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STROMAL CHANGES AND TRANSFORMING GROWTH FACTOR-β1 IN THE MAMMARY GLAND DURING THE DRY PERIOD OF DAIRY COWS

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STROMAL CHANGES AND TRANSFORMING GROWTH FACTOR-β1 IN THE MAMMARY GLAND DURING THE DRY PERIOD OF DAIRY COWS

By

Lindsey De Vries

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ABSTRACT

STROMAL CHANGES AND TRANSFORMING GROWTH FACTOR-β1 IN THE MAMMARY GLAND DURING THE DRY PERIOD OF DAIRY COWS

By

Lindsey De Vries

Our first objective was to determine if mammary remodeling during the dry period of dairy cows is characterized by a transient increase in stromal fibronectin, myofibroblasts, stromelysin (MMP-3), transforming growth factor-beta1 (TGF-β1) and its receptor, TGF-βR2. The second objective was to determine if TGF-β1 increases mammary stromal remodeling during the dry period. Mammary biopsies were obtained from nine cows at: late lactation, 1 week after dry-off, 3 weeks before expected calving, and 1 week before expected calving. For objective 2, tissue was incubated for 2 h with 0 or 5 ng TGF-β1/ml. Intralobular stromal area was greater at 1 week dry and 3 weeks before calving than at 1 week before calving (P=0.02). The number of fibroblasts was greater at one week dry than 1 week before calving (P=0.02). Stromal TGF-β1 was 15% lower during the dry period than in late lactation (P=0.02). The number of myofibroblasts and the expression of fibronectin, MMP-3, or TGF-βR2 did not differ. Incubation with TGF-β1 had no effect on the percent intralobular stroma area expression of fibronectin or MMP-3. The number of myofibroblasts increased by 19% in the intralobular stroma (P=0.03) and the percent of myofibroblasts tended to increase by 4% (P=0.06) in tissue incubated with TGF-β1. Based on these findings, TGF-β1 may be important in the activation of stromal cells during involution and mammogenesis of the bovine mammary gland.

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ABBREVIATIONS

LL – Late lactation

1 WD – One week after dry-off

3 BEC – 3 weeks before expected calving date

1 BEC – 1 week before expected calving date

PBS – Phosphate-buffered saline

MMP-3 – Matrix-metalloprotease-3

TGF-β1 – Transforming growth factor beta-1

TGF-βR2 – Transforming growth factor beta-2

SMA – Smooth muscle α-actin

ECM – Extracellular matrix

INTRODUCTION

Reducing the length of the dry period, the non-lactating period between successive lactations, may improve the efficiency of dairy production by increasing the number of productive days in a cow's life. However, it appears that the mammary gland requires time to remodel and replace cells between lactations. Many investigators who study bovine mammary remodeling in the dry period are focused on changes in the milk-producing epithelium, and do not measure changes in the surrounding stromal tissue. However, studies in mice show that in addition to providing structural support, stromal composition can influence epithelial cell fate. Thus, a better understanding of how mammary stroma remodels will be useful to develop strategies to effectively shorten the dry period. In chapter one, the literature pertaining to mammary development and remodeling is reviewed. The second chapter describes a study that was conducted to characterize changes in mammary stromal tissue during late lactation and the dry period.

Transforming growth factor beta-1 (TGF-β1) stimulates remodeling of mammary tissue of mice. If TGF-β1 can increase the rate of stromal remodeling in the bovine mammary gland, it may be useful to shorten the mammary remodeling time required during the dry period. In chapter three, the objective of the study described was to examine the effects of TGF-β1 on the stromal compartment from mammary explant tissue obtained during late lactation and the dry period. Overall, we expect our results to provide new insights into mammary remodeling during the dry period of dairy cattle.

CHAPTER 1

COMPREHENSIVE LITERATURE REVIEW

INTRODUCTION

The dairy industry is facing many challenges including rising feed costs and a growing need to reduce the impact agriculture has on the environment. To address these concerns and maximize efficiency, it is necessary to have a thorough understanding of the biology of milk production. A potential way to enhance efficiency of dairy production is to reduce or eliminate the dry period, the non-lactating period between lactations, just prior to parturition. During the dry period, a cow is consuming feed and producing wastes while not producing milk. However, current practices of eliminating or shortening the dry period reduce lifetime milk yield per cow (Kuhn et al., 2006; Rastani et al., 2005). Traditionally, scientists and managers have recommended a 40 to 60 d dry period for optimal milk quality and production (Coppock et al., 1974; Dias and Allaire, 1982). However, these recommendations are based primarily from milk production records rather than from an understanding of the mammary requirement of a dry period between lactations.

During the dry period, the mammary gland is physiologically active as it undergoes the processes of involution and mammogenesis. These processes have been studied extensively in rats and mice, but studies focused on dairy cows are limited (Capuco et al., 1997; Paape and Tucker, 1969). While some concepts of mammary involution from other species may apply to dairy cows, extrapolation must be done cautiously since, unlike many species studied, dairy cows are generally pregnant during involution after cessation of milk removal. For example, dairy cows do not exhibit

sloughing of the mammary epithelial cells during involution that is observed in mice (Holst et al., 1987). Dairy-specific research is necessary to develop strategies to shorten the dry period based on the biology of the gland, so that overall milk production is not compromised.

Milk production is a function of number and secretory activity of mammary epithelial cells, which are replaced and renewed for milk production in the dry period between lactations (Capuco et al., 1997). Because of the economic importance of mammary epithelial cells to milk production, most researchers have focused on the epithelial cells when studying the dry period. While the epithelial cells are important, they are only one component of the mammary gland, and they depend on other structures and surrounding cells to function and survive. Stromal tissue surrounding mammary epithelium not only provides structural support to the epithelial cells, but also plays an active role in mammary development and remodeling. A thorough understanding of changes during the dry period begins with an understanding of mammary gland function, and the interactions between stromal and epithelial compartments that take place during development and remodeling.

BOVINE MAMMARY GLAND ANATOMY

General Anatomy

The bovine mammary gland consists of four separate and distinct quarters that are attached to the body by a strong suspensory system of ligaments. The median suspensory ligament divides the mammary gland into visually distinct halves down the centerline of the cow, and the deep lateral suspensory ligaments provide lateral support to the gland.

Each quarter has one teat that is fed by a cistern and a network of ducts that branch into the alveoli. Milk is synthesized in the epithelial cells of the alveoli, which form small clusters, or lobules. The alveoli drain into the intralobular ducts that flow into the interlobular ducts that open up into gland cistern. The gland cistern is a large cavity that serves to collect milk that is secreted from the alveoli between milkings. The gland cistern is continuous with the teat cistern, and they are linked to the external environment by the streak canal, which opens to allow milk removal and closes to prevent milk leakage and to reduce the risk of pathogens from entering the gland (Klaas et al., 2005).

Parenchyma and Stroma

The tissue components of the mammary gland are generally categorized as stroma and parenchyma. The stroma houses the lymphatic, circulatory and innervation systems of the mammary gland and provides the supportive structures and tissues. The parenchyma is the glandular, milk-synthesizing region of the mammary gland. Fibroblasts, endothelial cells and the extra-cellular matrix are generally considered part of the stromal tissue, whereas epithelial cells make up the majority of the parenchymal tissue.

Mammary parenchyma contains many alveoli lined with epithelial cells that are connected to a basement membrane (Nguyen and Neville, 1998). Surrounding the alveoli is a layer of myoepithelial cells which, in response to oxytocin, aid in ejecting milk produced by the epithelial cells from the gland. During late pregnancy, alveolar epithelial cells begin to develop extensive synthetic machinery, including the rough endoplasmic reticulum and Golgi apparatus, as the gland prepares for milk synthesis.

The rate of epithelial cell proliferation increases right before parturition, declines after parturition, and remains relatively low throughout lactation. Secretory activity of the mammary epithelium increases until d 90 of lactation and then remains constant throughout late lactation (Sorensen et al., 2006). The number of epithelial cells appears to play an important role in determining lactation persistency, and if the rate of epithelial cell proliferation does not balance the rate of apoptosis in late lactation, there is an overall decline in milk production (Capuco et al., 2001).

There are two types of stromal tissue; interlobolular stroma is the tissue found between the lobules of alveoli, and the intralobular stroma is found within the lobules. Interlobular stroma is denser, containing more collagen fibers than the intralobular stroma (Atherton et al., 1998). The intralobular stroma is separated from the parenchymal tissue by the basement membrane. The regulation and coordination of changes in the mammary gland can occur through interactions between the epithelium and the closely connected intralobular stroma.

The extracellular matrix (ECM) is a network of proteoglycans, glycoproteins, glycosaminoglycans, minerals and other proteins including fibronectin, laminin, entactin, and collagen (Adams & Watt, 1993). The ECM functions to support surrounding cells as and is a storehouse of factors and proteins involved in cell survival or apoptosis (Farrelly et al., 1999). Mammary ECM provides the framework and three-dimensional structural support for the ducts and alveoli, and is also integrally involved with mammary cell function including proliferation, survival, differentiation, and movement (Doljanski, 2004).

Important interactions and communication occur between the epithelial cells and the surrounding ECM, and alterations to the composition of the ECM can influence the phenotype of epithelial cells. For example, differentiation of bovine mammary progenitor cells into a secretory epithelial cell phenotype was greatest when cells were grown on a dense, heterogeneous, but not homogenous matrix of ECM proteins including collagen and laminin (Holland et al., 2007). In fact, they found that a homogenous matrix of laminin or collagen did not induce differentiation, and suggested that microenvironment of the mammary gland is important to epithelial cell differentiation. Thus, the overall composition of the ECM appears more important to cell differentiation than one specific component independently.

The basement membrane ECM is intimately associated with the epithelial cells through integrin receptors, which mediate signals between the epithelium and stroma (Klinowska et al., 1999). The basement membrane separates the epithelial compartment from the stromal compartment and is composed primarily of collagen IV , laminin, and fibronectin, which are believed to be synthesized from both compartments. The basement membrane provides epithelial cells structure and support, and is required for cell signaling via integrins (Klinowska et al., 1999). The close attachment of epithelial cells to the basement membrane may be related to the importance of the ECM and stromal composition for epithelial cell survival.

Fibroblasts are found throughout the stroma and contribute to the synthesis of ECM proteins, growth factors and proteinases (Warburton et al., 1982). Fibroblasts also help direct the branching patterns of the epithelium (Berdichevsky et al, 1994).

The cytokine, transforming growth factor-beta1 (TGF-β1) can induce the differentiation of both fibroblasts and mammary epithelial cells into myofibroblasts or to a fibroblastic phenotype, respectively, in culture (Miettinen et al., 1994). Unlike fibroblasts, myofibroblasts express the contractile protein smooth muscle α-actin (SMA), which is believed to increase motility of the cells (Grinnell, 1994). Myofibroblasts are characterized by spindle-like morphology, abundant presence of rough endoplasmic reticulum and expression of fibronectin, particularly a fibronectin isoform with the extra-domain A (Eyden, 2008).

MAMMARY DEVELOPMENT

The mammary gland undergoes number of changes during growth and development throughout the course of a dairy cow's life. During the early stages of life, the gland is generally undergoing growth, development, and differentiation of cells and structures; also known as mammogenesis. Mammogenesis continues through the first gestation as structures mature and prepare for lactation. At the onset of lactation, or lactogenesis, epithelial cells develop secretory machinery to synthesize milk. The capacity to maintain the ability to synthesize milk during lactation is known as galactopoiesis. Towards the end of lactation, milk production gradually declines. At cessation of milk removal, or dry-off, the gland undergoes a period of rapid changes and functional regression towards a pubertal state (loss of epithelial cells, collapsing of alveoli) known as involution. If the animal is pregnant, the period of involution is

followed by another period of mammogenesis as the mammary gland prepares for lactation.

Bovine mammary development begins early in the fetal stage of life with the mammary band first appearing in the embryo around d 30. Development continues with the formation of the mammary bud around d 43, early teat formation by d 65, and the mammary fat pad becomes evident by d 80. From this point in fetal growth until birth, mammary growth is isometric. Postnatal mammary growth in heifers is allometric through puberty. During the allometric growth phase associated with the peripubertal period, the mammary epithelium that forms the rudimentary ductal system and elongates into the mammary fat pad.

As the mammary gland grows and ductal structures elongate into the fat pad, intralobular stroma must also be synthesized and remodeled to allow space for, and provide support to the parenchymal tissue. Remodeling of the stroma is stimulated by endogenous hormones and growth factors. Heifers ovariectomized at 3 mo of age had abnormal development of alveolar ducts and altered stromal composition compared to intact heifers after 2 mo of development. Intact heifers had more complex branching, less interlobular stroma, and less parenchymal fibronectin than ovariectomized heifers, indicating that the hormones released before puberty from the ovaries can alter the composition of the ECM (Berry et al, 2003).

Pregnancy and Lactation

During pregnancy, when levels of both estrogen and progesterone are high, the growth of mammary gland ductal system resumes and lobulo-alveolar structures develop

in preparation for lactation (Sheffield, 1988). As parturition approaches, blood levels of progesterone remain high until 2 d prepartum, when they drastically decline. Estradiol gradually increases from 26 to 5 d prepartum, drastically increases from 5 d prepartum to 1.5 d prepartum, and then decreases at parturition (Smith et al., 1973). While estrogen and progesterone concentrations decrease in the periparturient period, blood concentrations of prolactin and glucocorticoids increase.

