Correction of mucopolysaccharidosis type IIIB fibroblasts by lentiviral vector-mediated gene transfer

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Mucopolysaccharidosis type IIIB (MPS IIIB; or Sanfilippo syndrome type B) is a lysosomal disease, due to glycosaminoglycan storage caused by mutations on the α -N-acetylglucosaminidase (NAGLU) gene. The disease is characterized by neurological dysfunction but relatively mild somatic manifestations. No effective treatment is available for affected patients. In the present study, we evaluated the role of a lentiviral vector as the transducing agent of NAGLU cDNA in MPS IIIB fibroblasts. The vector expressed high transduction efficiency and high levels of enzymic activity, 20-fold above normal levels, persisting for at least 2 months. PCR experiments confirmed the integration of the viral vector into the target genome. The NAGLU activity restored by virus infection was sufficient to normalize glycos-

aminoglycan accumulation, which is directly responsible for the disease phenotype. Metabolic labelling experiments on transduced fibroblasts exhibited, in the medium and in cellular lysates, polypeptide forms of 84 and 80 kDa respectively related to the precursor and mature forms of the enzyme. The enzyme secreted by transduced MPS IIIB fibroblasts was endocytosed in deficient cells by the mannose 6-phosphate system. Thus we show that lentiviral vectors may provide a therapeutic approach for the treatment of MPS IIIB disease.

Key words: α -N-acetylglucosaminidase, lysosomal diseases, Sanfilippo B syndrome, third-generation lentiviral vector.

INTRODUCTION

Mucopolysaccharidosis III (MPS III) or Sanfilippo syndrome results from the absence of one of four lysosomal enzymes that are sequentially involved in the degradation of glycosaminoglycan (GAG) heparan sulphate: heparan *N*-sulphatase (MPS IIIA), α -N-acetylglucosaminidase (NAGLU; MPS IIIB), acetyl-CoA: α -glucosaminide-*N*-acetyltransferase (MPS IIIC) or *N*-acetylglucosamine 6-sulphatase (MPS IIID) [1]. Each MPS III subtype is inherited as an autosomal recessive disorder. The clinical presentation of MPS III is predominantly characterized by severe dysfunction of the central nervous system (CNS) resulting in progressive mental retardation. The mechanism by which heparan sulphate storage leads to CNS degeneration is not known.

MPS IIIB results from an unsuccessful removal of the *N*-acetylglucosamine residues from heparan sulphate due to a deficiency in NAGLU (EC 3.2.1.50). The consequence is an accumulation of this GAG in tissues of affected subjects and its abnormal urinary excretion. Patients are characterized by hyperactivity, aggressive behaviour and severe CNS degeneration [1]. Affected children, usually diagnosed at 2–8 years of age, were found to suffer from mental retardation progressing throughout childhood, sleep disorders, hepatosplenomegaly, hirsutism, vasomotor instability, dysmorphism, dysostosis and delayed development. Death occurs, typically, during the second or third decade of life, although patients with a more attenuated phenotype have been reported.

NAGLU protein has been purified from urine [2], liver [3] and placenta [4]. The enzyme biosynthesis has been studied in cultured fibroblasts [5] and in cultured kidney cells [6] showing a precursor form of 84 kDa, which progresses after processing to a mature form of 80 kDa. The gene encoding NAGLU has been characterized [4,7] as follows: it is located on chromosome 17q21.1, is approx. 8.5 kb in length and contains six exons coding for a protein of 743 amino acids. The isolation and characterization of the NAGLU genomic structure has made it possible to start mutational analysis. So far more than 80 defects have been elucidated, showing genetic heterogeneity [7–13]. However, only a few alterations have been reproduced by site-directed mutagenesis and expressed *in vitro* [8,13].

Recombinant wild-type NAGLU has been expressed and characterized in CHO-K1 cells [14–16] and COS cells [13] and shown to be synthesized as a 84–83 kDa precursor polypeptide which is cleaved to a 80–77 kDa mature protein. The recombinant enzyme has been reported to be poorly mannose 6-phosphorylated, compared with other lysosomal enzymes such as α -L-iduronidase and *N*-acetylgalactosamine 4-sulphatase [14–16]. A murine MPS IIIB model showing the chronic and progressive course of the human disease has recently been made by disrupting the NAGLU gene in exon 6 [17]. This model will be very valuable for studies on the pathogenesis and for the development of therapies for the MPS IIIB disease.

