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Transient Expression of Functional Glucocerebrosidase for Treatment of Gaucher's Disease in the Goat Mammary Gland

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Abstract Gaucher disease (GD) is an orphan disease characterized by the lack or incapacity of glucocerebrosidase (hGCase) to properly process glucosylceramide, resulting in its accumulation in vital structures of the human body. Enzyme replacement therapy supplies hGCase to GD patients with a high-cost recombinant enzyme produced in vitro in mammalian or plant cell culture. In this study, we produced hGCase through the direct injection of recombinant adenovirus in the mammary gland of a non-transgenic goat. The enzyme was secreted in the milk during six days at a level up to 111.1 ± 8.1 mg/L, as identified by mass spectrometry, showing high in vitro activity. The milk-produced hGCase presented a mass correspondent to the intermediary high-mannose glycosylated protein, which could facilitate its delivery to macrophages through the macrophage mannose receptor. Further studies are underway to determine the in vivo delivery capacity of milk-hGCase, but results from this study paves the way toward the generation of transgenic goats constitutively expressing hGCase in the milk.

Keywords Gaucher disease · Glucocerebrosidase · Adenovirus · Recombinant protein · Milk · Glycosylation

Introduction

Glucocerebrosidase (hGCase, β-glucosidase, glucosylceramidase, E.C. 4.2.1.25) is an ubiquitous lysosomal enzyme responsible for the cleavage of glucosylceramide in ceramide and glucose. Its deficiency characterizes Gaucher disease (GD), resulting in accumulation of glucosylceramide inside macrophages [1, 2]. GD is the most common hereditary lysosomal storage disorder, with macrophage accumulation mainly in liver, spleen, bones, and bone-marrow. More than 300 mutations and polymorphisms in the *GBA1* gene (1q21) are associated with GD, with a prevalence of 1:75,000 in general people and 1:600 carriers in Ashkenazi Jews [3].

There are three distinct manifestations of GD: Type 1, which constitutes 94 % of all cases, is considered non-neuropathic, while the less-frequent Types 2 and 3 manifest signs of neurological impairment [4]. Only Type 1 and non-neuropathic Type 3 GD are treatable, using preferably an enzyme replacement therapy (ERT) with bi-weekly intravenous infusion employing three commercial recombinant hGCase forms: imiglucerase (Cerezyme®, Genzyme Corporation, Cambridge, MA, USA), velaglucerase alfa (VPRIV®, Shire Human Genetic Therapies, MA, USA), and taliglucerase alfa (Elelyso®, Protalix Biotherapeutics, Carmiel, Israel). The reduced number of patients and the

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high cost of manufacture make recombinant hGCase one of the most expensive drugs commercially available [5].

Adenovirus is considered the most effective vector for gene delivery in vivo [6], representing 21.7 % of all the vectors used in gene therapy clinical trials in humans [7]. Despite concerns due to the strong host immune response against adenoviral proteins [8], E1- and E3-deleted regions replication-defective type 5 adenovirus remains the first choice as a delivery vector due to the elevated transduction capability in dividing and non-dividing cells and high rate of transgene expression [6]. Its construction involves simple DNA cloning steps, and high titers of recombinant adenoviruses can be obtained using E1-expressing cell lineages in 4–5 weeks [9].

The use of the mammary gland as a potential target for adenoviral vectors was first described by Yang et al. [10], resulting in the efficient intracellular delivery of the β-Galactosidase gene to the mammary epithelial cells both in vitro and in vivo. To date, six different proteins have been expressed through adenoviral infusion in the mammary gland: human growth hormone in mice [11] and goats [11, 12]; human erythropoietin in mice [14] and goats [15, 16]; human lactoferrin in goats [13] and rabbits [17]; human β-nerve growth factor in rabbits [18] and goats [19]; envelope glycoprotein E2 of classical swine fever virus in goats [20, 21]; and human anti-thrombin in goats [22] and rabbits [23]. The average time of recombinant protein secretion is 10–20 days, and levels up to 3.5–4.8 g/L have been reported [14, 23].

