Treatment of the mouse model of mucopolysaccharidosis type IIIB with lentiviral-NAGLU vector

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The Sanfilippo syndrome type B (mucopolysaccharidosis IIIB) is an autosomal recessive disorder due to mutations in the gene encoding NAGLU (α -N-acetylglucosaminidase), one of the enzymes required for the degradation of the GAG (glycosaminoglycan) heparan sulphate. No therapy exists for affected patients. We have shown previously the efficacy of lentiviral-NAGLU-mediated gene transfer in correcting *in vitro* the defect on fibroblasts of patients. In the present study, we tested the therapy *in vivo* on a knockout mouse model using intravenous injections. Mice (8–10 weeks old) were injected with one of the lentiviral doses through the tail vein and analysed 1 month after treatment. A single injection of lentiviral-NAGLU vector resulted in transgene expression in liver, spleen, lung and heart of treated mice, with the highest level reached in liver and spleen. Expression of 1 %

normal NAGLU activity in liver resulted in a 77 % decrease in the GAG content; more remarkably, an expression of 0.16 % normal activity in lung was capable of decreasing the GAG level by 29 %. Long-term (6 months) follow up of the gene therapy revealed that the viral genome integration persisted in the target tissues, although the real-time PCR analysis showed a decrease in the vector DNA content with time. Interestingly, the decrease in GAG levels was maintained in liver, spleen, lung and heart of treated mice. These results show the promising potential and the limitations of lentiviral-NAGLU vector to deliver the human NAGLU gene *in vivo*.

Key words: gene therapy, glycosaminoglycan, lentiviral vector, mouse model, α -N-acetylglucosaminidase, Sanfilippo type B.

INTRODUCTION

Sanfilippo syndrome type B [MPS (mucopolysaccharidosis) IIIB] is an autosomal recessive disorder due to mutations in the gene encoding NAGLU (α -N-acetylglucosaminidase; EC 3.2.1.50), one of the enzymes required for the degradation of heparan sulphate [1]. It is heterogeneous at the molecular level, with more than 80 mutations of the NAGLU gene already described in [2–6]. The enzyme defect results in the accumulation of heparan sulphate in the tissues of affected subjects and its abnormal urinary excretion. Affected children are usually diagnosed at 2–8 years of age and they present with sleep disorders, hepatosplenomegaly, hirsutism, vasomotor instability, dysmorphism, dysostosis, delayed development and mental retardation progressing throughout childhood; death occurs, typically, during the 2nd or 3rd decade of life, although patients with a more attenuated phenotype have been reported [7,8].

At present, there is no definite treatment for MPS IIIB [1]. The development of a knockout mouse model of the disease [9], showing a biochemical phenotype generally similar to that of the human disorder, provides a good model for the development of new treatments. This model was used to evaluate the potential of enzyme replacement therapy based on an unphosphorylated form of recombinant NAGLU [10] to verify the efficacy of intracranial injections of adeno-associated vectors for the correction of the neurological impairment [11] and, more recently, to test the therapeutic effect of retrovirally transduced bone marrow [12].

A number of viral vectors have been used in the last few years for gene therapy of lysosomal disorders; more recently, lentiviral vectors were shown to be effective by intracranial delivery into

murine models of metachromatic leukodystrophy [13] and MPS VII, β -glucuronidase deficiency [14–16]. Recently, we demonstrated that fibroblasts from patients with MPS IIIB or MPS I, infected with lentiviral vectors, produced consistent levels of a correctly processed protein whose expression is time-persistent and sufficient to reverse abnormal GAG (glycosaminoglycan) accumulation [17,18]. In the present study, we used this late-generation lentiviral vector containing the human NAGLU cDNA driven by a CMV (cytomegalovirus) promoter for the *in vivo* transduction of the therapeutic gene into the murine MPS IIIB model by intravenous administration.

MATERIALS AND METHODS

Materials

Tissue culture reagents and fetal bovine serum were purchased from Sigma (Milan, Italy) and the 4-methylumbelliferyl substrates from Calbiochem (San Diego, CA, U.S.A.). Alcian Blue, heparan sulphate, Protein A-acrylic beads, *p*-nitrophenyl phosphate, alkaline phosphatase-labelled goat anti-mouse Ig were obtained from Sigma. Reagents for immunostaining, including Vecstatin Elite ABC reagent, peroxidase substrate solution and biotinylated rabbit IgG were obtained from Vector Laboratories (Burlingame, CA, U.S.A.). For the standard PCRs, the buffer was obtained from PerkinElmer Life Sciences (Milan, Italy), *Taq* polymerase from Invitrogen SRL (Milan, Italy), dNTPs from Amersham Biosciences (Milan, Italy) and primers from PRIMM (Milan, Italy). Probes and master mix for real-time quantitative PCR were obtained from Applied Biosystems (Milan, Italy).

Abbreviations used: CMV, cytomegalovirus; GAG, glycosaminoglycan; GFP, green fluorescent protein; MPS, mucopolysaccharidosis; NAGLU, α -N-acetylglucosaminidase.