A number of viral vectors have been proposed in recent years for the gene therapy of diseases. More recently, lentiviral vectors appear to be excellent tools to deliver cDNAs in several tissues

Abbreviations used: BMT, bone marrow transplantation; CMV, the immediate early enhancer/promoter of the human cytomegalovirus; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAG, glycosaminoglycan; GFP, green fluorescent protein; MPS, mucopolysaccharidosis; NAGLU, α -N-acetylglucosaminidase; PPT, polypurine tract and termination sequences from *pol* of HIV-1-enhancing nuclear translocation; WPRE, the post-transcriptional regulatory element from the woodchuck hepatitis virus.

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since they can integrate into the genome of both dividing and non-dividing cells. Primate- and non-primate-derived lentiviral vectors that give a good performance in a large number of cells and tissues are now available [18–24]. Recently, a lentiviral system was also used for two lysosomal diseases. For MPS VII in β -glucuronidase-deficient mice, a complete or significant reduction in the pathology was observed [25]. In metachromatic leukodystrophy [26], following transduction of a functional arylsulphatase A gene into the brain of adult mice, a sustained and persistent expression of the transgene was obtained.

In the present study, we used a late-generation improved lentiviral vector for the *in vitro* transduction of human NAGLU cDNA into deficient fibroblasts. The aim was to evaluate transfer efficiency, the ability to reduce GAG accumulation and the persistence of expression and biosynthetic pathway of the recombinant enzyme in transduced cells.

MATERIALS AND METHODS

Cells

MPS IIIB fibroblasts were obtained from a Sanfilippo B patient characterized previously (patient 15 [13]); cells were cultured in plastic dishes by using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (all media and supplements from Life Technologies). In all cases, cells were maintained in a humidified atmosphere containing 5% CO₂/95% air at 37 °C.

Lentiviral vector containing NAGLU cDNA

All cloning steps were performed according to standard methods [27]. The 2.5 kb human NAGLU cDNA, containing the entire coding region and the 3'-untranslated region, was excised from pCMV-huNAGLU plasmid (CMV, the immediate early enhancer/promoter of the human cytomegalovirus) [8] by digestion with *Eco*RI and was treated with Klenow polymerase to form blunt ends. The purified blunted fragment was then digested with *Bam*HI and subcloned into the plasmid pRRLsinPPT.CMV.WPRE (PPT, polypurine tract and termination sequences from *pol* of HIV-1 enhancing nuclear translocation; WPRE, the post-transcriptional regulatory element from the woodchuck hepatitis virus) [28] after digestion of this vector with *Xba*I, followed by treatment with Klenow polymerase to create blunt ends and a second digestion with *Bam*HI. The self-inactivating (sin) gene transfer construct, obtained after subcloning, was pRRLsinPPT.CMV.NAGLU.WPRE, whose partial structure is reported in Figure 1. Recombinant lentiviral vectors were packaged as described previously [29]. Briefly, 293T cells were plated at 60–70% confluence in 10 cm tissue culture plates containing Iscove's modified Dulbecco's medium (IMDM)/10% (v/v) FCS approx. 24 h before transfection. Co-transfection was performed by standard calcium phosphate procedure by using four different constructs: 6.5 μ g of pMDLg/pRRE, a multiple-attenuated packaging construct containing Rev response element sequence and coding for HIV-1 *gag* and *pol* genes, 2.5 μ g of pRSV-Rev, expressing Rev protein, 3.5 μ g of pMD2.G, which produces VSV.G protein and 10 μ g of the appropriate gene transfer vector (pRRLsinPPT.CMV.NAGLU.WPRE or pRRLsinPPT.CMV.GFP.WPRE as control construct; GFP, green fluorescent protein). After 16 h the medium was replaced with complete IMDM and 24 h later 293T-conditioned media were filtered and ultracentrifuged at 50 000 g for 140 min at room temperature to concentrate the vector. Viral content was determined by immunocapture of HIV-1

Figure 1 Structure of the proviral form of lentiviral vector containing NAGLU cDNA

The schematic representation shows the most important features of the internal expression cassette for NAGLU cDNA. From the 5'- to the 3'-end: *cis*-acting sequences of HIV-1 (5'-LTR, with the largely deleted U3 derived from the self-inactivating construct; 5'-ss, splice donor site; Ψ , packaging signal; mut GAG, truncated HIV-1 *gag* gene; 3'-ss, splice acceptor site); 3'-LTR region.

p24 antigen by using the HIV-1 p24 Core Profile ELISA Kit (NEN).