Considering the high levels of recombinant protein expression associated with the short time needed to obtain high titers of a recombinant adenoviral vector, this system offers a fast way to evaluate potential targets prior to the production of transgenic animals constitutively expressing recombinant proteins in the mammary gland [14], allowing for the full characterization of a desired protein in the milk of non-transgenic animals, saving time and money. In this study, a recombinant adenoviral vector was used to successfully produce hGCase both in vitro, in bovine mammary epithelial cells, and in vivo, in the milk of a non-transgenic goat. The identity of the protein was confirmed through mass spectrometry, and rhGCase in vitro activity was characterized using the β-glucocerebrosidase-specific 4-MUG cleavage assay [24].

Materials and Methods

Adenoviral Vectors Construction

A replication-defective type 5 adenovirus carrying the hGCase gene (rAV-hGCase) was generated using the AdEasy™ adenoviral vector system (Stratagene, USA).

Briefly, the hGCase cDNA (NM_001005741) was in vitro synthesized and ligated in the XhoI site of the pAdTrack-CMV vector (pAdT–hGCase). The resulting plasmid was recombined with the pAdEasy™ vector in BJ5183 electro-competent cells (Agilent, USA) resulting in the final vector pAdE-pAdT-hGCase containing the hGCase cDNA regulated by a CMV promoter, in tandem with the green fluorescent protein (GFP) gene, under another CMV promoter and the adenoviral genome with E1- and E3-deleted genes.

Primary and Virus Stock Generation

The purified pAdE-pAdT-hGCase vector was linearized with PacI to expose its ITRs (Inverted Terminal Repeats) and transfected into human embryonic kidney 293 cells (HEK-293; Life Technologies, USA) using Lipofectamine 2000 (Life Technologies, USA) for the production of the primary viral stock of hGCase recombinant adenovirus (rAV-hGCase). Further amplification in 60 confluent 10-cm dishes and the titration assay were done in HEK-293 cells cultured at 37 °C and 5 % CO_2, monitoring the transduction by the evaluation of the GFP expression.

In Vitro Expression of hGCase in HEK-293 and MAC-T Cells

Circular pAdT-hGCase and pAdE-pAdT-hGCase were transfected to HEK-293 cells using Lipofectamine 2000 (Life Technologies, USA) in a 6-well plate. After 72 h, the medium was assayed for hGCase expression to verify the expression capacity of both vectors.

Bovine mammary epithelial cells (MAC-T) [25] were cultured in 6-well plates under 5 % CO_2 at 38.5 °C in DMEM with 10 % fetal calf serum. When cell culture reached 90–95 % confluence, cells were transduced with rAV-hGCase in media without serum at a multiplicity of infection (MOI) of 25. Cells were monitored for GFP expression 24 h later, and the medium was collected and assayed for hGCase 72 h post-transduction.

In Vivo Expression of hGCase in the Milk of Non-transgenic Goats

A 10-month-old crossbred female goat was submitted to a hormonal protocol to induce lactation. The animal received estradiol (0.25 mg/kg) and progesterone (0.75 mg/kg) every other day during 13 days and daily doses of prednisolone (0.4 mg/kg) from days 14 to 16 associated with daily massage of the mammary gland until the start of lactation. Prior to adenovirus infusion, udders were milked and washed twice with phosphate-buffered saline (PBS) through the infusion in the teat canal using a catheter coupled to a syringe. The rAV-hGCase was infused in

EGTA at 36 mM in PBS at 1×10^9 GTU/mL. Milk was collected from day 0 (before rAV-hGCase infusion) until day 10 and stored at −80 °C until further analyses. Total milk proteins were measured using Qubit 2.0 (Invitrogen, USA), accordingly to manufacturer's instructions.

hGCase Detection and Quantification

Milk samples were mixed 1:1 (v/v) with protein extraction buffer (7 M urea, 0.1 M DTT, 0.05 % Triton X-100, 25 mM NaCl, 20 mM HEPES–NaOH at pH 7.6), centrifuged for 20 min at 4 °C at 15000×g, with the fat layer discarded afterward. Fifty micrograms of total proteins were separated in a 12 % SDS-PAGE, transferred to nitrocellulose membrane, and immunodetected with an anti-human β-glucosidase monoclonal antibody (sc166407, Santa Cruz Biotechnology, USA) and an anti-mouse HRP secondary antibody (NA931 V, GE Healthcare, USA). Blots were developed with ECL (GE Healthcare, USA) and exposed to an ECL Hiperfilm. Semi-quantification of hGCase in milk was determined by densitometry using ImageJ software v.148 [26], with a standard curve with different levels of imiglucerase (Cerezyme®, Genzyme Corporation, USA) in duplicate.

