Short Communication: Ability of Cultured Mammary Epithelial Cells in a Bicameral System to Secrete Milk Fat

I. Ernens,*†‡¹ R. Clegg,* Y.-J. Schneider,† and Y. Larondelle‡

*Hannah Research Institute, Hannah Research Park, Ayr, KA6 5HL, Scotland, UK

†Laboratoire de Biochimie Cellulaire, and

‡Unité de Biochimie de la Nutrition, Institut des Sciences de la Vie, Université catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium

ABSTRACT

Mammary epithelial cells from lactating cows were cultured onto inserts coated with type I collagen. Every second day, the rates of fatty acid synthesis and secretion were determined by measuring the amount of [¹⁴C]-labeled sodium acetate incorporated into lipids over a 4-h period. The [¹⁴C]-containing lipids were identified by thin layer chromatography fractionation. In parallel, the integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance. The integrity increased progressively to reach a maximum after 8 d of culture. Cells incorporated acetate into lipids; 1.34% of acetate was incorporated into lipids produced by freshly isolated cells. This percentage decreased to 0.5% after 2 d of culture. Moreover, this capacity decreased with the duration in culture; on d 8, the rate of incorporation dropped to about 3% of that on d 2. In the cell extracts, the [¹⁴C]-labeled lipids were mainly triglycerides, although the proportion of diglycerides and phospholipids progressively increased as a part of total newly synthesized lipids. The proportion of triglycerides decreased 0.66 times between d 2 and 8 when the proportion of diglycerides and phospholipids increased 1.33 and 2.18 times, respectively. About 28% of the newly synthesized lipids were secreted within 4 h of incubation. Around 65 to 85% of these labeled lipids were found in the apical compartment, suggesting a partially vectorial secretion. But 58 to 80% of labeled lipids found in the apical and basolateral medium were free fatty acids. Functional tight junctions and incorporation of labeled fatty acids into triglycerides are not compatible with an inferred status of complete dedifferentiation of the cell layer. Moreover, triglyceride secretion seems compromised, probably due to the lack of an appropriate cell environment and cell shape.

Key words: lipid secretion, lipogenesis, mammary cell, transepithelial electrical resistance

Most of the lipids in milk are triacylglycerols present in fat globules, in which the lipids are enclosed within a membrane derived from the secretory epithelial mammary cell. Although the general outlines of lipid droplet formation, growth, movement, and secretion are known, virtually no molecular details of any of these processes have been elucidated. One major obstacle to molecular studies is the paucity of established in vitro mammary epithelial models that secrete or can be induced to secrete milk fat globules (Heid and Keenan, 2005). Delabarre et al. (1997) developed an in vitro system using mammary epithelial cells obtained from lactating cows. These differentiated cells were cultured onto type I collagen-coated inserts. In this culture system, the cells reorganized functional tight junctions and secreted a high level of CN vectorially for 2 wk. In the present study, a similar bicameral in vitro system was used to evaluate the ability of mammary epithelial cells to keep or recover their capacity to synthesize and secrete lipids.

Mammary glands from lactating Holstein-Friesian cows were obtained from a commercial abattoir 10 to 15 min after slaughter. Blocks of tissue were collected from the median zone of one of the anterior glands. Mammary epithelial cells were isolated from these blocks by an enzymatic dissociation procedure modified from that described by Delabarre et al. (1997). Briefly, small pieces of tissue were digested in M199 medium with 3% heat-inactivated fetal bovine serum (Invitrogen, Paisley, UK) supplemented with pronase (5.5 U/mL), hyaluronidase (450 U/mL), and DNase I (100 U/mL), purchased from Roche (Lewes, UK). The cell suspension resulting from digestion was filtered through a sterile 100- μ m filter (Lockertex, Warrington, UK). The resulting filtrate, composed primarily of single cells, was resuspended in Dulbecco's MEM/nutrient mix F-12 (1:1, vol/vol; Invitrogen), without L-glutamine and with pyridoxine, supplemented with 10% heat-inactivated fetal bovine serum, 5 mM L-glutamine, and 2.5 mM sodium acetate. The following hormones were added to the culture medium on the day of use: insulin (5 μ g/mL), hydrocortisone (1 μ g/mL), and prolactin (5 μ g/mL), purchased from Sigma-Aldrich (Poole, UK).

Received April 8, 2006.

Accepted August 21, 2006.

¹Corresponding author: isabelle.ernens@crp-sante.healthnet.lu

Figure 1. Change in DNA content after different durations of culture. This representative chart corresponds to the mean $\pm$ SEM from 3 independent experiments ($n = 9$).

