ABSTRACT

Bovine mammary tissue was collected by surgical biopsy at intervals during involution for histological and ultrastructural observation. In lactating tissue (d 0 of involution, collected 8 h after the final milking), alveolar epithelial cells had marked ultrastructural evidence of lactation, including protein-containing secretory vesicles, lipid droplets, extensive rough endoplasmic reticulum, and numerous mitochondria. By d 2 of involution, alveolar epithelial cells contained large vacuoles apparently formed by coalescing of protein-containing secretory vesicles and lipid droplets. Large vacuoles were observed in epithelial cells until about the 3rd wk of involution. By d 2 of involution, the Golgi apparatus generally was not apparent. Rough endoplasmic reticulum and mitochondria were observed throughout the period studied, although in reduced amounts compared with their presence in lactating tissue. A marked increase in lysosomal or cytosegresomal structures in epithelial cells was not observed. There was no evidence of extensive sloughing of epithelial cells from the basement membrane. There was a progressive increase in the interalveolar area and a concurrent decrease in the alveolar luminal area as involution progressed. Ultrastructural examination showed that alveolar epithelial cells at d 21 and 30 of involution appear to be functionally active but not secreting milk components.

INTRODUCTION

The early nonlactating period in the dairy cow is a time of active involution of the mammary gland (18). This active involution is characterized by changes in the composition of secretion; milk components generally decrease and other components increase in concentration (5, 20, 22, 23). Specific changes in tissue structure and function have not been extensively defined in the dairy cow. Histological and ultrastructural changes in involuting mammary tissue have been described in the rat, mouse, and rabbit (2, 3, 4, 7, 15, 19, 21). In those species, autophagocytic and heterophagocytic mechanisms of tissue degeneration apparently play a major role in the process of mammary involution (2). Formation of lysosomes and cytosegresomal structures (containing cytoplasmic organelles) have been interpreted as evidence of autophagocytosis of alveolar epithelial cells (2). This degeneration often is accompanied by dissociation of epithelial cells from the basement membrane (3, 4, 15). Infiltration of mononuclear leukocytes into involuting tissue has been associated with heterophagocytosis of degenerating cells and cellular debris (2, 7, 16, 21).

This study describes histological and ultrastructural changes in bovine mammary tissue during involution. Observations made on involuting tissue in the bovine are compared with previous descriptions of mammary involution in rodents.

MATERIALS AND METHODS

Mammary tissue was obtained by surgical biopsy (17) from four Holstein cows (two pregnant, two nonpregnant). Quarters were biopsied consecutively on d 0 (left front, LF), 2 (right front, RF), 4 (left rear, LR), 7 (right rear, RR), 10 (LF), 14 (RF), 21 (LR), and 30 (RR) of mammary involution. Signs of clinical
mastitis were not observed in any of the quarters. The d 0 sample was taken 8 h after the last milking. Tissue was immediately immersed in 2 or 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 15 min, then minced into 1-mm cubes and placed in fresh fixative for 3 to 4 h at 4°C. The tissue was postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for 1 h at 22°C. Following dehydration in an ascending graded acetone series of once each in 10, 25, 50, 75, 95% and three times in 100% acetone (10 min incubation in each), the tissue was infiltrated with one part resin (50:50 Araldite:Epon resin) in one part 100% acetone for 1 h, then three parts resin in one part 100% acetone for 12 h, and finally embedded in pure resin.

Sections (1 μm) for light microscopy were made on a Reichert OM-2 ultramicrotome, mounted on glass slides, and stained with 0.5% toluidine blue in 1.0% borax. Sections were examined using an Olympus BH-2 microscope. Thin sections (60 to 70 nm) were cut with a diamond knife, mounted on uncoated copper grids, and stained with uranyl acetate and lead citrate. Thin sections were examined in either an Hitachi H-500 or JEOL 100-C transmission electron microscope.

RESULTS

Histology

Secretory epithelial cells at d 0 (considered to be lactating tissue) were cuboidal and contained many small vacuoles in the apical half of the cell. The alveolar lumen occupied most of the tissue area and little interalveolar tissue was present (Figure 1). By d 2 (ca. 56 h after the last milking) many secretory cells contained several large vacuoles (Figure 2) that often occupied a major portion of the intracellular space making the epithelial cells bulge into the lumen. Small vacuoles that appeared to be coalescing were observed (Figure 2). By d 4, most of the smaller vacuoles were no longer visible. The large vacuoles persisted through d 14 (Figure 3), then decreased in incidence on d 21. By d 30, few epithelial cells had large vacuoles (Figure 4).