In vitro infections and transduction efficiency

For dose-effect experiments, MPS IIIB fibroblasts cultured in three sets of 6 cm plates up to subconfluency (2×10^5 cells) were infected by incubation for 14 h with different amounts of NAGLU vector (1, 10 and 100 ng of p24) in 2 ml of fresh culture medium; DMEM was then replaced and the cells were fed with 3 ml of fresh medium and incubated for an additional 2 days before harvesting for biochemical tests. To evaluate transduction efficiency, control infections were performed as described above, by using 20 ng of GFP vector; after incubation, fibroblasts, grown on coverslips, were fixed with 3.7% formaldehyde in PBS, washed once with 0.1 M glycine in PBS, twice with PBS and once with H₂O. The fluorescence was visualized under an Axioplan fluorescence microscope (Zeiss). In expression persistence experiments, MPS IIIB fibroblasts, infected as above with 20 ng of p24, were subcultured and maintained in culture until aging impaired cell growth; samples were collected at different times.

Alu-PCR for detection of the integrated form of the lentiviral construct

Cell extracts were prepared as described elsewhere [30] with minor modifications. Briefly, transduced and untransduced fibroblasts from subconfluent 6 cm plates (2×10^5 cells) were harvested and washed twice with 0.9% NaCl; cells were then resuspended in 50 μ l of PCR buffer (PerkinElmer): 10 mM Tris (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, containing 0.45% Nonidet-P40, 0.45% (v/v) Tween 20. After incubation with proteinase K (0.2 mg/ml) at 55 °C for 2 h and inactivation of the enzyme by boiling for 5 min, samples of 10 μ l were subjected to Alu-PCR reactions in a total volume of 100 μ l containing 1 $\times$ buffer (PerkinElmer), 0.4 mM of each dNTP (Pharmacia Biotech), 0.8 μ M of each forward and reverse primer, 5% DMSO and 5 units of *Taq* polymerase (Gibco BRL). Two different pairs of primers were used: one at the 5'-end and the other at the 3'-end of the vector genome. Primers for 5'-end were Alu 3'-sense [30] and 5NC2 antisense: 5'-GAGTCCTGCGTCG-AGAGAG-3' [31]; the other pair at the 3'-end was Alu-278 antisense [30] and WPRE sense: 5'-CTTCCATGGCTGCTCC-C-3' [31]. The PCR procedure required a 10 min initial denaturation step (95 °C), followed by 30 cycles at 94 °C for 1 min, 57 °C (for the 5'-Alu-PCR) or 49 °C (for the 3'-Alu-PCR) for 1 min, and 72 °C for 1 min. After this first amplification, a nested PCR was performed by using 10 μ l of the first PCR product in a final volume of 100 μ l using two different internal primers in the

vector genome. For the 5'-nested PCR the primers were LTR9 (sense: 5'-GCCTCAATAAAGCTTGCTTGC-3') and U5PBS (antisense: 5'-GGCGCCACTGCTAGAGATTTT-3'), amplifying a fragment of 121 bp [31]. For the 3'-nested PCR the primers used were Δ nef (sense: 5'-CGAGCTCGGTACCTTTAAGACC-3') and LTR8 (antisense: 5'-TCCCAGGCTCAGATCTGGTCTAAC-3') amplifying a fragment of 166 bp [31]. The nested PCR conditions were similar to the first amplification, but differing in concentrations of dNTP and primers (0.2 mM and 0.5 μ M respectively), and in the annealing temperature which was 55 °C for the 5'-nested PCR and 58 °C for the 3'-nested PCR. As control, a nested PCR was performed by using 1 μ l of non-amplified lysate from transduced fibroblasts.

