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IMMUNOHISTOCHEMICAL STUDY OF THE STROMAL CELLS IN THE LACTATING BOVINE MAMMARY GLAND

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ABSTRACT

The bovine mammary gland is a special gland characterized by high secretory activity. During lactation the cellular and fibrous components of the interstitial tissue septa are exposed to store accumulated secretory products. The aim of this study was to find and study the cells in the stroma of the bovine lactating mammary gland. For this purpose, the immunohistochemical methods and antibodies against the smooth muscle actin, vimentin, and desmin were used. The myoepithelial cells (MEC) which stained with smooth muscle actin (SMA), were found supporting the secretory units and the intralobular ducts. Coexpression of the SMA and desmin were found in the smooth muscle cells of the blood vessels. The fibroblasts (myofibroblasts) and free cells positive to vimentin were located in the connective tissue septa. The results of this study on the mammary glands indicated that smooth muscle cells (SMC) were altered in the lactating mammary gland, with additional cells such as fibroblasts (myofibroblasts) participated in the storage and after milk let-down they allowed the mammary glands to return to their original state.

Key words: bovine; immunohistochemistry; mammary gland; stromal cells;

INTRODUCTION

The cells of the mammary gland are in intimate contact with other cells and with the extracellular matrix, both of which provide not only a biochemical context, but a mechanical context as well. Cells within the mammary gland respond to changes in the stiffness of their environmental structures, serving as an important role for cellular force and mechanosignaling events in the normal development and differentiation of the gland at puberty, pregnancy, lactation, and involution [23].

Studies carried out with anti-smooth muscle actin (SMA), anti-desmin and anti-vimentin antibodies in several glandular organs confirmed the heterogeneity of stromal

cells [10]. The heterogeneity of stromal cells was confirmed after using anti-alpha SMA, anti-desmin and anti-vimentin antibodies in salivary, sweat and mammary glands [5, 11]. Antibodies to keratin and smooth muscle actin have been described in myoepithelial cells but they are not specifically stained by antibodies to vimentin nor desmin [21, 24]. The smooth muscle-specific proteins and vimentin were found in the myoepithelium and stromal myofibroblasts of normal and malignant mammary glands [10, 15]. Smooth muscle actin was observed in the stroma of neoplastic breast tissue to identify myofibroblasts [3]. In carcinoma cases examined, numerous brightly stained elongated stromal cells were positive for SMA whereas no immunoreactivity was detected in fibroblasts of normal human breast tissue [10].

The loose fibrovascular stroma of the mammary gland was positive for vimentin and basal cells covering the ductal epithelium for alpha-smooth-muscle actin suggesting myoepithelial cells [7]. These cells are characteristized by their high metabolic activity and have an important function. They play the central role in milk ejection during lactation, and actively participate in mammary morphogenesis and are assumed to influence the proliferation, survival and differentiation of luminal cells [4, 18]. Myoepithelial cells, which tightly surround ductal epithelium and loosely encase the luminal epithelial cells, are specifically designed for contractility, and likely create mechanical events within the gland that differ between ductal and alveolar cells and with hormone status [23].

The interaction between stromal cells and tumor cells is known to play a major role in cancer growth and progression. The mammary stromal fibroblasts express and produce cytokines which plays roles in mammary cancer [9]. Studies relative to a stromal components of the mammary gland, mainly those involved in the ejection of milk and the recoil of the gland, are scarce. The objective of this study was to localize supporting and contractile cells in the stroma of the bovine lactating mammary gland and to appraise their role in the process of milk ejection.

MATERIALS AND METHODS

Mammary gland tissue samples were obtained from five lactating cows (Holstein) at the slaughter house. The samples were placed in 0,1 mol phosphate buffered 10 % formalin for 24 hours at room temperature, dehydrated and embedded in paraffin. The sections of thickness 5 mm were deparaffinised and rehydrated. For immunohistochemical study sections were immunostained for their reactivity with monoclonal antibodies. For this purpose, the histological sections were pre-treated with 3 % H_2O_2 in methanol for 30 min to reduce endogenous activity and preincubated with 2 % goat serum to mask nonspecific binding sites. Afterwards the sections were incubated at 4 °C overnight with the primary antibodies (Table 1). Sections were then incubated with biotinylated secondary antibody for 45 min. Tissue samples were then incubated with the streptavidin-biotin peroxidase complex method. The peroxidase activity was visualized with 0.05 % 3´.3´- diaminobenzidine (DAB) and 0.03 % v/v H_2O_2. Some sections were counter-stained with Mayer's hematoxylin. Negative controls were performed by omitting the primary antibody.

