary epithelial cells during involution (Jaggi et al., 1996; Lund et al., 1996; Chapman et al., 2000; Wiesen and Werb, 2000). In rodent, following the removal of the suckling stimulus, milk synthesis in mammary gland is decreased and mammary cells undergo an extensive programmed cell death and tissue restructuring process (Strange et al., 1992; Marti et al., 2000). Atwood et al. (1995) have reported that hormonal control in epithelial cells in vitro affects apoptotic cell death and tissue remodeling in rats. Programmed cell death is also observed in human mammary luminal epithelial cells when cells are deprived of serum in culture (Harris et al., 1995).

Tumor necrosis factor α (TNF α), a 17 kDa polypeptide secreted by activated macrophages (Old, 1987), plays a role in a variety of cellular responses including proliferation, cellular differentiation, cytotoxicity, and cell death (Aoki et al., 1998; Lee et al., 2000; Quentmeier et al., 2000). Some studies have reported the presence of TNF α in milk during different physiological stages. The TNF α in milk is increased after weaning, peaked at the mid-dry period, and is undetectable at parturition (colostrum). Thereafter, TNF α reappears and is kept at the mid-level concentration in normal milk during lactation (Rewinski and Yang, 1994). In addition, macrophages and T lymphocytes producing TNF α are mainly present

* Tel: 82-2-961-9208, Fax: 82-2-964-1079
E-mail: shkim@khu.ac.kr

in the mammary gland during the dry period (Tatarczuch et al., 2000). In mammary carcinogenic cells, the addition of TNF α causes induction of cell death (Kumar and Mendelsohn, 1994). However, the role of TNF α in normal lactating bovine mammary cells still remains unclear.

No study has characterized the regressed bovine mammary tissue with respect to the apoptotic process, although apoptosis is a key cellular mechanism controlling cell turnover in a variety of tissue. As involution progresses, the rodent mammary epithelial cells are dissociated from the basement membranes. In contrast, most of bovine mammary epithelial cells remain attached to the basement membranes during involution (Holst et al., 1987). Nevertheless, the occurrence of apoptosis is not ruled out in involuted bovine mammary cells, because phagocytosis by macrophage in bovine mammary tissue does occur to remove cellular debris including dead epithelial cells during involution (Hurley, 1989; Oliver and Sordillo, 1989). In general, apoptotic cells are phagocytozed by adjacent epithelial cells and interstitial macrophages in the last stage of apoptosis (Walker et al., 1989; Marti et al., 2000). There is evidence only in ruminant that goat mammary cells undergo apoptosis during involution (Quarrie et al., 1994). However, it is not known whether mammary epithelial cells in cattle undergo apoptotic process during involution. Therefore, this study focused on the occurrence of apoptosis in bovine mammary tissue during involution and the induction of apoptosis in lactating mammary cells by TNF α.

Materials and Methods

Mammary tissue sampling

Mammary tissues from rear quarters were collected from at least 3 cows on the different lactational and involutional stages. Signs of clinical mastitis were not observed in any of the biopsied quarters. A portion of each tissues was immediately immersed in phosphate-buffered saline (PBS) buffer, and fixed in formation for histological studies, and the remaining tissues were quick frozen in liquid nitrogen and stored at -70℃ until analysis by Northern blotting.

RNA extraction and Northern blot analysis

Total RNA was extracted from mammary tissues and mammary acini by the guanidine thiocyanate and phenol extraction method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated from total RNA using oligo dT cellulose columns (5 Prime → 3 Prime Inc.). RNA concentrations were determined spectrophotometrically. Poly(A)$^+$ RNA (6 μg/lane) was fractionated by electrophoresis on a denaturing gel and transferred to a nylon membrane. After prehybridization, hybridization was performed for 17 h at 42℃. The membrane was washed twice at room temperature in a solution containing 5X SSPE and 0.5% SDS, followed by washing twice at 37℃ in a solution containing 1X SSPE and 0.1% SDS. The membrane was exposed to X-ray film (Kodak) with an intensifying screen at -70℃.

Probes were labeled with the Multiprime DNA Labeling Systems (Amersham). The full length rat TRPM-2 cDNA was kindly provided by Dr. Tenniswood (University of Ottawa, Ottawa, Canada). The bovine β-casein cDNA was a gift of Dr. MacKinlay (University of New South Wales, Kensington, Australia). The bcl-2 and bax cDNA were supplied by Oncogene Corp. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was obtained from ATCC.