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Methods

Large-scale vector production and assays

Lentiviral vector containing the human NAGLU cDNA was prepared as described previously [17,19,20]. For the large-scale preparation, 5×10^6 293T cells/dish were seeded in 24 dishes 6 h before transfection. A total of 22.5 μ g of plasmid DNA was used for the quadrant transfection of one dish: 10 μ g of the transfer vector plasmid pRRLsin.PPT.CMV.NAGLU.WPRE (PPT stands for polypurine tract and termination sequences from *pol* of HIV-1-enhancing nuclear translocation and WPRE for the post-transcriptional regulatory element from the woodchuck hepatitis virus), 6.5 μ g of the packaging plasmid pMDLg/pRRE, 2.5 μ g of pRSV-Rev construct and 3.5 μ g of the envelope plasmid pMD2.G. The viral content was determined by immunocapture of HIV-1 p24 antigen using the HIV-1 p24 core Profile ELISA kit from NEN (Cologno Monzese, Italy). For each large-scale preparation of lentiviral-NAGLU vector, transfection of 1.2×10^8 293T cells yielded 8–20 μ g of HIV-1 p24 antigen. To test the viral preparation, 50 ng of p24 was used to infect 10^5 defective fibroblasts in 6 cm plates. Transduction of deficient fibroblasts with each preparation resulted in a mean enzymatic activity of 60.4 ± 23.1 nmol $\cdot$ h $^{-1}$ $\cdot$ (mg of protein) $^{-1}$ (over the range 38.4–84.4); untreated fibroblasts showed a mean activity of 0.33 ± 0.17 (over the range 0.18–0.61).

Transduction efficiency *in vivo*, as measured by using similar doses of a lentiviral vector expressing the GFP (green fluorescent protein) gene, was reported to reach up to 21% efficiency of GFP-positive cells found in the liver of treated mice [20].

Experimental animals: genotyping

The mouse model of MPS IIIB was created by targeted disruption of exon 6 of the corresponding mouse NAGLU gene [9]. The biochemical phenotype of mutant mice is generally similar to that of the human disorder, the only exception being that affected mice do not show increased urinary excretion of heparan sulphate. The animal studies were approved by the Italian Ministry of Public Health. The mice were genotyped by polymerase reaction analysis performed on DNA samples extracted from tail clipping exactly 1 month after birth. PCRs were performed in a total volume of 100 μ l containing 500 ng of template DNA extracted from tail clipping, 1 $\times$ PCR buffer from PerkinElmer, 0.2 μ M of each dNTP, 2.5 units of *Taq* polymerase and two sets of the following primers (0.5 μ M each): (i) MXXDe1F, sense primer starting at position 6773 of the murine NAGLU gene, GenBank[®] accession number AF003255 (5'-GCTCCTACTC-AGAAGTGTCTACAACCTGCTC-3'), and MXXDe1R, antisense primer starting at position 7279 (5'-GAGGCTGGTAGTAATC-AGCCACCAGTCTCTG-3'). Amplification yielded a 537 bp fragment in the presence of the normal allele, no band in the presence of the mutant allele; (ii) Neo1F, sense primer starting at position 202 in the Neo ORF (5'-GTGGCTGGCCACGACG-GGCGTTCCTTGCG-3'), and M7R, antisense primer starting at position 7454 of the murine NAGLU gene (5'-GAGGAA-GATCTTCTTGGAGAGGTCCACGGTG-3'). Amplification yielded no band in the presence of the normal allele but yielded a 1200 bp band in the presence of the mutant allele. Cycling conditions were the following: 96°C for 5 min, 35 cycles at 95°C for 45 s, 64°C for 35 s and 72°C for 2 min.

In vivo gene therapy: intravenous injections of lentiviral-NAGLU vector

Homozygous mutant mice (8–10 weeks old) obtained from heterozygote matings were used for all *in vivo* transduction experiments. A volume of 200–300 μ l of vector in PBS was injected

into the tail vein; controls were mock-injected. At the time of killing, the mice were killed with CO₂ and the organs (brain, liver, spleen, lung, heart, thymus and kidney) were removed and immediately frozen in liquid nitrogen or subdivided into parts, one of which was fixed in 4% (w/v) paraformaldehyde overnight and treated for immunohistochemistry as described in the next paragraph.