Mass Spectrometry

Fifty micrograms of total milk proteins from day 3 after injection and 1 μg of control glucocerebrosidase (imiglucerase, Cerezyme®) were separated through SDS-PAGE and stained with Coomassie Brilliant Blue. Milk from an induced and not injected goat was used as control. Excised gel bands were destained for 3 h in 50 % methanol/5 % acetic acid. In-gel digestion was performed according to Schevchenko et al. [27]. Peptide mixtures were chromatographically separated in a nanoLC Ultra 1D Plus system (Eksigent, USA) equipped with an AS-2 autosampler (Eksigent, USA) connected to a hybrid LTQ-XL Orbitrap Discovery mass spectrometer (Thermo Fischer Scientific, USA). Precolumns (150 μm internal diameter, one end blocked with a Kasil frit) and analytical capillary columns (100 μm internal diameter, one end pulled to a 5-μm-internal-diameter tip) were in house packed with 2 and 20 cm of reversed phase packing material (5 μm ODS-AQ C18, Yamamura Chemical Lab, Japan), respectively. Samples were loaded into the columns by the autosampler, with a flow rate of 1 μL/min for 15 min (10 μL injection volume). Chromatographic method with 120 min applied the following gradient of solvent A (5 % acetonitrile and 0.1 % formic acid in water) and B (90 % acetonitrile and 0.1 % formic acid in water): 0–5 % B in 5 min; 5–25 % B in 60 min; 25–50 % B in 20 min; 50–80 % B in 15 min; 80 % B isocratic for 5 min; 80–5 % B in 1 min; 5 % B

isocratic for 14 min with a flow rate of 400 nL/min. Samples were electrosprayed directly from the tip of the analytical column in a nano-electrospray ion source (Thermo Fischer Scientific, USA). LTQ-XL Orbitrap Dicovery instrument was operated in a data-dependent mode. Full MS1 scans were collected in the Orbitrap, with a mass range of 400–1600 m/z at 30,000 resolution. The eight most abundant ions per scan were selected to CID MS2 in the Ion trap. Dynamic exclusion was enabled with a repeat count of 1, repeat duration of 30 s, exclusion list of 100, and exclusion duration of 30 s.

Tandem mass spectra were analyzed using PatternLab for Proteomics platform [28]. MS2 spectra were searched with the Comet software [29], using a non-redundant database containing forward and reverse sequences from *Capra hircus* reference proteome (obtained at http://www. uniprot.org) and the sequence of human glucocerebrosidase (SP|P04062|GLCM_HUMAN). The search space included all fully tryptic and half-tryptic peptide candidates. Carbamidomethylation on cysteine was used as static modification. Data were searched with 50 ppm precursor ion tolerance and 1 Da fragment ion tolerance. The validity of the peptide spectrum matches (PSMs) generated by Comet was assessed using Search Engine Processor (SEPro; 28). Identifications were grouped by charge state and tryptic status, and a Bayesian discriminator was generated using Comet XCorr, DeltaCN, DeltaMass, ZScore, number of peaks matched, and Spec Count Score values. A cutoff score was established to accept a FDR of 1 % based on the number of decoys. A minimum sequence length of six residues was required, and results were post-processed to only accept PSMs with <10 ppm precursor mass error.

hGCase In Vitro Activity Assay

Recombinant hGCase activity was assayed using 4-methylumbelliferyl glucopyranoside (4-MUG; Sigma-Aldrich, USA) as substrate. Reactions were performed using 10 μL of milk in 190 μL of 100 mM potassium phosphate buffer, pH 5.0, containing 0.15 % (w/v) Triton X-100 (Sigma) and 0.125 % (v/v) taurocholate (Sigma) in the presence of 3 mM 4-MUG, for 60 min at 37 °C. Reactions were stopped by the addition of 1 mL of 0.1 M glycine, 0.1 M NaOH, pH 10. The amount of 4-MUG was recorded with a Spectrofluorophotometer RF5103 PC (Shmimadzu, Japan), with 340 nm excitation and 448 nm emission wavelengths [30].

