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Differential expression of living mammary epithelial cell subpopulations in milk during lactation in dairy cows

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ABSTRACT

Epithelial cells are shed into milk during lactation, and although they generally reflect the cellular characteristics of terminally differentiated luminal cells, previously the detection of more primitive cells was described in human milk where a cell population of epithelial lineage was detected expressing markers typical of progenitor cells. In this investigation we report the development of flow cytometry analysis to allow multiparametric assessment of mammary epithelial cells observed in milk. Cells collected from milk samples of 10 healthy dairy cows were directly analyzed for 6 different markers: CD45, CD49f, cytokeratin 14, cytokeratin 18, presence of nucleus, and cell viability. Milk samples were collected in 3 different periods of lactation: early lactation (EL = d 0–30), mid-lactation (ML = d 90–120), and late lactation (LL = 210–250). Here we identify the differential expression of precursor or differentiated cell markers (or both) in mammary epithelial cells present in bovine milk. Myoepithelial cells, as indicated by cells staining positively for cytokeratin 14^+/cytokeratin 18^-, were observed to increase from EL to LL with a high correlation with nuclear staining inferring potential proliferative activity. Furthermore, a significant increase in $CD49f^+$ and cytokeratin 14^+/cytokeratin 18^+ positive cells was observed in LL. This assay is a sensitive approach for evaluating the variations in the frequency and features of living epithelial cells, whose reciprocal balance may be significant in understanding mammary gland cellular function throughout lactation. These observations suggest that mammary epithelial cell immunophenotypes could be investigated as biomarkers for mammary gland function in dairy cows.

Key words: milk, bovine, epithelial cell, cell precursor

INTRODUCTION

Heterogeneous cell populations are present within the mammary secretions of all mammals. In most animals, immune cell types are predominant, in particular lymphocytes, polymorphonuclear neutrophils, and macrophages. Most studies of the cellular fraction of milk report the total count of somatic cells without taking into account the cell viability and cell types. Bovine species have a relatively lower percentage of epithelial cells observed in milk compared with humans (Boutinaud and Jammes, 2002). Mammary epithelial cells (**MEC**) are also found in milk, caused by shedding during the lactation phase, but the range of cell frequency may differ from total SCC if only the live cell fraction is analyzed. The identification of milk epithelial cells with the features of viable and mature alveolar epithelial cells has been reported. In milk, immune cells are generally accepted as a defense against inflammation or infectious diseases (or both) of the gland. However, very little is known with respect to the epithelial compartment shed in milk, and appreciable suppositions have been made concerning the origin and regulation of these cells (Boutinaud and Jammes, 2002). A renewed interest in this observation has occurred following the discovery that epithelial cells are present that have some immunophenotypes previously associated with precursor cells, and in milk from humans (Cregan et al., 2007; Thomas et al., 2011). In those studies, it has been described how human milk presents different cell populations exhibiting epithelial characteristics (identified by the detection of different cytokeratins; **CK**) but also expressing specific markers of progenitor cells such as nestin or p63.

The functional mammary cellular unit is a multicellular complex system organized into alveolar structures connected by a common ductal system. Cell types include an inner layer of $CK18^+$ luminal cells and an outer layer of $CK14^+$ myoepithelial cells. The luminal cells in the alveoli can produce milk, whereas the contractile myoepithelial cells are responsible for forcing

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the secreted milk to the teat cistern (Martignani et al., 2010).

Cells in milk have been demonstrated to be able to generate cell colonies of different mammary cell subsets. The functional role of these cells has been postulated to affect neonatal development and breast pathology (Hassiotou et al., 2013; Twigger et al., 2013).

In dairy science, the total amount of somatic cells, usually called SCC, in milk is affected by different factors, such as species, breeds, lactation phase, milk yield, individual animal differences, and management practices (Rupp et al., 2000). Both SCC and composition affect milk quality, but their relationship is not always apparent apart from the case of high SCC corresponding to a high concentration of neutrophils in milk. Usually, it is difficult to analyze cell composition because SCC is a total count that does not consider the concentration of any other cell types present in the secretion (Li et al., 2014).