The cells were plated in plastic culture flasks for 30 min to allow the attachment of contaminating fibroblasts. The nonattached cells were collected and seeded onto type I collagen-coated homemade inserts (5 cm²) made of a microporous filter of poly(ethylene terephthalate) with approximately 1.6×10^6 pores (1- μ m diameter); the insert was stretched firmly across the inner of 2 concentric polycarbonate rings (3×10^6 cell/insert). The cells were incubated with 1.8 and 2.8 mL of the culture medium described above, in the apical and basolateral compartments, respectively, to maintain fluid integrity. The transepithelial electrical resistance of the cell layer was measured daily within an Endohm chamber using an epithelial voltohmmeter (EVOM) resis-

tance meter (World Precision Instruments, Sarasota, FL).

The rates of fatty acid synthesis and secretion were determined by measuring the amount of [¹⁴C]-labeled sodium acetate incorporated into lipids over a 4-h period using a modification of the method described by Vernon and Finley (1988). Briefly, the cells were incubated in culture medium supplemented with 1 μ Ci/mL of [1-¹⁴C]acetate (ICN, Doornveld, Belgium) plus 2.5 mM unlabeled sodium acetate. After 4 h of incubation, the medium was collected and the cells from each insert were lysed by the addition of 300 μ L of guanidine isothiocyanate buffer (0.1 M EDTA, pH 8.0, containing 5 M guanidine isothiocyanate). Lipids of the lysate were extracted by addition of an equal volume of water-saturated chloroform. The upper aqueous layer was removed for the determination of DNA content according to the method of Labarca and Paigen (1980). Lipids were recovered through evaporation of the chloroform layer. Lipids of the media were obtained through the same procedure but using the mixture of 2 mL of medium with 50 μ L of GIT-RNA buffer [10 mM EDTA, containing 4 M guanidine isothiocyanate, 50 mM Tris-HCl (pH 7.6), 2% (wt/vol) sarkosyl (sodium lauryl sarkosinate), and 2% (vol/vol) β -mercaptoethanol] and 2 mL of water-saturated chloroform. The dried extracts were counted in a Packard 1600 TR liquid scintillation analyzer (Packard Instruments Company, Meriden, CT) and the rate of lipogenesis was expressed as nanomoles of acetate incorporated per microgram of DNA in a 4-h period.

The lipids obtained by chloroform extraction of the cell lysates and media were redissolved in 200 μ L of chloroform:methanol (4:1, vol/vol). When possible, an equivalent of 5,000 dpm of sample was fractionated by thin-layer chromatography (TLC) on a Silica Gel 60 plate (Merck, Darmstadt, Germany) in a hexane:diethyl

Table 1. Distribution of radioactivity (%) in lipid classes in the cells following the incubation of bovine mammary cells with sodium [1-¹⁴C]acetate for 4 h at different stages of culture¹

	Stage of culture				
	Freshly isolated cells	Day 2	Day 4	Day 6	Day 8
Total lipids ²	33.5 $\pm$ 0.6	12.5 $\pm$ 0.4	2.2 $\pm$ 0.2	0.33 $\pm$ 0.02	0.26 $\pm$ 0.04
Triglycerides	87.8 $\pm$ 1.0	76.4 $\pm$ 0.8	65.6 $\pm$ 1.4	63.1 $\pm$ 2.3	50.4 $\pm$ 5.6
Free fatty acids	1.9 $\pm$ 0.8	0.3 $\pm$ 0.1	2.4 $\pm$ 0.6	1.0 $\pm$ 0.9	2.0 $\pm$ 1.0
Cholesterol	0.8 $\pm$ 0.6	1.1 $\pm$ 1.1	3.8 $\pm$ 2.3	4.7 $\pm$ 2.9	4.0 $\pm$ 1.5
1,3 diglycerides ³	2.4 $\pm$ 0.5	4.1 $\pm$ 0.5	6.9 $\pm$ 0.6	7.0 $\pm$ 0.7	9.3 $\pm$ 2.6
1,2 diglycerides ³	2.4 $\pm$ 0.4	4.9 $\pm$ 0.5	5.3 $\pm$ 1.3	4.0 $\pm$ 1.4	2.7 $\pm$ 2.2
Phospholipids + monoglycerides ³	5.6 $\pm$ 0.3	14.8 $\pm$ 0.5	17.8 $\pm$ 1.1	21.6 $\pm$ 0.4	32.4 $\pm$ 1.6

¹Means of 3 isolations $\pm$ SEM ($n = 3$).

²Rate of lipid accumulation in the cells after different durations of culture expressed in nanomoles of acetate incorporated per 4 h per microgram of DNA.