Data Analysis

Data regarding milk total protein and hGCase concentration were analyzed using the Tukey's test, for $P < 0.05$.

Graphs and statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software, Inc.).

Results

Virus Generation

Adenoviral DNA was produced by recombining the vectors pAdT-hGCase, which contains the human glucocerebrosidase cDNA under the control of a CMV promoter plus a GFP reporter under another *in tandem* CMV promoter, with pAdEasy™ vector, that accommodates the adenovirus serotype 5 genome and has double deletion for *E*1 and *E*3 genes. rAV-hGCase was packaged into the *E*1-expressing HEK-293 cells and the final titer of 2.5×10^9 GTU/mL was obtained through viral propagation in sixty confluent 10-cm cell culture dishes.

In Vitro Expression of hGCase

To verify the correctness of the plasmid constructs containing hGCase, both circular pAdT-hGCase and pAdE-pAdT-hGCase were transfected into HEK-293 cells and the medium assayed for hGCase expression. Both plasmids promoted transgene expression, as shown in Fig. 1a. To

access the capability of mammary epithelial cells to produce hGCase through adenoviral transduction in vitro, bovine MAC-T cells were infected with rAV-hGCase at a MOI of 25. Cells showed high GFP expression 24 h after transduction (Fig. 1b) and hGCase was detected in the culture medium 72 h later in three sizes: 61.1, 65.1 and 68.8 kDa, probably correspondent do different glycosylation patterns (Fig. 1c).

In Vivo Expression of hGCase

The mammary gland of a 10-month-old non-transgenic goat was in vivo transduced with 36 mM EGTA in PBS containing 1×10^9 GTU/mL of rAV-hGCase via the teat duct. Total milk proteins were daily measured, and the levels were significantly higher ($P < 0.05$) on days 1 and 2 than the other days (Fig. 2a). Milk production decreased 56 % in the first day and 70 % in the second day post-infection, but returned to the same level as before rAV-hGCase inoculation on day 3, increasing this day forward (Fig. 2b). A specific band of 63.3 kDa corresponding to hGCase was detected in the milk 24 h post-injection (Fig. 2c), reaching its maximal level on day 2 (111.1 ± 8.1 mg/L), and was nearly undetectable from day 6. No signs of clinical impairment or distress were
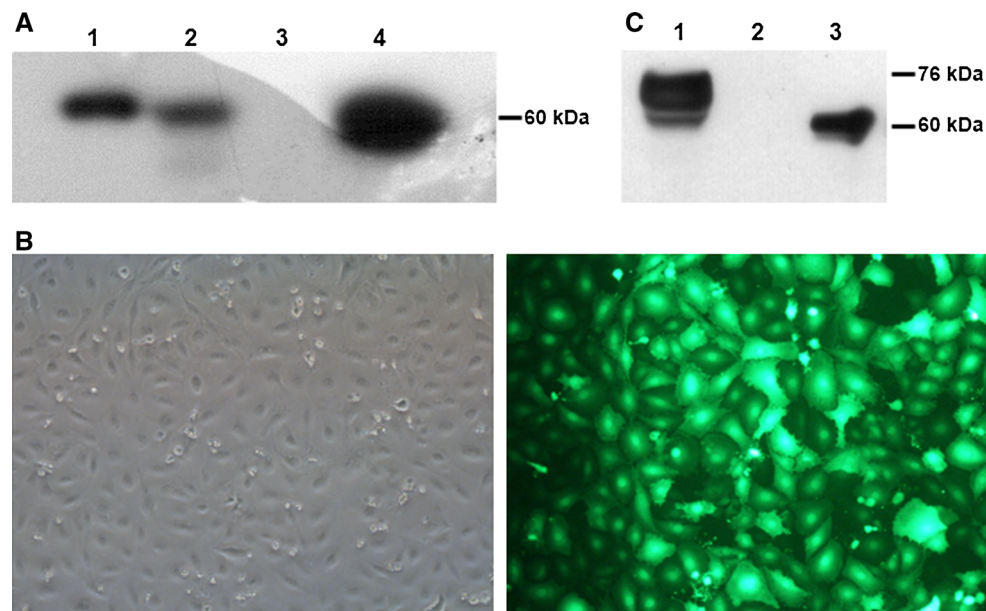

