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Temporal and spatial heterogeneity in milk and immune-related gene expression during mammary gland involution in dairy cows

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ABSTRACT

The aim of this study was to investigate heterogeneity in tissue morphology, milk protein and immune-related gene expression, and apoptosis of epithelial cells in the lactating and involuting mammary glands of the dairy cow. Mammary tissue from different regions of the gland (alveolar, cisternal, and peripheral) was collected postmortem from nonpregnant, pasture-fed, Holstein-Friesian primiparous cows in mid-lactation that were killed at different time points postmilking: 0, 6, 12, 18, 24, 36, and 72 h (n = 6 per time point). The *CSN1NS1* and *LALBA* mRNA was decreased in alveolar, cisternal, and peripheral tissue by 12 to 36 h postmilking. In contrast, lactoferrin (*LF*) and mammary serum amyloid A3 (*M-SAA3*) mRNA was increased in these regions by 36 to 72 h. During lactation, more variability was present in gene expression in alveolar tissue between cows and between quarters within a cow, than within quarters. Histological analysis indicated the alveolar tissue from lactating cows was mostly uniform in structure; however, in situ hybridization indicated that although most of the alveolar tissue expressed milk proteins, the level of expression varied within and between alveoli. This heterogeneity became more pronounced with involution and with increasing regions of alveoli expressing lactoferrin, indicating that alveoli enter involution asynchronously. The peripheral and cisternal tissue had more variability in gene expression between cows compared with the alveolar tissue. The *M-SAA3* signal was more intense in the cisternal tissue and less intense than the peripheral compartment compared with *LF* particularly in the earlier time points. In addition, between cows within the later time points, differences were observed in tissue morphology, the levels of milk protein and immune-related gene expression, and phosphorylated signal transducer and activator of transcription (STAT) 5-P and STAT3-P proteins, and degree of apoptosis, indicating that involution of the mammary gland occurs at different rates between cows. Understanding the mechanisms initiating the process of involution of the mammary gland provides an opportunity for enhancing milk production of the dairy cow.
Key words: dairy cow, lactation, mammary involution, apoptosis

INTRODUCTION

In dairy cows, the number and activity of the mammary epithelial cells (**MEC**) are the key factors determining milk production. The gradual decline in milk production that occurs following peak lactation is due to a steady decrease in MEC number via apoptosis (Capuco et al., 2001). The cessation of milk removal also initiates the involution process and increases cell death (Wilde et al., 1997; Singh et al., 2005), as well as increasing cell proliferation (Sorensen et al., 2006; Mallah et al., 2013). Initially, the physiological changes that occur following milk accumulation include distension of the mammary gland, a decline in the rate of milk secretion, increased tight-junction permeability and lactose efflux, and an inflammatory response (Hurley, 1989; Davis et al., 1999). The morphological changes that occur include a reduction in luminal alveolar area and an increase in the stromal area in the mammary gland (Holst et al., 1987; Hurley, 1989).

Widespread changes are also present at the molecular level (Singh et al., 2008), which include a decline in milk protein gene expression (Hurley, 1989; Good-

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man and Schanbacher, 1991; Singh et al., 2008) and cell survival signals (Singh et al., 2005). This is followed by an increase in multiple protective responses to oxidative stress and numerous immunomodulatory factors (Singh et al., 2008), including lactoferrin (*LF*) (Hurley, 1989; Goodman and Schanbacher, 1991) and mammary serum amyloid A3 (*M-SAA3*; Molenaar et al., 2009). Furthermore, the reciprocal activation (phosphorylation) of signal transducer and activator of transcription factors (**STAT**) 5 and STAT3 has been demonstrated in bovine mammary glands. This is associated with milk protein gene expression and the proportion of tissue with a lactating phenotype during early involution (Singh et al., 2016) and in response to altered milking frequency (Murney et al., 2015). The activation of STAT5 is a survival signal in MEC, suppressing STAT3-mediated apoptosis (Clarkson et al., 2006). At the onset of involution, prolactin signaling is downregulated via the inactivation of STAT5 (Schmitt-Ney et al., 1992; Philp et al., 1996), and pro-apoptotic STAT3 is activated (Chapman et al., 1999; Kritikou et al., 2003), which then activates acute phase response and inflammation-related genes (Clarkson et al., 2004) and is also linked to survival signaling (Chapman et al., 1999).