NAGLU and lysosomal enzyme assays

For the enzyme assays, 30 mg of the tissue was homogenized in 1 ml of 0.9% NaCl/0.2% Triton X-100, rotated for 2 h at 4°C and centrifuged to remove the debris. NAGLU activity was measured as described in [21]: 50–100 μ g of each lysate was added in duplicate to a solution containing 2 mM 4-methylumbelliferyl-NAGLU in 0.2 M sodium acetate buffer (pH 4.3). The cleaved substrate was quantified after a 17 h incubation at 37°C, and subsequent to the addition of glycine buffer (pH 10.5). Other lysosomal enzyme activities were determined by the method of Li et al. [9] by using 4-methylumbelliferyl substrates incubated with the appropriate amount of homogenate for 1 h at 37°C under the following conditions: α -L-iduronidase, 10 μ l of homogenate with 10 μ l of 2 mM substrate in 0.1 M formate (pH 3.2); β -glucuronidase, 50 μ l of homogenate with 50 μ l of 0.2 mM substrate in 0.1 M sodium acetate buffer (pH 4.3); β -hexosaminidase, 25 μ l of homogenate with 125 μ l of 0.24 mM substrate in 0.1 M sodium acetate buffer (pH 5). The amount of fluorescent product released in the enzyme reaction was determined from a standard curve using 4-methylumbelliferone.

Analysis of GAG (quantitative determination)

Soluble GAGs were precipitated from tissue homogenates with Alcian Blue for quantitative measurement [9,22]. The mice were killed and organs were dissected as described above. Frozen tissues were freeze-dried and, after weighing, resuspended in 0.9% NaCl/0.2% Triton X-100 (30 μ l/mg dry weight), rotated at 4°C overnight and centrifuged to remove the debris. GAGs were precipitated with Alcian Blue, the blue precipitate was dissolved, and absorbance was measured at 600 nm. Heparan sulphate from porcine intestinal mucosa was used as standard.

Immunohistochemistry

An enzyme-based immunohistochemical stain was used to detect the human NAGLU in cryosections of liver after viral treatment. For antibody staining of recombinant NAGLU, the liver was fixed overnight in 4% paraformaldehyde and embedded in paraffin. Sections (6 μ m thick), deparaffinized and rehydrated, were incubated with Protein A-purified anti-human NAGLU and biotinylated anti-rabbit antibody. Colour was developed using the ABC Elite Vector Staining kit and the horseradish peroxidase substrate by VIP kit. The presence of recombinant NAGLU was visualized by the magenta colour of the peroxidase reaction [10].

Biodistribution of viral genome: PCR assay

Genomic DNA for the PCR assay was purified from tissue homogenates by phenol–chloroform extraction and ethanol precipitation. The integrity of the DNA was verified by performing, for all the tissues, a PCR amplification with Neo1F and M7R primers (described in the Experimental animals: genotyping subsection). The extracted DNA was then subjected to PCR in a total volume of 100 μ l containing 1 $\times$ buffer, 0.2 mM of each dNTP and 0.5 μ M each of the forward and reverse primers NB17 and NB18 [2]. These primers were selected to amplify a 429 bp fragment from exon 6 of the human NAGLU gene that is present

Table 1 Dose-response after the intravenous injection of lentiviral-NAGLU vector in MPS IIIB mice

MPS IIIB mice (8–10 weeks old) were intravenously injected with a lentiviral-NAGLU vector at the indicated p24 doses for each mouse. Normal and heterozygous mice were mock-injected. After 1 month, mice were killed and tissues were analysed for NAGLU activity. Enzyme activity values represent the means $\pm$ S.D. for three animals. * $P < 0.05$ versus untreated mice; no symbol is used to indicate $P > 0.05$.

Lentiviral dose (μ g of p24)	NAGLU activity [nmol $\cdot$ (17 h) $^{-1}$ $\cdot$ (mg of protein) $^{-1}$]						
	Liver	Spleen	Lung	Thymus	Heart	Kidney	Brain
0	0.020 $\pm$ 0.023	0.050 $\pm$ 0.026	0.039 $\pm$ 0.023	0.023 $\pm$ 0.018	0.024 $\pm$ 0.016	0.027 $\pm$ 0.005	0.04 $\pm$ 0.0028
3	0.055 $\pm$ 0.037	0.070 $\pm$ 0.033	0.073 $\pm$ 0.025	0.046 $\pm$ 0.028	0.061 $\pm$ 0.030	0.049 $\pm$ 0.034	0.041 $\pm$ 0.037
6	0.095 $\pm$ 0.037*	0.107 $\pm$ 0.020*	0.070 $\pm$ 0.018	0.022 $\pm$ 0.015	0.082 $\pm$ 0.046	0.026 $\pm$ 0.024	0.021 $\pm$ 0.018
9	0.133 $\pm$ 0.045*	0.237 $\pm$ 0.08*	0.080 $\pm$ 0.016	0.052 $\pm$ 0.020	0.080 $\pm$ 0.043	0.016 $\pm$ 0.010	0.01 $\pm$ 0.008
12	2.62 $\pm$ 1.6*	32.5 $\pm$ 21.3*	0.168 $\pm$ 0.057*	0.079 $\pm$ 0.060	0.087 $\pm$ 0.029*	0.075 $\pm$ 0.038	0.045 $\pm$ 0.034
Normal mice	228.9 $\pm$ 105	117.3 $\pm$ 57	106.3 $\pm$ 41.6	53.3 $\pm$ 15.4	12.4 $\pm$ 2.86	76.8 $\pm$ 50.5	19.1 $\pm$ 2.27
Heterozygous mice	84.8 $\pm$ 49	24.5 $\pm$ 4.8	69.1 $\pm$ 39	33.9 $\pm$ 2.8	9.3 $\pm$ 2.4	22.7 $\pm$ 1	11.8 $\pm$ 2.6

only in the affected mice after lentiviral treatment but is absent from the genome of affected untreated mice. The PCR procedure required a 5 min initial denaturation step (96°C), followed by 35 cycles at 95°C for 45 s, 54°C for 35 s and 72°C for 2 min. The number of cycles was adjusted to obtain a non-saturating range of amplification of vector DNA.