Protein extracts and NAGLU activity assay

Transduced and untransduced fibroblasts were harvested for the enzyme assays, washed twice with 150 mM NaCl, resuspended in the same buffer and freeze-thawed 6 times. All infections were performed three times; each sample was assayed for total protein content by using the Lowry assay [32]. NAGLU activity was assayed by fluorogenic assay by the method of Chow and Weissmann [33]. Cell homogenates (25 μ l) were mixed with 50 μ l (0.2 mM) 4-methylumbelliferyl- α -N-acetylglucosaminide in 0.2 M sodium acetate buffer (pH 4.3). After incubation for 2 h at 37 °C, the reaction was stopped by adding 1.5 ml (0.5 M) glycine/NaOH (pH 10.3). The amount of 4-methylumbelliferone product released in the enzyme reaction was calculated by using a standard curve. One unit of NAGLU activity is the catalytic activity hydrolysing 1 nmol of substrate per hour.

Correction of 35 S-labelled GAG accumulation in transduced MPS IIIB fibroblasts

The lysosomal pool of newly made GAGs was measured according to Zhao and Neufeld [14]. Confluent fibroblasts in three identical sets of 6 cm plates, at 12 days from infection with 20 ng of p24, were starved for 16 h with sulphate-deficient DMEM (ICN, Aurora, OH, U.S.A.) supplemented with 10% (v/v) dialysed FCS, and labelled for 48 h at 37 °C in 2.5 ml of the same medium containing 25 μ Ci $\text{H}_2^{35}\text{SO}_4$ /ml. After labelling, the cells were washed 3 times in PBS both before and after trypsinization and extracted by 3 cycles of freezing and thawing in 10 mM sodium phosphate (pH 5.8) containing 0.5% Nonidet-P40. After centrifugation at 10000 rev./min (6200 g) for 5 min in an Eppendorf microfuge, the supernatants were used to determine radioactivity and protein concentration.

Biosynthetic labelling of NAGLU synthesized in transduced MPS IIIB fibroblasts

Three days after infection with 20 ng of p24, cells in 6 cm plates were labelled as described in [13]. In detail, cells were starved for 2 h with 1.5 ml methionine-cysteine-free medium (DMEM), supplemented with 2% (v/v) FCS, and labelled for 2 h (pulse) with 300 μ Ci of 35 S-Express Protein Labeling Mix (NEN Life Science Products) in the same medium. After labelling cells were chased for 24 h in DMEM supplemented with 0.3 g/litre unlabelled cysteine and methionine. The medium was then collected and concentrated to 0.5 ml by using Millipore Centricon Centrifugal Filter Device, with a YM-30 membrane, by spinning for 2 h at 3000 rev./min (2200 g) in a Beckman centrifuge CS-6R. Cells were washed 3 times with PBS and lysed on ice for 30 min in 1 ml of lysis buffer [10 mM Tris/HCl buffer, pH 7.4/150 mM NaCl/1 mM EDTA, pH 8.0/0.1% (v/v) Triton X-100] containing 0.2 mM PMSF, 1 μ M pepstatin A and 1 μ M leupeptin. Cell

lysates were clarified by centrifugation at 2200 g for 5 min in an Eppendorf centrifuge 541 SC. Lysates and media were incubated overnight with 1 μ l of polyclonal anti-NAGLU antibody; immune complexes were precipitated with 30 μ l of Protein A-agarose (Santa Cruz Biotechnology), washed 4 times with lysis buffer and analysed by SDS/PAGE (10% gel) followed by autoradiography.

