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RESEARCH ARTICLE

Improved retroviral vector design results in sustained expression after adult gene therapy in mucopolysaccharidosis I mice

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Abstract

Background Mucopolysaccharidosis I (MPS I) is a lysosomal storage disease due to α-L-iduronidase (IDUA) deficiency that results in the accumulation of glycosaminoglycans (GAG). Gene therapy can reduce most clinical manifestations, but mice that receive transfer as adults lose expression unless they receive immunosuppression. Increasing liver specificity of transgene expression has reduced immune responses to other genes.

Methods A gamma retroviral vector was generated with a liver-specific human α1-antitrypsin promoter and the canine IDUA cDNA inverted relative to the retroviral long-terminal repeat. Adult MPS I mice received the vector intravenously at 6 weeks of age and were assessed for expression via serial serum IDUA assays. Functional testing and organ analysis were performed at 8 months.

Results This vector resulted in high specificity of expression in liver, and serum IDUA activity was stable in 90% of animals. Although the average serum IDUA activity was relatively low at 12.6 ± 8.1 units/ml in mice with stable expression, a relatively high percentage of enzyme contained the mannose 6-phosphorylation necessary for uptake by other cells. At 6.5 months after transduction, most organs had high IDUA activity and normalized GAG levels. There was complete correction of hearing and vision abnormalities and significant improvements in bone, although the aorta was refractory to treatment.

Conclusions Stable expression of IDUA in adult MPS I mice can be achieved without immunosuppression by modifying the vector to reduce expression in the spleen. This approach may be effective in patients with MPS I or other lysosomal storage diseases. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords canine iduronidase; gene therapy; lysosomal storage disease; mucopolysaccharidosis; retroviral vector

Introduction

Mucopolysaccharidosis I (MPS I) is an autosomal recessive lysosomal storage disease with an incidence of 1 : 100 000 [1]. It is due to deficient

α-L-iduronidase (IDUA; EC 3.2.1.76) activity and results in the accumulation of the glycosaminoglycans (GAG) heparan and dermatan sulfate [2]. This results in a variety of clinical manifestations via the physical thickening of structures such as heart valves and airway components [2], the upregulation of proteases that degrade extracellular matrix proteins in the joints [3] and aorta [4], or other as yet unidentified mechanisms. MPS I can manifest as the relatively mild Scheie syndrome [Online Mendelian Inheritance in Man (OMIM) #607016] involving stiff joints, coarse facial features, hernias, visual impairment, deafness and cardiac valve disease without neurological involvement [2]. The severe Hurler syndrome (OMIM #607014) results in mental retardation and more profound systemic manifestations.

Available treatments for patients with MPS I include enzyme replacement therapy (ERT) [5] or hematopoietic stem cell transplantation (HSCT) [6]. All approaches rely at least in part upon uptake of mannose 6-phosphate (M6P)-modified IDUA from the extracellular space by cells via the M6P receptor and translocation of enzyme to the lysosome. For ERT, this involves the intravenous (i.v.) injection of M6P-modified IDUA protein. For HSCT, blood-derived cells migrate into organs and secrete enzyme locally, although some enzyme can be secreted into blood.