Integration of the lentiviral construct: B2-PCR assay

DNA extracted from mice tissues was subjected to B2-PCR. In a first set of amplifications, primers based on the murine B2 family of highly repeated dispersed elements [23–25] were used together with primers derived from the 5'- or 3'-end of the vector. Reactions were performed on 100 ng of genomic DNA in a 100 μ l total volume containing 1 $\times$ buffer (PerkinElmer), 0.4 mM of each dNTP, 0.8 μ M each of the forward and reverse primers, 5% (v/v) DMSO and 5 units of *Taq* polymerase. Two different primer pairs were used; one at the 5'-end and the other at the 3'-end of the vector genome [20]. The primers for the 5'-end were B2 sense and 5NC2 antisense; the other pairs at the 3'-end were B2 antisense and WPRE sense. After the first amplification, a nested PCR was performed using 10 μ l of the first PCR product in a 100 μ l final volume using two different internal primers in the vector genome. For the 5'-nested PCR, the primers were LTR9 (sense) and U5PBS (antisense). For the 3'-nested PCR, the primers used were Δ nef (sense) and LTR8 (antisense) [20]. As the control, a nested PCR was performed using 10 ng of non-amplified genomic DNA from mice tissues.

Quantitative real-time PCR

Real-time PCR was performed on genomic DNA extracted from the liver and spleen of the 12 μ g-injected mice 1 month after treatment and 6 months after treatment. Primers (forward primer: 5'-TGAAAGCGAAAGGGAAACCA-3'; reverse primers: 5'-CCGTGCGCGCTTCAG-3') and the TaqMan probe (5'-VIC-AGCTCTCTCGACGCGAGACTCGGC-TAMRA-3') targeted the lentiviral 5'-untranslated region. Amplicon size was 65 bp. Each reaction contained 1 $\times$ TaqMan universal PCR master mix, 400 nM of each primer, 200 nM TaqMan probe and 300 ng of sample DNA in a final volume of 25 μ l. As standard, the diluted gene transfer vector was used (from 1 $\times$ 10⁶ copies to 1 copy/reaction in GeneAmp 1 $\times$ PCR buffer containing 20 ng/ μ l sheared salmon sperm DNA). The following cycling parameters were used: 2 min incubation at 50°C followed by 10 min at 95°C and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. All the reactions were performed in triplicate sets and the results were reported as the average of three reactions. Real-time PCR targeted for the mouse β -actin

gene was used for normalization of the DNA content; for this purpose, a β -actin-specific TaqMan probe was used (5'-VIC-CAGTCCGCATCCTCTTCCTCCC-TAMRA-3') and the following primers were employed: forward primer 5'-AGAG-GGAAATCGTGCGTGAC-3' and reverse primer 5'-CAATAG-TGACCTGGCCGT-3'. To obtain the vector copy number for each cell, the values were divided by the number of cells corresponding to 300 ng of genomic mouse DNA.

Transgene-specific immune response: ELISA

The presence of an immune response in mice against human NAGLU was evaluated by an ELISA of samples from affected mice after lentiviral-NAGLU treatment. For the detection of anti-NAGLU antibodies, the wells were coated with 0.2 μ g of recombinant NAGLU in 50 μ l of PBS (pH 5.8) and blocked with 0.1% Tween 20 in PBS. Serum samples from mice treated with lentiviral-NAGLU vector and untreated controls were diluted in PBS; after primary antibody binding, the samples were washed before the addition of the secondary antibody, alkaline phosphatase-labelled goat anti-mouse Ig. After washing, 200 μ l of the alkaline phosphatase substrate (*p*-nitrophenyl phosphate) was added and absorbance was measured at 405 nm on a Bio-Rad ELISA reader.

A titration curve was established in the serum of mice killed 1 month after treatment and in the serum of mice killed 6 months after treatment by using several dilutions of mice serum.