Interestingly, analysis of tissue sections during lactation in the beef cow and sheep mammary gland indicate the presence of a heterogeneous population of alveoli (Molenaar et al., 1992, 1996a). That is, the majority of the alveoli are functionally active and secrete milk; however, small regions of alveoli do not express lactation-associated milk protein genes but contain numerous fat-containing vesicles (vesicle-engorged alveoli; **VEA**), indicative of involution. Thus, milk protein genes, such as *LALBA* and *CSN1S1*, may be either switched on or off between adjacent alveoli (Molenaar et al., 1992; Farr et al., 1996), especially in the more heterogeneous peripheral alveolar tissue compared with the central secretory alveolar tissue. In addition, *LF* is expressed in alveoli where there is no expression of major milk proteins. These alveoli are considered to be quiescent and in the engorged state, and it is proposed that all MEC pass through the quiescent phase before becoming senescent (Davis et al., 1999).

The diversity in milk protein gene expression was also observed during involution of the mammary gland in both beef cows and sheep (Molenaar et al., 1992). The majority of alveoli have an involuting phenotype with small regions expressing milk protein genes. In sheep, there were local regions of alveoli with an inverse relationship between MEC apoptosis and milk secretory activity (Molenaar et al., 1996b), and also in cows and goats, MEC apoptosis may be localized to regressing alveoli (Wilde et al., 1997; Li et al., 1999).

Heterogeneity in tissue morphology and gene expression of the lactating and involuting mammary gland in dairy cows has not been studied extensively. If it occurs to any extent this local variation could result in sub-optimal dairy production and its potential manipulation offers a way of increasing production. This study examines variation in the mRNA expression of 2 milk protein genes (*LALBA* and *CSN1S1*), 2 involution/defense-associated genes (*LF* and *M-SAA3*), and the STAT transcription factors during lactation and in relation to apoptosis during mammary involution of dairy cows.

MATERIALS AND METHODS

Cows

Animal experimentation was conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). Mammary alveolar tissue samples were used from a subset obtained from an animal experiment described previously (Singh et al., 2016). Briefly, involution of the bovine mammary gland was induced by abrupt termination of milking in 42 nonpregnant Holstein-Friesian dairy cows at, or close to, their peak milk production and before mating in mid-lactation (average DIM, 89.1 $\pm$ 2.2). The primiparous cows were solely pasture-fed, milked twice daily from parturition, and had an average daily milk yield of 14.3 $\pm$ 0.3 kg/cow. The average SCC in composite (4 quarters) milk before the termination of milking was 159,000 $\pm$ 20,000 cells/mL. The cows were slaughtered at the Ruakura Abattoir (Hamilton, NZ) using standard commercial procedures (electrical stunning followed by exsanguination) at 0, 6, 12, 18, 24, 36, and 72 h (n = 6 per time point) after the last milking.

Mammary alveolar tissue (approximately 30 g) was collected from the middle of the upper one-third of the gland of a rear quarter of each animal, snap-frozen in liquid nitrogen, and stored at −80°C until required for subsequent RNA and protein extraction. Additional samples of alveolar tissue (10 to 30 g) were taken from 5 different random but distributed sites within each of the 4 udder quarters of cows (n = 3 cows, 60 samples in total) in the 6-h group to examine local variation in gene expression. Samples of peripheral tissue (referred to as capsular tissue by Farr et al., 1996) were also collected and snap frozen in liquid nitrogen and stored at −80°C until required for subsequent RNA extraction. Peripheral tissue is a 1 to 2 cm band of subcutaneous secretory mammary tissue surrounding the majority of the secretory alveolar tissue facing the skin and cisternal tissue, located between the teat and the secretory alveolar tissue.

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