RESULTS

Under a light microscope the bovine mammary gland demonstrated itself as a highly modified tubulo-alveolar apocrine gland. The branching ducts and alveoli were lined by an inner layer of the secretory luminal epithelial cells that produced milk and were surrounded by the contractile myoepithelial cells and the basement membrane. The secretory units drained into intralobular ducts, which left the lobule and opened into the interlobular ducts. Groups of tubuloalveolar secretory units forming lobules are separated by different mounts of connective tissue septa. The interalveolar connective tissue within the lobule was loose and rich in the capillary network. Interlobular connective

Table 1. Primary antibodies

Antibodies used	Donor	Code	Isotype	Dilution	Source
Smooth muscle actin	Mouse	M 851	IgG2a	1:200	Dako
Vimentin	Mouse	1074	IgG1	1:50	Imunotech
Desmin	Rabbit	PS 031	OgG1	1:50	Imunotech

tissue was usually dense and fibrous and contained small blood vessels and nerves.

Smooth muscle actin

The myoepithelial cells and smooth muscle cells were strongly stained with alfa-SMA whereas the secretory cells gave no positive staining for SMA (Fig. 1). The myoepithelial cells were seen to cover stromal surface of the epithelium of the secretory alveoli and the intralobular ducts. The typical location of the myoepithelial cells around the alveolar epithelium was well seen in cross sections through alveoli where the myoepithelial cells formed a continuous layer. In the intralobular ducts the myoepithelial cells formed an incomplete layer. Smooth muscle cells of blood vessels and pericytes of the capillaries revealed a strong reaction to SMA (Fig. 1). Numerous blood capillaries in the interalveolar space demonstrated a rich blood supply of the lactating mammary gland.

Vimentin

The vimentin was observed in fibroblasts (myofibroblasts) disposed in the connective tissue of the interalveolar and interlobular septa (Fig. 2). Vimentin was also observed in some cells of the external epithelial surfaces of the secretory alveoli and interlobular outlets. Some free cells, such as lymphocytes and macrophages were present in the connective tissue septa, and they also expressed different intensities of the positive reaction for vimentin (Fig. 2). In the intralobular ducts, the positive reaction for vimentin was seen in myoepithelial cells (MEC) forming a layer in the subepithelial space. The endothelial cells of blood vessels were strongly stained for vimentin.

Desmin

Desmin-positive smooth muscle cells were seen in the wall of the blood vessels located in the connective tissue of the inter-lobular space (Fig. 3). No positive reaction was observed in the myoepithelial cells. A few desmin-positive smooth muscle cells were observed closely associated with capillaries. These cells had the morphological features of pericytes. Coexpression of SMA and desmin was observed in the smooth muscle cells of larger blood vessels.