Gene therapy is currently being tested in animal models [7]. An obstacle to successful gene therapy for MPS I is the development of immune responses to the therapeutic protein. Indeed, expression was unstable or low after i.v. injection of gamma retroviral vectors (RV) [8,9], lentiviral vectors [10,11] or plasmid vectors [12,13] to adult MPS I mice without immunosuppression. These mice have an insertion into exon 6 of the 14-exon gene, and have very low levels of IDUA activity and fail to express much of the 653-amino acid protein [14]. These mice serve as a model for patients with severe deficiency of IDUA activity (Hurler syndrome) that have premature stop codons at W402X, Q70X or other sites and similarly fail to express many epitopes of the protein [15]. Pharmacological immunosuppression of MPS I mice after gene therapy with a canine IDUA-expressing RV resulted in stable serum IDUA activity after gene therapy to adult MPS I mice [9], suggesting that the loss of expression was probably due to an immune response. Because mice that lost expression did not develop antibodies but had a marked fall in the liver DNA copies to $<$10% and RNA levels to $<$1% of the level seen in immunosuppressed mice [9], this effect was likely due to a cytotoxic T lymphocyte (CTL) response. Furthermore, some mice that lost expression had positive CTL assays against IDUA-expressing cells (B. Wang and K. Ponder, unpublished data). Similarly, MPS I cats that received neonatal gene therapy with the same canine IDUA-expressing vector also lost expression, which was associated with a decrease in DNA copies to 10% and RNA to 0.3% of the levels found in cats that received the same dose of vector and were immunosuppressed, and was associated with a CTL response against autologous canine IDUA-expressing cells [16]. This immune response may be due to the fact that long-terminal repeat (LTR)-initiated transcripts in nonhepatic organs such as the spleen [17] can be translated into IDUA protein, which may allow expression to occur in antigen presenting cells (APC).

One approach to reduce an immune response is to perform gene therapy in newborns before the immune system is mature. This was effective in MPS I mice [8,10,18,19] but was not effective in MPS I cats [16], and may not be effective in humans with their relatively mature neonatal immune systems [20]. As noted above, transient immunosuppression was effective in adult mice [9] and neonatal cats [16] but increases the risk of infection and malignancy [21]. A third approach is transfer of transduced hematopoietic stem cells into irradiated recipients [22,23] but hematopoietic stem cell transfer has risks. Finally, organ-restricted expression might avoid expression in APCs and reduce subsequent immune responses [24–27]. Indeed, the latter approach resulted in some long-term expression of human IDUA from the albumin promoter of a lentiviral vector in MPS I mice [26]. However, the level of expression was only 1% of normal in liver, and GAG levels were not normalized.

In this study, we used the liver-specific human α1-antitrypsin (hAAT) promoter and inverted the expression cassette relative to the LTR of an RV to attempt to restrict IDUA expression after gene therapy to adult MPS I mice. We used the canine IDUA because that evoked an immune response in our previous study using an RV [9], and we wanted to examine the effect of vector design upon CTL responses without the confounding effect of changing the cDNA. In the present study, we report that this vector results in stable expression in most mice and the correction of many of the disease manifestations.

Materials and methods

All reagents were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA) unless otherwise stated. Hepatocyte growth factor (HGF) was purified as described previously [28].

Construction of reverse-hAAT-cIDUA

A gamma RV designated reverse-hAAT-cIDUA contained the 420-bp hAAT promoter from hAAT-WPRE-767 [29], the synthetic intron with adjacent exonic sequence from pCMS-EGFP (BD Biosciences, San Jose, CA, USA), the canine IDUA cDNA [30], and the bovine growth hormone polyadenylation site from pcDNA3.1(−) (Invitrogen, Carlsbad, CA, USA). This was assembled with the expression cassette inverted relative to the LTR as detailed in the supplementary methods section and as shown in Figure 1B. A replication-incompetent amphotropic RV was prepared as previously described [29] using GP+AM12 packaging cells [31]. Titers were determined by transduction of NIH 3T3 cells *in vitro* followed by determination of the RV DNA copy number 1 week later [8], and the vector was found to be negative for