Changes in the hormonal milieu during the periparturient period stimulate terminal differentiation of the mammary gland, in order for lactation to commence upon parturition. Interestingly, when lactation was induced in 7 non-pregnant dairy cattle in by administering a series of estradiol and progesterone injections, twice daily for 7 days, peak milk production was comparable to production from previous pregnancy-induced lactations. These data suggest that the progesterone and estrogen injections promoted lobulo-alveolar development sufficiently to support lactation (Smith and Schanbacher, 1973). When dexamethasone and reserpine, known to increase prolactin, were added to the injection schedule, lactation induction was more successful, and milk production was greater than cows not given reserpine (620 kg vs. 1,229 kg milk in 100 d of treatment) (Collier et al., 1977).

Once initiated, galactopoiesis continues as long as there is a regular suckling stimulus and/or milk removal and a steady supply of metabolic substrates. However, in dairy cows there is a gradual decline in milk production after peak lactation, despite regular milk removal and sufficient metabolic supply.

The Dry Period

Dairy cattle are unique in that following their first lactation they are nearly always pregnant during late lactation and the dry period. Concurrent pregnancy alters the hormonal profile of the cow and thus may alter the process of involution. During mammary involution in pregnant dairy cattle, the alveolar structures are not completely degraded and function is not completely lost, as they are in species that are not pregnant at cessation of milk removal (Capuco et al., 2003).

Involution

Concurrent pregnancy during the dry period of the dairy cow likely slows the normal involution process at cessation of milk removal. In mice, progesterone implants delayed involution of alveolar structures was at weaning (Feng et al., 1995). These investigators suggested that high levels of progesterone during a concurrent pregnancy may affect the normal involution process.

Holst and Hurley (1987) described the changes in tissue biopsied during mammary involution in dairy cows. By d two of involution, epithelial cells contained large vacuoles that persisted until d 14. The vacuoles appeared to come from coalescing secretory vesicles, and compressed the cytoplasm and cell nuclei. At d two epithelial cells in involuting tissue also had fewer mitochondria, fewer intact rough endoplasmic reticuli and no detectable Golgi apparatus. By d 21, the alveoli appeared to have collapsed, leaving very little lumen area (Holst et al., 1987). Stromal area increased from 38% at d 0 dry to a maximum of 48% at d 14 dry (Sordillo and Nickerson, 1988).

As the alveoli collapse, decreasing the lumenal and epithelial area, the stroma is likely remodeled through degradation and synthesis of stromal proteins. Rabot et al (2007) found that the mRNA abundance of matrix metallo-proteases (MMPs) and their inhibitors in mammary tissue was low during lactation (early, mid and late lactation) compared to mammogenesis around puberty and involution 28 d after dry-off in non-pregnant cows. These investigators also found that expression of proteinases were expressed concomitantly with their corresponding inhibitors, regardless of stage of lactation or development. These data indicate that the tight regulation of proteinease degradation is likely influenced by other factors, other than just proteinase inhibitors (Rabot et al., 2007).

Singh et al (2005) examined the mRNA abundance of integrins involved in epithelial-ECM signaling, as well as pro and anti-apoptotic factors in mammary tissue obtained from primiparous, non-pregnant cows. These cows were dried off at 92 DIM and slaughtered at 0, 6, 12, 18, 24, 36, 72 and 192 h after dry-off. The mRNA expression of integrins β1, α6, and α5, and the anti-apoptotic factor Bcl-2 decreased within 24 h of the last milking. Epithelial apoptosis was increased at 72 h after dry-off. The pro-apoptotic factor, αBax, increased over 8 d following dry-off. Integrins are involved in epithelial cell signaling to the ECM, and the decline in integrin mRNA abundance with the concomitant increase in pro-apoptotic factors and decrease in anti-apoptotic factors indicates that reduced stromal-epithelial interaction is associated with apoptosis in involution of the bovine mammary gland (Singh et al., 2005).

Wilde et al (1997) observed an increase in epithelial apoptosis 7 d after dry-off by measuring terminal deoxynucleotidyl transferase dUTP nick-end-labeled cells. They also

found that regions of the mammary gland with collapsed alveoli and reduced histological integrity had higher percentages of apoptotic cells: 7.2% of cells were apoptotic in collapsed regions vs. 0.6% where alveoli were intact (Wilde et al., 1997). Increased alveolar apoptosis observed during involution in dairy cows is consistent with changes observed during involution in mice, where alveolar apoptosis increased within 2 d of dry-off (Lund, 1996).

Post-lactational involution is well-characterized in mice and rats, and has been used as a model for involution in the cow (Capuco et al., 2002; Paape and Tucker, 1969; Strange et al., 1992). In mice, two distinct phases have been used to describe involution when milk removal ceases. Lund et al (1996) characterized the first phase by apoptosis of the epithelium, and the second phase by degradation of the basement membrane surrounding the epithelium. Transgenic mice expressing an auto-activating isoform of the proteinase MMP-3 exhibited accelerated involution and increased degradation of basement membrane proteins, collagen IV and fibronectin (Sympson et al., 1994). These results were consistent with previous study by Talhouk et al (1992), who found that MMP-3 expression was preceded by the expression of its inhibitor, TIMP-1 after cessation of milk removal. TIMP-1 implants delayed involution at cessation of milk removal by maintaining the integrity of the basement membrane. In the absence of the exogenous TIMP-1 implants, Talhouk et al (1992) observed an increase in MMP-3 around regressing alveoli with degraded basement membranes. Whereas some of the changes in the mammary glands of mice during involution and remodeling may be consistent with dairy cattle, comparisons should be made cautiously.

Mammogenesis

The latter part of the dry period in pregnant animals is a time of rebuilding and remodeling as parturition approaches. The mammary gland responds to pregnancy hormones towards the end of gestation, and redevelops in preparation for the next lactation. In contrast to involution, stromal area decreases and lumen area increases during mammogenesis (Capuco and Akers, 1999; Sordillo and Nickerson, 1988). During mammogenesis in the dry period, epithelial cell are renewed in numbers and in secretory function. Capuco et al (1997) characterized 98% of mammary epithelial cells as secretory 7 d before parturition, while none of the epithelial cells were classified as secretory 35 d before parturition. Norgaard et al (2008) found an increase from 6% to 11% of epithelial cells expressing Ki-67, a marker of proliferation from -48 d to -16 d before calving, suggesting that the rate of epithelial cell proliferation also increases during latter part of the dry period.

From dry-off to parturition, the overall number of epithelial cells does not change; however, there is an appreciable amount of cell turnover in both epithelial and stromal cell compartments. Capuco et al (1997) compared cows given a typical 60 d dry period with cows continued on a daily milking schedule until parturition. Tritiated thymidine ([^{3}H]Tdr) incorporation, used as an indicator of DNA synthesis and thus proliferation, was measured in tissue slices collected from dry cows and lactating cows at -53, -35, -20 and -7 days prepartum. At 53 d prepartum there was no difference in the percent of epithelial cells labeled with ([^{3}H]Tdr) between lactating and dry cows, but by approximately 35 days prepartum, the rates of epithelial cell proliferation and turnover were significantly higher in dry cows than in lactating cows. For both treatment groups

the rate of ([^{3}H]Tdr) incorporation by mammary tissue slice was highest one wk before parturition. When cows were milked continuously between lactations, the mammary gland also had fewer epithelial cells at 7 d prepartum than cows that were dried-off (76% vs. 84% of total cells), indicating that the dry period is important to allow the gland to replace senescent cells and produce new cells (Capuco et al., 1997).

MAMMARY REMODELING

During the dry period, the stroma must remodel to accommodate changes in the shape and size of the alveoli. The breakdown of intralobular stroma requires the tightly orchestrated activities of proteinases through their activators and inhibitors. Proteinases are generally secreted from stromal cells in a latent form and require activation by other proteinases or growth factors. As proteinases degrade the stroma, cytokines and growth factors may be released. These growth factors may play a role during the remodeling of the ECM by regulating expression of proteinases. For example, the release of growth factors includes the TGF-β family that is present in a bound form in the ECM (Green and Lund, 2005). TGF-β is produced by stromal and epithelial cells, and increases production of proteinases through paracrine and autocrine activity. Therefore remodeling of the ECM is likely dependent on a balance of proteinases and proteinase inhibitors, mediated by growth factors such as TGF-β.

TRANSFORMING GROWTH FACTOR-β1

The TGF-β family includes a wide range of multifunctional polypeptides including inhibins, activins, anti-mullerian hormone, growth differentiation factor, and bone morphogenic proteins. These polypeptides play an important role in cell signaling and communication for processes including cell growth, differentiation, apoptosis, tissue development, and tissue repair.

Thirty years ago, de Larco and Todara first purified a 25kDa polypeptide which increased cell proliferation of murine fibroblasts in the presence of epidermal growth factor (EGF) (de Larco and Todaro, 1978). This polypeptide became known as part of a family of transforming growth factors (TGFs) because they induced a transformed phenotype in fibroblasts, characterized by anchorage independent growth, loss of contact inhibition, and loss of density dependent growth in culture (de Larco and Todaro, 1978). Initially TGFs were only found in neoplastic cells and tissues, but in 1981 TGFs were isolated in the submaxillary glands of mice (Roberts et al., 1981). Since this discovery, a variety of TGF-related proteins have been isolated and purified from multiple species.

The TGFs were initially divided into two classes: type α (TGF-α), which binds to epidermal growth factor receptor (EGFR), and type β (TGF-β), which does not bind to EGFR (Roberts et al., 1982). Further isolation revealed that TGF-β could enhance murine sarcoma fibroblast colony proliferation in the presence of either TGF-α or EGF; however, the addition of EGF to TGF-α alone did not enhance cell proliferation (Anzano et al., 1982).

TGF-β is composed of two polypeptide chains cross-linked by disulphide linkages (Dart et al., 1985; Roberts et al., 1982). Multiple isoforms of TGF-β have been identified and isolated since its discovery (Cheifetz et al., 1987; Derynck et al., 1988). Presently, a number of isoforms have been identified, and include TGF-β1 and TGF-β2, which were isolated from human placenta, porcine platelets, and bovine kidney (Assoian et al., 1983; Frolik et al., 1984; Roberts et al., 1983).

TGF-β chains are synthesized with an inactive latent associated peptide (LAP) bound at the C-terminus. Another protein, latent TGF-β binding protein (LTBP), facilitates the movement of the TGF-β-LAP complex and regulates its secretion into the ECM (Flaumenhaft et al., 1993). TGF-β is activated through post-translational modifications whereby the LAP and LTBP are cleaved off by proteases including thrombospondin, αVβ6 integrin, plasmin, and matrix-metalloproteases (Annes et al., 2004; Lyons et al., 1990; Ribeiro et al., 1999). This activation process provides a critical point of regulation of TGF-β prior to receptor binding and signal transduction in the cell.

Early work by Massague and Like (1985) using labeled TGF-β, revealed 280 kDa and 70-90 kDa proteoglycan receptors that would specifically bind TGF-β without cross-reacting with EGF or insulin. The biological activity of TGF-β was dependent upon these two receptors acting in coordination to produce a signal within the cell. The multiple receptor dependency of TGF-β was confirmed in a study using mutant mink lung cells, selected for their lack of response to TGF-β treatment because of a defect in one or both TGF-β receptors (Laiho et al., 1990).

By 1990, nine different membrane proteins were identified with TGF-β binding properties, including three main receptors; TGF-βRI, TGF-βR2, and TGF-βR3

(Massague, 1992). Receptor binding affinity and specificities of different TGFs was determined using iodinated TGF-β1 and affinity labeled receptors. An 85 kDa (type II) receptor, displayed high affinity for TGF-β1 (Cheifetz et al., 1987). TGF-β1 binds to TGF-β receptor II (TGF-βR2) which phosphorylates serine and threonine residues of TGF-βRI, and transmits the signal to the cell (Wrana et al., 1994). A third type of receptor, TGF-βR3 or betaglycan is a proteoglycans identified as a binding site of TGF-β (Fukushima et al., 1993). Receptor availability is also a limiting factor in the TGF-β pathway, for example, as the presence of the receptors increased, binding of TGF-β1 also increased (Plaut and Maple, 1995).

The major intracellular signal transduction pathway of TGF-β involves the Smad protein family. Following activation by ligand bound TGFBR2, TGF-βRI phosphorylates a receptor-regulated Smad protein (Smad2 or Smad3) which associates with Smad4 or Smad4b and translocates into the cell nucleus where the complex can regulate transcription and bind to DNA and activate genes specifically targeted by TGF-β (Nakao et al., 1997). Treatment of a mink lung epithelial cell line with TGF-β resulted in the phosphorylation and subsequent accumulation of Smad2, Smad3 and Smad4 in the nucleus (Nakao et al., 1997). Once in the cell nucleus, Smads interact with transcription factors to induce changes in gene expression. Mutations of the Smad proteins inhibited changes normally induced by TGF-β, including the expression of smooth muscle α-actin (SMA) by a mouse mammary epithelial cell line, or fibronectin and plasminogen activator inhibitor-1 in mink lung epithelial cell lines.

Early studies of TGF-β function in cell lines revealed that it had either an inhibitory or stimulatory effect on cell proliferation, depending on the cell type (Dart et

al., 198; Roberts et al., 1985; Roberts et al., 1986; Sporn et al., 1986). For example, the dosage of TGF-β that inhibited growth of rat fibroblast-derived cells, stimulated colony formation of normal rat kidney cells in culture. EGF and platelet-derived growth factor could alter the effects of TGF-β and influence its activity as either an inhibitor or stimulator of cell growth, indicating that TGF-β works in concert with other growth factors (Roberts et al., 1985). TGF-β can also slow down cell cycle, as incubation with 60 pM TGF- β increased doubling time of lung carcinoma cells from 21 to 36 hours, and the doubling time from 31 to 46 h of normal rat kidney cells (Roberts et al., 1985).