Apoptosis in situ end-labeling assay

Serial sections (5 μm) were cut from paraffin-embedded tissue and mounted on glass slides. The sections were deparaffinized and rehydrated. The end-labeling of fragmented DNA was performed essentially as described by Wijsman et al. (1993). Briefly, tissue sections were immersed in 3% H_2O_2 for 30 min to block endogenous peroxidase and digested by incubation with 20 μg/ml proteinase K for 30 min. The slides were washed in Buffer A (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.005% bovine serum albumin) for 5 min, and were incubated with Buffer A containing 0.01 mM dATP, dCTP and dGTP (Promega), 0.01 mM biotin-11-dUTP (Clontech Laboratories Inc.), and 20 units/ml E.coli DNA polymerase I (Promega) at 37℃ for 1 h. Sections were washed twice in PBS for 5 min, and incubated with horseradish peroxidase-conjugated avidin (Novocastra Lab.). Staining was developed in diaminobenzidine-H_2O_2. The specimens were then mounted with Permount. Sections were examined using an Nikon microscope. Rat intestine section was used as positive control. In negative control, DNA polymerase I was omitted during the incubation step of mammary tissue section. The apoptotic cell labeling index was calculated after counting more than 500 nuclei in randomly selected areas of three tissue sections from each cow. The labeling index was defined as the ratio positive nuclei: total number of nuclei counted, and was expressed as a percentage.

Mammary acinar culture

Alveolar mammary epithelial cells were isolated from tissue samples in the presence of enzyme solution containing 400 U/ml collagenase, 400 U/ml hyaluronidase, and 5% fetal bovine serum. Mammary acinar suspensions (equivalent to 3×10^6 cells) in the presence of TNF α were placed on 60 mm plastic dishes, which were coated with type I collagen gel. For Northern blotting, mammary acini were harvested at indicated time points and stored at -70℃ until analysis. Secretion of milk protein was measured by a pulse-chase method. Alveoli were pulse labeled with [^{3}H] lysine (1 μCi/ml) for 20 min and incubated further for 90 min with