RESULTS

In vivo expression of recombinant NAGLU: enzyme activity

Subsets of three affected mice were injected via the tail vein with different doses of lentiviral-NAGLU vector (3, 6, 9 and 12 μ g of p24 viral protein) as described in the Materials and methods section. All animals were 8–10 weeks old. Untreated affected mice (Table 1, 0 μ g of p24), normal mice and heterozygous mice were mock-treated. Mice were killed 1 month after treatment, and tissues were analysed for NAGLU activity. The results of these experiments are reported in Table 1. As shown in this Table, the NAGLU activity in all organs of untreated mice was nearly zero. In contrast, in animals treated with one of the doses of the vector, a dose-response was observed. At the highest viral dose (12 μ g), the liver, spleen, lung and heart of the injected mice showed a significant increase in enzyme activity compared with those of untreated animals, with the highest levels present in the liver and spleen. A NAGLU activity of 2.62 nmol (17 h) $^{-1}$ $\cdot$ (mg of protein) $^{-1}$ was reached in the liver, corresponding to 1.14% of the normal control; in the spleen, the value of 32.5 nmol

Figure 1 Biodistribution of lentiviral-NAGLU vector in injected mice

MPS IIIB mice were injected with 12 μ g of p24 lentiviral dose and killed 1 month after treatment. Biodistribution of the lentiviral gene transfer vector into the mouse genome was evaluated by a single PCR amplification using human NAGLU cDNA-specific primers and genomic DNA extracted from the various tissues. The integrity of the DNA was determined by amplifying a 1200 bp region of the neo-gene belonging to the mutant allele of the MPS IIIB mice (bottom panel).

(17 h)⁻¹ · (mg of protein)⁻¹ corresponded to 27.7 % of the normal control (Table 1). For the lung and heart, the restored NAGLU activities were less consistent, with the values corresponding to 0.16 and 0.7 % of the values found in normal controls respectively. Lentiviral-NAGLU vector did not mediate recombinant NAGLU expression in the thymus, kidney or brain, the values of the enzyme activity being very similar to those found in untreated animals (Table 1).

Biodistribution and integration of lentiviral-NAGLU vector

To determine the biodistribution of lentiviral-NAGLU vector administered through the tail vein, various organs of the mice were collected 1 month after injection with the 12 μ g dose. The presence of the vector genome, as determined by the PCR analysis described in the Materials and methods section, is reported in Figure 1. Lentiviral vector DNA was detected at the highest level in the liver and spleen of injected mice; a much lower level of vector DNA could be detected in the lung, whereas no signal was recovered from the heart, kidney, thymus and brain of the affected mice and from all tissues of the untreated controls. To verify the integration of the vector DNA into the host genome, a double round of PCR amplification was performed on DNA extracted from the liver and spleen of injected and untreated mice. Limiting amounts of the amplified products and non-amplified genomic DNA were used as templates for a second round of PCR using nested primers at the 5'- or 3'-end of the vector genome (Figure 2). As shown in this Figure, the expected bands (160 bp for the 3'-nested PCR and 120 bp for the 5'-nested PCR) were obtained only from the amplified products, indicating that the vector genome was integrated in the mouse DNA.

In vivo expression of recombinant NAGLU: immunohistochemical localization in the liver

To confirm the presence of recombinant NAGLU in the liver tissue from the lentiviral-treated mice, immunohistochemical staining was performed as described under the Materials and methods section. Immunohistochemical staining for NAGLU demonstrated that the recombinant protein was present in the 12 μ g-treated mice (Figure 3A), whereas the untreated affected mice had no detectable protein (Figure 3B). The presence of human NAGLU enzyme, visualized by the brown colour of the peroxidase

Figure 2 Proviral integration after systemic delivery: nested PCR

To verify the integration of the lentiviral DNA into the host genome, two rounds of PCR were used. A first B2-specific PCR performed on DNA extracted from liver (L) or spleen (S) of 12 μ g-injected mice 1 month after treatment was followed by a nested PCR. In the first set of amplifications, primers based on the murine B2 family of highly repeated dispersed elements were used together with primers derived from the 5'- or 3'-end of the vector. Limiting amounts of the amplified products and non-amplified genomic DNA were then used as templates for a second round of PCR using nested primers at the 5'-end (5'-nested) or 3'-end (3'-nested) of the vector. Control, genomic DNA subjected only to nested PCR; L, liver; S, spleen.

reaction (Figure 3A, arrows), allowed us to estimate that approx. 3–4 % of the liver cells from the treated mice were transduced and that most of them were Kupffer cells, as determined based on their morphology.

Correction of the phenotype after lentiviral treatment: levels of soluble GAG

To study the lentiviral-mediated correction of lysosomal storage in the MPS IIIB affected mice, the GAG dosage was performed on tissues from the 12 μ g p24-injected mice. The results are reported in Table 2. As shown in this Table, the GAG content in the injected mice was substantially decreased, corresponding to a decrease of 77 % in the liver, 46 % in the spleen, 29 % in the lung and 16 % in the heart, whereas no effect was found in the kidney or brain (Table 2).

