Short communication: Opposing effects of lactoferrin on the proliferation of fibroblasts and epithelial cells from bovine mammary gland

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ABSTRACT

Lactoferrin is present in several physiologic fluids, including milk and colostrum. Recently, evidence has accumulated that lactoferrin acts as a regulator of cell proliferation. Lactoferrin mRNA and protein levels in bovine mammary glands are known to markedly increase after cessation of milking. To clarify the role of bovine lactoferrin (bLF) in mammary involution and remodeling during dry periods, we investigated whether bLF affects the proliferation of cultured cells derived from bovine mammary gland and examined the mechanism underlying the proliferative response to bLF. Addition of bLF to the culture medium increased the proliferation of bovine mammary stromal fibroblasts (bMSF), but decreased that of bovine mammary epithelial cells (bMEC). Proliferation was significantly increased in the bMSF treated with bLF (100 µg/mL or greater) as compared with unstimulated cells. The maximal proliferative effect of bLF on bMSF occurred at 1,000 µg/mL, such that the proliferation of the bLF-stimulated bMSF was approximately 2.5 times that of unstimulated cells. The bLF increased the production of proliferating cell nuclear antigen and rapid phosphorylation of the p44/p42 mitogen-activated protein kinase in bMSF, but not in bMEC. The bLF-induced proliferation and production of proliferating cell nuclear antigen in bMSF was suppressed by U0126, a specific inhibitor of mitogen-activated protein kinase. Furthermore, treatment with bLF for 24 h decreased the mRNA levels of the 3 isoforms of transforming growth factor β in bMSF (16–66%) but upregulated those in bMEC (122–157%). These opposite effects of bLF on the proliferation of epithelial and fibroblast cells and their expression of transforming growth factor β may play a crucial role in bovine mammary involution and remodeling.
Key words: cell proliferation, lactoferrin, mammary epithelial cell, mammary stromal fibroblast

Short Communication

A nonlactating (i.e., dry) period between lactations is important for optimal milk production in dairy cows. During the dry period, mammary epithelial components undergo remodeling (Capuco et al., 1997; Sorensen et al., 2006) and the composition of mammary stromal tissue changes (De Vries et al., 2010). Fibroblasts, which are responsible for the synthesis and remodeling of the extracellular matrix, are the predominant cell type in mammary stroma. Interactions between mammary epithelial cells and the extracellular matrix are important for the functional differentiation of these cells (Barcellos-Hoff et al., 1989; Delabarre et al., 1997). Furthermore, cross-talk between epithelial and stromal cells occurs and the growth factors and cytokines produced by these cells influence the functions of other cell types (Cunha and Hom, 1996; Liotta and Kohn, 2001). In fact, hepatocyte growth factor derived from mammary stromal fibroblasts stimulates the growth of cultured murine mammary epithelial cells (Sasaki and Enami, 1999). Therefore, the epithelial-stromal interaction likely plays a key role in mammary involution and remodeling during dry periods.

Lactoferrin is an iron-binding glycoprotein belonging to the transferrin family (Anderson et al., 1989). Lactoferrin is produced mainly by glandular epithelial cells and is found in many physiologic fluids, including milk and colostrum (Steijns and van Hooijdonk, 2000). Lactoferrin is thought to be an important component of the mammalian host defense mechanism because of its potent antimicrobial, antiviral, and immunomodulatory activities (Hasegawa et al., 1994; Caccavo et al., 2002). In addition to the role of lactoferrin in host defense, accumulating evidence indicates that this glycoprotein has pleiotropic regulatory effects on cell migration, differentiation, and proliferation and on cytokine secretion (Ward et al., 2005; Tang et al., 2010). In particular, bovine lactoferrin (**bLF**) exerts both stimulatory and inhibitory effects on cell proliferation; bLF stimulates the proliferation of osteoblasts, bone marrow stromal cells, and myoblasts (Byatt et al., 1990; Cornish et al. 2004; Grey et al., 2004), but decreases the viability or

proliferation of mammary epithelial cells, polyp fibro-blasts, and breast cancer cell lines (Riley et al., 2008; Nadolska et al., 2010; Duarte et al. 2011). In addition, bLF induces apoptosis in human stomach cancer cell lines (Xu et al., 2010). The effect of bLF on cellular proliferation seems to be cell-type-specific, but the underlying mechanism is unknown.

