Domestic Animal Endocrinology 65 (2018) 49–55

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.journals.elsevier.com/domestic-animal-endocrinology

Excess deposition of collagen in mammary glands of tamoxifen-treated Holstein heifers is associated with impaired mammary growth

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ARTICLE INFO

Article history:
Received 7 February 2018
Received in revised form 26 April 2018
Accepted 11 May 2018

Keywords:
Tamoxifen
Mammary
ECM
Stroma
Estrogen

ABSTRACT

It is established that the ovary and estrogen are essential to bovine mammary development with the onset of puberty. Recent studies have shown that ovariectomy in the very early prepubertal period, well before onset of puberty, also dramatically impairs mammary growth. Similarly, prepubertal heifers treated with the antiestrogen tamoxifen (TAM) also exhibit markedly impaired mammary growth in correspondence with reduced estrogen receptor α (ESR1) expression. Our objective was to evaluate the effect of TAM on the mammary stroma and specifically to determine if the reported decrease in mammary development was related to changes in TAM-induced alterations in the stroma surrounding the mammary parenchyma. Briefly, 16 Holstein heifers calves were randomly assigned to one of 2 treatment groups: TAM-injected or control. Calves were administered TAM (0.3 mg kg^{1} d^{1}) or placebo from 28 to 120 d of age. At day 120, calves were euthanized and udders removed. Mammary tissue from near the boundary between the parenchyma and surrounding mammary fat pad was collected for histology and morphometric analysis, expression of selected extracellular matrix–related genes, and quantitation of stromal collagen deposition by study of Sirius Red-stained tissue sections imaged with polarized light. Compared with tissue from control heifers, TAM heifers frequently exhibited areas with abundant fibroblasts and mesenchymal cells especially within the intralobular stroma, as well as less complex ductal structures. Among the array of extracellular matrix–related genes tested, only a small difference ($P < 0.05$) in expression of laminin was found between treatments. The relative tissue area occupied by stromal tissue was not impacted by treatment. However, the deposition of collagen within the stromal tissue was more than doubled ($P < 0.0001$) in TAM-treated heifers. These data suggest that blocking ESR1 expression with TAM allows for excessive collagen deposition in the stroma surrounding the developing epithelial structures and that this interferes with both the degree of overall mammary parenchymal development, as well as the pattern of normal ductal morphogenesis.

1. Introduction

Progressive growth and development of mammary ducts in the peripubertal mammary gland depends on hormonal and growth factor mediation of interactions between the epithelial ducts and the surrounding stromal tissues to produce the tubulo-alveolar structures present in the mature functional mammary gland at the onset of lactation [1]. Estrogen and growth hormone are especially critical in regulation of ductal elongation and morphogenesis [2,3]. In an earlier study [4], we showed that prepubertal heifers treated with tamoxifen (TAM) exhibited a 50% reduction in overall mammary development (reduced

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https://doi.org/10.1016/j.domaniend.2018.05.006

mass and DNA content). These reductions were similar to those observed in heifers ovariectomized at similar ages [5,6]. Together these results strongly support the relevance of estrogen and/or estrogen signaling in early prepubertal mammary development in the bovine. While the impairment in mammary growth after TAM treatment was not related to either location or proportion of epithelial cells expressing estrogen receptor α (ESR1) or progesterone receptor, there was 623% more ESR1 expression in control (CON) vs TAM calves at the protein level (quantitative immunocytochemistry) and a significant reduction in ESR1 mRNA. Interestingly, there was no significant difference in circulating concentrations of estradiol in TAM- and placebo-treated heifers [4]. Moreover, Geiger et al [7] noted that increased mammary growth in enhanced-fed preweaning heifers was positively correlated with increased expression of ESR1. These data suggest that loss of ESR1 expression at least partially explains the negative effects of ovariectomy (OVX) and TAM treatment in prepubertal heifers and alternatively that increases in ESR1 expression is associated with improved mammary growth in heifers.