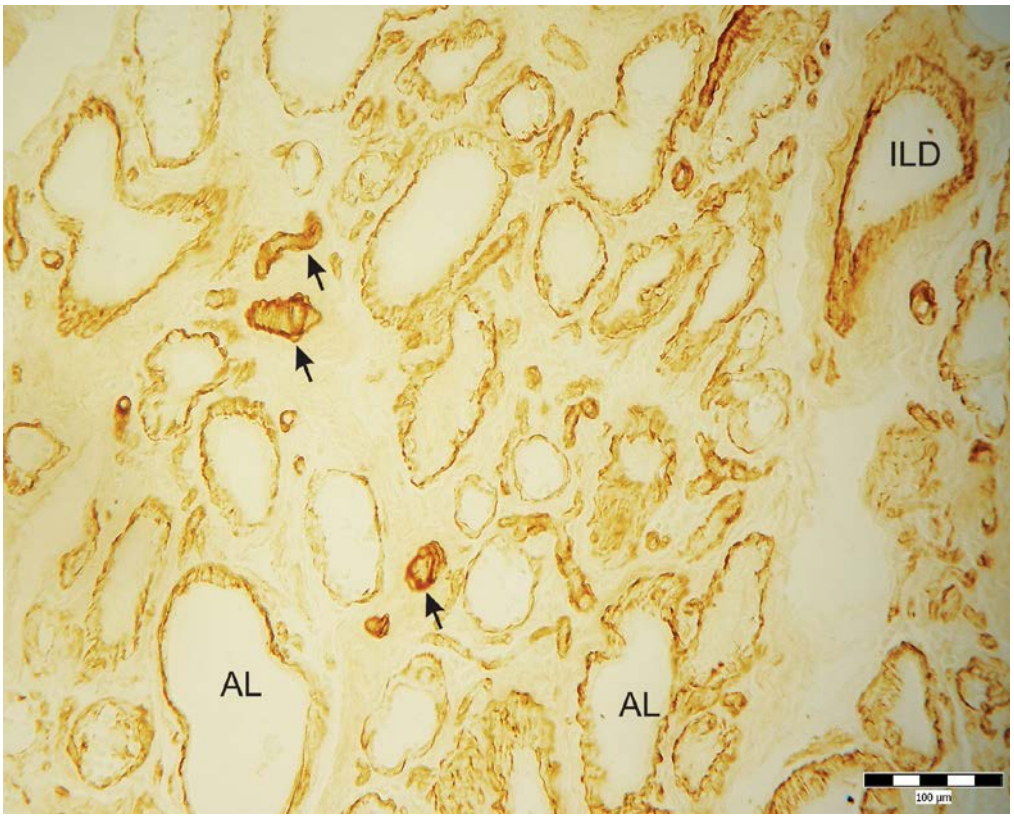

Fig. 1. Immunostaining for SMA. The myoepithelial cells form a complete layer on the periphery of alveoli (AL) and intralobular ducts (ILD). Positive reaction for SMA was strong also in SMC of capillaries and arterioles (arrows)

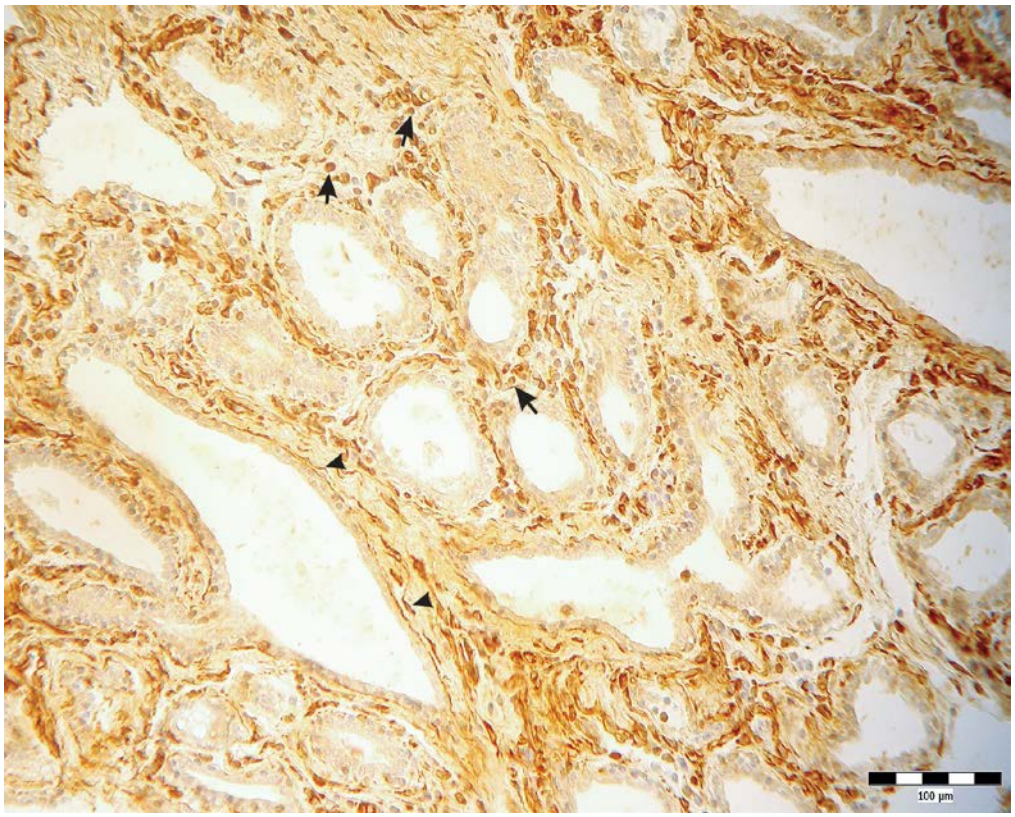

Fig. 2. Immunostaining for vimentin. Strong positive reaction was seen in the fibroblasts (arrowheads). Numerous free cells express positive reaction for vimentin (arrows)