Correction of the phenotype after lentiviral treatment: lysosomal enzyme activity

Since it was reported [9] that the MPS IIIB knockout mouse shows a secondary increase of lysosomal enzyme activity (β -hexosaminidase and β -glucuronidase) both in the liver and brain or only in the liver (α -L-iduronidase), these lysosomal activities were measured in the 12 μ g of p24-injected mice 1 month after treatment. We demonstrated that the lentiviral-NAGLU injection was capable of partially decreasing these enzyme activities in the liver tissue (Figure 4), with particular relevance to β -hexosaminidase activity, which reached values very similar to those found in heterozygous mice or in normal mice, resulting in a decrease of approx. 40 %. As expected, no decrease in α -L-iduronidase, β -glucuronidase or β -hexosaminidase activities was observed in the brain of injected mice.

Immune response of mice treated with lentiviral-NAGLU vector

To determine the presence of anti-recombinant NAGLU mouse IgG, blood was collected from the 12 μ g-treated mice at different time points. In all the analysed sera (Figure 5A), a humoral response was not detected 1 week post-injection; the levels slightly increased at later time points. At 1 month after treatment, the

Figure 3 Immunohistochemical localization of recombinant NAGLU in liver

Immunostaining was performed on paraffin-embedded liver sections from a mouse treated with 12 μg of lentiviral vector and killed 1 month later. (A) A $\times 40$ magnification of the treated mouse; (B) the untreated control. The presence of human NAGLU enzyme was visualized by the brown colour of the peroxidase reaction (arrows): most of the positive cells were Kupffer cells. The tissues were counterstained with haematoxylin, which stains the nuclei blue.

Table 2 GAG levels in tissues from lentiviral-NAGLU-treated mice

MPS IIIB mice (8–10 weeks old) were intravenously injected with 12 μg of lentiviral-NAGLU vector corresponding to 0.75×10^9 T.U. (transducing units, calculated as endpoint titre on HeLa cells). Normal and heterozygous mice were injected with PBS. After 1 month, mice were killed and tissues were analysed for the GAG content. Data are means $\pm$ S.D. for three animals. * $P < 0.01$ versus untreated mice; † $P < 0.05$ versus untreated mice; no symbol is used to indicate $P > 0.05$.

Mouse	GAG level ($\mu\text{g}/\text{mg}$ tissue)					
	Liver	Spleen	Lung	Heart	Kidney	Brain
Untreated	7.12 ± 1.09	4.11 ± 1.0	3.49 ± 0.44	2.4 ± 0.15	14.9 ± 3.27	7.35 ± 3.0
12 μg -treated	$1.67 \pm 0.6^*$	$2.25 \pm 0.27^\dagger$	$2.5 \pm 0.34^\dagger$	$2.0 \pm 0.10^\dagger$	14.2 ± 2.27	12.9 ± 3.28
Normal	0.61 ± 0.14	1.17 ± 0.27	0.55 ± 0.15	1.14 ± 0.82	0.51 ± 0.1	2.26 ± 1.27

Figure 4 Correction of the phenotype after lentiviral treatment: lysosomal enzyme activities

MPS IIIB mice were injected with 12 μg of p24 viral vector; 1 month later, liver (L) and brain (B) were assayed for lysosomal enzyme activities with fluorimetric assays. Each data point represents the mean $\pm$ S.D. for three mice. Values on the left refer to β -hexosaminidase activity and values on the right to β -glucuronidase and α -L-iduronidase activities. Decrease in β -hexosaminidase was statistically significant at $P = 0.001$; decrease in β -glucuronidase was statistically significant at $P = 0.01$. White bar, untreated mice; black bar, treated mice; dotted bar, heterozygous mice; striped bar, normal mice.

Figure 5 Immune response of mice treated with lentiviral-NAGLU vector

MPS IIIB mice (two for each subset) were intravenously injected with lentiviral-NAGLU vector at the 12 μ g dosage and killed 1 month ($\square$) or 6 months ($\circ$) after treatment. All values are averages at the indicated time points. (A) The anti-NAGLU antibody levels as measured by an ELISA. The mice serum was tested at 1:50 dilution (open symbols) or at 1:500 dilution (closed symbols). (B) The titration curves in the serum of mice killed 1 month ($\diamond$) or 6 months ($\blacklozenge$) after treatment.

antibody titre obtained was very low, with the maximum dilution of antiserum determined to be 50 times (Figure 5B).

Long-term (6 months) follow-up of lentiviral-NAGLU therapy

To evaluate whether a single administration of lentiviral vector into the peripheral blood of the mouse led to a long-term expression of the NAGLU gene, MPS IIIB mice were injected with 12 μ g of viral dose and examined after 6 months. Except for the spleen, no significant NAGLU activity was detected in any of the tissues analysed (Table 3); however, a statistically significant decrease in the GAG content was found in the liver, spleen, lung and heart of the injected animals, the decrease being 35, 36, 44 and 34 % respectively (Table 3). B2-PCR analysis revealed the persistence of the viral genome in the liver, spleen and lung of the analysed mice, indicating that the integration of the vector was still present after 6 months (results not shown).