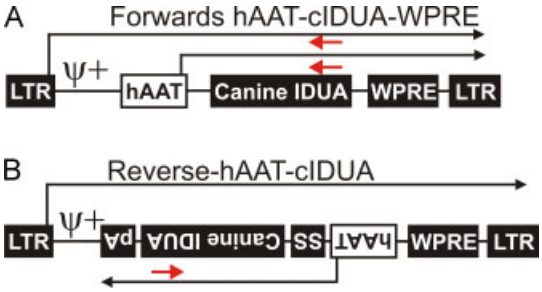

Figure 1. Retroviral vectors. (A) Forward-hAAT-cIDUA-WPRE. This previously described Moloney murine leukemia virus-based RV [8] contains 5′ and 3′ intact long-terminal repeats (LTR), an extended packaging signal (ψ+), the 420 bp human α1-antitrypsin promoter (hAAT), the 2.2-kb canine IDUA cDNA, and WPRE. Transcription of the canine IDUA cDNA can be driven by the liver-specific hAAT promoter or the nonspecific 5′-LTR promoter. The arrows above the canine IDUA sequence represent the position of the 3′ reverse primer used for reverse transcription. (B) Reverse-hAAT-cIDUA. The hAAT-canine IDUA expression cassette was inverted relative to the LTRs to prevent the 5′-LTR from directing expression of the canine IDUA cDNA. A synthetic splice site (SS) with adjacent exonic sequences and a bovine growth hormone polyadenylation sequence (pA) were added

replication-competent retrovirus using a marker-rescue assay [29].

Animals

All animal studies were approved by the authors' Institutional Review Board. Six week-old MPS I mice [14] in a C57BL/6 background were injected intraperitoneal (i.p.) with five doses of 5 mg/kg of HGF with 15 mg/kg of dextran sulfate per dose at 0, 3, 6, 9 and 12 h. Mice were injected via the tail vein with 2–5 doses of 300 µl of either the previously described RV designated hAAT-cIDUA-WPRE [8] (hereafter referred to as forward-hAAT-cIDUA-WPRE vector) or with reverse-hAAT-cIDUA at 30–48 h after the first dose of HGF, as shown in the legend to Figure 2. The cumulative dose was $0.5–1.7 \times 10^{10}$ transducing units (TU)/kg. Heterozygous normal and untreated MPS I mice were used as controls. Serum was obtained from the right retroorbital plexus or the tail vein.

Evaluation of bone, heart, eye and ear

Bone radiographs and bone mineral density (BMD) were performed as described previously with the same machinery. The diameter of the femur was measured at a point approximately two-thirds of the distance between the superior portion of the greater trochanter and the distal end of the epicondyles as described previously [8], and the ratio to that in normal mice was determined. Echocardiography was performed using inhaled isofluorane anesthesia with a Vevo 770 echocardiography machine (VisualSonics, Toronto, ON, Canada), which is a different machine than this laboratory had used previously. The internal diameter of the aorta was determined at the ascending aorta at a position that corresponds to our previous measurements. The maximum length of the aortic insufficiency jet was measured by pulse wave Doppler mapping, and a value of 0.5 mm or more was considered evidence of aortic insufficiency. Electroretinography (ERG) [32,33] and auditory-evoked brainstem responses (ABR) [34] were performed by a different investigator (K.K.O.) with a different apparatus than was previously used by this laboratory for MPS I mice, although they followed the same protocol. The magnitude of the ERG signal was less than in our previous results [8] for reasons that are unclear. ABR determined the decibel sound-pressure level required to elicit a brainstem response. By convention, animals that did not respond to any threshold at a particular frequency were denoted by the maximum feasible threshold of 110 db. Prior to sacrifice, serum was collected, and animals were transcardially perfused with 20 ml of saline.