The concentration of bLF in milk increases steadily as lactation progresses (Rainard et al., 1982; Cheng et al., 2008). In addition, bLF mRNA and protein levels in bovine mammary glands markedly increase after milking stops (Welty et al., 1976; Rejman et al. 1989; Schanbacher et al. 1993; Wilde et al. 1997). Therefore, the elevated bLF levels in mammary glands are likely to be associated with mammary involution and remodeling. In fact, bLF decreases the expression of casein and the proliferation of bovine mammary epithelial cells (**bMEC**) in vitro (Riley et al., 2008). Although lactoferrin is secreted by glandular epithelial cells (Steijns and van Hooijdonk, 2000), an immunohistochemical study has shown that bLF is localized in stromal areas during involution of the bovine mammary gland (Hurley and Rejman, 1993). Therefore, the bLF secreted by bMEC may penetrate the basement membrane and thus influence the functions of stromal cells. However, how bLF affects the proliferation of bovine mammary stromal fibroblasts (**bMSF**) remains to be clarified.

In the current study, we focused on bMSF to explore the roles of bLF in mammary involution and remodeling. To this end, we established primary cultured bMSF and their clones from bovine mammary glands and compared the effect of bLF on the proliferation of these cells with that on bMEC (Nakajima et al., 2008) in vitro. We also investigated potential differences in proliferation mechanisms between bMSF and bMEC.

The study was approved by the Animal Care Committee of NARO Hokkaido Agricultural Research Center. Two Holstein cows in midlactation were euthanized by intravenous overdose injection of pentobarbital sodium (100 mg/kg of BW). Mammary tissues were removed within 20 min of death.

Primary bMSF were prepared by using a modification of a method used to isolate periodontal ligament fibroblasts (Okubo et al., 2010). In brief, mammary tissues obtained from lactating Holstein cows were dispersed in 120 U/mL of collagenase (Sigma Chemical, St. Louis, MO). The cell suspension was filtered through a 150-μm nylon mesh filter to remove undigested tissue fragments and debris. The filtrate then was centrifuged at $80 \times g$ for 10 min at room temperature. Cell clumps were removed and the supernatant was centrifuged at $120 \times g$ for 10 min at room temperature. The pelleted cells were washed twice with Dulbecco's modified Eagle's medium (**DMEM**; Sigma Chemical) containing 10% (vol/vol)

fetal calf serum (Hyclone, Logan, UT), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma Chemical). Cells then were seeded into culture dishes (100 mm in diameter) coated with type I collagen and containing DMEM supplemented with 15% (vol/vol) fetal calf serum, 10 ng/mL of fibroblast growth factor 1 (R&D Systems, Minneapolis, MN), 5 μg/mL of heparin (Sigma Chemical), 100 U/mL of penicillin, and 100 μg/mL of streptomycin and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells obtained from passages 3 to 5 were used in subsequent studies.

To establish populations derived from single cells, the primary bMSF described previously were seeded into wells (1 cell/well) of a 96-well plastic plate coated with type I collagen (Becton Dickinson, Lincoln Park, NJ). The cells were maintained in DMEM supplemented with 15% (vol/vol) fetal bovine serum (Hyclone), 10 ng/mL of fibroblast growth factor 1 (R&D Systems), 5 μg/mL of heparin, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The culture medium was changed every 2 d, and each well was monitored for cell growth and confluence. Once a well became confluent, the cells were expanded into a plastic dish (100 mm in diameter) coated with type I collagen. Four bMSF clones (CL1 through CL4) were established and used for additional experiments. Cells obtained from passages 6 to 10 were used in subsequent studies.

The isolation of bMEC clone was described previously (Nakajima et al., 2008). In brief, the mammary tissue from lactating Holstein cow was dispersed with 120 U/mL of collagenase. The cell suspension was centrifuged at $80 \times g$ for 10 min at room temperature. The centrifuged cells and cell clumps were washed twice with RPMI-1640 medium (Sigma Chemical) supplemented with 10% (vol/vol) fetal bovine serum. The cells then were seeded into plastic dishes with RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 5 μg/mL of insulin, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. After the cells became 80% confluent, we performed a limiting dilution of these cells and established cloned bMEC. The cloned bMEC were maintained in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 5 μg/mL of insulin, 100 U/mL of penicillin, and 100 μg/mL of streptomycin on noncoated plastic culture dishes.

Proliferation of bMSF and bMEC was determined by using a Cell Counting Kit 8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The bLF purified from milk was obtained from Wako (Osaka, Japan; purity greater than 95%). In most cases (Figures 1, 2B, and 3C), 5×10^3 cells were plated in each well of a 96-well tissue culture plate and cultured for 24 h in DMEM supplemented with 10% (vol/vol) fetal bovine serum. Next, the cells were cultured in