Fig. 1 In vitro expression of hGCase in HEK-293 and MAC-T cells. **a** Culture media from HEK-293 cells transfected with plasmid DNA from rAV-hGCase after 3 days post-transfection. *Lane 1* pAdT–hGCase, *lane 2* pAdE–pAdT–hGCase, *lane 3* mock transfected cells, *lane 4* imiglucerase (Cerezyme®, Genzyme Corporation, Cambridge, MA, USA). **b** GFP expression of MAC-T cells transduced with rAV-hGCase at a MOI of 25, 24 h after transduction. Cells under bright field (*left*) and fluorescent UV-light (*right*). **c** Culture media from MAC-T cells transduced with rAV-hGCase (MOI of 25), 72 h post-transduction, showing three distinct glycosylation patterns for adenovirus-produced hGCase. *Lane 1* rAV-hGCase, *lane 2* non-transduced cells, *lane 3* imiglucerase (Cerezyme®, Genzyme Corporation, Cambridge, MA, USA)
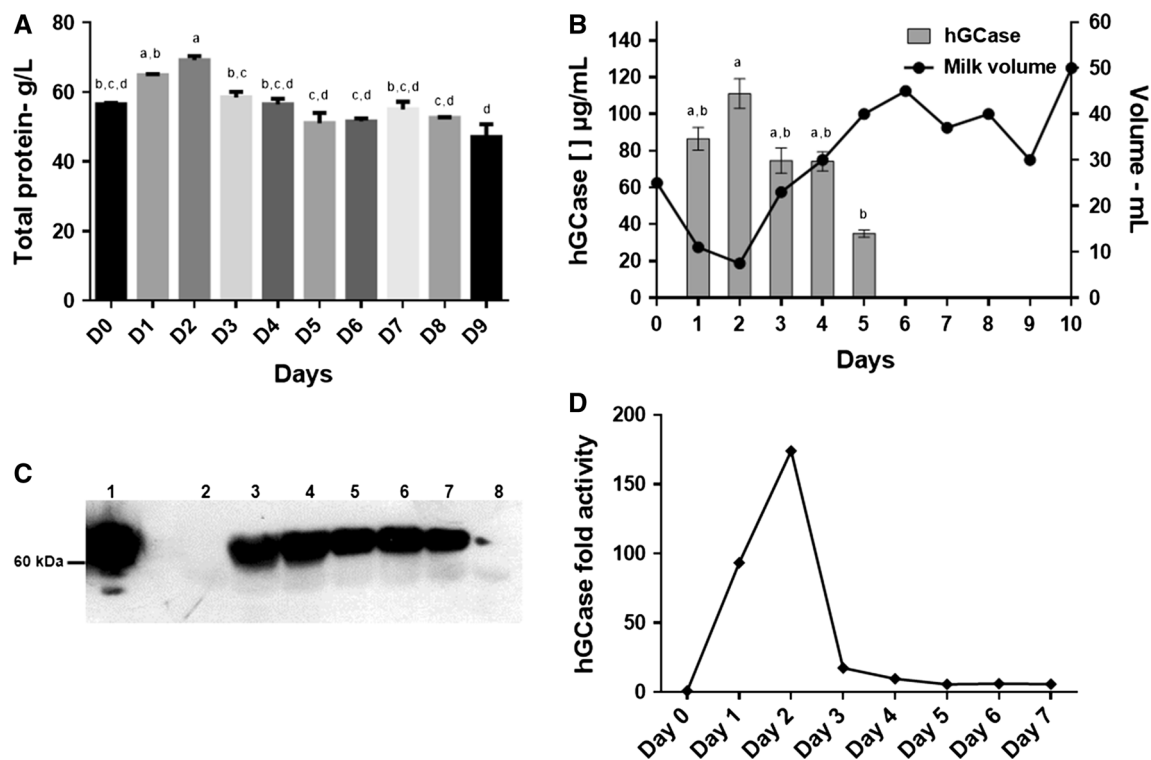
Fig. 2 Expression of hGCase in the mammary gland of a non-transgenic goat. A solution of PBS-EGTA containing an adenovirus harboring the hGCase gene was injected through the teat duct, and the milk collected during the next 10 days. a Total milk proteins concentration before (D0) and after (D1–D9) rAV-hGCase intra-mammary injection. Columns with different superscripts differ, for $P < 0.05$. b Milk secretion and hGCase concentration after rAV-hGCase transduction of the mammary gland. Columns with different superscripts differ, for $P < 0.05$. c Western blot showing the hGCase-specific band in the milk. Lane 1 imiglucerase (Cerezyme®, Genzyme Corporation, Cambridge, MA, USA), lane 2 milk from day 0 (before adenovirus injection), lanes 3–8 milk from days 1 to 6 after transduction. d hGCase in vitro activity using the 4-MUG cleavage assay in the milk

