

Rapid report

Expression of cDNA-encoded human acid α -glucosidase in milk of transgenic mice

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Abstract

Enzyme replacement therapy is at present the option of choice for treatment of lysosomal storage diseases. To explore the feasibility of lysosomal enzyme production in milk of transgenic animals, the human acid α -glucosidase cDNA was placed under control of the α_{S1} -casein promoter and expressed in mice. The milk contained recombinant enzyme at a concentration up to 1.5 μ g/ml. Enzyme purified from milk of transgenic mice was internalized via the mannose 6-phosphate receptor and corrected enzyme deficiency in fibroblasts from patients. We conclude that transgenically produced human acid α -glucosidase meets the criteria for therapeutic application.

Keywords: Enzyme replacement therapy; Lysosomal storage disease; Recombinant enzyme

Transgenic production of therapeutic proteins in mammalian milk can be an attractive strategy to provide low cost treatment for rare diseases [1,2]. We have investigated whether this strategy, originally designed for the production of secretory proteins, is also suitable for the manufacturing of lysosomal enzymes to be used for enzyme replacement therapy for lysosomal storage diseases. The production of human acid α -glucosidase was taken as prototype. Deficiency of acid α -glucosidase (acid maltase; EC 3.2.1.3) leads to glycogen storage disease type II (GSD II; Pompe disease; acid maltase deficiency), an autosomal recessive disorder with an estimated incidence of 1 in 100 000 in the Caucasian population and 1 in 50 000 in southern China and Taiwan [3]. The disease is clinically and genetically heterogeneous. The common clinical feature is generalized muscle weakness. In the severe early onset cases also the heart is involved.

A therapy for GSD II is not available yet, but there is

optimism about the applicability of receptor-mediated enzyme replacement therapy [4–9]. The receptor aimed at is the mannose 6-phosphate receptor which is crucial for the intracellular targeting of most lysosomal enzymes and for the endocytosis and lysosomal delivery of exogenous mannose 6-phosphate containing (lysosomal) proteins [10]. In the past, we have demonstrated correction of lysosomal glycogen storage in cultured fibroblasts and muscle cells of GSD II patients after uptake of mannose 6-phosphate containing acid α -glucosidase via this receptor [4,5]. Moreover, uptake was demonstrated in heart and skeletal muscle of mice receiving acid α -glucosidase intravenously [7]. Similarly, administration of mannose 6-phosphate containing β -glucuronidase [11] and α -L-iduronidase [12] to animals deficient in these enzymes remedied associated clinical features. Most of all, we were encouraged by the therapeutic efficacy of enzyme replacement therapy for Gaucher disease type I aimed at the mannose-receptor [13]. Thus, we have set out to develop methods for production of human recombinant, mannose 6-phosphate containing, acid α -glucosidase [8,9].

We present a first evaluation of transgenic production

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of human mannose 6-phosphate containing lysosomal enzymes in milk. The method takes advantages of an expression vector developed to target expression of foreign secretory proteins to the mammary glands of mice, rabbits, sheep, goats and pigs [1,2]. For our pilot studies we have used mice as model system.

To obtain lactation-specific expression of a human acid α -glucosidase transgene, we inserted the human acid α -glucosidase cDNA [14] in an expression cassette derived from the regulatory sequences of the bovine α_{S1} -casein gene [15,16]. The 25.5-kb *NotI* expression module (Fig. 1) excised from plasmid p16,8 α glu was microinjected into pronuclei of fertilized mouse oocytes (CBA/BrA \times C57Bl/6), according to Hogan et al. and Platenburg et al. [16,17]. Using Southern blot analysis of genomic DNA prepared from tail biopsies of the newborn mice, 11 transgenic mice were identified. Ten of these founder mice transmitted their transgenes in a Mendelian fashion, one did not. The copy number of the transgene in the 10 mouse lines was estimated to vary from five to twenty, comparing the intensities of the hybridization signals on mouse-tail DNA-blot using the human acid α -glucosidase cDNA as probe (data not shown).