In addition to its effects on cell phenotype and growth, TGF-β also plays a role in the production of ECM proteins. TGF-β treatment increased fibronectin and collagen incorporation into the ECM of cell cultures from chick embryos. Interestingly, the ECM may also, in turn, modulate the mitogenic effects of TGF-β1. For example, when fibronectin was added in the presence of TGF-β, it stimulated proliferation of rat kidney cells in culture, and the addition of a peptide sequence that blocked fibronectin receptors reduced the activity of TGF-β on rat kidney cell proliferation (Ignotz and Massague, 1986). The diverse range of responses elicited by TGF-β has stimulated considerable interest. In a review, Massague (1990) summarized the activity of TGF-β to include the regulation of synthesis of ECM proteins and proteinase inhibitors, as well as regulation of epithelial cell adhesion to the ECM through up-regulation of integrin receptors (Massague, 1990).

TGF-β in the Mammary Gland

Mammary remodeling includes synthesis and degradation of the ECM as well as cell proliferative and apoptotic events. The role of TGF-β in these processes has generated interest in the effects of TGF-β in normal and neoplastic mammary remodeling and development. Immunostaining of endogenous TGF-β1 in normal murine mammary tissue showed more TGF-β1 in the periductal mature ECM than in newly synthesized tissue, indicating the ECM may sequester TGF-β for times of remodeling when the ECM is degraded by proteinases (Silberstein et al., 1992).

Reversible inhibition of mammary ductal growth and morphogenesis was observed in 5-wk-old virgin mice, with slow release mammary implants containing TGF-β. After 4 d, glands with TGF-β implants had fewer end buds and reduced elongation compared to control glands with implants containing bovine serum albumin. The effect was reversed and normal growth rates were resumed 10 d after implants were removed (Silberstein and Daniel, 1987). This inhibitory effect of TGF-β1 was specific to the epithelium, as DNA synthesis was inhibited in the surrounding epithelial cells but not stromal cells (Daniel et al., 1989).

Mice with a homozygous null TGF-β3 mutation had reduced terminal end bud units in the mammary gland relative to wild-type mice. Transplanted mammary epithelium of these mice with the TGF-β3 mutation extended further into a wildtype recipient fat pad during pubertal development. The TGF-β3 null mice also experienced delayed onset of mammary development (Ingman and Robertson, 2008). Transgenic mice that over-expressed TGF-β1 also had impaired mammary development, similar to the TGF-β3 mutant mice (Pierce et al., 1993). TGF-β1 transgenic mice had reduced

lateral ductal branching during early development as a result of TGF-β1 over-expression (Pierce et al., 1993).

The effect of stromal TGF-β1 on alveolar development was investigated in mice with a mutant form of TGF-βR2, the receptor which TGF-β1 commonly binds to. TGF-βR2 transgenic mice had increased lateral ductal branching, disorganized ductal structure, and increased stromal expression of plasminogen activator inhibitor (PAI-1), an inhibitor of proteinases that degrade ECM proteins (Joseph et al., 1999). These results indicate that stromal remodeling during ductal development is dependent on TGF-β1-induced alteration of stromal proteins and proteinases.

The effects of TGF-β1 on mammary remodeling are likely dependent on the stage of development. Jhappan et al (1993) observed normal ductal development, but impaired lobuloalveolar growth in late pregnancy in transgenic mice that over-expressed TGF-β1. These mice also had greater production of TGF-β1 in the periductal stroma during pregnancy than wildtype controls (Jhappan et al., 1993; Pierce et al., 1993). TGF-β implants that produced inhibitory effects on ductal growth and morphogenesis in virgin mice did not impair alveolar DNA synthesis in pregnant mice given the same dosage, further suggesting that the role of TGF-β as an inhibitor of epithelial cell proliferation is dependent on stage of development (Daniel et al., 1989).

Whereas TGF-β1-null mice do not survive long enough to examine them during lactation and mammary development, TGF-β3-null mice do. The rate of epithelial apoptosis in the wildtype mammary gland increased rapidly following a large peak of TGF-β3 mRNA and protein expression, at the cessation of removal from the gland. However in TGF-β3-null mice, this increase in apoptosis was not as pronounced as in the

wildtype mice. Similar effects were seen when mammary glands from mice with a null TGF-β3 mutation were transplanted into a wildtype host (Nguyen and Pollard, 2000).

TGF-β1 in the Bovine Mammary Gland

The role of TGF-β1 has not been studied as extensively in cattle as in mice. However, several studies have demonstrated the effects of TGF-β1 in the bovine mammary gland. Immunofluorescent staining of bovine mammary epithelial cells treated in culture with 2 ng TGF-β1/ml showed significant evidence of apoptosis and autophagy markers relative to untreated controls (Gajewska et al., 2005).

The effects of TGF-β on mammary tissue were demonstrated in prepubertal heifers using in vivo implants. TGF-β1 altered mammary stromal composition by increasing fibronectin protein expression. In addition, TGF-β1 increased stromal cell proliferation yet had no effect on epithelial cell proliferation or apoptosis (Musters et al., 2004).

The presence of TGF-β1 and its receptors in the mammary gland is dependent upon stage of lactation or development. TGF-β1 receptor binding is highest in early stages of mammary development, and higher in lactating pregnant cows than non-lactating pregnant cows (Plaut & Maple, 1995). TGF-β1 mRNA expression in the bovine mammary gland was greatest during involution and induced mammogenesis of heifers and lowest during lactogenesis and galactopoiesis (Plath et al., 1997). Using in situ hybridization Maier et al. (1991) found that TGF-β1 mRNA was highest in the intralobular stroma surrounding the epithelium, and in the epithelial cells. Based on these studies, TGF-β1 is likely an important mediator of intralobular stromal and epithelial

remodeling that occurs during involution and mammogenesis in dry dairy cows. A proposed model of the effects of TGF-β1 on mammary remodeling is presented below in Figure 1.1. We hypothesize that remodeling events during involution and early mammogenesis are characterized by increased TGF-β1, TGF-βR2, proliferation of fibroblasts, differentiation of myofibroblasts, and increased ECM protein synthesis. We also expect that exogenous TGF-β1 would increase these remodeling events, particularly during involution and mammogenesis.

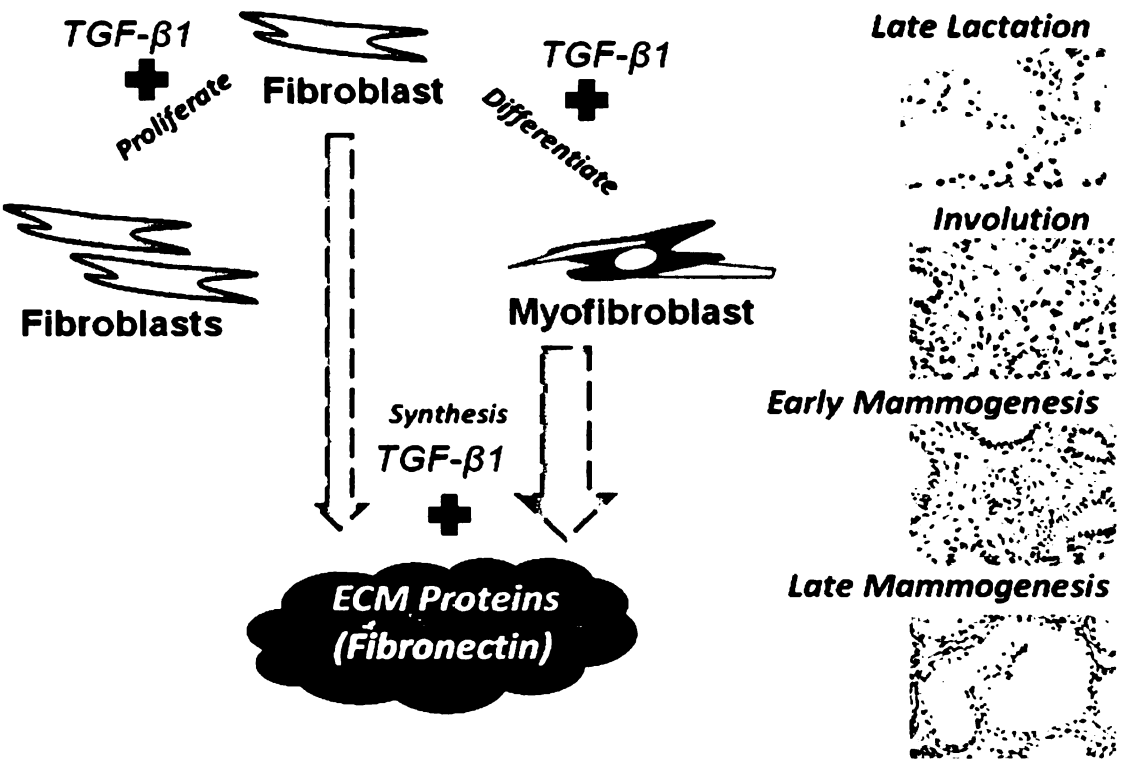

Figure 1.1. Proposed model of mammary remodeling during the dry period.
We expect that transforming growth factor-β1 will increase the proliferation and differentiation of fibroblasts and myofibroblasts, and the synthesis of stromal proteins and proteinases during involution and mammogenesis in the dry period of dairy cows.

CHAPTER 2

CHARACTERIZATION OF MAMMARY STROMAL REMODELING DURING THE DRY PERIOD

ABSTRACT

The bovine mammary gland undergoes extensive remodeling during involution and mammogenesis between lactations. Changes in mammary epithelium were well characterized during the dry period, but few studies in dairy cows have characterized changes in stromal tissue. We hypothesized that involution and early mammogenesis in the dry period are characterized by a transient increase in stromal fibronectin, myofibroblasts, stromelysin, TGF-β1, and TGF-βR2. Our objective was to determine if changes occur in the stromal area, number of myofibroblasts, and the expression of fibronectin, MMP-3, TGF-β1, and TGF-βR2 in the stroma during involution and mammogenesis. Tissue was biopsied from nine Holstein cows at: late lactation (LL), 1 week after dry-off (1 WD), 3 weeks before expected calving date (3 BEC), and 1 week before expected calving date (1 BEC). The number of activated fibroblasts was greater at 1 WD than 1 BEC (2,718±270 to 1,800±270 cells/mm^2, P=0.02). The percentage of intralobular stromal area was greater (P<0.05) at 1 WD (32%) and 3 BEC (37%) than 1 BEC (25%). TGF-β1 decreased 15% from LL to the dry period (P=0.02). The percentage of myofibroblasts, and the percentage of stromal area expressing the proteins fibronectin, MMP-3, or TGF-βR2 expression was not different among biopsies. These results support the hypothesis that stromal expression of TGF-β1 and fibroblast proliferation is important for remodeling during the dry period.

INTRODUCTION

A dry period of 45 to 60 days between lactations is generally recommended to prevent milk production losses in a subsequent lactation (Coppock et al., 1974; Klein and Woodward, 1943; Swanson, 1965). Reducing the length of the dry period would improve efficiency of dairy production be reducing the number of unproductive days in the lifetime of a cow. The current dry period length recommendations are largely based on optimizing milk production, and are determined using records from subsequent lactations rather than an understanding of the biological requirements in the mammary gland. Strategies to reduce the length of the dry period and improve efficiency should come from a basic understanding of changes in the mammary gland.

The typical dry period in a dairy cow is characterized by two main phases: active involution after cessation of milk removal, followed by a period of redevelopment prior to the next lactation (Hurley, 1989). During involution, changes occur in the stromal and parenchymal compartments. The stroma is the connective tissue that provides structural support to the parenchyma, and includes fibroblasts, adipocytes, and endothelial cells, while the parenchymal tissue contains epithelial cells, which line the lumen of alveoli and connect to the basement membrane. The stromal area increases and parenchymal area decreases during involution after milk removal ceases in dairy cows (Capuco et al., 1997; Sordillo and Nickerson, 1988). There is also an increase in the rate of epithelial cell apoptosis from 2.4% in late lactation to 4.8% after dry-off (Wilde et al., 1997).

Milk production is likely a function of the number and secretory activity of epithelial cells in ruminants (Knight and Peaker, 1984); thus many investigators have

focused on the role of the epithelial cells in the mammary gland during the dry period. , Other cells types and tissues however, also may play a critical role during remodeling of the mammary gland and should not be overlooked.

The parenchyma is closely linked to the stroma through the extracellular matrix (ECM) which surrounds the basement membrane. There is extensive evidence to support a role for the stroma in regulating and coordinating changes in the mammary parenchyma of mice (Joseph et al., 1999; Silberstein et al., 1992). An understanding of changes in the stromal tissue surrounding the epithelium during the dry period of dairy cows may provide insight into new strategies to manage or shorten the dry period without compromising milk production.

The composition of the stroma changes during involution, and may be influenced by the proteinases that degrade proteins in the ECM. The mRNA expression of proteinases and their inhibitors, including matrix-metalloproteases MMP-2 and MMP-14, were greater in involuting bovine mammary tissue during involution after cessation of milk removal (Rabot et al., 2007). MMP-3, a proteinase produced by stromal fibroblasts, degrades ECM components including collagen and fibronectin. In mice, gene expression of MMP-3 was greater in mammary tissue during involution and gestation during lactation or in virgin mice (Sorrell et al., 2005). MMP-3 was implicated in alveolar apoptosis by degrading the basement membrane supporting the alveoli (Sympson et al., 1994). MMP-3 was also shown to mediate mammary remodeling and ductal morphogenesis, likely by degrading the intralobular stroma proteins to allow elongation of the ducts (Simian et al., 2001). Whereas the effects of MMP-3 were well characterized

in mice, the presence and activity of MMP-3 has not been studied in the dry period of dairy cattle.