To quantify the amount of integrated vector sequences, a quantitative PCR analysis was performed. The DNA copy number was determined in the liver and spleen by normalizing it to the β -actin sequence of the mouse genome. As shown in Table 4, the vector DNA was much lower in tissues of mice killed 6 months

Table 3 Long-term (6 months) follow up of lentiviral-NAGLU treatment

MPS IIIB mice (8–10 weeks old) were intravenously injected with 12 μ g of lentiviral-NAGLU vector. Untreated age-matched controls were mock-injected. After 6 months, mice were killed and tissues analysed for NAGLU activity and GAG content. Data are means $\pm$ S.D. for three animals. * $P < 0.05$ versus untreated mice; no symbol is used to indicate $P > 0.05$.

Mouse	Tissue	NAGLU activity [nmol $\cdot$ (17 h) $^{-1}$ $\cdot$ (mg of protein) $^{-1}$]	GAG level (μ g/mg tissue)
Untreated	Liver	0.019 $\pm$ 0.020	10.0 $\pm$ 2.15
	Spleen	0.067 $\pm$ 0.018	5.0 $\pm$ 0.81
	Lung	0.096 $\pm$ 0.027	4.84 $\pm$ 0.28
	Heart	0.068 $\pm$ 0.020	4.5 $\pm$ 0.20
Treated	Liver	0.028 $\pm$ 0.011	6.5 $\pm$ 0.40*
	Spleen	0.154 $\pm$ 0.044*	3.2 $\pm$ 0.28*
	Lung	0.115 $\pm$ 0.039	2.7 $\pm$ 1.09*
	Heart	0.072 $\pm$ 0.01	3.0 $\pm$ 0.88*

Table 4 Real-time PCR for quantitative assessment of lentiviral-NAGLU vector

Mice were treated with 12 μ g of p24 viral protein and killed at two time points after injection (1 and 6 months). Real-time PCR analysis was performed on DNA isolated from the liver and spleen. Copy numbers were obtained from known copies of positive control plasmid diluted into negative control mouse liver DNA. Results are expressed as number of copies/cell.

Tissue	Time after treatment	
	1 month	6 months
Liver	0.21 $\pm$ 0.08	0.0071 $\pm$ 0.003
Spleen	0.059 $\pm$ 0.028	0.0225 $\pm$ 0.0092

after injection compared with the tissues from animals analysed 1 month after treatment. Interestingly, the decrease in vector DNA was much greater in the liver tissue than in the spleen.

The immune response tests on the sera collected 6 months after treatment showed that the anti-NAGLU humoral response remained at similar levels to those obtained at 1 month after treatment (Figure 5A). At 6 months after treatment, the resulting antibody titre was very low, with the maximum dilution of antiserum determined to be 50 times (Figure 5B).

DISCUSSION

A number of viral vectors have been proposed in the last few years for gene therapy. Among these, lentiviral vectors appear to be promising tools: they integrate a sizeable gene expression cassette efficiently into the target cells, providing for a stable and inherited genetic modification; moreover, they can integrate into the genome of both dividing and non-dividing cells [26]. A third generation lentiviral vector was developed using only a fractional set of HIV genes: *gag*, *pol* and *rev*, with constructs non-functional outside the vector producer cells [19,27,28]. These late generation recombinant lentiviral vectors have significantly improved biosafety but still maintain high transduction efficiency and a broad targeting range; they were shown to be effective by intracranial delivery into murine models of metachromatic leukodystrophy [13] and β -glucuronidase deficiency, MPS VII [14–16].

We used an improved vector construct packaged by a quadri-transfection minimal system where the NAGLU cDNA is driven by the CMV promoter. We recently reported that this vector was

capable of restoring NAGLU enzyme activity and correct the metabolic defect in MPS IIIB-deficient fibroblasts [17].

In this paper, we report our data on lentiviral-NAGLU gene therapy *in vivo* for the murine model of MPS IIIB disease. Our results show that 1 month after a single administration of one of the vector doses, the transgene expression was mainly present in the liver and spleen of treated animals. At the highest viral dose used in the present study, the transgene expression was evident also in the lung and heart. PCR analyses confirmed the presence of vector DNA in the liver, spleen and lung of treated animals, showing higher copy numbers of vector DNA in the liver than in the spleen. These results are in agreement with previous biodistribution studies on intravenous injected lentiviral vectors [20,29].

The dose-response reported in the present study seems to suggest a threshold effect: in both liver and spleen, there was an almost linear response up to 9 μ g of viral vector followed by a nonlinear response when the dose was increased to 12 μ g. Although this phenomenon was never reported for lentiviral vectors, indeed it was found for adenoviral vectors [30].

In 12 μ g-treated mice, the recovered recombinant NAGLU activity ranged from the highest value in the liver (1.1 % of normal) to the lowest value in the lung (0.16 % of normal). It is possible that a limited population of cells (liver Kupffer cells and lung macrophages) stores most of the GAG in this mouse model and that these are the cells that were mainly transduced and corrected. Although minute, the recombinant NAGLU activity resulted in significant decrease of GAG accumulation: it is remarkable, in fact, that 0.16 % of the wild-type NAGLU activity achieved in the lung of treated animals was sufficient to bring a 29 % decrease in GAG level in that tissue. It is possible that complete normalization of GAG accumulation can be reached with higher doses of lentiviral-NAGLU vector, at least in the liver and spleen.