Endocytosis of NAGLU protein secreted by transduced MPS IIIB fibroblasts

Before starting the endocytosis experiments, MPS IIIB fibroblasts were plated in 6 cm dishes, transduced with 50 ng of p24 viral protein and subjected to one subculture. Endocytosis of NAGLU protein into deficient cells was performed by two different systems: co-culture with transduced fibroblasts or uptake of conditioned medium from transduced cells. For co-culture experiments, fibroblasts from confluent dishes were transferred to trans-well plates (0.45 μ m; Costar, Cambridge, MA, U.S.A.) in 1.5 ml of DMEM containing 10% (v/v) FCS; transduced fibroblasts were placed in the upper chambers and untransduced cells plated in the lower chambers, with or without 5 mM mannose 6-phosphate. After 72 h co-culture, fibroblasts from both chambers were harvested to test for NAGLU activity. For uptake experiments, three 6 cm plates of transduced fibroblasts at day 10 after the transduction were supplemented with 10 mM NH_4Cl in 3 ml of DMEM containing 1% (v/v) FCS and allowed to grow for an additional 48 h. Conditioned media were collected, dialysed overnight in PBS at 4 °C, filtered through 0.22 μ m Millipore filters and finally placed on a second population of untreated MPS IIIB fibroblasts with or without 5 mM mannose 6-phosphate. After 48 h endocytosis, recipient cells were harvested to measure NAGLU activity.

RESULTS

Lentiviral vector containing NAGLU cDNA

A third-generation VSV-pseudotyped lentiviral vector containing NAGLU cDNA was constructed and prepared by using a conditional packaging system [29]. Figure 1 shows the proviral form of the vector, with the features of the internal expression cassette for NAGLU cDNA. The packaging system contains the self-inactivating transducing constructs (sin) due to a 400 bp deletion including the enhancer and promoter from U3. It conserves only three of the nine genes of HIV-1 and relies on four separate transcriptional units for the production of transducing particles. This system offers biosafety and allows the production of high-titre HIV-derived vector stocks.

As an improving feature, this late-generation gene-transfer construct contains, upstream of the expression cassette, the structural elements in *cis* from *pol* of HIV-1 (PPT), which encompass the central polypurine tract and termination sequences. Previous studies have reported that they enable a significant improvement in gene transfer into a panel of human primary cells including peripheral blood lymphocytes, macrophages, fibroblasts and endothelial cells derived from umbilical cord. Transduction was reported to be enhanced in both dividing and growth-arrested cells [28].

Lentiviral-mediated NAGLU gene transfer and transduction efficiency

Three sets of MPS IIIB fibroblasts were exposed to 1, 10 and 100 ng of p24 viral preparation as described in the Materials and methods section and the enzyme activity was measured

Figure 2 Enzyme activity in fibroblasts after lentiviral vector-mediated transduction

Replicate plates of fibroblasts were exposed to the indicated doses of NAGLU vector (ng of p24) as described in the Materials and methods section; 2.5 days after infection, cell lysates were assayed for protein content and enzyme activity (mean $\pm$ S.D., $n = 3$). MPS IIIB, untreated patient fibroblasts; control, normal fibroblasts.

2 days after transduction (Figure 2). Transduced cells contained substantially higher levels of NAGLU activity (mean, 1.04, 7.6 and 33.6 units/mg of protein respectively) than untreated fibroblasts (Figure 2, MPS IIIB: mean, 0.2 unit/mg of protein). The activity level of transduced MPS IIIB fibroblasts was 0.6-, 4.4- and 19.6-fold respectively the activity of normal fibroblasts (Figure 2, control: mean, 1.72 units/mg of protein). p24 viral particles (20 ng) were chosen as the experimental dose for further studies.

Transduction efficiency was evaluated by detection of fluorescence after infection of MPS IIIB cells with 20 ng of lentiviral GFP; efficiency was calculated as the number of fluorescent cells over the total number of cells and was estimated to be 70% (results not shown).

Persistence of NAGLU expression

To assess the persistence of the NAGLU expression over the course of time, MPS IIIB fibroblasts were transduced with 20 ng of p24 and two identical sets of plates were maintained and examined for time periods ranging from 48 h to 60 days after infection; samples were collected at different times and lysates prepared to test for NAGLU activity as reported in the Materials and methods section. Enzyme activity showed an initial linear increase over the first 10 days, followed by a plateau phase (Figure 3A), and persisted at plateau levels for as long as 60 days, when fibroblasts showed aging and the experiment was concluded.