TGF-β1 is a cytokine that mediates stromal cell proliferation and epithelial cell apoptosis. In dairy heifers, mammary implants containing TGF-β1 up-regulated stromal fibronectin expression and increased stromal cell proliferation from 1.8% to 3.5% (Musters et al., 2004). In concert with ED-A fibronectin, an isoform of the stromal protein fibronectin, TGF-β1 induced differentiation of fibroblasts to myofibroblasts (Serini et al., 1998). Myofibroblasts are activated fibroblasts that express the contractile protein, smooth muscle α-actin (SMA). Myofibroblasts were also characterized by enhanced endoplasmic reticuli and an increased capacity to synthesize ECM proteins and proteinases relative to undifferentiated fibroblasts (Greiling, 1997; Phan, 2008). Thus, TGF-β1 may increase stromal protein synthesis by increasing the number of myofibroblasts.

TGF-β1 inhibits MMP-3 gene expression in murine fibroblasts, and thus the inhibitory role of TGF-β1 on mammary ductal growth may be related to an inhibition of MMP-3 (Kerr et al., 1990). The TGF-β type II receptor (TGF-βR2) is the predominant receptor for TGF-β1. Mice with a deletion of the gene encoding TGF-βR2 had disproportionate lobular-alveolar growth and epithelial apoptosis, demonstrating the importance of TGF-β1 for regulation of mammary ductal remodeling (Forrester et al., 2005). Using immunohistochemistry, TGF-βR2 was localized along the ductal and alveolar epithelium in heifers (Plaut et al., 2003). Little is known about the function or presence of MMP-3 in the bovine mammary gland during the dry period, or how TGF-β1

may be involved during the involution and remodeling of the intralobular stroma during the dry period.

The mRNA expression of transforming growth factor-β1 (TGF-β1) was greater during involution (3-4 weeks after cessation of milk removal) than during an induced lactation of non-pregnant cows (Plath et al., 1997). It is likely that TGF-β1 mediates epithelial cell apoptosis during mammary involution; however, temporal changes in the protein expression and localization of TGF-β1 have not been measured during the dry period of pregnant dairy cows.

Much of the current research pertaining to the dry period of dairy cattle has focused on epithelial cells or gene expression of combined stromal and parenchymal tissue, and consequently does not account for changes in the stroma. For this study, changes in activation of fibroblasts to myofibroblasts, and stromal composition were characterized from late lactation, through the dry period.

The objective of this study was to provide a characterization of mammary stromal changes during the dry period in the dairy cow. We expect that TGF-β1 and its receptor, TGF-βR2, are expressed in the bovine mammary gland during involution and mammogenesis of the dry period, as these are times of active remodeling. Further, we expect MMP-3 plays an active role in remodeling the stroma as alveolar structures change in size and shape during involution and mammogenesis. We also expect that fibroblasts and active myofibroblasts will increase with a concomitant increase in the stromal protein, fibronectin during dry period remodeling.

MATERIALS AND METHODS

Animals

All animal handling and surgical procedures were performed humanely under the approval of the MSU All University Committee on Animal Use and Care. Nine multigravid Holstein cows (five primiparous, four multiparous) impregnated between 90 and 105 DIM from the Michigan State University Dairy Cattle Teaching and Research Center were used to obtain mammary biopsies. Cows were dried off at 310 ± 12 DIM and had an average dry period length of 55± 5 days. A summary of lactation, gestation, and dry period lengths is presented in Table 2.1.

Experimental Design

Cows were biopsied from separate quarters of the mammary gland at four stages during late lactation and the dry period. Each quarter was only sampled once so that a previous biopsy would not influence subsequent biopsies. The quarters were randomly assigned to be biopsied at different stages starting in different seasons to average the potential seasonal variation that may have influenced mammary remodeling in the dry period. The first biopsy was obtained during late lactation (LL). The second biopsy was obtained one week after dry-off (1 WD) to correspond with a time of expected active involution. The third biopsy was obtained three weeks before expected calving date (3 BEC), during expected mammogenesis, and the final biopsy was obtained one week before expected calving date (1 BEC), during late mammogenesis or early lactogenesis.

Table 2.1. Summary of cow information		Mean	SD	Min.	Max.
Milk Production at Dry-Off (kg/d)		19.4	7.7	8.3	36.9
Total DIM at Dry-Off		310	12	282	324
Total Dry Period Length		55	5	49	66
Late Lactation	Milk Production (kd/d)	26.6	11.0	11.9	42.7
	Days in Milk	277	10	255	289
	Days in Gestation	190	3	182	193
1 Week Dry	Days Dry	7	0	7	7
	Days in Gestation	230	6	216	235
3 Before Expected Calving	Days Prepartum	19	2	15	22
	Days Dry	36	6	30	49
	Days in Gestation	259	1	258	260
1 Before EC	Days Prepartum	5	2	3	8
	Days Dry	50	7	46	63
	Days in Gestation	272	2	269	276
(n=9 cows)					

Biopsy Procedure

Biopsies were obtained using the method described by Farr et al (1996) with several modifications. In brief, cows were restrained and sedated with an intramuscular injection of xylazine hydrochloride (35-45 μg/kg of body weight, Rompun; Bayer Animal Health, Shawnee, KS). The biopsy site was shaved, washed with iodine, surgical iodine scrub, and 70% ethanol. Topical lidocaine gel was applied to the biopsy site before 3 ml s.c. lidocaine injection was administered to improve cow comfort. Approximately 1g of tissue was collected using a stainless steel, retractable biopsy tool (AgResearch, NZ). Sterile absorbable gelatin sponge (Gelfoam; Pfizer, NY, NY) was placed in the biopsy site when excessive bleeding occurred. The incision area was closed

with surgical staples and Autoclips (Becton, Dickinson and Company, NJ). A teat cannula was inserted after the first biopsy (LL) and the fourth biopsy (1 BEC) to minimize the formation of intramammary clots and improve cow comfort. Biopsies from lactating quarters were hand milked twice daily until the milk was free of visible blood to prevent blood clots. Lactating cows were sent to the milking herd to be milked twice daily beginning on the day of the biopsy procedure. Overall milk production, presented in Appendix 1, was not impacted by the biopsy procedure (Dover et al., 2007).

Tissue Processing

Biopsied tissue was immediately placed in cold media (Waymouth's Media 752/1 (Sigma, St Louis, MO), sterilized using a 0.1μm filter. The media was supplemented with 50 μg gentamicin (Invitrogen; Carlsbad, CA) and 100U penicillin – 100 μg streptomycin (Sigma, St Louis, MO) per ml. Biopsied tissue was rinsed in phosphate-buffered-saline (PBS) containing 100U penicillin – 100 μg streptomycin (Sigma, St Louis, MO), and 1.5 μg amphotericin B (Invitrogen; Carlsbad, CA) per ml, cut into 0.5 cm^2 sections and fixed overnight in buffered formalin. After 24 hours of formalin fixation, tissue was transferred to 60% ethanol, embedded in paraffin, cut into 5-μm tissue sections, and placed on silanized slides. One hematoxylin-and-eosin stained slide for each paraffin block was obtained for a general histological examination.

Immunohistochemistry (IHC)

Biopsied tissue sections were stained in set consisting of all biopsies of a cow, a positive control slide and a negative control slide where the primary antibody was

substituted with PBS. A broad-spectrum streptavidin-peroxidase Histostain-kit (Zymed Laboratories Inc., San Francisco, CA) was used according to the manufacturer's directions. Prior to immunohistochemistry, slides were incubated overnight at 60° C, deparaffinized in xylene, rehydrated through a graded series of ethanol washes (100%, 95%, 70%, and 60%) and rinsed in PBS. Endogenous peroxidases were blocked for 15 minutes in 3% hydrogen peroxide in methanol. With the exception of anti-MMP-3, sodium citrate antigen-retrieval was performed in a 100°C water bath for all antibodies (Jiao et al., 1999). Following antigen retrieval, sections were incubated for 10 min at RT in non-immune blocking serum block (Histostain kit; Zymed Laboratories Inc., San Francisco, CA). All sections were incubated with the primary antibody at room temperature.

Primary antibodies were selected for use based on how well they matched one or more of the following criteria: proven reactivity with bovine tissue, epitope sequence homology to bovine (determined with the Basic Local Alignment Search Tool 2.0, NCBI), previous use in mammary tissue, and/or proven use in IHC of paraffin-embedded tissue sections. The primary antibodies used were mouse monoclonal anti-smooth muscle alpha-actin (1:10,000; 45 min; A2547; Sigma, St Louis, MO), mouse monoclonal anti-fibronectin (50 μg/ml; 1 hr; MS-1351-R7, Lab Vision Products, Fremont, CA), rabbit polyclonal anti-TGF-β1(V) (1:100; 1 hr; SC-146, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-TGF-βR2 C-16 (1:100; 1 hr; SC-220, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and mouse polyclonal anti-MMP-3 (1:200; 45 min; 3523R-100; Biovision Inc., Mountainview, CA). Sections were washed in PBS and incubated with a broad-spectrum secondary antibody conjugated with streptavidin-

peroxidase (Histostain kit; Zymed Laboratories Inc., San Francisco, CA). For visualization of antibody binding site, slides were incubated for 3 minutes with 3', 3'-diaminobenzidine chromogen. All slides were counterstained with hematoxylin, dehydrated in a graded series of alcohols, cleared in xylene, and mounted with Histomount (Zymed Laboratories, San Fransisco, CA).

Photomicrograph Analysis

Stained slides were examined with a Nikon Eclipse 50i light microscope (Nikon Instruments Inc., Melville, NY). Five photomicrographs were taken per slide at 200X magnification, avoiding areas high in interlobular stroma or areas close to tissue section edges. Images were captured at a size of 2560 by 960 pixels with 32-bit per pixel depth and saved in tagged image file format (TIFF) in Image Pro Plus 5.1 (Media Cybernetics, Bethesda, MD). Each image represented a tissue area of 0.29 mm^2. Histological measurements were obtained using the free-hand area-of-interest tool to measure area or the manual tag tool for counting cells in Image Pro Plus. An example of the intralobular stromal area measurement is presented in Figure 2.1. Protein expression for TGF-β1, TGF-βR2, fibronectin and MMP-3 was determined by measuring the percent of intralobular stroma area stained by immunohistochemistry. Stromal cells with phenotypic characteristics of fibroblasts (paler nuclei, and not associated with blood vessels or other structures) were considered fibroblasts, although no specific antibody was used to distinguish them from other cell types. Fibroblasts expressing smooth muscle α-actin were considered activated fibroblasts, or myofibroblasts. These results are presented as a percentage of all fibroblast cells (fibroblasts and myofibroblasts) in the

intralobular stroma, and as the number of cells per area of intralobular stroma (cells/mm^2).

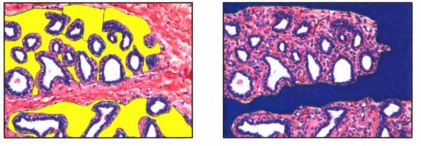

Figure 2.1. Example of intralobular stromal area measurement. Sample of intralobular stromal area measurement from hematoxylin and eosin stained images. Left: Example of intralobular stromal area highlighted yellow with the area-of-interest tool. Right: Interlobular stromal, highlighted blue in the same tissue section was not included in stromal area measurements.

Fibronectin, TGF-β1, TGF-βR2, and MMP-3 Expression

To determine the stromal protein expression of fibronectin, TGF-β1, TGF-βR2, and MMP-3, positive (brown) staining was selected in the intralobular stromal area using the eyedropper tool in the Count/Size dialog of Image Pro Plus. Using the eyedropper tool, settings for positive DAB staining were saved and applied to all biopsy stages within a cow, including the positive and negative control slides run within each set. The stained area was expressed as a percentage of the total intralobular stromal area measured in each image. Samples of staining are presented in Figure 2.2.

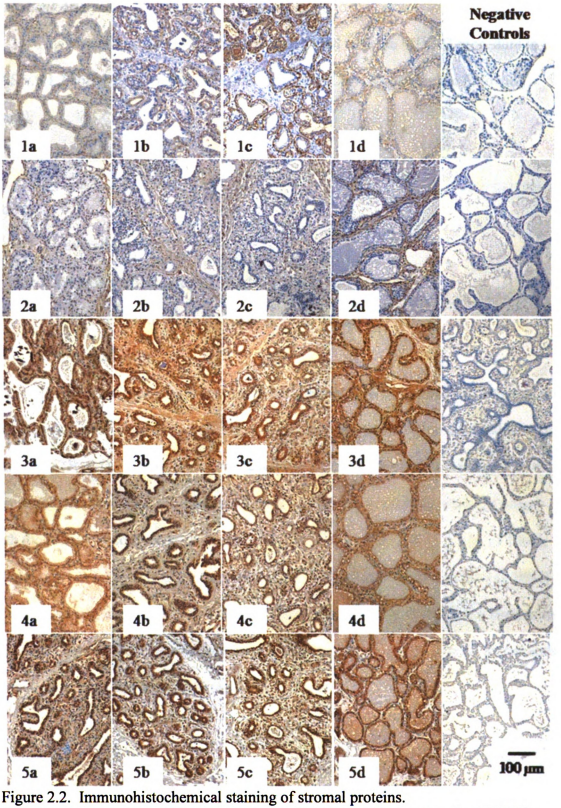

Figure 2.2. Immunohistochemical staining of stromal proteins.
Each panel represents a different protein (1= SMA; 2 = Fibronectin; 3 = MMP-3; 4 = TGF-β1; 5 = TGF-βR2) and stage (a = late lactation ; b = 1 week dry; c= 3 weeks before calving; d = 1 week before calving). Far right panels are negative controls.