In recent years, some studies of somatic cells by flow cytometry reported new understanding of the cell subpopulation organization, in particular for immune cells populations (Piepers et al., 2009; Albenzio and Caroprese, 2011). To our knowledge, however, few studies have investigated epithelial subpopulations in relation to the stage of lactation.

We described a 6-color immunophenotyping assay to investigate different living epithelial cell subpopulations present in 3 different phases of lactation in dairy cows. This approach provides the opportunity to further investigate the modification of these cells according to the physiological and pathological state or age of the animal, which may have some effect on production of milk quality.

MATERIALS AND METHODS

Farms and Animals

The trial was conducted at the farm of the Department of Veterinary Medical Science, University of Bologna (Ozzano Emilia, BO, Italy) and conducted according to European animal care guidelines. The experimental procedures were approved by the Ethics Committee of the University of Bologna. Cows in the transition period (from 21 d before the expected calving to 7 d after calving) were housed in a group pens, then moved to a free-stall pen for the rest of lactation. Cows were fed long grass hay and a concentrate mixture (on average 3 kg of concentrate for every 7 kg of hay) before calving and received a total mixed ration after calving, distributed once a day in the morning. After forestripping into a foremilk cup, paper tissues were used for udder cleaning. Water was available ad libitum.

Study Design

The general udder health status of all lactating cows was determined by analyzing the SCC. Based on these data, 10 Holstein-Frisian cows in good condition were chosen to analyze with apparently healthy mammary glands with low SCC values (<100,000 cells/mL). Samples were collected from 3 different periods: first period of lactation or early lactation (**EL**), d 0 to 30 after parturition; mid-lactation (**ML**), d 90 to 120 after parturition; and late lactation (**LL**), d 210 to 250 after parturition.

Milk Sampling and Processing

Quarter foremilk samples were obtained in accordance with the Veterinary Services Standards of the Italian National Health Service, branch of the Ministry of Health. Before morning milking, teats were scrubbed with 70% ethanol and the first 2 strips of milk were discarded. Aliquots of 200 mL of milk per udder were collected aseptically in a sterile 50-mL BD Falcon tube (BD, Heidelberg, Germany). Ten milliliters was used for SCC assessment according to International Dairy Federation standards (Hamann, 1996). Milk sample were diluted in PBS buffer at a 1:1 ratio to minimize the influence of lipids on pellet formation. Cells were isolated from the milk using 2 repeated centrifugation steps at 200 × *g* for 15 min at 4°C. Pellets were then washed in PBS to a final dilution of 0.5 to 1 × 10^6 cells/100 μL.

Flow Cytometry Analysis: Sample Processing

The determination of epithelial subpopulations in milk was carried out utilizing a 6-color flow cytometry assay: nuclear staining was evaluated with Vybrant DyeCycle Ruby stain (Life Technology, Thermo Fisher, Mumbai, India) and living cell with Live/Dead Fixable (Violet) Dead Cell Stain Kit (Life Technology, Thermo Fisher). Anti-CD45 antibody (VMRD Inc., Pullman, WA) was used to gate immune cells, anti-human-CD49f–FITC antibody (anti-h-α-integrin-6-FITC, Novus Biological, Littleton, CO), monoclonal anti-CK peptide 18 antibody (clone KS-B17.2, Sigma, St. Louis, MO), and anti-CK14 antibody (Covance, Life Technology, Thermo Fisher). Anti-CD45, anti-CK14, and anti-CK18 antibodies were labeled with, respectively, QDotS525, QDot605, and PE with SiteClick antibody labeling kits according to the manufacturer's instructions (Life Technology, Thermo Fisher). Briefly, 100-μL aliquot of the cell suspension was incubated with 1 μL of Violet stain for 30 min at 4°C in the dark; subsequently, cells were washed twice (250 × *g* for 5 min