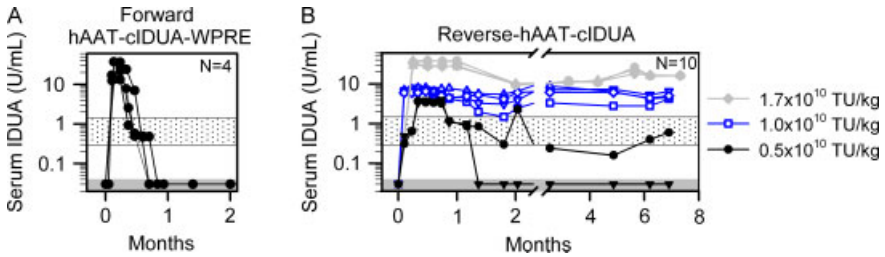

Figure 2. Serum IDUA activity after adult treatment in MPS I mice. Six-week old MPS I mice were treated with five doses of HGF given i.p. at 0, 3, 6, 9, and 12 h after initiation of HGF. (A) Forward-hAAT-cIDUA-WPRE. Four mice received two doses of 0.25×10^{10} TU/kg of forward-hAAT-cIDUA-WPRE given at 30 and 36 h after initiation of HGF for a cumulative dose of 0.5×10^{10} TU/kg. Each line represents serum IDUA activity for an individual mouse. (B) Reverse-hAAT-cIDUA. The low-dose group (closed black symbols) received 0.25×10^{10} TU/kg of reverse-hAAT-cIDUA given at 30 and 36 h after initiation of HGF, for a cumulative dose of 0.5×10^{10} TU/kg. The medium dose group (open symbols) received three doses of 0.33×10^{10} TU/kg of reverse-hAAT-cIDUA given at 30, 36 and 48 h after initiation of HGF for a cumulative dose of 1×10^{10} TU/kg. The high-dose group (closed grey symbols) received five doses of 0.33×10^{10} TU/kg of reverse-hAAT-cIDUA given at 30, 36, 40, 44 and 48 h after initiation of HGF for a cumulative dose of 1.7×10^{10} TU/kg. The groups were not treated concurrently. Each line represents serum IDUA activity for an individual mouse. Serum IDUA activity for heterozygous normal mice was determined experimentally and multiplied by 2 to give the expected value for homozygous normal mice; this mean ±2 SD for homozygous normal mice was 0.3–1.6 U/ml and is depicted by the stippled bar. Untreated MPS I mice had <0.04 U/ml as depicted by the grey bar

IDUA activity, β-hexosaminadase (β-hex) activity and GAG levels

Organs were homogenized in lysis buffer as described [8], and the same homogenate was used for enzyme and GAG assays. The total protein concentration was determined with the Bradford assay (BioRad Laboratories, Hercules, CA, USA). Unless otherwise specified, IDUA assays were performed with 4-methylumbelliferyl α-L-idopyranosiduronic acid (Toronto Research Chemicals, North York, ON, Canada) in 0.4 M formate buffer at pH 3.5 at 37 °C with a Fluoroskan Ascent microplate fluorometer from Thermo Electron Corporation (Milford, MA, USA) with excitation at 355 nm and emission at 460 nm, as described previously [9]. One unit of enzyme releases 1 nmol of 4-methylumbelliferone per hour and standards were dilutions of 4-methylumbelliferone. IDUA activity in organs from homozygous normal mice was assumed to be twice the level determined experimentally for the heterozygous normal mice. The IDUA enzyme activity was calculated in a slightly different fashion from before and gave values that were approximately 43% of those determined previously (see Supplementary Material). The β-hex assay used 4-methylumbelliferyl-acetyl-β-D-glucosaminide as described previously [8]. GAG levels were determined [35] using a sulfated glycosaminoglycan kit from Blyscan (Newtownabbey, UK) with chondroitin 4-sulfate as the standard.

To determine the percentage of IDUA in serum that contained M6P, a 1-ml volume column with the cationic-dependent M6P receptor [36] was used to capture M6P-modified enzyme, which was eluted with 5 mM M6P after washing the column with 5 mM glucose 6-phosphate as detailed in the Supplementary material. The percentage of enzyme with M6P was determined by evaluation of the different fractions.

Nucleic acid analysis

DNA and RNA were isolated as previously described [18] and primers and Taqman probes for canine IDUA [37] and mouse β-actin [29] were used. For DNA analysis, real-time polymerase chain reaction (PCR) analysis was performed using 100 ng DNA and Taqman technology with reagents from Applied Biosystems (Foster City, CA, USA). The standards were mixtures of the plasmid reverse-hAAT-cIDUA DNA with genomic liver DNA from an untransduced mouse [18]. For RNA analysis, reverse transcription (RT) was performed with the reverse canine IDUA (Figure 1) and reverse β-actin primers. Real-time PCR with Taqman technology was performed for canine IDUA with normalization to the β-actin signal. No signal for IDUA was observed using samples that did not receive RT or for RT-treated RNA from nontransduced controls.