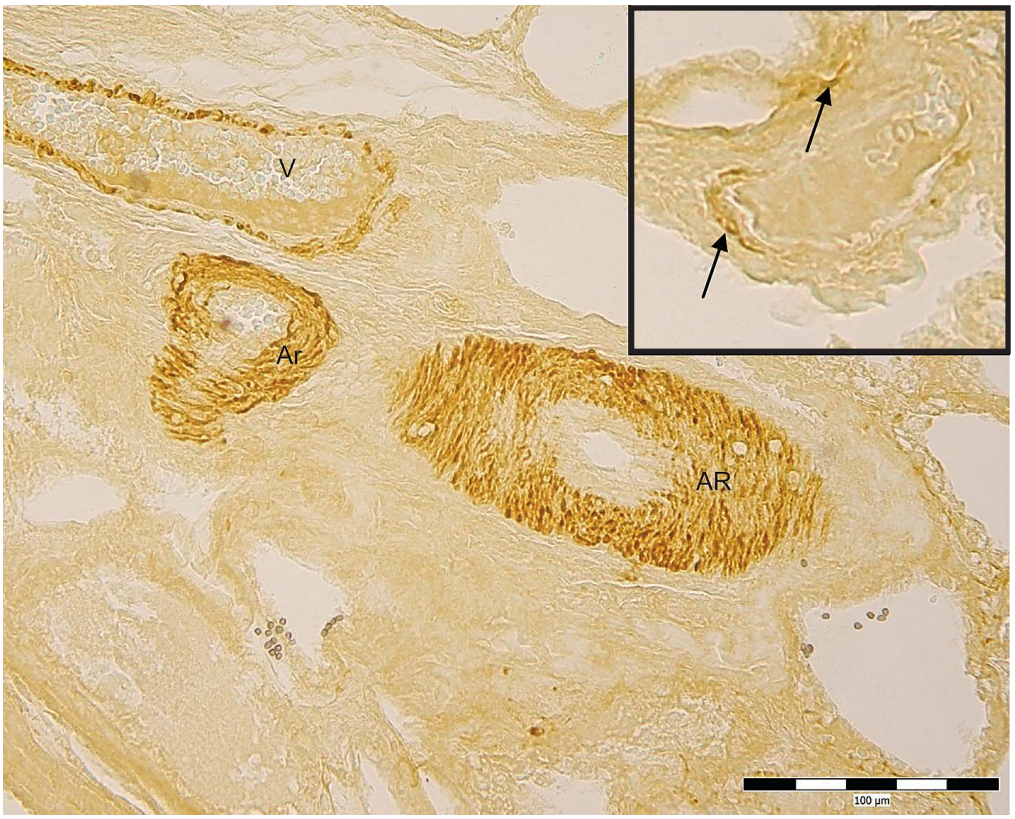

Fig. 3. Immunostaining for desmin. Positive reaction is seen in smooth muscle cells of the blood vessels: arterioles (Ar), arteries (AR) and veins (V). Top right: A few desmin-positive smooth muscle cells were observed closely associated with capillaries

DISCUSSION

Immunohistochemical studies carried out with SMA, desmin and vimentin antibodies in several glandular organs confirmed the heterogeneity of stromal cells. The smooth muscle-specific proteins and vimentin were found in the myoepithelium and stromal myofibroblasts of normal and malignant mammary glands [10]. Although SMA have been described in myoepithelial cells they are not specifically stained by antibodies to vimentin nor desmin [21, 24]. The myoepithelial cells and some stromal cells corresponding to myofibroblasts were stained with SMA thus hampering the identification of MEC [2, 5]. The MEC and stromal fibroblasts have epithelial and mesenchymal cells origin, which coordinate their expression of a set of smooth muscle markers while maintaining their specific original features. The dual nature of MEC and the phenotypic transition of fibroblasts to myofibroblasts are the examples of the plasticity of the differentiated cell phenotypes [10].