To verify NAGLU vector integration in transduced MPS IIIB fibroblasts, Alu-PCR reactions were performed, as described in the Materials and methods section, on cellular extracts prepared from untreated and transduced fibroblasts on day 10 after infection. Reactions included a first amplification using two

Figure 3 Time course of NAGLU lentiviral-mediated transduction

(A) Three 6 cm dishes of MPS IIIB fibroblasts were transduced with 20 ng of p24 as described in the Materials and methods section, grown to confluence and expanded into cultures. After infection, cell lysates were assayed at various time intervals for protein content and NAGLU activity. Values represent mean $\pm$ S.D. for each time point. (B) Alu-PCR analysis was performed on day 10 after infection to verify the integration of gene transfer-derived DNA. Cell extracts from replicate cultures were prepared as described in the Materials and methods section and used as a template for two different pairs of primers, one at the 3'-end and the other at the 5'-end of the vector genome. The first amplification (lanes 1–3) did not show any band. The nested reaction (lanes 4–7) amplified a single band of 166 or 121 bp at the 3'- or 5'-end of the vector DNA respectively. M, 100 bp marker; lanes 1 and 4, blank reaction; lanes 2 and 5, transduced cells; lanes 3 and 6, untreated fibroblasts; lane 7, control nested reaction using lysate not subjected to the first amplification.

different pairs of primers specific for Alu ubiquitous repeats and for the 3'- or 5'-end of the lentiviral vector genome respectively, followed by a nested PCR to amplify sequences from the vector DNA. As expected, both for 3'- and 5'-Alu-PCR no bands were visible after the first amplification (Figure 3B, lanes 1–3); discrete bands were obtained only after the nested PCR (Figure 3B, lanes 4–7). Reactions corresponding to the transduced MPS IIIB fibroblasts amplified a single band of 166 bp from the 3'-end and a 121 bp fragment from the 5'-end (Figure 3B, lane 5). In contrast, no fragments were visible for the untreated cells (Figure 3B, lane 6) and for the control using as a template cell lysate not subjected to the first amplification (Figure 3B, lane 7).

Correction of ^{35}S -labelled GAG accumulation in transduced MPS IIIB fibroblasts

To verify whether the GAG-impaired degradation could be successfully restored by treatment with the recombinant lentiviral vector, fibroblasts were incubated in the presence of $^{35}\text{S}\text{O}_4$ to measure the amount of GAG levels in transduced MPS IIIB cells

Figure 4 Correction of $^{35}\text{SO}_4$ -GAG accumulation in transduced MPS IIIB fibroblasts

Three sets of 6 cm dishes of MPS IIIB fibroblasts (untreated or transduced with NAGLU lentivirus) and normal fibroblasts were incubated in SO_4 -free medium overnight. $\text{H}_2^{35}\text{SO}_4$ (25 $\mu\text{Ci}/\text{ml}$) was added to each cell dish and left for 48 h. Cells were then harvested and assayed for $^{35}\text{SO}_4$ -GAG accumulation and protein content as described in the Materials and methods section. U, untreated fibroblasts; T, transduced cells; N, normal fibroblasts. Values represent mean $\pm$ S.D.

versus untreated MPS IIIB fibroblasts and to compare these levels with that of normal fibroblasts. As shown in Figure 4, in two different experiments, untreated MPS IIIB fibroblasts (U) accumulated consistently higher levels of labelled GAGs when compared with normal fibroblasts (N) (3.6- and 2.02-fold respectively); transduced cells (T) exhibited a substantial decrease in GAG quantities: 32 and 56% respectively to that found in untreated cells, resulting in very similar GAG levels to those observed in the normal control.

Metabolic labelling of NAGLU protein in transduced MPS IIIB fibroblasts

To investigate the biosynthetic pathway of lentivirally encoded human NAGLU, labelling experiments were performed in MPS IIIB fibroblasts 3 days after transduction. After a 2 h pulse followed by 24 h chase time, the medium and cells were harvested in order to check for the presence of precursor and mature forms of the enzyme respectively. Labelled NAGLU molecules were shown after immunoprecipitation with appropriate antibodies and separation by SDS/PAGE. The expected precursor protein of 84 kDa was identified in the medium from transduced MPS IIIB fibroblasts, which progressed to a mature polypeptide of 80 kDa, as shown in the cell lysates after 24 h of chase (Figure 5). Neither the medium nor cell lysates from untreated MPS IIIB fibroblasts showed any band related to precursor-mature forms of the enzyme (Figure 5).

