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The goat mammary glandular epithelial (GMGE) cell line promotes polyfucosylation and *N*,*N*′-diacetyllactosediaminylation of *N*-glycans linked to recombinant human erythropoietin

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Abstract

We have established a continuous, non-transformed cell line from primary cultures from *Capra hircus* mammary gland. Low-density cultures showed a homogeneous epithelial morphology without detectable fibroblastic or myoepithelial cells. The culture was responsive to contact inhibition of proliferation and its doubling time was dependent on the presence of insulin and epidermal growth factor (EGF). GMGE cells secrete caseins regardless of the presence or absence of lactogenic hormones in the culture media. Investigation of the total *N*-glycan pool of human erythropoietin (rhEPO) expressed in GMGE cells by monosaccharide analysis, HPLC profiling, and mass spectrometry, indicated significant differences with respect to the same protein expressed in Chinese hamster ovary (CHO) cells. *N*-Glycans of rhEPO-GMGE are core-fucosylated, but fucosylation of outer arms was also found. Our results also revealed the presence of low levels of sialylation (>95% Neu5Ac), *N*,*N*′-diacetyllactosediamine units, and possibly Gal-Gal non-reducing terminal elements.

Keywords: *N*-Glycosylation; Goat; Mammary gland; Epithelial cells; *N*,*N*′-diacetyllactosediamine; Gal-Gal; Polyfucosylation

Considerable effort has been directed toward the conceptual understanding of factors involved in mammary development and the mechanism of milk synthesis and secretion. *In vivo* experiments using ruminants are very costly and generally difficult to perform because they require a large commitment of animal resources and technical labour. Therefore, emphasis has turned towards the development of cell culture methods that will enable studies on growth regulation, hormonal responsiveness, and biochemical properties of mammary epithelial cells.

For a better comprehension of mammary gland physiology and gene regulation, several *in vitro* models of development and function based on primary culture systems have been used [1]. Whole organ and explant cultures have been valuable in identifying the role of specific hormones in both the growth and differentiation of mammary tissue and the induction of milk protein gene expression [2]. However, these cultures have a limited lifespan and are not useful for studies at the cellular level. Epithelial cells can also be isolated from mammary tissue, maintained in culture and induced to differentiate with lactogenic hormones. A major drawback of this system, in addition to the short lifespan of the cells, is the considerable amount of starting material required. Development of established mammary epithelial

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cell lines provides an alternative to primary cells. A number of established (non-malignant) mouse mammary epithelial cell lines exist [3–5]. However, extrapolation of findings from mouse cell lines to human or ruminant is difficult to justify.

As the transgenic goat has become a useful bioreactor for the biotechnology industry, a caprine mammary epithelial cell line would be useful for species-specific studies of protein expression and proliferation. In this regard, the main objective of this study was to establish a spontaneously immortalized goat mammary epithelial cell line. Special attention was paid to the clonal nature of this cell line in order to avoid further changes in its culture behaviour. The cells derived from a goat mammary glandular epithelium (GMGE)[2] were assayed for their morphological homogeneity, culture conditions, response to stimuli with lactogenic hormones, and for the *N*-glycosylation pattern of a recombinant protein expressed in these cells.

Materials and methods

Primary cell culture

Biopsies were aseptically taken from lactating (92 days post-parturition) Alpine goat (*Capra hircus*) mammary glands. Tissue samples were cut in small pieces of about 2 or 3 mm and digested with collagenase A (Boehringer Mannheim, Mannheim, Germany) at 5 mg/mL in DMEM-F12 (Gibco BRL, Eggenstein, Germany) media for 20 min with agitation for 10 s every 3 min. The digested tissue fragments were washed twice with phosphate-buffered saline (PBS) and digested with trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA, 1 mM in PBS) for 15 min at 37 °C with agitation for 10 s every 3 min. The digestion was stopped by the addition of one fifth of the volume of fetal bovine serum (FBS). After centrifugation, the cellular pellet was resuspended in DMEM-F12 containing 20% of FBS, gentamicin (50 μg/mL), epidermal growth factor (10 ng/mL) and insulin (10 μg/mL). The tubes were left to stand for 2 or 3 min, until the undigested fragments and the large cellular aggregates sedimented. The supernatant was seeded on 100-mm plates. After 18–24 h, the plates were washed twice with culture medium in order to remove the cells that had not adhered. After two passages, the culture medium was changed to a growth medium consisting of DMEM (Gibco BRL, Eggenstein, Germany) containing 10% of FBS, gentamicin (40 μg/mL), epidermal growth factor (10 ng/mL), and insulin (10 μg/mL).

