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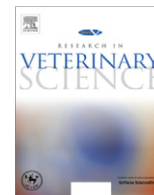

Evaluation of a replacement method for mammary gland biopsies by comparing gene expression in udder tissue and mammary epithelial cells isolated from milk

K. Krappmann, R. Weikard, C. Kühn *

Res. Unit Molecular Biology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

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ABSTRACT

Somatic cells isolated from milk offer an attractive non-invasive replacement of invasive udder biopsies for monitoring bovine mammary gland metabolism. However, for metabolic gene expression studies the mammary gland epithelial cells (MEC) isolated from milk have to be purified from the non-epithelial leukocyte fraction in milk samples. In our study, enrichment of MEC by using anti-cytokeratin peptide 18 (KRT18) antibody coated magnetic beads was evaluated. MEC showed a substantially increased expression of the epithelial-cell-specific KRT18 gene compared to udder tissue. The expression levels of genes specific for mammary gland epithelial cells (CSN3 and LALBA) showed a significant positive correlation in MEC and also in udder tissue. However, no significant correlation of the expression of a specific gene was found between udder and MEC samples. Therefore, MEC isolated from total milk samples via KRT18 antibodies probably do not reflect the true metabolic situation of the bovine udder. Thus, quantitative gene expression profiling of MEC isolated via KRT18 antibodies has to be interpreted carefully with respect to the situation in the udder.

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1. Introduction

In vivo-biopsies or tissue collection post-mortem are the common methods to get an inside-view into mammary gland metabolism. But these are invasive methods, and as for slaughter give only a single snap shot of the udder status and do not enable a comprehensive, dense monitoring over the entire lactation period of a cow. A surrogate approach replacing the invasive biopsy methods is the use of milk somatic cells to analyse the metabolic situation of the mammary gland. However, somatic milk cells mainly comprise immune cells like leukocytes, macrophages and lymphocytes (Concha, 1986). Only a minority of cells represents viable exfoliated cells from the mammary epithelium (Lee et al., 1980) that show alveolar cell characteristics (Boutinaud and Jammes, 2002).

Using microarray expression analysis, Hayashi et al. (2004) found similar expression patterns of bovine milk protein genes in mammary tissue samples collected post-mortem and total somatic cells in milk. Concordance between expression levels of mammary tissue samples and total somatic milk cells was also reported by Murrieta et al. (2006) who performed northern blot gene expression analyses to investigate the usefulness of somatic milk cells as basis to study lipogenesis in the bovine mammary gland. However, as outlined by Boutinaud et al. (2008), high amounts of non-epithelial leukocyte RNA contaminate the RNA preparation from total milk cells, which impedes mRNA quantification by qRT-PCR and subsequent relative quantification by normalisation to a reference gene. Consequently, for metabolic gene expression profiling, monitoring gene expression of mammary epithelial cells (MEC) isolated from milk should be favoured compared to total milk somatic cell expression, because the MEC represent the main source of metabolic activity in the mammary gland.

In our study, we evaluated the usefulness of MEC isolated from milk by an immuno-magnetic enrichment procedure to reflect the metabolic situation of the MEC in the bovine udder and thus, as an appropriate alternative to mammary biopsies for quantitative gene expression studies. Therefore, we comparatively analysed mRNA expression levels of selected genes that are characteristic for two major metabolic processes (milk protein synthesis and glucose metabolism) of the bovine udder in samples collected from mammary tissue and from in MEC captured directly from milk. We selected the κ-casein (CSN3) and α-lactalbumin (LALBA) genes, which represent two of the six major milk protein genes (Jenness, 1985) and are known to be expressed specifically in bovine mammary gland epithelial cells (Larson, 1979). Due its key function as main glucose-transporter in the bovine mammary gland (Zhao et al., 1996), the solute carrier family 2, member 1 (GLUT1) gene was included in our study.

* Corresponding author. Tel./fax: +49 38208 68709.
E-mail address: kuehn@fbn-dummerstorf.de (C. Kühn).

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2. Materials and methods

2.1. Animals and tissue sampling

Seven animals of a F_2 resource population established from a crossbreed between Charolais and German Holstein (SEGFAM resource population, Kuhn et al., 2002) were studied. The cows were milked twice a day in a commercial tandem milking parlour. All animals were kept under identical conditions in a loose stall barn in the dairy herd of the Leibniz Institute for Farm Animal Biology (FBN) Dummerstorf. All cows were fed the same total mixed ration (TMR) diet ad libitum, which contained grass and corn silage, hay, concentrates and a mineral and vitamin mix. The TMR had an energy content of 6.6 MJ of NEL/kg and a protein content of 147 g utilisable protein/kg of dry matter and was supplemented by 1 kg concentrates per 2 kg milk, when milk yield exceeded 17 kg/day (Hammon et al., 2010).

At the day of slaughter (30th day of the second lactation), a 2-l morning milk sample was taken directly from the milking parlour. The animal was slaughtered 2 h later in the registered slaughter house of the FBN Dummerstorf under conditions monitored and approved by the institutional authorities and by the responsible office of the State of Mecklenburg-Vorpommern, Germany. A mammary tissue sample was taken immediately, snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. Isolation and purification of MEC