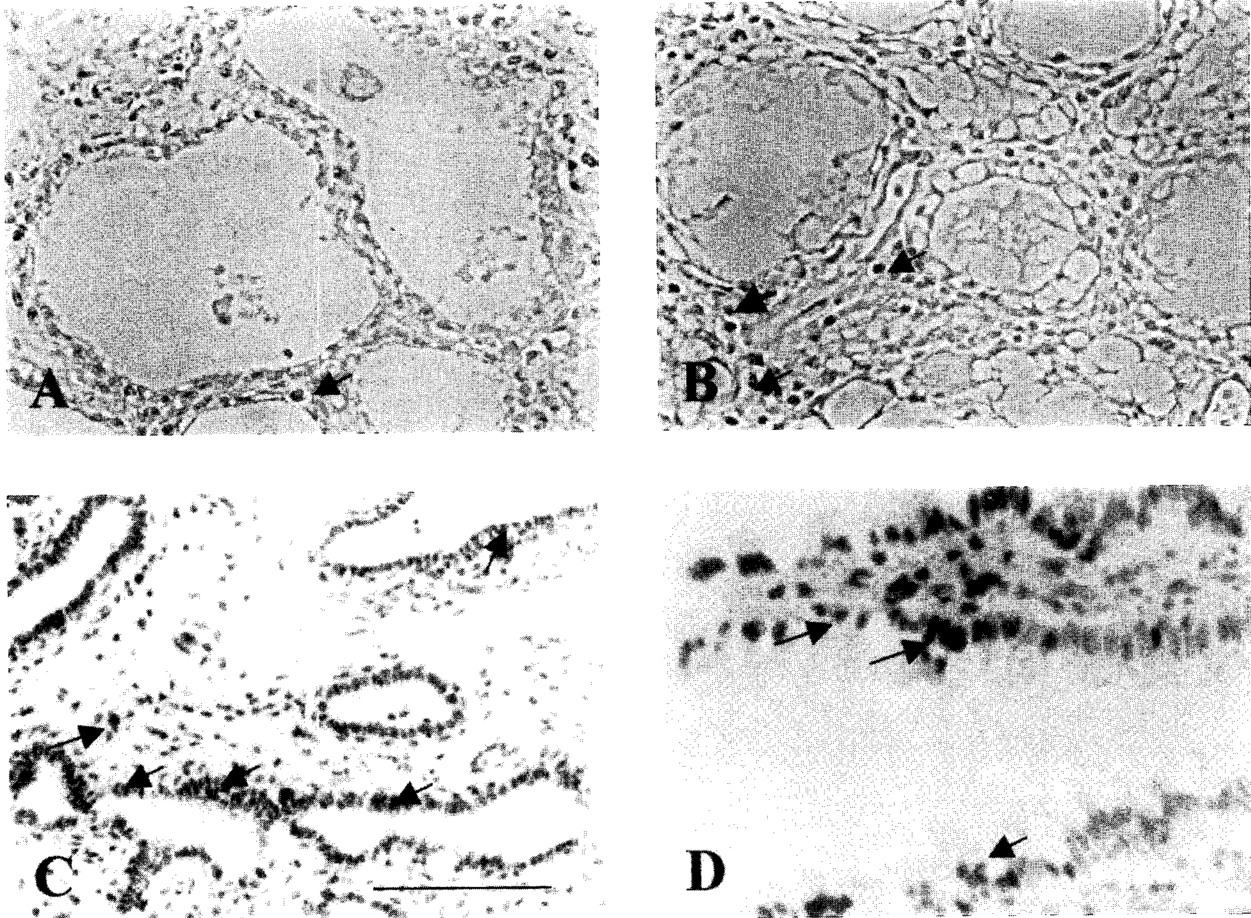

Fig. 1. Identification of apoptosis in bovine mammary epithelial cells. Apoptotic cells were observed mainly at day 14 (B) and day 35 (C) of involution by using apoptosis in situ end labeling assay. Arrows show representative apoptotic cells. A, Section of lactating mammary tissue. D, Positive control. Bar=100 µm.

chase medium. The specific activity (dpm/mg x 10^{-2} protein) was measured as described (Park et al., 1979). The amino acid uptake was also measured according to the procedure of Park et al. (1979), with [^{14}C] cycloleucine as a nonmetabolizable monitor. Uptake of cycloleucine was determined as follows: cycloleucine uptake (nmol/mg protein) = acini specific activity (dpm/mg protein)/ medium specific activity (dpm/nmol).

DNA fragmentation assay

Mammary acini were cultured in 6-well culture plates coated with collagen. After preincubation for 24 h to be attached, cells were incubated for various time periods with TNF α. Genomic DNA was prepared in a similar manner to that described by Tilly and Hsueh (1993). Mammary cells were lysed with a buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, and 1 mg/ml proteinase K at 37℃ for 20 h. Isolated DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA (2 µg/lane) was electrophoresed on a 1.2% agarose gels. After being stained with 0.5 µg/ml ethidium bromide, the DNA fragments were visualized by UV light.

Results & Discussion

The mammary gland is a final reproductive organ whose milk secretion is influenced by the number of secretory cells and the retention of synthetic capacity of cells (Wilde and Knight, 1989). In order to determine whether the mammary cells turnover by the apoptotic process, bovine mammary tissues were obtained at different physiological stages and were analyzed by the apoptosis in situ end labeling assay. Fig. 1 shows representative apoptotic cells in bovine mammary tissues during post-lactation. These apoptotic cells were identified mainly as mammary epithelial cells surrounding the acinar cell lining. At day 14 of involution, apoptotic cells were observed in the alveolar epithelial cell area. At day 35, apoptotic cells appeared in the interalveolar area of bovine mammary tissue. These apoptotic mammary cells tended to increase as involution progressed. In rodent, apoptosis in the mammary gland occurs rapidly after weaning and is completed by 8 days postweaning (Strange et al., 1992). Based on this morphological observation, the molecular evidence of apoptosis was sought to confirm

