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Monosialylated biantennary *N*-glycoforms containing GalNAc–GlcNAc antennae predominate when human EPO is expressed in goat milk

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Abstract

Recently, our group reported the expression of recombinant human erythropoietin in goat milk (rhEPO-milk) as well as in the mammary epithelial cell line GMGE (EPO-GMGE) by cell culture using the adenoviral transduction system. *N*-Glycosylation characterization of rhEPO-milk by Normal-Phase HPLC profiling of the fluorophore, 4-aminobenzoic acid-labeled enzymatically released *N*-glycan pool from rhEPO-goat milk, combined with MALDI, ESI-MS and LC/MS, revealed that low branched, core-fucosylated, *N*-glycans predominate. The labeled *N*-glycans were separated into neutral and charged fractions by anion exchange chromatography and the charged *N*-glycans were found to be mostly α2,6-monosialylated with Neu5Ac or Neu5Gc in a ratio of 1:1. Unlike the *N*-glycans from rhEPO produced in CHO cells, where the glycans are multiantennary highly sialylated, core-fucosylated oligosaccahrides, or even in the goat mammary gland epithelial cell line cultured *in vitro* in which multiantennary, core- and outer-arm fucosylated, monosialylated *N*-glycans are the most abundant species, a large proportion of the *N*-glycans from rhEPO-milk were monosialylated, biantennary, antennae mostly terminating with the more unusual GalNAc–GlcNAc motive and without outer-arm fucosylation. These findings, emphasizing the difference in the *N*-glycan repertoire between the rhEPO-milk and EPO-GMGE, are consistent with the principle that glycosylation is cell-type dependent and that the cell environment is crucial as well.

Keywords: Mammary gland; Epithelial cells; Goat milk; Erythropoietin; Glycosylation; Sialylation; Monosialylated biantennary *N*-glycans

The large-scale production of recombinant biopharmaceutical glycoproteins in the milk of transgenic animals is becoming more widespread due to the very promising economic production processes [1]. To date several proteins have been expressed in the milk of transgenic animals in quantities ranging from a few micrograms to several grams per liter. Some examples are: human lactoferrin in mice [2] and cows [3]; α1-antitrypsin [4] and C1 inhibitor [5] in rabbits; human anti-thrombin [6] and human tissue-type plasminogen activator [7,8] in goats; human Factor VIII [9] and human Protein C [10] in pigs; human α-glucosidase in mice [11] and rabbits [12] and human erythropoietin in rabbits [13] and pigs [14]. However, the functionality and specificity of mammary gland glycosylation is, as yet, incompletely understood in comparison with other mammalian cell lines.

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Human erythropoietin (hEPO) is a glycoprotein hormone with three potential *N*-glycosylation sites at Asn-24, -38, and -83, and one *O*-glycosylation site at Ser-126. The glycosylation on hEPO is essential for *in vivo* activity [15], as the unglycosylated hormone shows full *in vitro* but no *in vivo* biological activity [16]. Thus, the system for the production of biologically active EPO requires the biosynthetic machinery of mammalian cells like Chinese hamster ovary (CHO)[1] cell cultures, able to assemble the necessary tetrasialylated tetraantennary *N*-glycans to maintain the half life of the protein [17,18].

Recently, our group has expressed hEPO in the milk of mice and goats (*Capra hircus*) by adenoviral transduction of the mammary secretory epithelial cells [19,20]. An adenoviral vector, carrying the hEPO cDNA, allowed the expression of the recombinant glycoprotein at levels over 2 g/L in goat milk. However, the milk-derived recombinant hormone (rhEPO-milk), showed a lower molecular weight, more basic isoforms and a very low *in vivo* hematopoietic activity compared to the described homologous rhEPO produced in CHO cell cultures, presumably due to differences in the glycosylation of rhEPO produced in milk compared to that produced in CHO cell line [20]. Moreover, we have also established a continuous, non-transformed epithelial cell line (GMGE) from primary culture of goat mammary gland and expressed rhEPO in this system (EPO-GMGE). The recombinant protein expressed in GMGE cells showed an *N*-glycosylation pattern that significantly differed from that of the classical CHO-EPO, with core- and outer-arm fucosylation and low sialic acid content. The glycoprotein showed a reduced hematopoietic activity [21].

The aim of the present study was to characterize the *N*-glycosylation of hEPO, as a glycoprotein model, expressed in the milk of goats to compare with that already reported for the same glycoprotein expressed *in vitro* in the goat epithelial mammary cell line, GMGE and the classical CHO cells system.