The presence of human recombinant acid α -glucosidase in the milk of transgenic mice was demonstrated with a mouse polyclonal antiserum recognizing specifically the human and not the murine isoform [18]. Of the ten lines tested (Table 1), three were positive (#1672, #1673 and #1676). Line #1672 had the highest activity of 446 nmol MU/ml/h at midlactation, equivalent to 1.5 μ g acid α -glucosidase/ml. Expression levels were not related to the copy-number of the transgene, which suggests integration site-dependent transcription. In agreement with the activity data, human recombinant acid α -glucosidase was detected in the milk of lines #1672, #1673 and #1676 by immunoprecipitation followed by immunoblotting (Fig. 2). Panel A shows specifically the human isoforms, whereas both human and murine isoforms are shown in panel B.

Table 1

The activity of human recombinant acid α -glucosidase in the milk of transgenic and non transgenic animals

Line	α -Glucosidase activity ^a (nmol MU/ml/h)	(n)	Estimated amount ^b (μ g/ml)
1672	446	(5)	1.5
1673	162	(3)	0.5
1676	28	(6)	0.09
others	≤ 20	(21)	–
n.t.	≤ 20	(5)	–

^a Milk samples were collected with a hand-made milking apparatus 10 minutes after subcutaneous injection of 1 unit oxytocin (PitonS, Organon) and stored at -20°C until use. The acid α -glucosidase activity in the milk samples was determined with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside [18]. Milk samples were adjusted to an activity of 10 nmol 4 methylumbelliferone (MU)/h in a final volume of 200 μ l, by adding 10 mM sodiumphosphate pH 6.6, containing 0.9% NaCl and 1 mg/ml bovine serum albumin (PBS), and were incubated with protein A Sepharose beads (Pharmacia) to pre-absorb immunoglobulins. Thereafter, the recombinant acid α -glucosidase was precipitated with antibodies raised in mice and coupled to protein A Sepharose beads, precipitating human but not murine acid α -glucosidase. The activity of human acid α -glucosidase in the milk was calculated from the activity measured on the Sepharose beads with the artificial substrate as described [7,18]. ^b The amount of human acid α -glucosidase in the mouse milk was calculated based on a specific enzyme activity of 300 μ mol MU/mg/h [19]. (n): number of milk samples; others: includes transgenic lines #1671, 1675, 1677, 1678, 1679, 1680 and 1692; n.t.: non-transgenic lines.

The human acid α -glucosidase in the mouse milk is present in two isoforms with an apparent molecular mass of 110 and 76 kDa, like the precursor and mature forms of acid α -glucosidase present in human tissues. The murine isoforms are 95 and 73 kDa (Fig. 2B).

Expression of the human acid α -glucosidase transgene was mammary gland-specific and lactation-dependent as illustrated in Fig. 3 for the mammary gland, spleen, heart and skeletal muscle. Also no expression of the transgene was detected in liver, kidney, brain, lacrimal glands, sali-