³Components identified by comparison with the thin-layer chromatogram pattern obtained under the same conditions by Christie (1982).

ether:acetic acid system [80:20:2 (by vol)] for 70 min according to Christie (1982). Twenty micrograms of cholesterol, triolein, and oleic acid and 3 spots at 300, 600, and 1,200 dpm of [14 C]palmitic acid were applied on the plate as reference standards. The plate was exposed to a Kodak phosphor screen and scanned by using a phosphorimager 445 SI (Molecular Dynamics, Kemsing, UK), which gave a signal proportional to the radioactivity in each lipid spot. The signal was quantified with the ImageQuaNT Software (Molecular Dynamics). A standard curve was drawn from the 3 spots of [14 C]palmitic acid and used to convert the intensity of each spot into disintegrations per minute. The nonradioactive standards were visualized with iodine vapor.

The experiments were repeated 3 times on 3 lactating cows. Three replicates were conducted for each set of experiments. The results obtained from the 3 independent experiments were reproducible in terms of tight junction formation and DNA measurement. The results on lipid production showed a similar trend for all experiments, but the level was variable from one mammary gland to the other. This variability may be explained by our inability to control the variables of age, health, and lactation status between the animals used in this study.

The transepithelial electrical resistance value progressively increased during the first days of culture from 200 $\Omega \cdot \text{cm}^2$ to a maximum of 1,600 to 2,000 $\Omega \cdot \text{cm}^2$ reached on d 8 after seeding, indicating that the cells reorganized functional tight junctions, which reflects cell confluence, and also cell differentiation and polarization (data not shown).

The DNA content was determined after 0, 2, 4, 6, and 8 d in culture. The change in DNA was nonexistent between d 0 and 4 (Figure 1). After the fourth day, DNA increased linearly to double after 8 d of culture. This late increase in DNA content could be due to the proliferation of contaminating mesenchymal cells or some dedifferentiated epithelial cells.

The mammary cell layers incorporated acetate into lipids. The rate of incorporation was variable from one gland to another (range from 8.5 to 33.5 nmol of acetate incorporated per 4 h per μg of DNA), but their capacity to synthesize lipids rapidly decreased with the duration of culture (Table 1). The [14 C]-containing lipids of the cell extracts were predominantly triglycerides throughout the culture period of 8 d (Figure 2). The distribution of radioactivity in the various lipid classes is shown in Table 1. These data indicate that within the cell, the [14 C]-labeled triglycerides decreased progressively with the days in culture, whereas the labeled phospholipids and diglycerides increased as a proportion of total newly synthesized lipids.

Figure 2. Phosphorimager image of thin-layer chromatogram of the major classes of newly synthesized lipids in cell homogenates and media after different durations of culture. FFA_{standard} = [14 C]-palmitate; TG = triglycerides; CHOL = cholesterol; 1.2DG and 1.3DG = 1,2 and 1,3 diglycerides; PL = phospholipids; MG = monoglycerides. * = components identified by comparison with the thin-layer chromatogram pattern obtained under the same conditions by Christie (1982). The other spots were identified through comparison with standard compounds. The labeled spots were detected with a phosphorimager; the nonradioactive standards were visualized with iodine vapor.

Figure 3. Rate of lipid secretion in the cells after different durations of culture. A = apical medium; B = basolateral medium; A+B = sum of the 2 compartments. This representative chart corresponds to the results obtained from one mammary gland ($n = 3$). Data are expressed as mean $\pm$ SD in nanomoles of acetate incorporated in 4 h per microgram of DNA.

Interestingly, the amount of labeled lipids was higher in the apical compartment than in the basolateral compartment (Figure 3), suggesting the presence of a partially vectorial secretion of lipids. The extracellular lipid composition revealed a different trend than the intracellular one (Table 2). In particular, the labeled lipids were predominantly free fatty acids and not triglycerides. Some radioactivity was associated with triglycerides more than with free fatty acids only for freshly isolated cells. Lipid separation by TLC (Figure 2) suggested that in general, a mixture of lipids was present in the apical medium with free fatty acids as the major component. By contrast, free fatty acids were the only lipids found in the basolateral medium.

These experiments demonstrate that cells isolated from lactating bovine mammary tissue can utilize exog-

enous acetate for fatty acid and lipid biosynthesis. Moreover, the lipogenic capacity decreased rapidly with the duration of culture, in accordance with Kinsella (1968), who described a rapid drop in the lipid production with dispersed lactating mammary cells.

Differentiated mammary tissue incorporated fatty acids mainly into triglycerides, in contrast with nondifferentiated (from virgin or early pregnant animals) or dedifferentiated (from late pregnant or lactating animals) mammary tissue, which mainly incorporates fatty acids into phospholipids and diglycerides (Kinsella and Heald, 1972). In the present study, the proportion of labeled triglycerides remained higher than the other cellular lipid classes for all the experiments, even though the proportion of labeled phospholipids and diglycerides progressively augmented, possibly to meet the structural demands of cell proliferation. During the 8 d in culture, triglycerides were the most abundant of the newly synthesized cellular lipids contrasting with the idea of a complete dedifferentiation of the cell layer.