Endocytosis of NAGLU protein secreted by transduced MPS IIIB fibroblasts into deficient cells

To verify the capacity of transduced MPS IIIB cells to secrete the NAGLU enzyme to the extracellular environment providing protein for cross-correction of untransduced cells, endocytosis experiments were performed by co-culture or by classical uptake. The results are shown in Table 1. After co-culture of transduced/untransduced fibroblasts, recipient cells exhibited a NAGLU activity (0.5 unit/mg of protein) 1.6-fold higher than that found

Figure 5 Biosynthetic labelling of NAGLU after infection of MPS IIIB fibroblasts

MPS IIIB fibroblasts, infected or uninfected with NAGLU lentiviral vectors, were pulse-labelled for 2 h and harvested after a 24 h chase period as reported in the Materials and methods section. The medium was collected from all plates and concentrated as described. NAGLU polypeptides were immunoprecipitated from the medium and cell lysates, separated by SDS/PAGE and detected by autoradiography. Positions of molecular-mass markers are shown on the left. U, untreated fibroblasts; T, transduced fibroblasts; M, the medium; C, cell lysate. Arrows on the right correspond to the 84 kDa precursor form and the 80 kDa mature form of retrovirally encoded NAGLU.

Table 1 Endocytosis of NAGLU protein released from transduced MPS IIIB fibroblasts into a second population of deficient recipient cells

Experiments were performed in trans-well plates (A) or in 6 cm dishes (B) as described in the Materials and methods section. Co-culture was for 72 h and uptake was for 48 h. Activity is expressed as units/mg of protein (mean $\pm$ S.D.).

MPS IIIB fibroblasts	NAGLU activity	β -Galactosidase activity
Untreated cells	0.32 $\pm$ 0.06	695 $\pm$ 40
Transduced cells	32.43 $\pm$ 5.52	700 $\pm$ 35
Recipient cells after		
Co-culture with transduced cells (A)	0.51 $\pm$ 0.10	751 $\pm$ 15
Co-culture + Man 6-P (A)	0.33 $\pm$ 0.07	735 $\pm$ 17
Uptake of conditioned medium (B)	2.84 $\pm$ 0.48	713 $\pm$ 15
Uptake + Man 6-P (B)	0.42 $\pm$ 0.07	633 $\pm$ 13

in untreated cells (0.3 unit/mg of protein). After uptake experiments using secretions obtained by NH_4Cl treatment of transduced fibroblasts, recipient cells exhibited a NAGLU activity of 2.8 units/mg of protein, 9-fold higher than the basal level. Taking into account the total enzyme units (11.1) provided to recipient cells and the total enzyme units (0.48) recovered after uptake, endocytosis was determined to be 4.3%. In both cases, the endocytosis of the recombinant protein was inhibited by the addition of mannose 6-phosphate. The expression of a control

enzyme, β -galactosidase, was not affected by the lentiviral-mediated gene expression of the NAGLU protein.

DISCUSSION

Mucopolysaccharidoses have been traditionally considered to be good candidates for therapy by an exogenously supplied enzyme that would allow metabolic correction through the well-characterized mannose 6-phosphate signal/receptor system [34] (reviewed in [1]). Therapeutic approaches include bone marrow transplantation (BMT), enzyme replacement therapy and gene therapy. Among the mucopolysaccharidoses, the Sanfilippo disorder MPS III is characterized by relatively mild somatic manifestations associated with severe progressive mental deterioration. This clinical presentation is probably related to the observation that heparan sulphate is the most abundant GAG in the CNS. It suggests that the treatment of Sanfilippo patients should be aimed at achieving efficient and rapid metabolic correction within the CNS. Allogeneic BMT and gene therapy via the pluripotent haemopoietic stem cells provide functional cells of the macrophage/monocyte lineage. They were able to colonize the CNS and are probably suitable for the treatment of these pathologies. BMT was given to a small number of children with MPS IIIB; allogeneic BMT performed on twins gave doubtful results [35]. Gene therapy using lentiviral vectors is a promising therapy for these disorders.