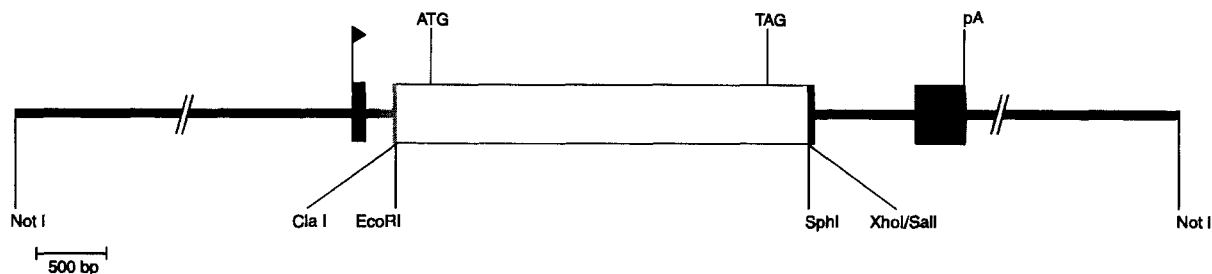


Fig. 1. Physical map of the 25.5 kb mammary gland-specific expression module for human acid α -glucosidase. To make this module, the acid α -glucosidase cDNA was excised from 2pSHAG2 [14] with *EcoRI* and *SphI* and subcloned in pKUN7 Δ C, a pKUN1 derivative [22]. From this plasmid the 3.3-kb cDNA-fragment was excised with *ClaI* and *XhoI* and inserted into the *ClaI* site and *XhoI*-compatible *SaI* site of the plasmid p16,8hF3ks replacing the lactoferrin cDNA [16]. The resulting plasmid p16,8 α glu consists of the human acid α -glucosidase cDNA flanked upstream by 14.2 kb of sequences, containing the promoter and untranslated first exon of the bovine α_{S1} -casein gene, and a hybrid α_{S1} -casein/immunoglobulin G intron [23]. The cDNA is flanked downstream by 8 kb of 3' bovine α_{S1} -casein sequences including the polyadenylation signal. Black boxes represent non-translated bovine α_{S1} -casein exons. Other bovine α_{S1} -casein sequences are indicated with a bold line. The hatched line and box represent immunoglobulin G intron and exon sequences. The open box represents the human acid α -glucosidase cDNA. The transcription initiation site (flag), the translational start site (ATG), the stop codon (TAG), and the polyadenylation site (pA) are indicated.

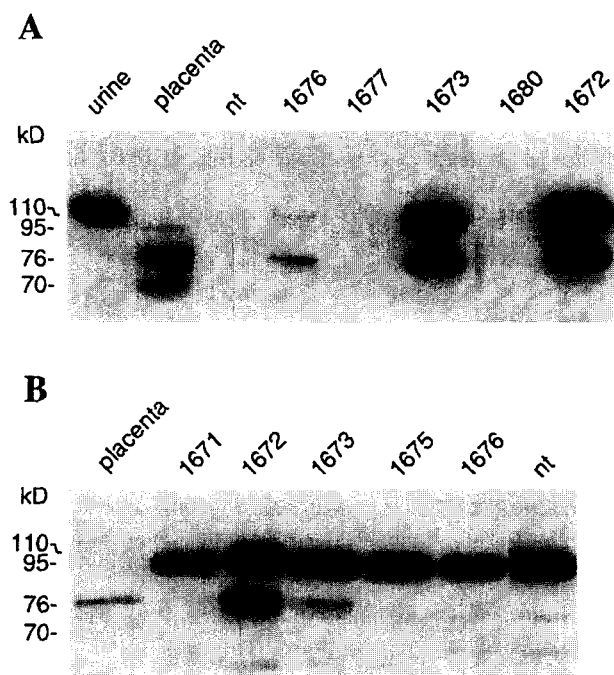


Fig. 2. Immunoblot analysis of acid α -glucosidase from milk of transgenic mice. The milk samples were adjusted to an acid α -glucosidase activity of 100 nmol MU/h in a final volume of 1 ml by adding 10 mM PBS and were cleared of immunoglobulins (see the legend of Table 1 for details). Acid α -glucosidase was subsequently precipitated with antibodies raised against human placental acid α -glucosidase complexed to protein A Sepharose beads. The Sepharose beads with the enzyme-immunoglobulin complex bound to it were boiled in sample buffer and applied to SDS-PAGE as described [19]. The proteins were subsequently blotted onto nitrocellulose filters and acid α -glucosidase was visualized on X-ray film after incubating the blots with rabbit anti-human placental acid α -glucosidase antibodies coupled to 125 I-protein A. Acid α -glucosidase purified from human urine (110 kDa) and human placenta (76 and 70 kDa) served as molecular mass markers. Panel A shows the human acid α -glucosidase isoforms immunoprecipitated with antibodies raised in mouse, not recognizing the mouse isoforms. Panel B shows both the human and the mouse isoforms immunoprecipitated with antibodies raised in rabbit, recognizing the human as well as the murine acid α -glucosidase isoforms. The numbers above the lanes refer to the transgenic lines from which the samples were taken; n.t. is a sample from a non-transgenic mouse. The non-expressing lines were chosen randomly.