The impaired mammary development we reported in TAM-treated heifers led us to evaluate stromal development and extracellular matrix (ECM) deposition and expression of a select group of ECM-related genes. These genes were selected based on a comparison between ovariectomized and ovary-intact heifers [6]. Our general observations of reduced complexity of ductal morphology [4] and ongoing recognition of the importance of the ECM in regulation of ductal development [8] also supported our evaluation of ECM-related proteins. We were also influenced by observations of altered expression of collagen and fibronectin and ECM-associated genes in mammary tissue of ovariectomized heifers [9,10] and reports of impacts of TAM on tissue fibrosis in breast cancer models [11].

Our primary objective was to determine if the marked impairment in overall mammary development we previously reported in TAM-treated heifers [4] was associated with alterations in the mammary stroma surrounding the developing ductal structures. Such results would suggest that estrogen signaling in the prepubertal heifer mammary gland regulates not just epithelial cells (as noted previously) but that estrogen signaling might also impact the mammary stroma to influence overall mammary development. This might well provide opportunities to modulate parenchymal development and possibly future milk production by targeting development of the stromal tissue within the developing mammary gland.

2. Materials and methods

2.1. Animals

For this evaluation, archived tissues from Tucker et al [4] were utilized. Briefly, all experimental procedures were conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (11–208 DASC). Sixteen female Holstein heifers were housed individually in calf hutches and randomly assigned to 1 of 2 groups: CON ($n = 7$) or TAM ($n = 8$). All calves were fed milk replacer twice daily and were weaned at 8 wk of age. One CON calf died during the trial. The data from this animal were omitted before statistical analysis. Calves were managed and reared following standard practices at the Virginia Tech Dairy Center.

2.2. Treatments

Because no information exists in the literature to suggest an optimal dose of TAM to use to suppress ESR1 action in the bovine mammary gland, we chose an amount equivalent to that administered to human breast cancer patients on TAM therapy [12]. Details describing solution preparation appear in Tucker et al [4]. Heifers were given daily subcutaneous injections of TAM (0.3 mg/kg) or the equivalent volume of excipient in placebo-treated heifers. Heifers were euthanized using Euthasol (Virbac Animal Health, Fort Worth, TX) and exsanguination at 121 ± 1 d of age.

2.3. Mammary tissue sampling

At slaughter, udders were removed and bisected medially into left and right hemispheres. The left fore quarter was used to collect samples of parenchyma for real-time quantitative polymerase chain reaction (RT-qPCR) and stored at −80°C until RNA was isolated. The left rear quarter was trimmed of excess mammary fat pad tissue, butterflied, and formalin-fixed for histological analysis and immunohistochemistry. After 24 h in fixative, tissue blocks for embedding and sectioning were collected from each of 3 zones. Zone 1 was just above the teat and gland cistern, zone 2 was approximately midway between the gland cistern and the outer edge of the parenchyma, and zone 3 was near the outer edge at the interface between the mammary parenchymal and mammary fat pad. For Sirius red staining (described in the following), only zone 3 sections were used. Following fixation tissues were stored in 70% ethanol.

2.4. Real-time quantitative polymerase chain reaction

Protocols for isolation and purification of RNA followed manufacture instructions based on use of the RNAse Mini Kit (catalog number 74,104; Qiagen, Valencia, CA) and DNAse 1 digestion (catalog number 79,254; Qiagen Inc). Purity of resulting RNA was evaluated with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE). Only samples with a ratio of optical density measurements (260 and 280 nm) greater than 1.8 were accepted. The integrity of 18S and 28S ribosomal RNA was evaluated with gel electrophoresis using a 1% agarose gel and visualized by ethidium bromide staining under UV light.

Reverse transcription involved synthesizing single stranded cDNA via the High Capacity cDNA Archive Kit (Life Technologies Corporation). Briefly, 4 μg of RNA was reverse transcribed to single-stranded cDNA in a final reaction volume of 40 μL using random primers. The cDNA and no reverse transcriptase controls products were diluted 1:100 in sterile nuclease-free water. A total of 2 μL of cDNA was