Statistical analysis

Analysis of variance (ANOVA) with Tukey's post-hoc analysis or the Student's *t*-test compared values in different groups using Sigma Stat software, version 3.1 (Systat Software, Inc., Point Richmond, CA, USA). Fisher's exact test determined if the frequency of an event differed between two groups.

Results

Generation of reverse-hAAT-cIDUA

The previously described RV designated hAAT-cIDUA-WPRE [8] that exhibited unstable expression after transfer to adult mice is referred to the forward-hAAT-cIDUA-WPRE vector and is shown in Figure 1A. It contains an intact LTR from the Moloney murine leukemia virus at the 5′- and 3′-ends, the liver-specific hAAT promoter, the canine IDUA cDNA, and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). We hypothesized that modification of the vector to reduce extrahepatic expression might reduce an immune response. We therefore generated the RV designated reverse-hAAT-cIDUA shown in Figure 1B, in which the expression cassette was inverted relative to the LTR, and splice and polyadenylation sites were placed in the appropriate orientation upstream and downstream of the canine IDUA cDNA, respectively. The canine cDNA was used, as that evoked a CTL response previously in mice. The WPRE was maintained in an orientation that would allow it to improve expression of the genomic LTR-initiated transcript in order to improve the titer. An amphotropic vector with a titer of 2×10^8 TU/ml was generated and shown to be replication-incompetent using a marker-rescue assay. The reverse-hAAT-cIDUA vector directed expression of IDUA activity in human hepatoma-derived HepG2 cells but did not result in expression in fibroblast-derived NIH 3T3 cells (data not shown).

Serum activity in MPS I mice after adult administration of reverse-hAAT-cIDUA

Six-week-old adult MPS I mice were injected with HGF to stimulate hepatocyte replication, as described in the Materials and methods and the legend to Figure 2. Four mice were treated with the forward-hAAT-cIDUA-WPRE vector (Figure 2A) with a cumulative dose of 0.5×10^{10} TU/kg. Despite strong initial expression at 23.9 ± 9.6 units (U)/ml at 1 week, the expression fell to nearly undetectable levels in all mice by 14 days, which was similar to previous results [8–9].

Ten mice were treated with the reverse-hAAT-cIDUA vector (Figure 2B). The low ($n = 2$), medium ($n = 4$), and high ($n = 4$) dose groups received a total of 0.5×10^{10}, 1×10^{10}, and 1.7×10^{10} TU/kg, respectively. The groups

were not treated concurrently, and differences in the timing of RV injections relative to the initiation of HGF (Figure 2B), which was due to our evolving information on the timing of hepatocyte replication after the initiation of HGF, are likely responsible for the failure to observe a linear relationship between the dose of RV and the expression levels. At 1 week after transduction, the mice achieved 0.65 ± 0.01 U/ml, 6.8 ± 0.7 U/ml and 34 ± 4.1 U/ml IDUA activity in serum in the low, medium and high dose groups, respectively. Nine of 10 (90%) reverse-hAAT-cIDUA-treated mice maintained stable expression long-term, which was statistically higher than with the forward-hAAT-cIDUA-WPRE vector ($p < 0.005$ with Fisher's exact test). The average long-term expression of serum IDUA was 12.6 ± 8.1 U/ml in the mice who received the medium or high dose of vector. This was lower than the long term expression of 35 ± 18 U/ml that was obtained using the enzyme assay reported here for mice that received the forward-hAAT-cIDUA-WPRE vector and were immunosuppressed [9]. These data demonstrate that most mice that received the reverse-hAAT-cIDUA vector maintained stable expression long-term without immunosuppression. However, this approach was not always effective because one of the low-dose reverse-hAAT-cIDUA-treated mice lost expression at a relatively late time (1.4 months after transduction).