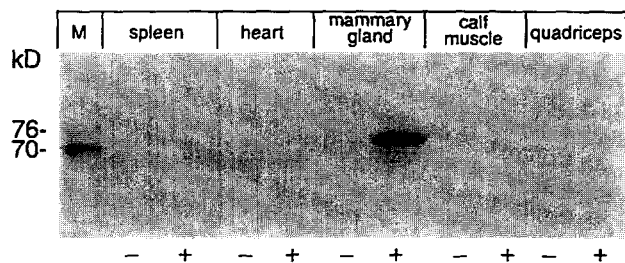


Fig. 3. Immunoblot analysis of tissue and lactation-specific expression of the human acid α -glucosidase transgene. Various tissues from a lactating (8 days post-partum) (+) and a non lactating (-) mouse of line #1672 were homogenized as described [7], and the human acid α -glucosidase isoforms were immunoprecipitated with antiserum raised in mice against human placental acid α -glucosidase as described in the legends of Fig. 2. Acid α -glucosidase purified from human placenta was applied in the first lane (M) to serve as molecular mass marker.

Table 2

Uptake of acid α -glucosidase purified from the milk of transgenic mice by enzyme-deficient fibroblasts

Celline	Additions enzyme	M6P	Acid α -glucosidase activity (nmol MU/mg/hr)	(n)
Normal	-	-	145	(12)
Deficient	-	-	≤ 0.1	(4)
Deficient	+	-	676	(8)
Deficient	+	+	5.5	(4)

Acid α -glucosidase was isolated from the milk of transgenic mice. The enzyme was added to the culture medium of enzyme deficient fibroblasts in a concentration equivalent to 1.4 μ mol MU/h in 1 ml of Ham's F10 medium supplemented with 10% fetal bovine serum, 3 mM PIPES and antibiotics (as described [24]), in the presence or absence of 5 mM mannose 6-phosphate (M6P). The cells were harvested after 22 h and homogenized. The acid α -glucosidase activity was determined with the artificial substrate 4-methylumbelliferyl- α -D-glucopyranoside. Protein concentrations were determined using the BCA protein assay (Pierce). (n) = number of assays.

vary glands, and lung (data not shown). The molecular mass of the recombinant enzyme in the mammary gland is the same as of mature lysosomal acid α -glucosidase in human tissues (76 kDa). All transgenic mice appeared healthy and reproduced normally.

To test the applicability of the recombinant acid α -glucosidase for enzyme replacement therapy, the enzyme was purified from transgenic mouse milk, essentially as described before [19], and added to culture medium of acid α -glucosidase deficient fibroblasts. Table 2 shows that the acid α -glucosidase activity of the deficient fibroblasts increased above control level after overnight incubation. Endocytosis was mediated by the mannose 6-phosphate receptor, since no corrective effect was obtained when the milk enzyme was administered in the presence of 5 mM mannose 6-phosphate.

In conclusion, the human recombinant acid α -glucosidase produced in the mammary gland of transgenic mice exhibits the proper characteristics for use in enzyme replacement therapy for GSD II and is an alternative for enzyme production in CHO-cells [8,9]. It is obvious, however, that higher expression levels of the recombinant enzyme in milk are needed for the industrial production of therapeutic enzyme. A way to increase the expression level is to use genomic rather than cDNA constructs [20]. Our preliminary results indicate that the expression can be upgraded by at least 100-fold by using a genomic construct [21]. We are encouraged to fully explore the possibilities to produce mannose 6-phosphate containing lysosomal enzymes in the milk of transgenic animals for treatment of lysosomal storage disease.

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