Statistical Analysis

Analysis of variance of the data was conducted with the MIXED procedure in SAS v.9.1.3 software (Statistical Analysis Software, Cary, NC) using the following model:

$$Y_{ij} = \mu + C_i + B_j + e_{ij}$$

where

μ = overall mean,

C = random effect of cow (i = 1to 9 cows),

B = fixed effect of stage at biopsy (j = 1 to 4 stages),

e = residual error.

The effect of parity (primiparous or multiparous) and the interaction between cow and stage at biopsy were evaluated, but removed from the model when not declared significant ($P>0.1$). Results were considered statistically significant at a probability of α less than 0.05. Statistical differences were based on the least squared means of biopsy with the following comparisons: lactation (LL) vs. dry period (1 WD, 3 BEC, 1 BEC), involution (1 WD) vs. late mammogenesis (1 BEC), and early mammogenesis (3 BEC) vs. late mammogenesis (1 BEC). Although comparisons were pre-planned, a Bonferroni-adjustment was applied because comparisons were non-orthogonal. Results are presented as least squared means ± standard error of the mean.

RESULTS

Intralobular Stromal Area & Tissue Characteristics

There was a significant effect of biopsy on the percent of stroma in the lobular area, or intralobular stroma (P=0.02). Intralobular stromal area was greater at 3 BEC (37% ± 4.7, P=0.005) and tended to be greater at 1 WD (32% ± 4.7, *P=0.08*) compared to 1 BEC (25% ± 4.7) (also presented in Figure 2.3). Tissue from LL biopsies was variable in structure and integrity. Areas of this tissue showed evidence of both intact and collapsing alveoli, including alveoli with incomplete basement membranes. The tissue obtained at other biopsies had more consistency and organization than the LL

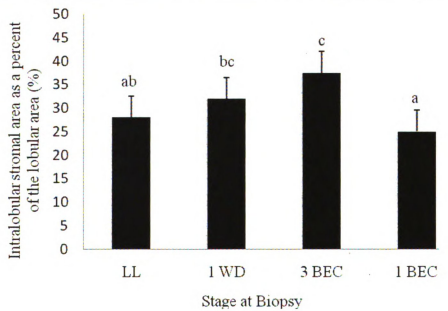

Figure 2.3. Intralobular stromal area during late lactation and the dry period. Stages at biopsy are late lactation (LL), 1 week after dry-off (1WD), 3 weeks before expected calving date (3 BEC), and 1 week before expected calving date (1 BEC). Stromal area in the lobule was greatest at 3 BEC, and significantly decreased at 1 BEC (P=0.02)

samples.

Fibronectin Expression

Fibronectin staining was present in all tissue sections, at all biopsy times. The interlobular stroma had more fibrous sheets with intense staining of fibronectin than the intralobular stroma. The lumen and epithelium did not stain positively for fibronectin however, a distinct band of fibronectin staining was observed along the basement membrane. The percent intralobular stromal area expressing fibronectin was not different among biopsies (Figure 2.4).

MMP-3 Expression

MMP-3 was also present in all tissue sections at all biopsy times. We observed more intense, darker staining for MMP-3 on the apical side of alveolar epithelial cells and in the interlobular stroma. Although intensity was not quantified in this study, we observed that the intralobular stroma stained diffusely with less intensity for MMP-3 than the interlobular stroma and epithelium. The percent intralobular stromal area expressing MMP-3 was not different among biopsies (Figure 2.4).

TGF-β1and TGF-βR2 Expression

TGF-β1 and TGF-βR2 were expressed in stromal and epithelial cells in tissue sections from all biopsies (Figure 4). We observed diffuse, lighter staining of TGF-β1 and TGF-βR2 in the intralobular stroma. The intralobular stroma expression of TGF-β1 decreased 15% from LL to the dry period (1 WD, 3 BEC, 1 BEC) (P=0.02). The

expression of TGF-β1 in the intralobular stroma was similar among dry period biopsies. The intralobular stromal expression of TGF-βR2 was similar at all biopsies.

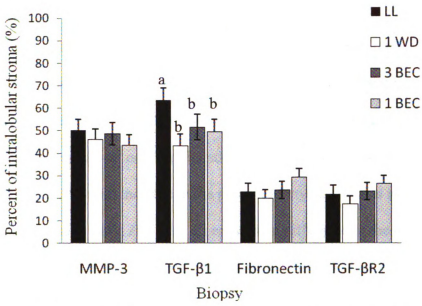

Figure 2.4. Percent of intralobular stromal area expressing proteins. Proteins measured are matrix-metalloproteinase-3 (MMP-3), transforming growth factor-β1 (TGF-β1), fibronectin, and TGF-β receptor2 (TGF-βR2). Percent of TGF-β1 in the stromal area decreased from late lactation to the dry period (P=0.02). There was no effect of biopsy on staining in the intralobular stroma for MMP-3, fibronectin, or TGF-βR2. Differing subscripts are significant at P<0.05

Fibroblasts and Myofibroblasts

Myoepithelial cell expression of smooth muscle α-actin (SMA) appeared as a distinct ring, delineating the basement membrane around the alveoli in tissue sections from all biopsies. SMA was also detected around blood vessels. Fibroblast-like stromal cells that expressed SMA were presumed to be activated myofibroblasts. While the percent of myofibroblasts was not significantly different among biopsies, there may have been a biologically important increase in the percent of myofibroblasts at 1 WD compared to LL (18% to 22%; P=0.11). There was no difference in the number of myofibroblasts per mm^2 of intralobular stromal tissue. Stage of biopsy tended to alter the number of fibroblasts (P=0.10), with a decrease of cells from 1 WD to 1 BEC (2718 ± 270 cells/mm^2, to 1800 ± 270 cells/mm^2, P=0.02). Changes in the number and percent of fibroblast and myofibroblasts are also presented in Table 2.2 and Figure 2.5.

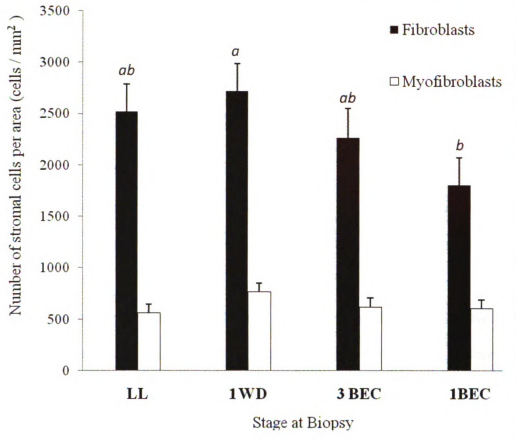

Figure 2.5. Number of fibroblasts and myofibroblasts.
Expressed as cells per area of intralobular stroma (cells/mm^2 of stroma) at stages of late lactation (LL), one week dry (1 WD), three weeks before calving (3BEC), and one week before calving (1 BEC). The number of fibroblasts decreased from 1 WD to 1 BEC (P=0.02). The number of myofibroblasts in the stroma was not significantly different. Overall effect of biopsy (P=0.1).
*Differing subscripts are significant at P<0.05.

DISCUSSION

The inductive nature of the stroma on epithelium was shown both in mammary cell cultures and in vivo in mice (Farrelly et al., 1999; Silberstein et al., 1992; Talhouk et al., 1992). For example, an increase in stromal proteinases relative to their inhibitors resulted in degradation of the basement membrane and loss of alveolar epithelial cell function during mammary involution in mice (Talhouk et al., 1992). This study was the first to characterize temporal changes in the stromal tissue during the dry period of dairy cattle.

As expected, the percent of intralobular stroma declined from 1 WD (involution) and 3 BEC (mammogenesis) to 1 BEC (late mammogenesis) (P=0.02). The decrease in stromal area we observed was consistent with previous studies that found the least proportion of stromal area was greatest one week prior to calving (Capuco et al., 1997; Sordillo and Nickerson, 1988).

Fibroblasts and myofibroblasts are associated with mammary remodeling in other species and therefore we expected to find an increase in stromal fibroblasts and myofibroblasts during involution and mammogenesis in dairy cows. The number of myofibroblasts did not change during the dry period (cells/mm^2 of intralobular stroma). Although there were no statistical difference detected, there may be a biological tendency for the percent of myofibroblasts to increase during the dry period relative to late lactation (P=0.11). The effect of stage at biopsy tended to alter the number of fibroblasts. There was a significant decrease in the number of fibroblasts from 1 WD to 1 BEC (2,718±270 vs. 1,800±270 cells/mm^2; P=0.02)

As parturition approaches, there is a decrease in both the number of fibroblasts and in intralobular stromal area. The decline in stromal area may relate to the decrease fibroblasts, which function to produce stromal proteins. While we expected differentiated myofibroblasts would contribute more to the synthesis of stromal proteins, it is possible that fibroblasts are contributing a greater share of protein abundance in the stroma, and consequently stromal area may depend on the number of fibroblasts. This question would require further testing of the capacity of bovine mammary fibroblasts and myofibroblasts to synthesis proteins at different stages in the dry period.

TGF-β1 mRNA expression was greater during periods of involution and mammogenesis in dairy heifers (Plath et al., 1997). Therefore we expected an increase in the abundance of TGF-β1 at 1 WD and 3 BEC relative to LL and 1 BEC. However, our results indicate that TGF-β1 was greater in LL than during the dry period, and there were no differences in the abundance of TGF-β1 among 1 WD, 3 BEC, and 1 BEC tissue samples. The greater number of fibroblasts observed at 1 WD may be due to increased abundance of TGF-β1 in the intralobular stroma at LL. Musters et al. (2004) demonstrated that exogenous TGF-β1 stimulated stromal cell proliferation in prepubertal heifers. Therefore, it is likely that endogenous TGF-β1 expressed during LL would contribute to the proliferation of fibroblasts during involution in the dry period of dairy cattle.

Our results of TGF-β1 expression may seem inconsistent with those of others. For example, Plath et al. (1997) found the abundance of mammary TGF-β1 mRNA to be greatest during involution and mammogenesis, while Norgaard et al. (2008) did not find any differences in abundance of TGF-β1 mRNA in lactation or the dry period of dairy

cows. A key difference between these studies may be that Plath et al. (1997) examined involution after induced lactation in virgin heifers, while Norgaard et al. (2008) obtained biopsies during lactation and the dry period of multigravid cows (Norgaard et al., 2008; Plath et al., 1997). However, neither of these studies examined changes in the abundance of TGF-β1 protein in the tissue, nor did their examinations provide a distinction between changes in the stromal tissue and parenchymal tissue. Our results provide new insight into changes in stromal expression of TGF-β1. An increase of TGF-β1 in the stroma during late lactation may contribute to the decline in milk production in late lactation, as TGF-β1 is known to slow epithelial proliferation (Gajewska et al., 2005). Further studies are necessary to test this hypothesis.

The ligand binding receptor in the TGF-β1 signal pathway is TGF-βR2. Expression of TGF-βR2 is necessary to maintain organization of ductal branching during mammary remodeling and development in mice (Joseph et al., 1999). Therefore, we expected an increase in TGF-βR2 during the dry period of dairy cows. As with TGF-β1, we did not observe the expected increase in abundance of TGF-βR2 during the dry period. However, our results for TGF-βR2 protein abundance were consistent with Norgaard et al (2008), who observed no differences in the mRNA expression of the receptor during late lactation and the dry period. While it was not directly quantified in this study, we observed stronger staining intensity for TGF-β1 and TGF-βR2 in the epithelial compartment than the stromal compartment. TGF-βR2 localization was consistent with previous literature that reported TGF-βR2 staining along the alveolar and ductal epithelium of heifers (Plaut et al., 2003).

Interestingly, the structural integrity of alveoli and their surrounding basement membrane in the LL tissue samples of this study were most heterogeneous of all biopsy time points. In mice, TGF-β promoter activity is increased in the absence of a basement membrane and repressed when a functional basement membrane is present (Streuli, 1991) It is possible that the incomplete basement membranes we observed in our samples increased the levels of TGF-β-promoter. Thus, the reduced integrity of the basement membrane we observed may explain, in part, the greater abundance TGF-β1 protein expression in late lactation. This elevated TGF-β1 in the intralobular stroma may, in time, contribute to reduced alveolar function and declining milk production at the end of lactation through.

Using immunofluorescence, Dickson and Warburton (1992) detected increased protein expression of MMP-3 by myoepithelial cells, in association with degradation of the basement membrane during murine mammary involution compared to pregnancy and lactation. The fact that we did not observe an increase in MMP-3 during involution and mammogenesis of the dry period may be consistent with the observation that unlike other species, there is no degradation or epithelial cell detachment from the basement membrane in the bovine mammary gland during the dry period (Capuco and Akers, 1999). However, based on our histological observations at LL, it appears that degradation of the basement membrane may be occurring in some regions of the mammary gland. While we did not quantify the abundance of MMP-3 in the epithelium, histological observations showed more intense staining in the epithelium than in stroma at all biopsy stages. Therefore it is possible that MMP-3 produced by the epithelium may contribute to degradation of the basement membrane, and thus increase TGF-β1 synthesis

in late lactation. In addition, the enzymatic activity of MMP-3in the stroma and epithelium was not measured in this study. Immunohistochemistry would not be sufficient to detect if remodeling during the dry period was mediated by enhanced proteinases activity, rather than an increase in proteinase abundance. This warrants further studies examining MMP-3abundance in the epithelium and activity in the stroma, particularly during late lactation and involution.