In vitro studies on Sanfilippo A fibroblasts exposed to up to 4 cycles of retroviral transduction showed that, although the first 2 cycles of exposure to the virus resulted in low-level transduction (1.7 and 2.1 % sulphamidase expression), the last two resulted in much higher levels (28 and 13 % respectively). In both cases correction of the phenotypic storage defect was achieved, as assessed by measurements of ^{35}S -labelled GAG accumulation [36]. In a recent study, a retroviral construct containing NAGLU cDNA was used to infect Sanfilippo B fibroblasts. Exposure to 5 cycles of virus infection resulted in a partial clearance of ^{35}S -labelled GAG storage and a 5.6-fold elevation of intracellular NAGLU activity compared with normal fibroblasts. However, only 0.36 % of the total NAGLU secreted from transduced MPS IIIB fibroblasts, endocytosed by a second population of MPS IIIB cells via the mannose 6-phosphate receptor pathway was observed [15].

Our approach to develop effective gene therapy for MPS IIIB is based on lentivirus-mediated gene transfer into 'knock-out' mouse models. As an initial step towards this goal, we have demonstrated that the Sanfilippo B phenotype can be efficiently corrected in primary skin fibroblast cultures by lentivirus-mediated gene transfer of the human NAGLU gene. Recombinant NAGLU was expressed in transduced fibroblasts at high levels: approx. 20- and 168-fold higher than those obtained in normal cells and untreated MPS IIIB fibroblasts respectively. These results confirm the findings that high levels of protein expression are obtained when the coding sequence is transcriptionally regulated by strong heterologous promoters [37]. CMV was used as NAGLU cDNA promoter within the transfer vector because it has been shown to be efficient in different systems [21,26]. However, since it has been reported that the use of strong, constitutive viral promoters does not always produce long-term transgene expression [38,39], transduced MPS IIIB fibroblasts were expanded for as long as possible, thus demonstrating the persistence of expression, at plateau levels, for at least 2 months after infection. Moreover, Alu-PCR experiments confirmed the integration of lentiviral-derived sequences into the target genome after the same period of time.

Correction of the enzymic deficiency was in turn demonstrated by correction of the mucopolysaccharide storage, as assayed by the intracellular level of ^{35}S -labelled GAGs and by normal maturation of the enzyme as shown by metabolic labelling experiments: a precursor form of 84 kDa in the conditioned medium and a mature form of 80 kDa found intracellularly.

Although the endocytosis experiments (Table 1) confirmed the results obtained in other laboratories [14–16], i.e. the secreted recombinant NAGLU is poorly taken up by Sanfilippo B fibroblasts probably due to scarce mannose 6-phosphorylation, future treatment of the murine MPS IIIB model by lentivirus-NAGLU could be successful through the systemic correction of the various somatic sites of pathology, either by direct viral infection or by mechanism(s) alternative to the mannose 6-phosphate signal/receptor system. Indeed, the advantage of lysosomal disease correction is that high levels of expression may not be necessary to correct the disease, as shown for β -glucuronidase: levels $\leq 1\%$ of wild-type in the liver of MPS VII mice were sufficient to decrease lysosomal storage material in these animals [40,41]. These findings imply that systemic delivery of a relatively small amount of enzyme, either by the expression of a newly introduced gene or by metabolic cross-correction, may be sufficient to prevent or metabolize accumulated storage materials in affected tissues. As for mechanisms alternative to the mannose 6-phosphate signal/receptor system, recent studies on 'knock-out' mouse models lacking the mannose 6-phosphate receptors have demonstrated that alternative mechanisms can also provide for enzyme transport to the lysosomes and that these mechanisms are cell type-specific [42]. In line with these findings, the recent report on enzyme replacement therapy of the Sanfilippo B murine model, making use of recombinant NAGLU, demonstrated that the protein was endocytosed *in vitro* by mouse peritoneal macrophages via mannose receptors and *in vivo* in the liver and spleen of treated animals by macrophages residing in those tissues [43].

CNS therapy may require neonatal treatment of MPS IIIB mice, as for MPS VII mice [41], implying that the blood-brain barrier is not completely formed, or might be partially achieved through colonization by haemopoietic cells [44] or may require direct injection of protein or gene into brain [24] or the development of technology to cross the blood-brain barrier [45].

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