The proportion of interalveolar connective tissue appeared to increase throughout the period studied, while the alveolar luminal area decreased, although these changes were not quantitated. By d 21 and 30 many alveoli appeared to have “collapsed” to a mass of cells with little or no apparent lumen (Figure 4). Upon ultrastructural examination of these structures, most cells maintained contact with the basement membrane and all cells had tight junctions with adjacent cells. At no time was there an indication of extensive sloughing, phagocytosis, or pyknosis of epithelial cells. The increased interalveolar space primarily was filled with fibrous connective tissue, although numerous cells were observed in that area. These cells probably included plasma cells, fibroblasts, and invading phagocytes and lymphocytes. Phagocytic cells were observed in the alveolar lumen during involution and were primarily macrophages, many of which contained ingested fat droplets (Figure 3, arrows).

Ultrastructure

Alveolar epithelial cells from d 0 of involution (lactating tissue, Figure 5) contained numerous secretory vesicles and fat droplets, extensive rough endoplasmic reticulum (RER), a well-developed Golgi apparatus, and many mitochondria. Microvilli were prevalent at the apical surface of cells. Tight junctions between cells were apparent, particularly near the apical ends of adjacent cells. Myoepithelial cells were present at the basal side of secretory epithelial cells.

The ultrastructure of secretory epithelial cells was markedly altered by d 2 of involution (Figure 6). Large vacuoles had formed in the cytoplasm of alveolar epithelial cells. These vacuoles often contained flocculent material or darkly staining casein micelles. Other vacuoles did not contain the darkly staining material and may have been composed of lipid. The large vacuoles occupied a major proportion of the intracellular space, often causing the cell to bulge into the alveolar lumen. Cytoplasm was compressed into a thin region around the vacuole with many mitochondria and other cellular organelles packed into a small area around the nucleus. The nucleus generally was located basally and was often misshapen by the presence of the large vacuoles. Some vacuoles (Figure 7) contained separate regions of protein and lipid, suggesting that the vacuoles may have arisen from coalescing of both fat droplets and
Figure 1. Light micrograph of lactating tissue (d 0 of involution) containing alveoli with large lumen (Lu) and small amounts of stromal area between alveoli. ×1500.

Figure 2. Light micrograph of mammary tissue on d 2 of involution. Epithelial cells contained large vacuoles (V) that may have arisen from coalescing of smaller vacuoles (arrows). ×1500.

Figure 3. Light micrograph of mammary tissue on d 14 of involution. Large vacuoles (V) still remained in the epithelial cells. Luminal area was reduced in many of the alveoli and interalveolar connective tissue was increased. Phagocytic cells were observed in the lumen (arrows). ×1500.

Figure 4. Light micrograph of mammary tissue on d 30 of involution. Epithelial cells contained few large vacuoles. Connective tissue between alveoli had increased dramatically compared to d 0 of involution. Some of the alveoli appeared as a mass of cells. General alveolar structure was intact. ×2500.
Figure 5. Electron micrograph of lactating tissue (d 0 of involution) containing many secretory vesicles (SV), several of which contain casein micelles (C). Extensive rough endoplasmic reticulum (RER) present. The Golgi apparatus (GA) was apparent and there were several mitochondria (M) present, although the mitochondrial membranes were difficult to distinguish. Tight junctions (arrow) were readily apparent. Mi = Microvilli, Lu = lumen, My = myoepithelial cell. ×10,000.

Figure 6. Electron micrograph of epithelial cells on d 2 of involution. Large vacuoles (V) occupied much of the intracellular space. The vacuoles had affected nuclear shape and forced the cytoplasm into a thin band along the outside of the cell. Little rough endoplasmic reticulum (RER) was present but there were still many mitochondria (M). The vacuoles contained both casein micelles (C) and flocculent material (F). My = myoepithelial cell, ×11,800.

Figure 7. Electron micrograph of epithelial cells on d 2 of involution. Large vacuoles contained both protein (P) and areas of lipid (L). X 10,000.