TGF-β1 induces the differentiation of fibroblasts into myofibroblasts, which have an increased production of the ECM proteins (Petrov et al., 2002). The expression of ED-A fibronectin preceded the expression of SMA by human dermal fibroblasts induced to differentiation by TGF-β1, indicating that stromal composition mediates this process (Serini et al., 1998b). In this study, we expected an increase in intralobular stromal fibronectin concomitant with increases in myofibroblasts during involution and mammogenesis in the dry period. In contrast, the percent of fibronectin protein expressed in the intralobular stroma was not different among biopsies.

While the percent of fibronectin did not change, the fact that it remained constant as the stromal area changed among biopsies may be relevant. If the change in stromal area were extrapolated to the entire gland, it would follow that although no differences were detected in the percent area, if the total stromal area was increased, total fibronectin would increase as well. However, this extrapolation would require verifying that the increase in stromal area observed was not merely an effect of dilution due to increased lumenal area, or an effect of distending ducts compressing the stromal tissue during the onset of lactogenesis.

Capuco et al (1997) found that hydroxyproline, a marker of collagen synthesis, was greatest during mammogenesis in the dry period of dairy cows, paralleling the time of greatest stromal area. These investigators suggested this is evidence that dry period stromal area changes are a direct result of an increase in ECM synthesis rather than a change in stromal area by compression or changes in elasticity (Capuco et al., 1997). The fibronectin changes we observed would also support the hypothesis. The increase in intralobular stromal area in the dry period is likely a result of the increased production of stromal proteins, since the percent of fibronectin in the stroma remained constant among biopsies, in spite of changes in intralobular stromal area.

Limitations

Immunohistochemistry staining was correlated highly with other methods to detect protein expression including fluorescent in situ hybridization (Couturier et al.). Despite this evidence, the methods for quantification of immunohistochemistry are still widely debated, and the interpretations of staining intensity and patterns as a measure of protein expression have not been fully agreed upon (Walker, 2006).

In this study, we used immunohistochemistry to quantify temporal changes in the number of cells and in protein expression of the bovine mammary gland. Takenoue et al. (2000) examined the accuracy of immunohistochemistry quantification by multiplying intensity scores by the number of cells positively expressing a protein of interest, and comparing these results to protein levels determined by Western blot and mRNA expression. They found that immunohistochemical analysis predicted the protein levels as measured by Western blot with higher accuracy than measurements of mRNA (Takenoue et al., 2000).

Abundance of mRNA is not always directly related to protein concentrations in the tissue because transcripts may not always be translated at equal rates and proteins may be modified after translation. Furthermore, proteins, unlike mRNA, are not always produced in the tissue where they may be localized. There also may be a dilution effect when mRNA is extracted from whole tissue samples, rather than isolated from specific tissue compartments. For example, we observed intense MMP-3 staining in the epithelial compartment, but weak staining in the stroma thus differences in stromal expression would likely have been masked by expression of the epithelial cells if the whole tissue samples were analyzed. Isolation of the intralobular stromal compartment in the immunohistochemical analysis used in this study allowed a more specific analysis of the localization and distribution of changes during the dry period.

In this project, we quantified the percent of intralobular stromal area stained, rather than the staining intensity in the tissue. We found high variability between stromal intensity measurements within similar tissue samples from the same cow. Therefore, we did not quantify or present differences in staining intensity. Even without this measurement, the percent area stained and localization of proteins still provides a valuable analysis of mammary changes during the dry period.

While repeated biopsies on a single cow over time should reduce animal variability, the use of a single biopsy at each time-point may have disadvantages. Akers et al (1990) reported large variation in the composition of involuting bovine mammary glands. We observed some variation in the LL tissue samples, thus it is possible that the tissue obtained may not be representative of the whole gland at this time-point. However, with the exception of the late lactation sample, the overall histology among cows in this

study at a similar time point was relatively homogenous. Slaughtering cows for a whole gland analysis may provide a fuller picture of the gland, but it would likely require more animals as it would not allow a comparison within an animal over time, and therefore it would also not be as cost-efficient as serial biopsies.

CONCLUSIONS

The results of this study demonstrate that the stromal tissue is dynamic, undergoing changes in composition as it remodels during the dry period. We have shown that intralobular stromal expression of TGF-β1 was greater during late lactation than during the dry period. We speculate that TGF-β1 may be responsible in initiating remodeling as milk production declines towards the end of lactation. In vivo and in vitro studies are needed to test this hypothesis. TGF-β1 may also be responsible for increasing stromal cell proliferation early in the dry period, as we have shown that the number of fibroblasts in the intralobular stroma is greater at 1 WD than at 1 BEC.

While we did not detect changes in the percent of TGF-βR2, MMP-3, or fibronectin in the intralobular stroma, the stromal area was greater at 1 WD than at 1 BEC. All of these proteins would have had to increase concomitantly with the stromal area to maintain a constant percent of the stromal composition, and thus there may have been an overall increase in these proteins and proteinases in the gland. Whether it is the total abundance of proteins in the stroma or the percent stromal composition that is important to remodeling and development remains unanswered. The activity of proteins expressed in the dry period should also be examined to understand mammary remodeling during the dry period. These questions require further investigation in order understand bovine-specific mammary changes during the dry period. .

CHAPTER 3

THE EFFECTS OF TGFβ-1 ON MAMMARY STROMAL REMODELING DURING THE DRY PERIOD

ABSRACT

Mammary remodeling during the dry period of dairy cows involves the coordination of changes in the stromal tissue and epithelium. During tissue remodeling, the presence of transforming growth factor beta-1 (TGF-β1) increases the differentiation of fibroblasts to myofibroblasts, which are characterized by the expression of the contractile protein smooth muscle α-actin (SMA). The objective of this study was to determine if TGF-β1 increases stromal remodeling in the mammary gland during the dry period by increasing the expression of the protease MMP-3, stromal cell production of fibronectin, and the differentiation of fibroblasts. Tissue was biopsied from 7 Holstein cows at 4 time points: late lactation (LL), one week after dry-off (1 WD), 3 weeks before expected calving (3 BEC), and one week before expected calving (1BEC). Explants of biopsied tissue were incubated for 2 h in Waymouth's media containing insulin, hydrocortisone, and 0 or 5 ng TGF-β1/ ml. Formalin fixed, paraffin embedded slides of tissue explants were stained by immunohistochemistry and analyzed with Image Pro Plus software. Incubation with TGF-β1 had no effect on the percent intralobular stroma area expression of fibronectin or MMP-3. The number of fibroblasts expressing SMA increased by 19% in the intralobular stroma ($P=0.03$) and the percent activated tended to increase by 4% ($P=0.06$) in tissue incubated with TGF-β1. Based on these findings, TGF-β1 may be important in the activation of stromal cells during involution and mammogenesis of the bovine mammary gland.

INTRODUCTION

Traditionally, the greatest milk yield is obtained from dairy cows when there is a 60 d dry period between successive lactations. A reduced dry period length comes at the cost of milk production in the subsequent lactation, and this compromise in milk production generally outweighs the benefit of continuously milking cows during the 60 d prepartum period. There continues to be interest in developing methods to shorten the dry period to reduce the environmental impact per unit of milk produced and increase the profitability of dairy production (Rastani et al., 2005). In order to develop strategies to reduce the length of the dry period, the biological mechanisms underlying the reduction in milk production need to be fully understood.

During the dry period, the mammary gland actively remodels in two main phases: involution after cessation of milk removal, followed by a phase of redevelopment, or mammogenesis prior to parturition (Hurley, 1989). The dry period is important for renewal and replacement of senescent epithelial cells, particularly during mammogenesis prior to parturition (Annen et al., 2007; Capuco et al., 1997; Sorensen et al., 2006). Most investigators who study the dry period focus on changes in the epithelium and do not examine remodeling in the surrounding intralobular stromal tissue, despite evidence that composition of the stroma is important to epithelial cell fate (Farrelly et al., 1999; Silberstein et al., 1992).

Fibronectin fragments were increased in the intralobular stroma during post-lactational involution in mice. In culture, these fibronectin fragments were able to induce protease activity and inhibit epithelial cell growth (Schedin et al., 2000). In addition, Holland et al (2007) demonstrated that the composition of the extracellular matrix (ECM)

could promote differentiation of bovine mammary progenitor cells into a secretory phenotype. Thus, alterations of the stromal composition may provide a key to reducing dry period length.

The cytokine, transforming growth factor-beta1 (TGF-β1), has been implicated in mammary development, and may mediate stromal changes during the dry period. A dual role of TGF-β1 has been identified in the murine mammary gland; it inhibits epithelial cell proliferation and stimulates synthesis of the ECM surrounding the ducts (Silberstein et al., 1990). Exogenous implants of TGF-β1 increased stromal cell proliferation and increased fibronectin expression in the intralobular stroma of dairy heifers (Musters et al., 2004). Plath et al (1997) found that TGF-β1 mRNA abundance was greater during involution and mammogenesis than lactogenesis or galactopoiesis of dairy heifers. Therefore, it is likely that TGF-β1 would also alter stromal composition and stromal cell populations during the dry period of dairy cows.

TGF-β1 influences normal mammary development, and deletion or mutations of the TGF-β1 gene results in abnormal mammary development and growth. TGF-β1 deficient mice exhibited delayed and impaired post-lactational involution in the mammary gland after weaning (Gorska et al., 2003). Transgenic mice that over-express TGF-β1 exhibit impaired lateral ductal branching during pubertal development and late pregnancy (Jhappan et al., 1993; Pierce et al., 1993). Mice with a mutant form of the main TGF-β1 receptor, TGF-βR2, exhibited an increase in stromal degrading proteinases and disorganized lateral branching of the alveoli (Joseph et al., 1999).

TGF-β1 alters stromal composition by increasing the differentiation of stromal fibroblasts into active myofibroblasts, which in turn produce ECM proteins and

proteinases. The myofibroblast phenotype is associated with the contractile protein, smooth muscle alpha-actin (SMA), and an increased synthesis of ECM proteins relative to fibroblasts (Petrov et al., 2002). SMA expression allows myofibroblasts to alter ECM organization and aids in cell motility (Grinnell, 1994). Incubation of human mammary subcutaneous fibroblasts with 5 ng/ml TGF-β1 for 7 d, increased the percent of myofibroblasts from 7.5% to 45.3% (Desmouliere et al., 1993). The presence of myofibroblasts in stroma can range from 6.1% of stromal cells in normal human mammary tissue to 68% in cultures obtained from mammary carcinoma tissue (Rønnov-Jessen et al., 1992).

Differentiation of myofibroblasts, induced by TGF-β1, may be important to production of ECM proteins in the intralobular stroma during tissue remodeling. Collagen synthesis increased in cultures with TGF-β1, concomitant with an increase in myofibroblasts. The increased rate of collagen synthesis was maintained in cultures even when TGF-β1 was removed from the media (Petrov et al., 2002). Therefore, the influence of TGF-β1 on stromal composition may be caused by an increase in proliferation and differentiation of fibroblasts to myofibroblasts.

In addition to synthesizing ECM proteins, stromal fibroblasts and myofibroblasts also produce proteinases, including matrix-metalloproteases (MMPs), which are involved in mammary remodeling (Dickson and Warburton, 1992; Noel and Foidart, 1998). The balance of MMPs and their inhibitors during involution coordinates the degradation of the basement membrane and ECM, and contributes to alveolar regression during murine mammary involution (Talhouk et al., 1992). Simian et al (2001) demonstrated that MMP-3 is necessary in mammary ductal branching in cultured murine epithelial cells. In

the bovine mammary gland, abundance of MMP-3 mRNA was greatest during pubertal mammogenesis and involution, 28 d after dry-off of non-pregnant heifers. Therefore the expression of MMP-3 is likely important to remodeling the stroma during involution and mammogenesis.

The effects of TGF-β1 on bovine mammary tissue during the dry period have not been examined. Based on the role of TGF-β1 in mammary development and tissue remodeling in mice and heifers, TGF-β1 is likely an important mediator of mammary remodeling in the dry period. We hypothesized that TGF-β would increase proliferation of myofibroblasts and stimulate mammary remodeling through increased production of MMP-3 and fibronectin. We expected that the effect of TGF-β would be greater during involution and mammogenesis.

MATERIALS AND METHODS

Animals

All animal handling and surgical procedures were performed humanely under the approval of the MSU All University Committee on Animal Use and Care. Biopsied mammary tissue was obtained from nine Holstein cows (four multiparous, five primiparous) impregnated between 90 and 105 DIM, from the Michigan State University Dairy Cattle Teaching and Research Center (see Chapter 2). Biopsied tissue from seven cows was used to study the effects of TGF-β1 during the dry period. Cows were dried off at 310 ± 12 DIM; (Mean DIM ± SD) and had an average dry period length of 55 ± 5 days (Mean days dry ± SD).

Experimental Design

Multiple biopsies were obtained from cows in a split-plot design, where the main plot was cow, and the sub-plot was the effect of TGF-β1 on tissue biopsied from separate quarters of the mammary gland at four time-points from late lactation through one week before expected calving date, as described in chapter two. Each quarter was only sampled once so that a previous biopsy would not influence subsequent biopsies. The quarters were assigned randomly and biopsied at: late lactation (LL), one week dry (1 WD), three weeks before expected calving date (3 BEC), and one week before expected calving date (1 BEC). Biopsied tissue sections from each sample were assigned to incubation treatments with or without 5 ng TGF-β1/ml. Two multiparous cows were removed from the study because the histology of the cultured tissue was not of sufficient quality to examine and evaluate changes, so a final total of seven cows were included in the study.