[1] *Abbreviations used:* 4ABA, 4-aminobenzoic acid; BHK, baby hamster kidney; CHO, Chinese hamster ovary; GMGE, goat mammary gland epithelium; DEAE, diethylaminoethyl; dHex (deoxy-hexose (fucose); DHB, dihydroxybenzoic acid; DMB, 1,2-diamino-4,5-methylene-dioxybenzene; DSA, *Datura stramonium* lectin; EPO, erythropoietin; ESI, electrospray ionization; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GNA, *Galanthus nivalis* lectin; h, human; Hex, hexose; HexNAc, *N*-acetyl-hexose; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LC/MS, Liquid chromatography-mass spectrometry; MAA, *Maackia amurensis* lectin; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; *m/z*, mass to charge ratio; NBT, nitro blue tetrazolium; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoylneuraminic acid; NP, normal-phase, NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PNGase, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase; Q, quadrupole; rh, recombinant human; SDS, sodium dodecylsulfate; SNA, *Sambucus nigra* lectin; TFA, trifluoroacetic acid; TIC, total ion current; TOF, time-of-flight.

Materials and methods

Production of EPO in goat milk

rhEPO was expressed in goat milk by adenoviral transduction and purified from the skimmed milk pool from three nuliparous 1-year-old Toggenburg goats milked on days 32–38 after the induction of lactation [20]. The purity was greater than 99% as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining.

SDS–PAGE and Western-blotting assay

SDS–PAGE analysis was performed as described by Laemmli [22] in 12% polyacrylamide gels. Proteins were transferred to nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) using a semidry electroblotter. After incubation with a monoclonal antibody (mAb) antiEPO-horseradish peroxidase (HRP), conjugated signals were visualized with the 3,3′-diaminobenzidine tetrahydrochloride detection reagent (Sigma, St. Louis, MO).

In vivo hematopoietic activity

The rhEPO hematopoietic activity was evaluated by the increase of reticulocyte numbers in B6D2F1 mice. At least six mice per experimental group were used. Mice were subcutaneously injected with 8, 10 or 12 μg of rhEPO samples. Four days post-injection, blood samples were collected and diluted with brilliant cresyl blue (1%, v/v) for reticulocyte counts. Ten fields per sample were counted. The relative hematopoietic activity of the inoculated dose was analyzed by regression for each sample using the 0 μg dose as the baseline control. Biological activity in milk-derived hEPO was analyzed by ANOVA comparing the effect of the three dose levels and the baseline control.

Proteolytic digestion and mass spectrometry analysis

N-Deglycosylated protein (50 μg) was digested with sequencing-grade trypsin (Promega, MA) at an enzyme/substrate ratio of 1/50 at 37 °C for 4 h. Digestions were stopped by adding aqueous trifluoroacetic acid (TFA) (1%, v/v). The proteolytic digest was separated by liquid chromatography (AKTA Basic, Amersham Pharmacia Biotech, Sweden) equipped with a splitter (LC-Packings, Netherlands) in a 0.3×100 mm PepMap column (LC-Packings, Netherlands). The column was equilibrated in solvent A (H_2O, 0.05% TFA) and the digest was eluted with a linear gradient of Solvent A and solvent B (acetonitrile, 0.05% TFA) from 0 to 80% over 100 min.

Mass spectrometric analysis of peptides

A hybrid quadrupole orthogonal acceleration tandem mass spectrometer (QTOF-2) from Micromass (Manchester, UK) fitted with a Z-spray nanoflow electrospray (ESI) ion source was connected online with the HPLC system described above. The mass spectrometer was operated with a source temperature of 80 °C and a drying gas flow of 50 L/h. The capillary and cone voltages were 3500 and 35 V, respectively. To acquire the liquid chromatography-mass spectrometric (LC/MS) and LC-MS/MS spectra, the first quadrupole was used to select automatically the precursor ion within a window of 4–5 Th. Argon was used as the collision gas. Data acquisition and processing were performed using a MassLynx system (version 3.5). The mass accuracy was below 100 ppm.

Oligosaccharide identification using lectin binding assays

The "DIG Glycans Differentiation Kit" (Roche, Germany) was used to identify specific carbohydrate motives. After separation on 12% SDS–PAGE, the glycoproteins (1 μg each) were bound to Immobilon membranes (Millipore, USA). The membranes were incubated at 25 °C for 1 h