Figure 8. Electron micrograph of epithelial cells on d 2 of involution. Smaller vacuoles (SmV) appeared to be coalescing to form large vacuoles (V) in an epithelial cell. Mitochondria (M) were still present along with small amounts of rough endoplasmic reticulum (RER). X 11,800.
secretory vesicles. Coalescing of multiple secretory vesicles (Figure 8) was observed, as was fusion of smaller vacuoles, perhaps leading to the formation of the large vacuoles. The large vacuoles did not appear to be surrounded by a continuous membrane; however, occasional vacuoles were observed with intact membranes. Smaller vacuoles remained, and the cytoplasmic space was occupied by one or two large vacuoles. Microvilli were less prevalent than in lactating tissue. Regions of intact RER still were present, but the Golgi apparatus usually was not identifiable. Mitochondria were present, although their numbers may have been reduced. Few structures resembling lysosomes or cytosegresomes could be identified.

Only minor changes were observed in tissue collected on d 7, 10, and 14 of involution. Flocculent material in intracellular vacuoles was not apparent, and most vacuoles appeared to contain only lipid (Figure 10 is d 14). Few lysosomal structures were observed. Mitochondria were present throughout this period. Tight junctions always were present and there was little evidence for detachment of epithelial cells from basement membranes. However, tight junctions of the d 4, 7, and 10 tissues were less densely stained and frequently were hard to identify. Microvilli were observed on the apical surface of some cells. Mononuclear leukocytes were observed between the basement membrane and the epithelial cells (Figure 10), as well as in the alveolar lumen.

Few vacuoles remained in alveolar epithelial cells by d 21 and 30 of involution (Figure 11). Those vacuoles that did remain usually were small and appeared to be fat droplets. These fat droplets were frequently associated with several mitochondria. Alveolar structures often appeared to have multiple layers of cells surrounding a small luminal space with the cells lining the lumen being narrow. Alveolar cells of two staining intensities (Figure 12) were observed including those with darkly staining cytoplasm and "pale" cells. Both cell types had more highly convoluted nuclei than those observed in early involuting tissue. Tight junctions were present between cells at the luminal edge. Numerous microvilli were observed at the apical surface of luminal cells. Epithelial cells at d 21 and 30 of involution often contained numerous mitochondria and some organized RER (albeit greatly reduced from d 0 tissue). Alveolar epithelial cells appeared as actively metabolizing cells but did not have the ultrastructural components associated with copious synthesis and secretion of milk components. There was little evidence for extensive loss of epithelial cells.

Although the progression of ultrastructural changes in alveolar epithelial cells was consistent among the cows studied, results suggest that the rate at which this progression occurred was variable among tissue slices from the same cow. For example, some alveolar cells on d 10 were devoid of vacuoles compared with the norm of extensive vacuolization on that day.

DISCUSSION

The most striking morphological feature of mammary involution in the bovine was the formation of large intracellular vacuoles in the alveolar epithelial cells. These vacuoles formed within the first 2 d after the last milking and seemed to arise by fusion and coalescing of both secretory vesicles and lipid droplets. Other cytoplasmic components generally were excluded from the vacuole. Cytoplasm was distributed in a thin layer around the vacuole, and the nucleus was displaced to the basal end of the cell by the growing vacuole. Vacuoles generally disappeared between 2 and 3 wk of involution. The means of loss of vacuole contents was not determined in this study but may have been by intracellular catabolism, expulsion of vacuoles into the lumen, or both.

Vacuoles containing protein granules have been observed in early involuting rat mammary epithelia and have been identified as stasis vacuoles (4). In the rat, the number of stasis vacuoles decreases as involution progresses until only a few are present 2 d after weaning, although some vacuoles remain at 6 d (15). Bovine mammary epithelia appeared to be exhibiting a similar trend, although the extent of vacuolization was greater and the period of vacuolization longer than those observed in the rat. Vacuolization appeared to be a major feature of the involuting bovine mammary gland, and the large vacuoles persisted for at least 2 wk following formation. Many of the large vacuoles appeared to contain lipid, which has not been reported as a major vacuole component in the rat. An accumulation of intracellular vacuoles also has been observed in
Figure 9. Electron micrograph of epithelial cells on d 4 of involution contain one or two large vacuoles (V). Mitochondria (M) and a small amount of rough endoplasmic reticulum (RER) were still present. Microvilli (Mi) were still observed. ×6600.

Figure 10. Electron micrograph of epithelial cells on d 14 of involution contain one or two large vacuoles (V) that appeared to contain mostly lipid. Mitochondria (M) and tight junctions (arrows) were still present. An invading mononuclear leukocyte (MnL) was present along the basement membrane. Microvilli (Mi) were still present near cell junctions. ×4000.
Figure 11. Electron micrograph of epithelial cells on d 30 of involution. Large vacuoles were no longer present. Some small vacuoles (V) that appeared to contain lipid were still present. Mitochondria (M) were darkly stained. An invading mononuclear leukocyte (MnL) was located along the basement membrane. Tight junctions (arrows) were readily apparent. ×6600.