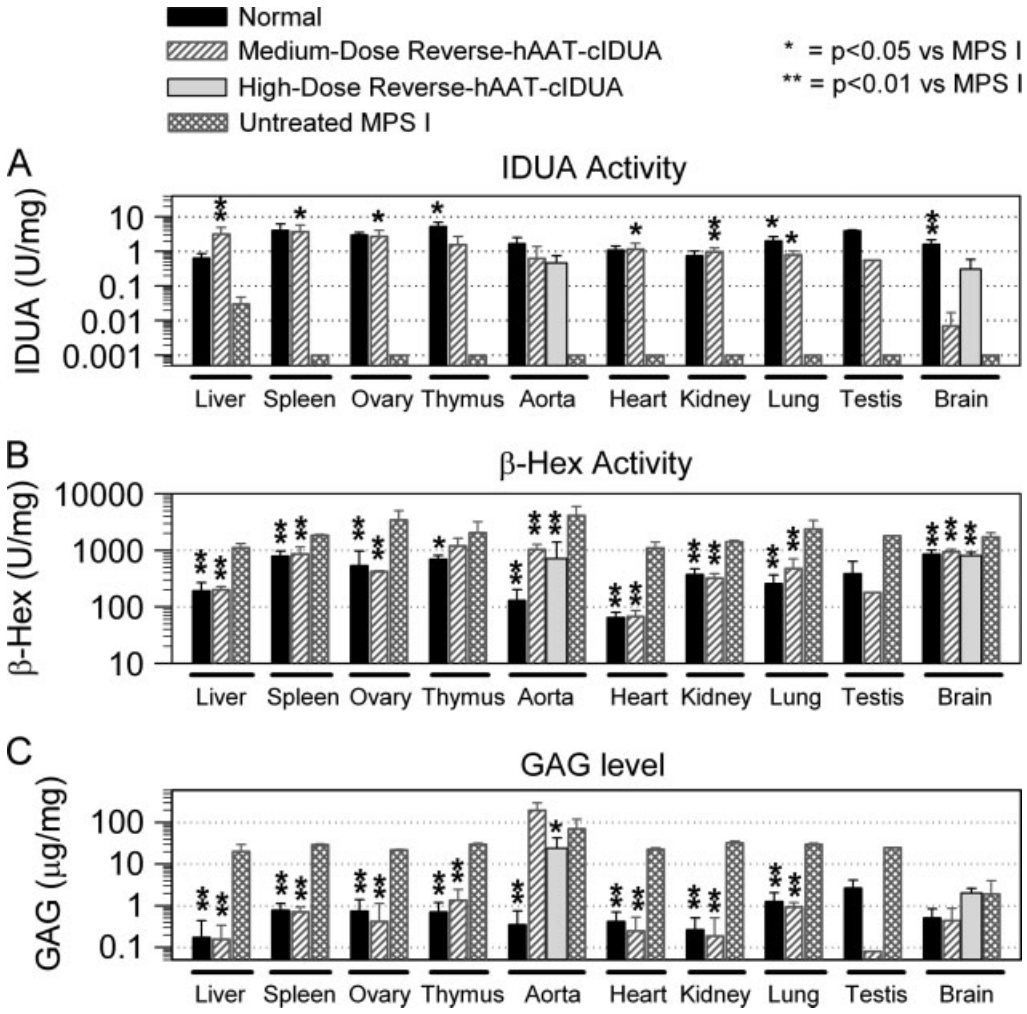

Figure 3. Lysosomal enzyme activity and GAG levels in organs. Some MPS I mice were treated with a medium dose (10^{10} TU/kg) or high dose (1.7×10^{10} TU/kg) of reverse-hAAT-WPRE at 6 weeks after birth. These are the same mice whose serum activity is shown in Figure 2. The medium ($n = 4$) and high ($n = 4$) dose groups had stable serum IDUA activity of 5.2 ± 1 and 20 ± 2 U/ml of IDUA activity in serum, respectively, at the time of sacrifice at 6.5 months after transduction, which was 8 months of age. For the high dose of reverse hAAT-cIDUA, only the aorta and brain were evaluated. Other age-matched MPS I ($n = 3$) and heterozygous normal ($n = 6$) mice were untreated. Organ homogenates were tested for enzyme activities and GAG levels, and the mean ± SD is shown. One asterisk (*) denotes $p < 0.05$ for the statistical comparison of that column with untreated MPS I mice using ANOVA with Tukey's post-hoc analysis, and two asterisks (**) denote $p < 0.01$. For the gonads, three females and one male were evaluated for the medium dose of reverse-hAAT-cIDUA, two females and one male were evaluated for MPS I mice, and three animals of each gender were evaluated for normal mice. (A) IDUA activity. IDUA activity in organs was normalized to total protein. Experimentally determined values in heterozygous normal mice were multiplied by 2 to give the expected value for homozygous normal mice. (B) β-Hex activity was normalized to total protein. For normal mice, values are from heterozygous normal animals without any correction factor. (C) GAG levels were normalized to total protein. For normal mice, values are from heterozygous normal mice without any correction factor