Kinetics of cell proliferation and dependence of GMGE cell line on growth factors

GMGE-3 cells were seeded at a density of 6.75×10^4 cells/well in 12-well plates and grown in DMEM containing 10% of FBS. Four experimental groups were assayed according to the growth factor supplemented in the culture medium: (1) DMEM containing 10% of FBS (group C), (2) DMEM containing 10% of FBS and 10 μg/mL of insulin (group I), (3) DMEM containing 10% FBS and 10 ng/mL of EGF (group E), (4) DMEM containing 10% of FBS, 10 μg/mL of insulin and 10 ng/mL of EGF (group El). Every 24 h, quadruplicate wells were dissociated with trypsin and aliquots were counted to determine the total cell count for each well. The average number of cells, calculated every 24 h, was used to plot kinetic proliferation curves for GMGE-3 cells.

Induction of milk protein gene expression

GMGE-3 cells were grown on plastic to confluency in DMEM supplemented with 10% of FBS, insulin (5 μg/mL) and EGF (10 ng/mL). For lactogenic hormone induction, the cells were maintained at confluency for 3 days in growth medium. GMGE-3 cells were then primed in DMEM supplemented with 0.5% w:v of AlbuMaxTM I (Gibco BRL, Eggenstein, Germany) and 10 μg/mL of insulin for 24 h. For hormonal induction, the priming medium was replaced with DMEM containing 0.5% w:v of AlbuMaxTM I and supplemented with: (1) insulin (5 μg/mL), (2) insulin (5 μg/mL), *o*-prolactin (5 μg/mL), and dexamethasone (0.1 mM), (3) insulin (5 μg/mL), *o*-prolactin (5 μg/mL), and hydrocortisone (1 μg/mL). Cultures were induced for up to 12 days without media changes.

Western blot analysis

Milk proteins were assayed both in cell extracts and in the culture medium. Milk proteins secreted into the culture medium were precipitated with trichloroacetic acid (TCA) from 1 mL of the culture medium and protein pellet washed extensively three times with cold acetone and further analyzed by Western blotting. For intracellular proteins, cells (area 8 cm^2) were washed in PBS and the proteins were extracted with TRI Reagent (Sigma, St. Louis, MO). The protein concentration of the samples was determined using the BCA (bicinchoninic acid) Protein Assay Reagent kit (Pierce and Warriner, Chester, UK).

One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [6] in 15% polyacrylamide gels with a 3% stacking gel. Proteins were transferred from the gels to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) at a current of 0.8 mA/cm^2 for 1 h using a semidry electroblotter [7]. After blocking non-specific binding with 3% bovine serum albumin (BSA) in PBS/Tween (0.1%), the nitrocellulose membrane was exposed to a polyclonal rabbit anti-goat caseins antibody diluted 1:200 in PBS. Primary antibodies were visualized by peroxidase-conjugated anti-immunoglobulin (Ig) antibodies and 3,3′-diaminobenzidine tetrahydrochloride detection reagent (Sigma, St. Louis, MO).

In vitro expression assay

GMGE-3 cells were seeded in 162 cm^2 culture flasks at a density of about 2.5×10^5 cells/cm^2 in DMEM supplemented with 10% of FBS, epidermal growth factor (10 ng/mL) and insulin (10 μg/mL). Confluent cells were infected with the adenoviral vector AdhEPO [8] at a multiplicity of infection (MOI) of 25. After 14 h, the growth medium was replaced with fresh DMEM without serum as the harvest medium. After 72 h, the medium was collected and the supernatant was assayed for rhEPO content.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out using PhastGel TM Dry IEF (Amersham Biosciences, Uppsala, Sweden). The dry gel was incubated in 5 mL of hydrate solution containing: 535 μL ampholine pH 2.5–5, 89.4 μL ampholine pH 3–10 (Pharmacia Amersham Biotech, Uppsala, Sweden), and 2.4 g urea. Protein, purified according to the method described by Toledo et al. [9] was dissolved in milliQ water to give a 1 mg/mL solution, and 4 mL of each sample were loaded per well. The glycoforms were

[2] *Abbreviations used:* 4ABA, 4-aminobenzoic acid; Ad, adenovirus; BCA, bicinchoninic acid; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMB, 1,2-diamino-4,5-methylene-dioxybenzene; DEAE, diethylaminoethyl; DHB, dihydroxybenzoic acid; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetic acid; EGF, epidermal growth factor; ESI, electrospray ionization; EPO, erythropoietin; FBS, fetal bovine serum; GLC, gas–liquid chromatography; GMGE, goat mammary glandular epithelial; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IEF, isoelectric focusing; MALDI, matrix-assisted laser desorption/ionization; MOI, multiplicity of infection; MS, mass spectrometry; MW, molecular weight; NP-40, nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNGase F, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F; Q, quadrupole; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TOF, time-of-flight.