observed in the animal during the course of the experiment (data not shown).

Mass Spectrometry

To further confirm the identity of the milk-expressed recombinant hGCase, the band corresponding to hGCase in day 3 milk was analyzed through LC–MS. Human glucocerebrosidase was identified, with a mass of 59660.2 Da (without post-translational modifications; Fig. 3a), corresponding to the migration pattern observed in the Western blot, and a coverage of 35.26 % (Fig. 3b). The hGCase isolated in the gel band was not purified, co-migrating mainly with goat albumin and butyrophilin.

hGCase In Vitro Activity

The presence of an active form of the milk-expressing glucocerebrosidase was measured through the 4-methy-lumbelliferyl-β-D-glucopyranoside, which specifically detects hGCase activity due to fluorescence release in the presence of sodium taurocholate [24]. The transduction of

the mammary gland with rAV-hGCase produced an active enzyme, with an increase up to 173 times on hGCase activity comparing to basal levels (Fig. 2d), reached on day 2 post-infection.

Discussion

Extensively applied as an efficient delivery system for gene therapy in humans and animals, adenovirus has been used to target the mammary gland of animals over the past two decades [10]. Its use as an effective recombinant protein expression platform in the mammary gland is more recent, starting with the recombinant human growth hormone expression in mice and goats described by Sanchez et al. [11]. Considering the successful expression of hGH in non-transgenic milk, six different recombinant proteins were produced applying this system using mice, rabbits, and goats as hosts [11, 17, 21]. In this study, the expression of human glucocerebrosidase is described in bovine mammary epithelial cells and in the milk of a non-transgenic goat using a recombinant adenoviral vector (Fig. 4).

A

Locus	MolWt	#Unique Pep	#SeqCount	#SpecCount	Coverage	Description
tr|B3VHM9|B3VHM9_CAPHI	66251.8	97	166	1321	0.8182	Albumin (Fragment)
tr|A3EY52|A3EY52_CAPHI	59209.8	30	30	124	0.3897	Butyrophilin subfamily 1 member A1
sp|P04062|GLCM_HUMAN	**59660.2**	**29**	**29**	**93**	**0.3526**	**Glucosylceramidase**