In the liver of treated mice, only 3–4 % of the liver cells appeared to be transduced, as shown by immunohistochemistry tests. This implies that the recombinant NAGLU activity found in liver, which gives rise to the decrease in GAG, is quite probably secreted; the secreted protein is taken up by the untransduced MPS IIIB mouse cells, resulting in the correction of GAG accumulation. The lack of a visible gradient of immunoreactivity around the positive cells could be due to the limitations of the immunohistochemistry technique and not due to a real lack of partially corrected cells. The secretion-recapture mechanism could apply also to the other organs metabolically corrected in the present study, i.e. spleen, lung and heart. Our results are in agreement with recent work on the intravenous administration into immunodeficient mice of lentiviral vectors carrying either an intracellular marker gene (GFP) or a gene of a secreted molecule (Factor IX): high transduction of the liver, spleen and bone marrow was obtained, with levels of Factor IX in the murine plasma (1–2 % of normal) that would be therapeutically useful if achieved in haemophilia B patients [20].

The efficacy of lentiviral gene therapy in the liver was confirmed by the decrease in activity of three lysosomal enzymes: β -hexosaminidase, β -glucuronidase and α -L-iduronidase. This finding is in agreement with the results of Yu et al. [10] that show a decrease in these lysosomal activities after short-term enzyme replacement therapy in MPS IIIB mice. Secondary increases in the lysosomal enzyme activity occur in lysosomal storage diseases, and gene or enzyme therapy often normalize these levels. For the MPS VII murine model, enzyme replacement decreased the increased α -galactosidase, β -galactosidase and β -hexosaminidase activity levels [14] and gene therapy decreased mRNA levels of two lysosomal enzymes in the brain [31]. It is currently unknown whether these secondary changes occur as a result of

increased transcription or decreased protein degradation. A recent study [32] on this has suggested that both transcriptional and post-transcriptional mechanisms are involved in the increase in enzyme activity.

In the present study, no correction was obtained in the brain of mice treated with lentiviral-NAGLU vector; this was as expected since intravenous injections cannot cross the blood-brain barrier. Indeed, intracranial injections of viral vectors resulted in neurological correction of lysosomal storage in some murine models [11,13–16,31]. A direct injection of the lentiviral-NAGLU vector is expected to result in the correction of lysosomal storage in MPS IIIB murine brain. However, less invasive procedures based on new technologies to cross the blood-brain barrier need to be developed. In this respect, the use of hyperosmotic drugs [33] or bone marrow stem cells [34] could be alternative avenues of approach. Recently, transplantation of retrovirally transduced bone marrow was reported to have a therapeutic effect on brain of affected MPS IIIB mice [12].

With the same type of lentiviral vector, a widespread transduction of non-parenchymal liver and spleen cells was recently demonstrated, suggesting that antigen-presenting cells were also efficiently transduced by lentiviral vectors [35,36] and probably triggered an immune response to the transgene product. In our case, however, 4 weeks after the 12 μ g p24 viral treatment, the anti-NAGLU immune response did not prevent the transgene expression and its distribution to non-transduced cells, as demonstrated by the metabolic correction of GAG accumulation achieved in the liver, spleen, lung and heart.

Long-term (6 months) lentiviral-NAGLU treatment of MPS IIIB mice showed a persistent effect in the liver, spleen, lung and heart. It could well be that, in these tissues, the low level of NAGLU activity recovered after treatment was sufficient to maintain the decreased GAG levels; alternatively, the decreased GAG at 6 months could be due to a very slow rate of reaccumulation of the heparan sulphate. PCR analysis showed that the vector DNA was still integrated in the liver, spleen and lung tissues; however, the DNA copy numbers proved to be halved in the spleen (in agreement with the residual NAGLU activity present in this organ), whereas a more significant decrease was evident in the liver. This could be due to either the turnover of transduced Kupffer cells or the induction of cytotoxic T-lymphocytes, resulting in the clearance of transduced cells. The data on the humoral response obtained after 6 months seem to exclude this hypothesis: the level of anti-NAGLU antibodies was apparently low, not much higher than that obtained 1 month after treatment. In a recent study on GFP and Factor IX driven by CMV in the same lentiviral system, the massive immune response directed against the transgenes was reported to be responsible for a clearance of the transduced cells in long-term treatment; selective targeting of the lentiviral expression to hepatocytes using an albumin promoter limited the immune response to the transgene [36]. Further studies are required to clarify this aspect of lentiviral-NAGLU gene therapy.

In conclusion, our results demonstrate the therapeutic potential of lentiviral-NAGLU vector for the correction of the pathology after intravenous gene therapy. The use of tissue-specific promoters and/or the application of techniques capable of overcoming the blood barrier would result in more effective long-term therapy.

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