Vimentin was expressed in a number of cells of loose fibrovascular stroma of the ovine mammary gland [7]. In the mammary gland of pregnant rats, Warburton et al. [25] observed a much weaker reaction for vimentin in the MEC of developing alveolar buds than in the main ducts. Gould et al. [6] found in humans, vimentin-positive cells in 8 of 12 normal breasts and in 12 of 20 of fibrocystic disease. According to the authors, these cells in most cases appeared to be myoepithelial. In the bovine mammary gland vimentin was found in numerous cells located in the connective tissue septa. Though the main cells positive for vimentin were fibroblasts and/or myofibroblasts, some cells located covering secretory epithelium showed also positivity for vimentin. According to their location and shape, these cells correspond to myoepithelial cells. Michalczyk et al. [12] observed in the human mammary gland an intensive reaction for vimentin in MEC. There was a four-fold increase in vimentin protein levels in lactating tissue relative to resting tissue, and this may be related to the increased cellular activity of the myoepithelial cells which surround secretory alveoli [12]. A positive reaction for vimentin in these cells have been described in both the resting and lactating gland. Positive cells for both vimentin and SMA were found also in the connective tissue cells in canine mammary tumors [3] and in breast carcinomas [15].

Myofibroblasts are a unique group of smooth-muscle-like fibroblasts that have a similar appearance and function regardless of tissue of residence. In the canine connective tissue of mammary tumors Destexhe et al. [3] described myofibroblasts positive for both vimentin and alfa-actin. In normal canine mammary tissues, mixed tumors or complex adenomas have been observed by some authors with immunopositive reactions to vimentin in every type of myoepithelial cells and cells of mesenchymal tissues [3, 19].

Structural changes in the mammary gland during milk production leads to an increase in the activity of vimentin positive MEC and the appearance of a number of free cells, including macrophages (V-type) which under certain conditions are immuno-positive to vimentin [18]. Vimentin was also observed on the cell surfaces of activated macrophages, but also in apoptotic T lymphocytes, aged neutrophils and platelets [13]. Secreted vimentin could play a role in mediating the movement of circulating blood cells across the endothelium, a process in which activated macrophages and activated platelets participate [26]. The detailed functions of macrophages are extensive, as their precursor cells can respond to a variety of physiological situations and mature along a spectrum of phenotypes [17]. Mor–Vaknin et al. [14] demonstrated that activated human macrophages secrete vimentin into the extracellular space. The studies of Ingman et al. [8] revealed a role of macrophages in collagen fibrillogenesis and in the organization of the structure of terminal end buds. Besides macrophages, other types of cells may be present in the stroma of mammary gland. Radu et al. [20] reported on the *in vitro* isolated Cajal-like interstitial cells from human inactive mammary-gland stroma which expressed c-kit/CD117 and vimentin.

Smooth muscle actin detected in smooth muscle cells (SMC) of the bovine mammary gland was found irregularly scattered in the connective tissue septa between the alveoli or lobules and along the intralobular ducts. SMA positively stained myoepithelial cells were found to be a stable cellular supporting and contractile component of secretory units of the bovine mammary gland. The position of SMC in the bovine mammary gland was same as in other species [1, 4, 22].

The positive reaction for desmin in the bovine mammary gland, was found in SMC of blood vessels located in thick interlobular connective tissue septa and around the blood capillaries. The desmin was found in a similar position also in a mammary fibroadenoma in a lamb [7]. Few positive spindle cells in the interalveolar space may present pericytes of blood capillaries. In the normal condition, the

desmin-positive pericytes are located around the endothelial cells of the capillary plexus and of larger vessels in the intermediate mesenchyme [16]. In this position pericytes stabilize vessel wall, participate in the regulation of blood flow microcirculation and influence endothelial proliferation, survival, migration and maturation.

CONCLUSIONS

The results of this study showed that the stroma of the bovine lactating mammary gland contains a population of various types of cells. Immunohistochemical studies carried out with anti-alpha SMA, anti-desmin, anti-vimentin antibodies revealed a heterogeneity of stromal cells located in the supporting connective tissue. It has been shown that the cellular components play a significant part of the lactating mammary glands of cattle. Supporting and contractile components are involved in the ejection of milk and recoil of the mammary gland.

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