B

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001  MEFSSPSREE  CPKPLSRVSI  MAGSLTGLLL  LQAVSWASGA  RPCIPKSFGY  SSVVCVCNAT  060
061  YCDSFDPPTF  PALGTFSRYE  STRSGRRMEL  SMGPIQANHT  GTGLLLTLQP  EQKFQKVKGF  120
121  GGAMTDAAAL  NILALSPPAQ  NLLLKSYFSE  EGIGYNIIRV  PMASCDFSIR  TYTYADTPDD  180
181  FQLHNFSLPE  EDTKLKIPLI  HRALQLAQRP  VSLLASPWTS  PTWLKTNGAV  NGKGSLKGQP  240
241  GDIYHQTWAR  YFVKFLDAYA  EHKLQFWAVT  AENEPSAGLL  SGYPFQCLGF  TPEHQRDFIA  300
301  RDLGPTLANS  THHNVRLLML  DDQRLLLPHW  AKVVLTDPEA  AKYVHGIAVH  WYLDFLAPAK  360
361  ATLGETHRLF  PNTMLFASEA  CVGSKFWEQS  VRLGSWDRGM  QYSHSIITNL  LYHVVGWTDW  420
421  NLALNPEGGP  NWVRNFVDSP  IIVDITKDTF  YKQPMFYHLG  HFSKFIPEGS  QRVGLVASQK  480
481  NDLDAVALMH  PDGSAVVVVL  NRSSKDVPLT  IKDPAVGFLE  TISPGYSIHT  YLWRRQ
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Fig. 3 Mass spectrometry analysis of the milk-produced hGCase. The specific band of hGCase (63.3 kDa) was excised, submitted to in-gel digestion, and analyzed by LC–MS. **a** Top three proteins identified in the excised gel band, with their respective molecular weights, peptide, sequence and spectral counts, and sequence coverages. **b** Sequence coverage representation of hGCase (in *blue*)
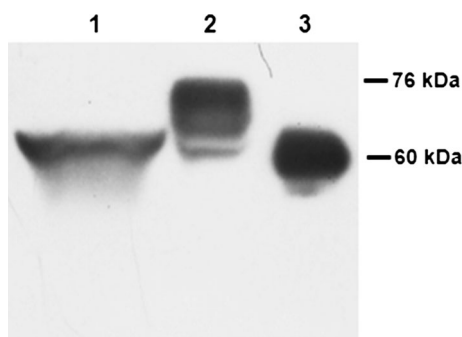

Fig. 4 Western blot showing the different sizes of the hGCase, probably correlated with its glycosylation patterns. *Lane 1* hGCase produced in the milk through intramammary adenovirus injection, with a size of 63.3 kDa, *lane 2* hGCase expressed in MAC-T cells, showing three distinct bands of 61.1, 65.1, and 68.8 kDa, *lane 3* imiglucerase (Cerezyme®, Genzyme Corporation, Cambridge, MA, USA), with a size of 60 kDa due to its remodeled glycosylation moieties

To access the viability of the constructed DNA vectors to produce hGCase, we first transfected in HEK-293 cells both the shuttle vector pAdTrack-CMV and the recombined pAdTrack-CMV with adenovirus backbone vector pAd-Easy with hGCase cDNA, resulting in a 63.3 kDa protein expressed in the cell culture medium. We then proceeded with adenovirus packaging, amplification and transduction of bovine MAC-T cells intending to verify the transduction efficiency and recombinant protein expression of hGCase in mammary epithelial cells. The rAV-hGCase

showed high transduction efficiency, as practically all the cells became green 24 h after transduction. The Western blot showed three distinct band patterns of 61.1, 65.1, and 68.8 kDa corresponding to hGCase. The three proteoforms could be attributed to differential glycosylation resulting from hGCase maturation inside the cell: immediately after synthesis, the protein has a high-mannose type oligosaccharides, with a molecular weight of 64 kDa; in the Golgi apparatus, the addition of complex-type oligosaccharides with sialic acid terminal residues can occur, increasing its molecular weight to 69 kDa; and finally in the lysosome, the protein is processed by exoglycosidases to its mature form of 59 kDa [31, 32]. Our findings are in accordance to previous studies, which also found two or three glycosylation types of hGCase when expressing it in GSK [33] and CHO [34] cells.

A total of 1×10^9 GTU/mL of adenovirus was injected in the mammary gland of a non-transgenic goat. This titer was defined based on previous publications comparing different titers that developed the highest protein expression using 1×10^9 GTU/mL in goats [11, 13] and 4×10^8 GTU/mL in rabbits [17, 18]. Likewise, it is well established that in the mammary gland EGTA causes a temporary disruption of the epithelial tight junctions, allowing adenovirus to enter in the mammary epithelial cells through CAR in the basolateral surface of the cells [15]. Total milk proteins were higher in the first two days post-infection ($P < 0.05$), probably due to the increased expression of hGCase, GFP, and adenoviral proteins along with increased

immune response against adenoviral vector [8]. The milk yield of the transduced udder was reduced only in days 1 and 2, probably due to a small inflammatory response resulted from udder washing and virus inoculation, as no signs of mastitis was observed, which generally results in severe milk yield reduction in affected udders [35, 36].