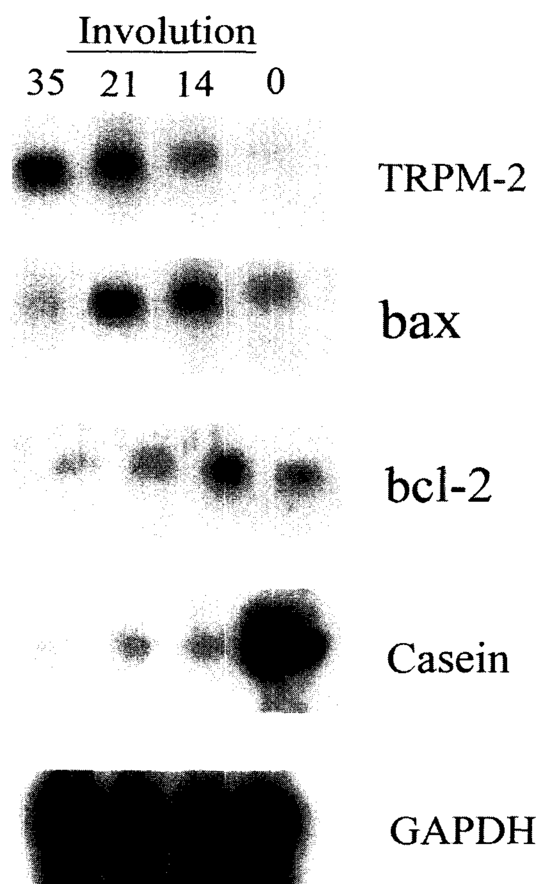

Fig. 2. The expression of apoptosis-related genes in bovine mammary tissue. Poly(A)$^{+}$ RNA (6 μg/lane) was fractionated on a denaturing agarose gel by electrophoresis and transferred to a membrane. After hybridization with labeled cDNA, the membrane was visualized by autoradiography. As a control, the membrane was probed with GAPDH cDNA. (0) lactation; (14) day 14 of involution; (21) day 21 of involution; (35) day 35 of involution.

the occurrence of apoptosis in bovine mammary tissue. TRPM-2 was used as a marker of tissue remodeling. As shown in Fig. 2, the expression of TRPM-2 mRNA was not detected in the lactating bovine mammary tissue. The expression of TRPM-2 transcript appeared to increase as involution progressed. The level of TRPM-2 mRNA message in mammary tissue at day 35 of involution was 10-fold higher than that of the lactating tissue.

TRPM-2 mRNA in rodent mammary tissue begins to be detected after weaning and is expressed at the highest level at day 2 of involution. TRPM-2 mRNA is not detected at day 8 of involution (Strange et al., 1992; Guenette et al., 1994). Bax, an inducer of apoptosis, was expressed strongly in the bovine mammary tissue after the cessation of milking. Interestingly, at day 35 of post-lactation, the expression of bax disappeared. However, apoptosis still actively occurred until 35 day of involution (Fig. 1), indicating that bax is an early response gene in the apoptotic process (Selvakumaran et al., 1994). The other proapoptic genes may be involved in induction of apoptosis in bovine mammary tissue during

Table 1. Milk protein secretion and amino acid uptake in bovine mammary acinar culture in the presence of TNF α

Secretion and uptake	TNF α (ng/ml)		
	0	10	100
Secreted protein[1] (dpm/mg x 10^{-2})	28.8 · 2.7^{a}	12.8 1.5^{b}	9.4 · 0.7^{b}
Amino acid uptake[2] (nmol/mg protein)	35.6 · 3.3^{a}	18.2 · 2.7^{b}	15.2 · 1.3^{b}

[1]Mammary acini were preincubated with basic 1X MEM in the presence of TNF α for 24 h at 37℃ in a 5% CO_2 atmosphere. The basic MEM was then replaced with pulse medium containing [^{3}H] lysine (1 μCi/ml). After 15 min incubation for pulse label, acini were recovered and incubated at 37℃ for 90 min in chase medium. The specific activity (dpm/mg protein x 10^{-2}) of secreted protein was determined. Each value is the mean of three observations with seven replications (culture dishes)/observation. [2]Mammary acini were preincubated in 1X MEM containing of TNF α at 37℃ for 90 min in a 5% CO_2 atmosphere. The MEM was then replaced with treatment media containing 0.5 mM unlabeled cycloleucine and 0.5 μCi/ml [^{14}C]cycloleucine. Incubation was continued for 30 min. Cells were harvested and pooled (three dishes for each treatment). After recovery of acini and washing with BSS, specific activities of acini were determined. Uptake of cycloleucine was calculated as: cycloleucine uptake (nmol/mg protein) = acini specific activity (dpm/mg protein)/medium specific activity (dpm/nmol). Each value is the mean of three observations with four culture dishes/ observation. ^{a,b}Means (n=3) within a row of each stage with different superscripts differ significantly ($p<0.05$).

the late phase of involution. The expression of bcl-2 mRNA, an inhibitor of apoptosis, in lactating bovine mammary tissue was maintained by day 14 of post-lactation, and declined thereafter. The induction of these apoptosis-related genes during involution may be regulated by a variety of mechanisms, such as apoptotic signal stimulation of transcriptional events, and the rate of apoptosis-related gene degradation (Klock et al., 1998; Bailey and Griswold, 1999).

In contrast with the expression of these apoptosis-related genes, the casein mRNA transcript was strongly expressed in lactating mammary tissue, and sharply decreased as involution progressed (Fig. 2). At day 35 of involution, casein mRNA in bovine mammary tissue was not detected at all. The induction level of these genes was normalized with the steady-state level of GAPDH mRNA as housekeeping gene. Holst et al. (1987) has reported that the bovine alveolar epithelial cells do not secrete milk components at day 21 and 30 of involution. The process of active involution is to be completed by day 30 after the cessation of milking (Oliver and Sordillo, 1989). These results suggest that the apoptosis and tissue remodeling process occur gradually in bovine mammary tissue after the cessation of milking compared with that in rodent involuting mammary tissue.