Biopsy Procedure

Biopsies were obtained using the method described by Farr et al (1996) with several modifications, described in chapter two. In brief, cows were restrained and sedated with an intramuscular injection of xylazine hydrochloride (35-45 μg/kg of body weight, Rompun; Bayer Animal Health, Shawnee, KS). The biopsy site was shaved, washed with iodine and with surgical scrub (Betadine Surgical Scrub; PurduePharma, Stamford, CT), and 70% ethanol. Approximately 1g of tissue was collected using a stainless steel, retractable biopsy tool (AgResearch; Hamilton, NZ). The incision area was closed with surgical staples and Michel clips. A teat cannula was inserted into lactating quarters immediately after biopsy to drain accumulated blood. The cannula was removed after approximately 5 minutes. Quarters were hand-milked twice daily after biopsy to remove clots until the milk was free of visible blood.

Tissue Processing

Biopsied tissue was immediately placed in cold media, sterilized using a 0.2μm filter (Waymouth's Media 752/1; Sigma, St Louis, MO), with 50 μg gentamicin per ml (Invitrogen; Carlsbad, CA) and 100 U penicillin – 100 μg streptomycin per ml (Sigma, St Louis, MO). Biopsied tissue was washed three times in phosphate-buffered-saline (PBS) containing; 100 U penicillin – 100 μg streptomycin per ml (Sigma, St Louis, MO), and 1.5 μg Amphotericin-B per ml (Invitrogen; Carlsbad, CA) and cut into 0.5 cm^3 sections. Tissue sections were placed on floating siliconized lens paper (Casey et al., 2000). The tissue was incubated for two hours in Waymouth's media with 5-bromo 2-deoxyuridine (BrdU) and either 0 ng TGF-β1/ml or 5 ng TGF-β1/ml (240-B, R&D

Systems, Minneapolis, MN) (Sudlow et al., 1994). After culture, tissue was placed in formalin for 24 hours, transferred to 60% ethanol, embedded in paraffin, cut into 5μm tissue sections, and placed on silanized slides. One hematoxylin-and-eosin stained slide for each paraffin block was obtained for a general histological examination (Figure 3.1).

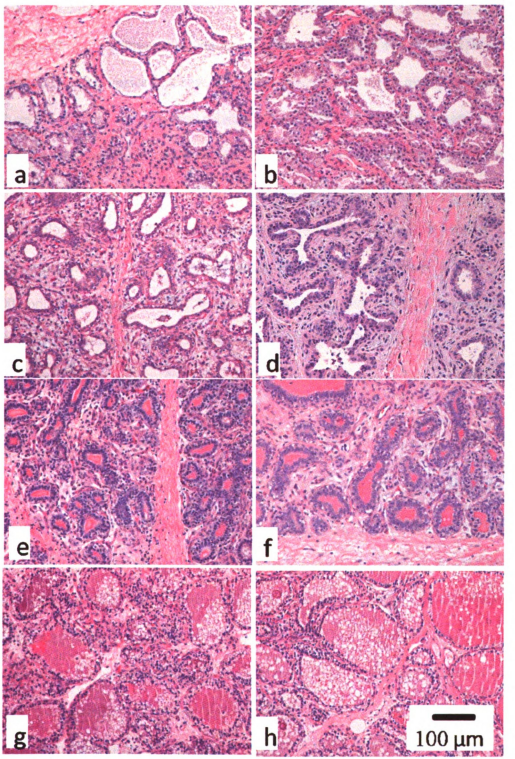

Figure 3.1. Hematoxylin and eosin staining (200X) of mammary tissue incubated with and without transforming growth factor-β1 (TGF- β1). Images are of tissue incubated with 0 ng TGF- β1/ml (a,c,e,g) or 5 ng TGF- β1/ml (b,d,f,h). Tissue was obtained at late lactation (a,b), one week after dry-off (c,d), three weeks before expected calving date (e,f) or one week before expected calving date (g,h)

Immunohistochemistry

Biopsied tissue sections were stained in a set consisting of 0 and 5 ng TGF-β1/ml treated tissues for all biopsies of a cow, a positive control slide and a negative control slide where the primary antibody was substituted with PBS. A broad-spectrum streptavidin-peroxidase Histostain-kit (Zymed Laboratories Inc., San Francisco, CA) was used according to the manufacturer's directions. The primary antibodies used were: mouse monoclonal anti-smooth muscle alpha-actin at 1:10,000 applied for 45 min (A2547; Sigma, St Louis, MO), mouse monoclonal anti-fibronectin clone IST-3 at 1:10,000 applied for 1 hour (F0791, Sigma, St Louis, MO), and rabbit polyclonal anti-MMP-3 at a 1:200 dilution applied for 45 min (3523R-100; Biovision Inc., Mountainview, CA).

Photomicrograph Analysis

Five photomicrograph images were captured with a Nikon Eclipse 50i light microscope (Nikon Instruments Inc., Melville, NY) at 200X, and analyzed using Image Pro Plus 5.1 (Media Cybernetics, Bethesda, MN) analysis software. Protein expression for fibronectin and MMP-3 was determined by measuring the percent of intralobular stroma area stained by immunohistochemistry. Stromal cells with phenotypic characteristics of fibroblasts (pale nuclei, and not associated with blood vessels or other structures) were considered fibroblasts, although no specific antibody was used to distinguish them from other cell types. Fibroblasts expressing SMA were considered to be activated fibroblasts, or myofibroblasts. Myofibroblast differentiation was determined by comparing the number of fibroblast expressing smooth muscle α-actin to the total

number of fibroblasts in the intralobular stroma. These results are presented as a percent of all fibroblasts and as a cell number per area of intralobular stroma, i.e. cells/mm^2.

Statistical Analysis

Analysis of variance of the data was conducted with the MIXED procedure in SAS v.9.1.3 software (Statistical Analysis Software, Cary, NC) using the following model:

$$Y_{ijk} = \mu + C_i + T_j + B_k + e_{ijk}$$

where μ = overall mean,

C = random effect of cow (i = 1to 9 cows),

T = fixed effect of treatment (j = 2; 0 or 5 ng TGF-β1/ml),

B = fixed effect of stage at biopsy (k = 1 to 4; LL, 1 WD, 3 BEC, 1 BEC),

e = residual error.

The effect of parity (primiparious or multiparous) and the interaction between biopsy and treatment were evaluated, but removed from the model when not declared significant (P>0.1). Results were considered statistically significant at a probability of α less than 0.05, and are presented as least squared means ± standard error of the mean.

RESULTS

General tissue histological characteristics were consistent among cows. Tissue sections obtained during LL biopsy were the least consistent within cow, as some areas appeared disorganized making stromal areas less distinguishable. Typical staining patterns for MMP-3, fibronectin and SMA are shown in Figure 3.2.

MMP-3 and Fibronectin Expression

Fibronectin staining was present in all tissue sections at all biopsy times. We observed lighter intensity staining of fibronectin in the intralobular stroma than in the interlobular stroma, and there was no positive staining in the epithelium. MMP-3 was also present at all stages. We observed darker MMP-3 staining surrounding the epithelial cells and in the interlobular stroma than in the intralobular stroma. TGF-β1 did not alter the percent intralobular stromal area stained for fibronectin or MMP-3 at any biopsy stage (Figure 3.2, P=0.35).

Fibroblasts and Myofibroblasts

Positive staining for smooth muscle α-actin was present in the myoepithelial cells, endothelial cells, and some fibroblast-like stromal cells (Figure 3.2). Stromal cells with a fibroblast phenotype were considered myofibroblasts. The percent of myofibroblasts tended to be higher (26.1 ± 2.6% vs. 22.0 ± 2.6%) in tissue incubated with TGF-β1 relative to the control (*P=0.06*). The number of myofibroblasts per mm^2 of intralobular stroma increased by 19% (640 ± 77 cells/mm^2 to 763 ± 77 cells/mm^2; *P=0.03*) in tissue

explants incubated with TGF-β1 relative to the control (Figure 3.4). The interaction between stage at biopsy and treatment on the number of myofibroblasts pr area was not significant (P=0.38). Treatment with TGF-β1 had no effect on the number or percent of fibroblasts in the intralobular stroma (Table 3.1 and Figure 3.3).

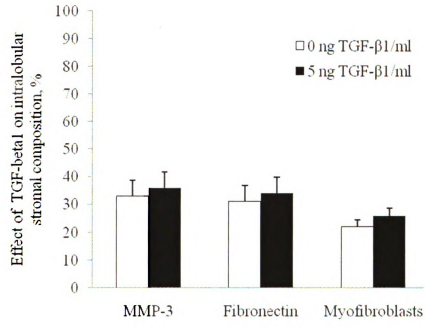

Figure 3.2 Effect of TGF-β1 on intralobular stromal composition. No significant effects of TGF-β1 were observed between the percent of stroma staining with matrix metalloproteinase (MMP-3) or fibronectin. There was also no significant effect of TGF-β1 observed on the percent of myofibroblasts (SMA-expressing fibroblasts) in the intralobular stroma. The effect of TGF-β1 did not differ between biopsies for any of the three variables shown here.

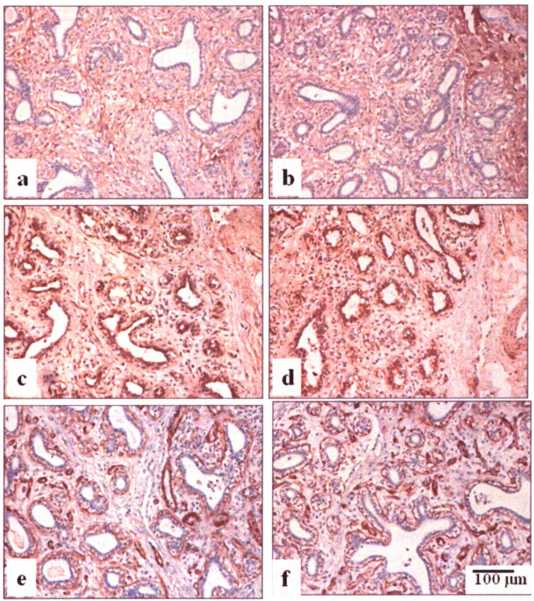

Figure 3.3 Immunohistochemical images (200X) of bovine mammary tissue at 1 week dry (1WD). (a) Fibronectin staining with 0 ng TGF-β1/ml (b) Fibronectin staining with 5 ng TGF-β1/ml (c) MMP-3 staining with 0 ng TGF-β1/ml (d) MMP-3 staining with 5 ng TGF-β1/ml (e) SMA staining with 0 ng TGF-β1/ml (f) SMA staining with 5 ng TGF-β1/ml

Table 3.1. Effects of Transforming growth factor-β1 (TGF-β1) on fibroblasts in the intralobular stroma.		0 ng TGF-β1/ml		5 ng TGF-β1/ml		Tukey Adj. P-value
		Least Squared Mean	SE	Least Squared Mean	SE	
Stromal cells (cells/mm^2)		2274	152	2193	153	.46
Activated stromal cells (cells/mm^2)		640	77	763	77	*.03**
Activated stromal cells (%)		22.0	2.6	26.1	2.6	*.06***
Late Lactation	Fibroblasts (cells/mm^2)	2236	200	2424	200	.97
	Myofibroblasts (cells/mm^2)	710	109	811	109	.90
	% Myofibroblasts	24	3.6	25	3.6	.99
1Weeks Dry	Fibroblasts (cells/mm^2)	2670	200	2374	200	.78
	Myofibroblasts (cells/mm^2)	516	109	762	109	.11
	% Myofibroblasts	16	3.6	25	3.6	.18
3 Weeks Before Expected Calving	Fibroblasts (cells/mm^2)	2221	212	1879	212	.73
	Myofibroblasts (cells/mm^2)	589	115	695	116	.91
	% Myofibroblasts	21	3.8	27	3.8	.72
1 Weeks Before Expected Calving	Fibroblasts (cells/mm^2)	1968	212	2096	226	.99
	Myofibroblasts (cells/mm^2)	744	116	782	121	.99
	% Myofibroblasts	27	3.8	28	4.0	1.0

* Significance ($P < 0.05$)
** Tendency to significance ($0.05 < P < 0.1$)

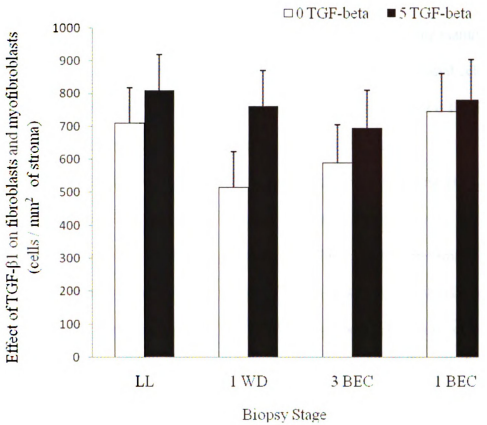

Figure 3.4. Effect of TGF-β1 on fibroblasts and myofibroblasts. Expressed as number of cells per area of intralobular stroma at late lactation (LL), one week dry (1 WD), three weeks before calving (3 BEC), and one week before calving (1 BEC). Overall treatment effect at P=0.03

DISCUSSION

Based on the effects of implanted TGF-β1 polymer pellets in the mammary gland of growing heifers, we expected that TGF-β1 plays would promote stromal cell proliferation and alter stromal composition (Musters et al., 2004). This is the first study to examine the effect of TGF-β1 during the dry period of dairy cows.