The protein hGCase was secreted in the milk as soon as 24 h after viral inoculation, and its expression was detected during 6 days (not quantifiable on day 6, but a faint band was present in the Western blot). All the previous reports of adenovirus injection in the mammary gland of goats related the duration of expression between 7 and 25 days [11, 13, 15, 16, 19–22]. Despite differences in duration, levels of recombinant proteins generally drop significantly after day 6 post-injection [13, 20], what could be explained by the high immune response against E1–E3 adenoviral particles [8].

A peak of expression of 0.11 g/L was reached on day 2, the day with the smallest milk production and highest total protein content ($P < 0.05$). Although higher levels of recombinant protein expression were achieved in previous publications using adenovirus-mediated transient expression [15, 16, 22], similar levels were observed in the expression of human growth hormone in goats [11] and human nerve growth factor beta in rabbits [18] and goats [19]. Lower expression levels of recombinant hGCase compared with other proteins in CHO cells were reported by Novo et al. [34], which also discussed the scarce data about hGCase expression in CHO cells and associate the low-level expression to the fact that hGCase is a membrane-associated glycoprotein with absent or low-level secretion under natural conditions.

Despite the fact that the levels of protein obtained in this study are not sufficient to propose it as an alternative to the in vitro cell culture-based sources of hGCase currently available (CHO cells for Cerezyme®, carrot cells for Elelyso®, or human fibroblasts for VPRIV®), the system was adequate to characterize the protein identity through mass spectrometry and in vitro activity using the 4-MUG cleavage assay. These data confirmed that it was possible to produce an active hGCase in goat's milk, which has stimulated our group to produce a transgenic cloned goat constitutively expressing hGCase in its milk (Martins et al. under revision).

Recombinant hGCase administered to Gaucher patients needs to be taken up by macrophages. This usually happens through the Trojan horse macrophage mannose receptor (MMR), due to an interaction with α-mannosyl residues that need to be exposed in the glycoprotein [37]. When expressed in CHO cells, imiglucerase (Cerezyme®) is glycosylated with terminal complex oligosaccharides, which overlaps its α-mannosyl residues, requiring further enzymatic deglycosylation to sequentially remove the terminal sialic acid, galactose, and N-acetyl-glucosamine sugars and improve its intracellular delivery [38] Interestingly, the milk-derived

hGCase showed a SDS-PAGE migration profile containing an unique band of 63.3 kDa, higher than the 60 kDa band of imiglucerase, and similar to the 64 kDa hGCase described as high-mannose intermediate [31, 32]. This profile could be attributed to the differential glycosylation pattern of the mammary gland that tends to produce higher high-mannose N-glycosylation than complex-type oligosaccharide N-glycans, as observed in recombinant lactoferrin produced in cow's milk [39] and in the only two transgenic milk-derived recombinant proteins approved to human use in Europe and USA: anti-thrombin III (ATryn®, REVO Biologics, USA) [40] and the human C1 esterase inhibitor (Ruconest®, Pharming, Holland) [41]. Differences in the glycosylation pattern between in vitro and in vivo expressed proteins were described in studies with recombinant human erythropoietin (rhEPO) produced through adenovirus transduction in mammary epithelial cells in mice [14] and goats [15]. In both studies, adenovirus-transduced mammary epithelial cells showed a heavily glycosylated rhEPO when kept in vitro (both in a cell line as in a primary isolate) and a lower extent glycosylation profile when expressed in vivo in the mammary gland, due to a lower sialic acid content and O-glycosylation sites occupancy in the milk active mammary gland, possibly a result from the saturation of the glycosylation machinery with milk proteins. On the other hand, this "fail" in the glycosylation machinery can be extremely useful for hGCase produced in the mammary gland, which has a molecular weight similar to the observed in human fibroblasts after the incorporation of the high-mannose type oligosaccharides [32]. The trend for the mammary gland to introduce high-mannose and low-sialic acid N-glycans could facilitate the direct recognition of hGCase by the MMR, or at least decrease the deglycosylation steps necessary to expose its mannose residues. Further studies are necessary to confirm the glycosylation profile of rAV-hGCase and to address its capacity to penetrate macrophages through its mannose receptor.

Compliance with Ethical Standards

Ethical approval All animal care and use were conducted in strict accordance with the Animal Research Committee guidelines of University of Fortaleza.

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