TNF α stimulates cell death in various cells including mammary tumor cells (Marr et al., 1998). The level of TNF α in milk is increased as involution progresses (Rewinski and Yang, 1994). Based on these previous results, the present study investigated whether TNF α is involved in induction of apoptosis and the loss of functional activity in bovine mammary cells. The milk protein secretion and the rate of amino acid uptake in mammary acinar cells shown in Table 1 represent the functional activity of mammary cells. Relative level of milk protein secretion in acinar cells in the absence of

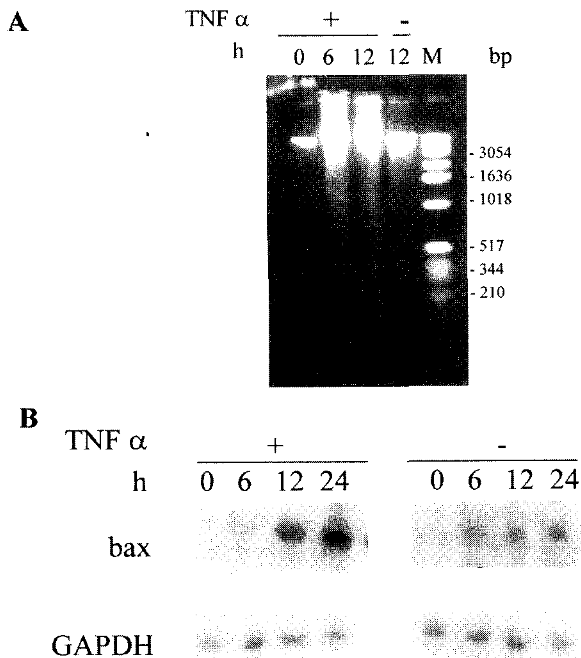

Fig. 3. The effect of TNF α on apoptosis induction in lactating bovine mammary acini. A, DNA fragmentation pattern in lactating mammary acini treated with TNF α. Mammary acini in 6-well dishes were exposure to TNF α (100 ng/ml) for indicated times. DNA fragmentation assay was carried out as described under "Materials and Methods". B, Northern blot of the bax mRNA expression in lactating mammary acini. Cells were treated with TNF α (100 ng/ml) or vehicle (-) for indicated periods of time. Total RNA (30 μg/lane) was extracted and electrophoresed on a denaturing agarose gel. After transferring a membrane, hybridization was conducted with ^{32}P-labeled bax cDNA. The signal was revealed by autoradiography.

TNF α was about 2 fold higher than that of the counterpart cells in the presence of TNF α (100 ng/ml). The decrease of protein secretion in lactating acinar cells treated with TNF α implies reduced functional activity and casein production in mammary cells (Choi et al, 1988). Amino acid uptake in mammary acini in the presence of TNF α (10 ng/ml) was also significantly decreased by 50%. Thus, the decreased availability of intracellular amino acid precursors induced by TNF α indicates reduction of synthesis of total milk proteins. These results suggest that TNF α may be involved in the reduction of cellular functional activity and the loss of mammary acinar cell.

In order to determine whether the reduced functional activity induced by TNF α is related with apoptosis in normal bovine mammary acini, DNA fragmentation pattern and the level of bax mRNA were investigated. The bax gene was used as a marker for apoptosis, because TNF α has been known to regulate the level of bax mRNA in various cells (Pulliam et al., 1998). In colorectal adenocarcinoma TNF α induces apoptosis resulting from the up-regulation of bax (Koshiji et al., 1998). As shown in Fig. 3, mammary cells were shown to induce DNA fragmentation pattern and expression of bax mRNA was enhanced by 12 h after the exposure to TNF α, whereas the cells in the absence of TNF α had no bax RNA transcripts or DNA fragmentation, suggesting that the pathway mediated by TNF α may regulate the bax-dependent apoptotic process in bovine mammary cells. The molecular mechanism of TNF α in normal bovine mammary cells in regulation of apoptosis remains to be elucidated.

Taken together, these data reveal that bovine mammary cells undergo bax-dependent apoptotic process gradually after the cessation of milking and that TNF α may be required to induce the loss of functional activity and apoptosis in lactating bovine mammary cells. Further investigation on the molecular mechanism of apoptosis in bovine mammary gland should provide additional information regarding the mammary cell turnover in the transition from lactation to involution for potential lactation performance.

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