Figure 12. Electron micrograph of epithelial cells on d 30 of involution. Pale (P) and darkly (D) staining cells were found in d 21 and 30 tissue. Arrows designate tight junctions. Microvilli (Mi) were apparent at the luminal surface of epithelial cells. ×9900.
involuting mammary tissue in the pig and the goat (1, 14).

A major ultrastructural feature of the rodent involuting mammary tissue apparently is the presence of increased numbers of lysosomes and the formation of cytosegresomes. These cytosegresomes are thought to play a role in the autophagocytic activity of the involuting mammary gland (2). In the rat, the number of cytosegresomes increased until d 3 of involution (2). Lysosomes only occasionally were observed in the bovine mammary epithelial cells during involution and rarely were observed in lactating cells. Cytosegresome-like structures were less prevalent than lysosomes in the bovine tissue, although some of those structures observed were in the bovine tissue and that mechanism of cytoplasmic restructuring may occur to a limited extent. Many densely stained mitochondria frequently were associated with small lipid droplets in the d 21 and 30 tissue. The dense staining often made it more difficult to distinguish mitochondria from lysosomes. Mitochondria and lipid droplets have been associated similarly in colchicine-treated tissue from lactating cows (12).

Involution in the rodent mammary gland is marked by the disengagement of epithelial cells from the basement membrane and the extension of myoepithelial cells to fill the gaps left by the sloughed epithelial cells (19). This process was not observed in the bovine tissue and there was little loss of alveolar epithelial cells during involution. This is consistent with the relative lack of epithelial cells in mammary secretions during involution (10), although phagocytosis of lost cells by invading macrophages cannot be ruled out. Invading macrophages have been implicated in removal of luminal milk fat globules in involuting mammary tissue (9) and also may be involved in removal of cellular debris.

Tight junctions between epithelial cells always were observed. On d 4, 7, and 10 of involution (when the vacuoles were the largest), the tight junctions were less densely stained than at other times. The decrease in staining intensity may have been an indication of a loss of integrity in the tight junctions. Concentrations of serum albumin increase in mammary secretions during involution (8), suggesting a loosening of the tight junctions and an opening of the paracellular pathway. However, the increase in serum proteins also may arise from breaking of tight junctions between epithelial cells during passage of leukocytes into the lumen. Phagocytic leukocytes are found in increasing numbers in mammary secretions during involution (6, 10). Immunoglobulins also increase in secretions from involuting mammary glands, perhaps in part because of increased selective transport of immunoglobulins during early involution (20).

Cytoplasmic organelles declined in the involuting cells, particularly those involved in the extensive milk protein synthesis and secretion function of the lactating cell, such as the RER, Golgi, and secretory vesicles. The remaining RER was more sparse and the Golgi apparatus usually was not distinguishable by d 2 of involution. The rapidity with which the secretory vesicles began fusing suggests that there was an equally rapid alteration of the cytoskeletal components involved in transport of secretory vesicles through the cell. A disruption of microtuble structure previously has been noted in involuting mammary tissue (11) and, in part, might result in the fusion of secretory vesicles.

Despite the apparent breakdown of the protein synthesis and secretion pathway associated with milk proteins, the involuting epithelial cells maintained intact cellular organelles involved in metabolic and secretory function. Ribosomes and segments of RER were present at all stages, as were mitochondria and microvilli. Appearance of cells was markedly altered from that of epithelial cells in lactating tissue, but nonetheless the epithelial cells seemed to be functioning.

The structure of alveoli at d 30 of involution (light microscope level) resembled the solid mass of cells identified in alveolar buds in the developing mammary gland of the rat (13). The observation of at least two cell types (darkly staining and pale cells) in the cell mass also was similar to the observation of differently staining cells in the alveolar buds in rats (13). The involuted bovine mammary gland seemed to have differentiated from a lactating state into a nonlactating state that had the potential for redeveloping the ductule and alveolar structures. The alveolar epithelial cells in that nonlactating state appear to be capable of metabolic activity.
Because of the remaining intact structures after the previous lactation, a fairly short time may be required to redevelop a structurally complete gland that is prepared for lactogenesis.

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