In the current study, we incubated explant cultures with 0 or 5 ng TGF-β1/ml for 2 h. We found that TGF-β1 increased the number of myofibroblasts in the intralobular stroma by 19% ($P=0.03$). In addition, we found that the percent of myofibroblasts tended to be higher in the intralobular stroma of tissue treated with TGF-β1 than in controls ($P=0.06$). Incubation with TGF-β1 did not alter the total number of fibroblasts in the intralobular stroma (Table 1). Therefore, TGF-β1 likely induces the differentiation of fibroblasts to myofibroblasts during the dry period. These findings are consistent with our expected results and with other studies that found increased expression of SMA by fibroblast cells treated with TGF-β1 in culture (Rønnov-Jessen and Petersen, 1993).

TGF-β1-induced differentiation of myofibroblasts may be influenced by the composition of the ECM. TGF-β1 increases fibronectin synthesis in the ECM, especially the fibronectin isoform, ED-A fibronectin (Serini et al., 1998). ED-A fibronectin synthesis preceded expression of SMA by human mammary fibroblasts in culture with 10 ng/ml TGF-β1, indicating that fibronectin is involved in the activation of fibroblasts (Serini et al., 1998a). In contrast to Serini et al (1998), 5 ng TGF-β1/ml treatment in this study did not increase in stromal fibronectin ($P>0.1$); however, we did not directly measure the ED-A fibronectin isoform. If TGF-β1 selectively increased the ED-A fibronectin in our study, we would likely have not detected the difference. The

fibronectin antibody used in this study recognized an epitope within the 4th repeat of the type III region of human fibronectin, and consequently would bind indiscriminately to fibronectin with or without the ED-A spliced region. Therefore it is not clear whether the increase in SMA induced by TGF-β1 was mediated by an increase in ED-A fibronectin synthesis.

TGF-β1 treatment had no effect on the protein expression of MMP-3. We expected that TGF-β1 would increase proliferation of fibroblasts and differentiation to myofibroblasts, which in turn would increase the production of MMP-3 during involution in mammogenesis. Since SMA production by differentiating fibroblasts occurs rapidly in the presence of TGF-β1, it is reasonable to expect an effect of TGF-β1 during a 2 h incubation. SMA mRNA expression was present within 4 h of incubation with 2 ng TGF-β/ml in rat lung fibroblast cultures (Roy et al., 2001). However, the 2 h incubation with TGF-β1 used for this study may not have been sufficient to observe differences in MMP-3 or fibronectin synthesis in the stroma. Ignotz and Massague (1986) did not observe a difference in ECM fibronectin synthesis by embryonic chick fibroblasts until after 4 h in culture with TGF-β1. Similarly, human fibroblasts cultured with 2 ng TGF-β1/ml did not show increases in fibronectin synthesis until after 6 h in culture (Thannickal et al., 2003). We observed an increase in myofibroblasts within 2 h incubations with TGF-β1, but perhaps the 2 h incubation with TGF-β1 was not sufficient for newly differentiated myofibroblasts to translate enough fibronectin or MMP-3 to detect significant differences. Since the stromal response to TGF-β1 is likely mediated through fibroblast activation, it is possible that a longer incubation period would have allowed activated fibroblasts time to synthesize stromal proteins in response to TGF-β1.

Based on our results, a 2 h incubation time was sufficient to detect changes in stromal cell activation by TGF-β1. Increasing the length of explant culture incubation time to 24 h may have allowed time for stromal protein expression however, we observed compromised histological quality of the tissue sections, and assessment and localization of immunohistochemical staining could not be determined consistently between biopsies and cows.

A dosage-dependant biphasic role of TGF-β1 was demonstrated using heifer parenchymal tissue showed in organoid cultures (Ellis et al., 2000). Those investigators found a stimulatory effect of TGF-β1 on cell proliferation at low concentrations (up to 500 pg/ml), and an inhibitory effect at higher concentrations (1-5 ng/ml) (Ellis et al., 2000). At the dose of 5 ng TGF-β1/ml, NOG-8 mammary epithelial cell lines underwent morphological changes to a mesenchymal phenotype and increased the production of fibronectin in culture (Plaut et al., 2003). The 5 ng/ml dose of TGF-β1 for this study was expected to inhibit epithelial cell proliferation and stimulate stromal cell proliferation and ECM production. SMA expression was detected, therefore 5 ng TGF-β1/ml was sufficient to detect differences in myofibroblast differentiation, in bovine mammary tissue however, the dose was not sufficient detect differences in ECM composition within 2 h.

CONCLUSIONS

TGF-β1 may mediate stromal remodeling in vivo during the dry period of dairy cows. TGF-β1 alters intralobular stromal remodeling in the bovine mammary gland by increasing the number of fibroblasts expressing SMA, and therefore likely to be myofibroblasts. While changes in MMP-3 and fibronectin were not detected in this study, further studies with longer incubation times or using in vivo implants are necessary to understand the potential for TGF-β1.

While there were no differences detected in the activity of TGF-β1 between biopsies on fibronectin and MMP-3, there is still potential for TGF-β1 administration to increase activation of myofibroblasts, increasing the rate of protein synthesis in the intralobular stroma, and thus potentially reducing the time required for dry period mammary remodeling. Further studies are needed to determine the optimal dose and length of treatment to maximize the effects of TGF-β1 on stromal composition. Based on the evidence presented in studies involving heifers and mice, TGF-β1 has a potent effect on stromal composition and mammary remodeling, and based on this study we believe that TGF-β1 does impact mammary remodeling during the dry period.

CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

Mammary remodeling during the dry period of dairy cows involves the coordination of changes in the stroma and epithelium. Transforming growth factor beta-1 (TGF-β1) is a cytokine that can influence interactions between stroma and epithelium through the extracellular matrix, and acts with opposing activity by inhibiting epithelium and promoting stromal development in mice (Daniel et al., 1989). My research showed that TGF-β1 mediates mammary gland remodeling in the intralobular stroma during the dry period of dairy cows.

My first experiment demonstrated that mammary stromal tissue undergoes changes in composition of cell types during late lactation and the dry period. The percent of intralobular stromal area decreased from 1 WD and 3 BEC to 1 BEC. This decrease in intralobular stromal area may be attributed to a decline in stromal proteins or a change in stromal elasticity or compression from distended lumen during lactogenesis.

One theory of mammary remodeling is that stromal area becomes compressed from increased pressure during lactogenesis as lumenal space increases and fills with milk or colostrum. This theory is based, in part, on the fact that there is little change in mammary DNA during the periparturient period (Capuco et al., 1997). To fully investigate this question would likely require measurements of intralobular stromal density and mass throughout the dry period, and potentially sacrificing a large number of animals to obtain sufficient tissue. We have shown that the composition of the stroma changes at different stages, and the changes we observed might cast some doubt on theory that the stroma merely compresses during late gestation and lactation. For

example, we observed that stromal fibronectin protein expression of remained constant as stromal area increased or decreased. In contrast to what we observed, if compression were causing the changes in stromal area we would have expected that during compressed periods LL and 1 BEC) the percent area of intralobular staining for fibronectin would be greater.

The number of fibroblasts per area of intralobular stroma decreased from 2,718 cells/mm^2 at 1 WD to 1,800 cells/mm^2 at 1 BEC (P=0.02). Based on these results, the number of fibroblasts is not likely constant at all stages in the stromal tissue. During involution (1 WD), the density of fibroblast cells in the stroma is greater than stromal tissue from cows in the later stages of mammogenesis (3 BEC) or early lactogenesis (1 BEC). Fibroblasts are involved in the synthesis of stromal proteins, proteinases, and growth factors. The increase in fibroblasts at a time when relative stromal area is also increased, suggests that fibroblasts are contributing to the increase in stromal area. Therefore, it follows that at a time when stroma is being synthesized, as expected during involution and mammogenesis, more fibroblasts would be present in the tissue than times when stromal tissue may be less active, such as during lactation. Methods to increase stromal fibroblast proliferation at dry-off may increase stromal synthesis, and thus speed up involution and potentially shorten the dry period.

TGF-β1 has been shown to stimulate fibroblast proliferation, and therefore may be initiating the increase we observed in fibroblasts in the stroma. Intralobular stromal TGF-β1 protein expression was greater during LL than in the dry period, yet not different between sampling times in the dry period. The increased presence of TGF-β1 in late lactation preceded the period of time when fibroblast cell density was also greatest.

Because TGF-β1 is known to increase stromal cell proliferation, it may have initiated an increase in fibroblast population early in the dry period. To determine if TGF-β1in late lactation did, in fact, initiate stromal cell proliferation in the early dry period, additional biopsies from late lactation and early involution after cessation of milk removal would have to be examined.

There was no effect of stage at biopsy on the number of myofibroblasts or the percent of intralobular stroma expressing the protein fibronectin, the proteinase MMP-3, or the TGF-β1 receptor, TGF-βR2 ($P>0.1$). While there was no change in the percent of intralobular stroma expressing these proteins, there may be changes in the expression of these proteins in other compartments of the mammary gland that were not measured in this study, such as the parenchyma (lumen and epithelium). It is possible that during involution and mammogenesis, stromal proteins such as fibronectin are both synthesized and degraded concurrently to balance the change in shape as alveolar structures collapse and re-form. In this study, the antibodies used for immunohistochemistry were not sufficient to differentiate between fragments and whole proteins. Many antibodies are developed against specific sequences of amino acids from proteins of interest, and consequently the antibody will bind to those sequences regardless of whether the protein is intact. Thus, a technique or antibodies that are sensitive to distinguish between fragmented and intact proteins might provide more insight into the changes in activity and function of proteinases and proteins during the dry period .

In the second experiment, we demonstrated that TGF-β1 can influence stromal remodeling during the dry period by increasing the number of myofibroblasts from 640 to 760 ± 77 cells per mm^2 ($P=0.03$). Like fibroblasts, myofibroblasts produce ECM

proteins, but unlike fibroblasts, they have an enhanced Golgi apparatus and endoplasmic reticulum consistent with increased protein synthesis (see review by Phan, 2008) in tissue incubated with TGF-β1. Based on these findings, TGF-β1 may be important in the activation of stromal cells during involution and mammogenesis of the bovine mammary gland.

We did not observe a significant effect of TGF-β1 on MMP-3 or fibronectin protein expression in the intralobular stroma during late lactation or the dry period. We expected that MMP-3 and fibronectin would increase in response to TGF-β1 treatment, particularly at 1 WD and 3 BEC, corresponding with periods of involution and mammogenesis, respectively. However, the mammary explants were cultured with 0 or 5 ng TGF-B1/ml for a period of only 2 hours. Petrov et al (2002) demonstrated that the effect of TGF- β1 on protein synthesis occurred indirectly through an increase in fibroblast activation to myofibroblasts. These investigators observed enhanced protein synthesis by the myofibroblast cells, which was maintained after TGF-β1 treatment was removed (Petrov et al., 2002). Thus, while we observed an effect of TGF-β1 on myofibroblast populations, our incubation period may have been too short to observe the transcription and translation of protein or proteinases by newly differentiated myofibroblasts.

The potential exists for TGF-β1 to increase the rate stromal remodeling through increased activation and proliferation of stromal fibroblasts. Further investigation of the effects of TGF-β1 on involution and mammogenesis is needed in the presence of the full milieu of growth factors and hormones in the mammary gland to determine its potential effectiveness as a method to reduce dry period length.

Milk production is a function of number and secretory activity of epithelial cells, and generally there is no net loss in epithelial cells from the beginning to the end of the dry period (Capuco et al., 1997). Thus, it is important to prevent a net loss in epithelial cells or any inhibition of epithelial proliferation from the beginning to the end of the dry period. Because TGF-β1 is a known inhibitor of epithelial cells, this may pose some concern for its application in a dry period management scheme. In vivo administration of exogenous TGF-β1 implants in heifers demonstrated that TGF-β1 could alter stromal remodeling without inhibiting epithelial cell populations (Musters et al., 2004). Thus, administration of TGF-β1 during involution may be useful in determining its potential in mammary remodeling of pregnant dairy cows.

APPENDIX 1

Serial Mammary Biopsies in Cows Do Not Alter Overall Milk Production

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Serial biopsies of the mammary gland are useful in studies of mammary biology; however, many researchers assume the cow's long term milk production might be impaired. The objective of this study was to determine the effect of mammary biopsies during late lactation through the dry period on overall milk production. Six multigravid cows were biopsied approximately 275-290 days in milk (biopsy 1), 7 days after dry off (biopsy 2), 3 weeks (biopsy 3) and 1 week (biopsy 4) before expected calving date. Animals were sedated with xylazine hydrochloride, and the biopsy site was numbed with lidocaine gel before lidocaine injection. A biopsy tool (AgResearch, Hamilton, NZ) powered by a cordless drill was used to obtain approximately 1 g of mammary tissue per biopsy. Immediately after biopsies 1 and 4, a teat cannula was inserted into the biopsied quarter to drain any accumulated blood. For approximately 3 days, cows were hand stripped at each milking until blood clots were no longer observed in the milk. Cows joined the milking herd after biopsy 1 and 4. Analysis of milk yields during the lactation prior to and following the biopsies indicate that milk production in biopsied cows was not different from the herd ($P>0.1$). At 30-60 days in milk, primiparous cows averaged 35 kg/d of milk prior to biopsy and 48 kg/d in the lactation following biopsy; for multiparous cows, milk yield averaged 50 kg/d prior to biopsy and 54 kg/d in the lactation following biopsy. The use of teat cannulae after biopsy appears to improve cow comfort. Results show that serial mammary biopsies do not impair milk production in dairy cows.

Keywords: Mammary Gland, Biopsy, Milk Production

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