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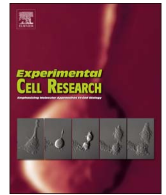

Inflammatory responses of stromal fibroblasts to inflammatory epithelial cells are involved in the pathogenesis of bovine mastitis

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ABSTRACT

Hypernomic secretion of epithelial cytokines has several effects on stromal cells. The contributions of inflammatory epithelial cells to stromal fibroblasts in bovine mammary glands with mastitis remain poorly understood. Here, we established an inflammatory epithelial cell model of bovine mastitis with gram-negative lipopolysaccharide (LPS) and gram-positive lipoteichoic acid (LTA) bacterial cell wall components. We characterized immune responses of mammary stromal fibroblasts induced by inflammatory epithelial cells. Our results showed that inflammatory epithelial cells affected stromal fibroblast characteristics by increasing inflammatory mediator expression, elevating extracellular matrix protein deposition, decreasing proliferation capacity, and enhancing migration ability. The changes in stromal fibroblast proliferation and migration abilities were mediated by signal molecules, such as WNT signal pathway components. LPS- and LTA-induced inflammatory epithelial cells triggered different immune responses in stromal fibroblasts. Thus, in mastitis, bovine mammary gland stromal fibroblasts were affected by inflammatory epithelial cells and displayed inflammation-specific changes, suggesting that fibroblasts play crucial roles in bovine mastitis.

1. Introduction

Mastitis is one of the most critical diseases in dairy production. Mastitis is a mammary gland inflammation caused by changes in metabolism, physiological trauma, or contagious or environmental pathogenic microorganisms [1]. Invasive pathogens are the main causes of mastitis. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are two of the most important pathogens causing bovine mastitis [2]. Lipopolysaccharides (LPS), the major structural elements of the *E. coli* cell membrane, trigger strong immune responses. Lipoteichoic acid (LTA), an important cell wall component of *S. aureus*, causes septic shock and multiple organ apoptosis. Therefore, these two important antigens might be used as conditional factors to induce inflammatory responses.

Neutrophils are the first line of defense against bacterial invasion in the bovine mammary gland [2]. During bacterial invasion, bovine mammary gland epithelial cells (BMEs) initiate neutrophil recruitment through inflammatory and immune responses, including increased pro-inflammatory cytokine secretion [3,4]. Tumor necrosis factor alpha (TNF-α) is the main pro-inflammatory cytokine producing inflammatory responses and promoting the release of various inflammatory mediators by multifarious cell types [5]. TNF-α induces fibroblasts to produce granulocyte macrophage colony stimulating factors and promotes granulocyte and macrophage progenitor maturation [6]. Proteins of TNF and the TNF receptor family play important roles in the control of cell death, proliferation, autoimmunity, the function of immune cells, and the organogenesis of lymphoid organs [7].

Dairy cow mammary glands mainly consist of BMEs and bovine mammary stromal fibroblasts (BMFs). BMEs play an important role in inducing a relevant innate immune response in mammary glands, act as sentinels, and signal invading mastitis-causing pathogens [2]. In bovine mastitis, bacterial infection first occurs in the epithelial cells of mammary gland crypts [1,2,8]. During the early stages of infection, epithelial cells release immunological factors to prevent the production and development of inflammation and also release inflammatory mediators to affect adjacent cells [1,2,8]. Fibroblasts are important stromal cells which activate and interact with infiltrating immune cells in a dynamic and site-specific manner. Fibroblasts also define the

Abbreviations: LPS, lipopolysaccharide; LTA, lipoteichoic acid; *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; BMEs, bovine mammary gland epithelial cells; BMFs, bovine mammary stromal fibroblasts; ECM, extracellular matrix; TNF-α, tumor necrosis factor-alpha; CCK-8, Cell Counting Kit-8; IL-6, interleukin- 6; IL-8, interleukin- 8; CXCL2, Chemokine (C-X-C motif) ligand 2; CCL5, Chemokine (C-C motif) ligand 5; α-SMA, α-smooth muscle actin; PDGFR-α, growth factor receptor α; PDGFR-β, growth factor receptor β; EGFR, epidermal growth factor receptor; GSK-3β, Glycogen synthase kinase-3β; HMGB1, high mobility group box-1 protein; ERK, extracellular signal-regulated kinase

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external tissue features, provide positional memory, and regulate the switch from resolving to persistent inflammation [9]. The regional identity of fibroblasts can be modified by inflammation [10–13]. Therefore, targeting fibroblasts and the stromal microenvironment are likely important methods in treating inflammation.

Toll-like receptor 2 recognizes LTA, whereas toll-like receptor 4 recognizes LPS [14,15]. Meanwhile, ligand binding activates a range of signaling molecules and releases transcription factors [16,17]. Released transcription factors mediate cytokine, chemokine, and growth factor expression levels [18]. Inflammatory mediators of abnormal expression induces inflammatory cell repair. Many abnormal and uncontrolled repair mechanisms may lead to fibrosis and extracellular matrix (ECM) deposition. During the early phase of bovine mastitis, inflammatory mediators released from epithelial cells stimulate fibroblasts and other stromal cells [19]. Activated fibroblasts are also key inflammation mediators and mediate the development of inflammation in bovine mastitis.

Better knowledge of inflammatory epithelial cell reactions to BMFs could eventually lead to novel therapeutic approaches to combat the spread of inflammation during the early phase of bovine mastitis. With that aim in mind, this study used an in vitro model of mastitis to investigate the mechanism of inflammatory epithelial cell effects on BMF. The proliferation, apoptosis, migration, secretion of inflammatory cytokines, and ECM deposition of BMFs cultured in conditioned medium from LPS- or LTA- induced inflammatory BMEs were investigated.