Unlike explants and organoids (Shamay et al., 1987; Jerry et al., 1989), the *in vitro* model used in this study allows work with purified mammary epithelial cells and the study of effects of various factors, specifically on this cell population. Moreover, the bicameral system provides easy access to the apical and basal surfaces of the cells and to the corresponding culture media, which helps in the study of vectorial processes, such as lipid secretion. This system also allows adequate gas and nutrient exchange, which is important in explant culture to induce lactogenesis (Jerry et al., 1989). But, this system did not allow maintenance of the correct three-dimensional shape of the mammary cells and the interactions with nonepithelial cells, which might be important for maintaining lipid synthesis and secretion. Indeed, the proper cellular environment and cell shape are required for the induction and maintenance of functional differentiation in mammary cells *in vitro* (Zoubiane et al., 2004). After 2 d in culture, the cells were

Table 2. Distribution of radioactivity (%) in lipid classes in the medium (apical + basolateral) following the incubation of bovine mammary cells with sodium [^{14}C]-acetate for 4 h at different stages of culture¹

	Stage of culture				
	Freshly isolated cells	Day 2	Day 4	Day 6	Day 8
Triglycerides	44.3 $\pm$ 8.7	9.1 $\pm$ 4.8	4.6 $\pm$ 3.9	7.0 $\pm$ 5.6	9.0 $\pm$ 7.1
Free fatty acids	33.6 $\pm$ 5.6	72.5 $\pm$ 8.0	80.0 $\pm$ 9.0	69.7 $\pm$ 10.4	57.8 $\pm$ 11.1
1,3 diglycerides ²	3.8 $\pm$ 0.4	5.6 $\pm$ 1.4	5.7 $\pm$ 2.7	8.9 $\pm$ 2.8	11.6 $\pm$ 3.0
1,2 diglycerides ²	5.4 $\pm$ 1.2	6.0 $\pm$ 1.8	5.1 $\pm$ 2.4	7.5 $\pm$ 2.9	10.2 $\pm$ 3.6
Phospholipids + monoglycerides ²	8.7 $\pm$ 4.8	6.7 $\pm$ 2.1	5.5 $\pm$ 2.4	7.6 $\pm$ 2.4	11.1 $\pm$ 3.5

¹Means of 3 isolations $\pm$ SEM ($n = 3$).

²Components identified by comparison with the thin-layer chromatogram pattern obtained under the same conditions by Christie (1982).

able to synthesize a quantifiable amount of lipids; yet only 7% of these lipids were present in the medium and in the form of free fatty acids. This limited capacity to secrete triglycerides most probably leads to an abnormal intracellular lipid accumulation. The cell response could then be a rapid diminution of synthesis, which was observed by a diminution of [^{14}C]acetate utilization.

This *in vitro* mammary epithelial system is conceptually limited for the study of lipid secretion. Moreover, it can be used to investigate, at early time points, molecular processes involved in lipid biosynthesis and tight junction organization.

REFERENCES

- Christie, W. W. 1982. The analysis of simple lipid classes. Pages 93–97 in *Lipid analysis*. W.W. Christie, ed. Pergamon Press, Oxford, UK.
- Delabarre, S., C. Claudon, and F. Laurent. 1997. Influence of several extracellular matrix components in primary cultures of bovine mammary epithelial cells. *Tissue Cell* 29:99–106.
- Heid, H. W., and T. W. Keenan. 2005. Intracellular origin and secretion of milk fat globules. *Eur. J. Cell Biol.* 84:245–258.
- Jerry, D. J., R. K. Stover, and R. S. Kensinger. 1989. Quantitation of prolactin-dependent responses in porcine mammary explants. 67:1013–1019.
- Kinsella, J. E. 1968. Lipid biosynthesis by bovine mammary cells in vitro. *J. Dairy Sci.* 51:1968–1970.
- Kinsella, J. E., and C. W. Heald. 1972. Na 1- ^{14}C stearate and Na 2- ^{14}C acetate metabolism and morphological analysis of late prepartum bovine mammary tissue. *J. Dairy Sci.* 55:1085–1092.
- Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102:344–352.
- Shamay, A., E. Zeelon, Z. Ghez, N. Cohen, A. G. Mackinlay, and A. Gelter. 1987. Inhibition of casein and fat synthesis and α -lactalbumin secretion by progesterone in explants from bovine lactating mammary glands. *J. Endocr.* 113:81–88.
- Vernon, R. G., and E. Finley. 1988. Roles of insulin and growth hormone in the adaptations of fatty acid synthesis in white adipose tissue during the lactation cycle in sheep. *Biochem. J.* 256:873–878.
- Zoubiane, G. S., A. Valentijn, E. T. Lowe, N. Akhtar, S. Bagley, A. P. Gilmore, and C. H. Streuli. 2004. A role for the cytoskeleton in prolactin-dependent mammary epithelial cell differentiation. *J. Cell Sci.* 117:271–280.