Immediately after collection of milk samples (2 l milk), aliquots of 125 ml were distributed into 250-ml tubes (Corning Incorporated), and 100 ml of 4 °C diethylpyrocarbonate (DEPC) treated phosphate buffered saline (PBS) buffer prepared from distilled water were added to each milk sample. The samples were defatted by 20 min centrifugation at 2800*g* at 4 °C. Afterwards, the fat layer was discarded, and the skim milk was carefully removed. The pellet and the remaining supernatant fraction (1 ml) were mixed with 800 μl of 4 °C DEPC–PBS and transferred into a 2-ml tube. After adding 200 μl EDTA (0.5 M pH 8.0, 4 °C), the samples were centrifuged at 14,000*g* for 1 min at 4 °C. The supernatants were discarded, and the pellets were resuspended in 200 μl 4 °C DEPC–PBS. After merging the resuspended pellets, the suspension was centrifuged at 5100*g* for 5 min at 4 °C. Thereafter, the supernatant was discarded, and the pellet was finally resuspended in 1.25 ml 4 °C PBS containing 1% bovine serum albumin (BSA, Roth). For the separation of immune cells and MEC, mammary epithelial cell specific anti-cytokeratin peptide 18 antibody (Clone KS-B17.2, Sigma–Aldrich) coated beads (Dynabeads® Pan Mouse IgG, Invitrogen) were used. The separation was performed as described by Boutinaud et al. (2008). Briefly, 25 μl of Dynabeads® were transferred to a 1.5-ml tube and washed twice with 1% BSA–PBS to remove the preservative. The Dynabeads® were resuspended in 1 ml 1% BSA–PBS and transferred to a 1.5-ml tube containing 3 μl of KRT18 antibodies. The suspension was incubated for 30 min at 4 °C on a Dynal Sample Mixer (Model MXIC1). Then, the tube was placed in the magnetic particle concentrator (Dynal MPC®-E, Dynal A.S.) for 30 s. After another washing step and aspiration of the supernatant containing unbound antibodies, the antibody-coated Dynabeads® were resuspended in 250 μl 1% BSA–PBS. These 250 μl were added to the 1.25 ml cell suspension and incubated for 1 h at 4 °C on the Dynal Sample Mixer. Finally, the specifically bound cells were collected by magnetic incubation for 1 min. The bead bound cell pellet was washed and immediately used for RNA extraction.

2.3. RNA extraction from udder and MEC

Total RNA was purified from udder and MEC using the NucleoSpin® RNA II (Macherey–Nagel) kit essentially according to the manufacturer's recommendations. RNA isolation from udder was performed using 30 mg tissue.

For RNA preparation from MEC, the freshly prepared bead bound cells were resuspended in 600 μl of buffer RA1 (supplied with the NucleoSpin® RNA II kit) and 6 μl of β-mercaptoethanol. The MEC sample was placed in a magnetic particle concentrator for 30 s, and the supernatant was filtrated according to the protocol of the NucleoSpin® RNA II kit (centrifugation at 11,400*g* for 1 min). Thereafter, RNA binding conditions were adjusted by adding 600 μl of 70% ethanol.

For both, RNA extraction from udder and from MEC, on-column DNase digestion was carried out with twice the amount of enzyme compared to the manufacturer's instructions. Finally, the RNA was eluted with 60 μl of nuclease-free water. Using 1 μl of the eluate, RNA concentration was measured with the Qubit™ Fluorometer (Invitrogen) according to the manufacturer's instructions. The RNA was stored at −80 °C until further processing.

2.4. Reverse transcription and purification of cDNA

RNA from both sources (udder and MEC of the same animal) was transcribed into cDNA by using the SuperScript III First-Strand Synthesis System for reverse transcription polymerase chain reaction (Invitrogen) according to the manufacturer's instructions. Briefly, the reaction volume of 10 μl contained 60 ng RNA, 1 mM dNTP mix, 5 μM Oligo$(dT)_{20}$ and 2 pmol/μl of each gene-specific primer (Table 1). The samples were preincubated at 65 °C for 5 min. For reverse transcription (RT), 10 μl cDNA synthesis mix comprising 2 × RT buffer (included in the kit), 10 mM $MgCl_2$, 20 mM DTT, 4 U/μl RNaseOut™ and 20 U/μl SuperScript™ III reverse transcriptase were added to each sample. The cDNA synthesis was carried out at 50 °C for 50 min and terminated by incubation at 85 °C for 5 min. After incubation with 1 μl *Escherichia coli* RNase H (2 U/μl) at 37 °C for 20 min, the cDNA was purified with the NucleoSpin® Extract II kit (Macherey–Nagel), eluted in 100 μl DNase free water and stored at −80 °C.

2.5. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with each cDNA sample in duplicate by using LightCycler® FastStart DNA MasterPLUS SYBR Green I on a LightCycler® 2.0 Real-Time PCR System (Roche). The qRT-PCR reaction mix (5 μl) contained 2 × LightCycler® FastStart DNA MasterPLUS SYBR Green I Master Mix and 0.4 pmol/μl of gene-specific forward and reverse primer (Table 2). Five microliters of cDNA (1:3 diluted with DNase free water) were added to the reaction mix. The qRT-PCR conditions for each gene are listed in Table 2. The gene expression levels were quantified by using a calibration standard curve, which was generated from a serial dilution of the respective gene-specific PCR fragment covering six orders of magnitude (10^1–10^6 copies) and was included simultaneously with the samples. After quantification, melting curve analyses were carried out to ensure the specificity

Table 1
Gene-specific primers for reverse transcription.

Gene	Primer sequence 5′ → 3′	Gene region	mRNA sequence reference
KRT18	GATGGTTTGCCTGGAGTTGCTG	Exon 7	NM_001192095
CSN3	CTCAGGTGGGCTCTCAATAAC	Exon 4, 3′ UTR	NM_174294
LALBA	CAAAGAAGCAAAACGAGGTAG	Exon 4, 3′ UTR	NM_174378
GLUT1	GTGGAGTAATAGAAAACAGCGT	Exon 9	NM_174602

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