2. Materials and methods

2.1. Cell isolation and culture

BMEs and BMFs were obtained from Holstein cow mammary tissues as previously described [20]. Mammary tissues were individually sampled from eight Holstein dairy cows. All mammary glands were healthy with no obvious evidence of infection. Fresh tissues were digested in a solution of Enzyme Cocktail (ISU ABXIS, Seoul, Korea) and incubated at 37 °C overnight in a humidified 5% CO_2 incubator. Digested tissue was filtered and centrifuged to yield pellets containing epithelial organoids and supernatants containing fibroblasts. The isolated epithelial cells and fibroblasts were seeded on cell culture dishes and cultured in complete DMEM/F12 medium (GIBCO BRL, Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL, Grand island, NY) at 37 °C in an incubator with 5% CO_2. Cells were passaged by digestion with 0.15% trypsin and 0.02% EDTA. BMEs and BMFs were almost pure after three to four separation and culture passages. By the fifth passage, extracted BMEs or BMFs were used as cell pools in all subsequent experiments. Both BMEs and BMFs were prepared by mixing equivalent amounts of cells from all individuals. All reagents were purchased from Sigma-Aldrich Canada (Oakville, ON), unless otherwise stated.

2.2. Establishment of inflammatory cell model

An inflammatory cell model of mastitis was established with LPS- or LTA-treated BMEs. Tumor necrosis factor-alpha (TNF-α) released in the medium and mRNA expression in the treated cells were utilized as indicators for LPS- or LTA-induced immune responses. Before stimulation with LPS and LTA, confluent BMEs monolayers were cultured in fresh medium without FBS for 24 h at 37 °C. Then, the medium was replaced with complete medium containing LPS or LTA at final concentrations of 10 ng/µl or 20 ng/µl according to recommended concentrations. After stimulation for 3 h, 6 h, 12 h, and 24 h with LPS or LTA, cell culture supernatants were discarded and replaced with fresh medium without FBS. The culture was incubated again for 24 h. Finally, cell culture supernatants were collected for TNF-α secretion measurement and BMEs were harvested for RNA extraction and TNF-α real-time PCR analysis. BMEs cultured in media without FBS and without stimulation were designated as controls. The inflammatory cell model of mastitis was established under the shortest treatment period exhibiting significantly upregulated TNF-α expression in BMEs. BMEs treated with LPS and LTA were designated as LPS-BMEs and LTA-BMEs, respectively.

2.3. Indirect co-culture experiments

We investigated the effects of inflammatory epithelial cells induced by LPS or LTA on BMFs with an indirect co-culture model utilizing conditioned medium. In brief, LPS-BMEs or LTA-BMEs were rinsed with PBS. Fresh serum-free DMEM/F12 medium was then added to the culture dishes. Cells were cultured for another 24 h at 37 °C under 5% CO_2. Then, the medium was collected and cells debris was moved by centrifuge. Finally, the medium was used at a 9:1 ratio with fresh DMEM/F12 and 10% FBS as the conditioned medium. BMFs cultured in conditioned medium collected from un-stimulated BMEs were taken as control.

For indirect co-culture, BMFs were cultured in LPS-BME- or LTA-BME-conditioned media. After day 3 of culture, cell culture supernatants were collected and BMFs were harvested for analysis.

2.4. RNA extraction and real-time PCR

Total RNA was isolated from cells washed twice with PBS. Cells were re-suspended in ice-cold TriZol solution (TransGene, Shanghai, China). Cells were passed through a ribonuclease-free 20-gauge needle 10 times for cell disruption. Total mRNA extraction was performed with the RNA Easy Kit (TransGene) according to the manufacturer's instructions. Total mRNA concentration was measured with a spectrophotometer (ND 2.0; NanoDrop Technologies, Wilmington, Delaware), and 1 µg of RNA was reverse-transcribed into cDNA with the TransScript II First-Strand cDNA Synthesis SuperMix (TransGene).

Primers were designed based on sequences from the National Center for Biotechnology Information Database. Specificity was determined with Primer-BLAST. The primers are listed in Supplementary Table S1. All PCR primers were synthesized by Sangon Biotech (Shanghai, China). Real-time PCR was performed with an iQ5 light cycler (Bio-Rad, Hemel Hempstead, UK) using TransStart Probe qPCR SuperMix (TransGene) in 25-µl reactions containing 1 µmol/L concentrations of each forward and reverse primer (Sangon Biotech). PCR conditions were as follows: activation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s. Melt curves were generated to estimate primer specificities. A standard curve was generated with reference cDNA to determine the starting quantity of mRNA in each sample. The expression of each target gene was normalized to that of GAPDH, the reference gene. Comparative Ct method was employed to quantify normalized target gene expression relative to the calibrator. Data were expressed as relative gene expression $=2^{-\Delta\Delta Ct}$.

2.5. Cell viability assay

Cell viability was determined with CCK-8 kits (Beyotime, Shanghai, China) according to the manufacturer's protocol. BMFs were evenly seeded into 96-well plates with 100 µl complete medium at a density of approximately 2×10^3 cells/well and incubated for 24 h. Then, the complete medium was replaced with BME-, LPS-BME-, or LTA-BME-conditioned medium. BMFs were then cultured in conditioned medium for 0, 1, 2, and 3 days. Before the cell viability assay, the medium was replaced with 100 µl fresh medium containing 10 µl of CCK-8 solution and incubated for an additional 3 h at 37 °C in a humidified incubator. The absorbance of samples in triplicate wells was measured at 450 nm wavelength with a microplate reader (Bio-Rad, Hercules, CA) at

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