Table 1. Summary of results of specialized testing

Organ	Test performed	Normal (p-value[a] versus MPS I)	Untreated MPS I (p-value[b,c] versus RV-treated)	Medium dose RV-treated (p-value[d] versus normal)	High dose RV-treated (p-value[d] versus normal)
Bone	Femur diameter (% normal)[e,f]	$100 \pm 7\%$ ($n = 4$) $p < 0.01$	$127 \pm 8\%$ ($n = 3$) $p < 0.01$[b]	$107 \pm 4\%$ ($n = 4$) NS	ND
	Bone mineral density (g/cm^2)[f]	0.0535 ± 0.002 ($n = 4$); $p < 0.01$	0.0631 ± 0.001 ($n = 3$); $p < 0.01$[b]	0.0535 ± 0.003 ($n = 4$); NS	ND
Ear	ABR at 5 kHz (dB)[f,g]	47 ± 10 ($n = 9$) $p < 0.01$	98 ± 7 ($n = 9$) $p < 0.01$[b] $p < 0.01$[c]	64 ± 6 ($n = 4$) $p < 0.05$	44 ± 17 ($n = 4$) NS
	ABR at 10 kHz (dB)[f,g]	33 ± 8 ($n = 9$) $p < 0.01$	98 ± 6 ($n = 9$) $p < 0.01$[b] $p < 0.01$[c]	52 ± 9 ($n = 4$) $p < 0.05$	33 ± 18 ($n = 4$) NS
	ABR at 20 kHz (dB)[f,g]	48 ± 13 ($n = 9$) $p < 0.01$	100 ± 10 ($n = 9$) $p < 0.05$[b] $p < 0.01$[c]	77 ± 12 ($n = 4$) $p < 0.05$	44 ± 24 ($n = 4$) NS
	ABR at 40 kHz (dB)[f,g]	65 ± 13 ($n = 9$) $p < 0.01$	110 ± 0 ($n = 9$) $p < 0.05$[b] $p < 0.01$[c]	84 ± 7 ($n = 4$) NS	68 ± 28 ($n = 4$) NS
Eye	Electroretinogram a-wave (μV)[f]	132 ± 34 ($n = 9$) $p < 0.01$	27 ± 10 ($n = 9$) $p < 0.01$[b] $p < 0.01$[c]	148 ± 30 ($n = 4$) NS	132 ± 73 ($n = 4$) NS
	Electroretinogram b-wave (μV)[f]	331 ± 77 ($n = 9$) $p < 0.01$	118 ± 36 ($n = 9$) $p < 0.01$[b] $p < 0.01$[c]	405 ± 157 ($n = 4$) NS	325 ± 142 ($n = 4$) NS
Aorta	Ascending aorta diameter (mm)[f]	1.6 ± 0.1 ($n = 5$) NS	2.3 ± 0.5 ($n = 7$) NS[b]	2.3 ± 0.7 ($n = 4$) NS	ND
Heart	Length of aortic regurgitation jet (mm)[f]	0.3 ± 0.4 ($n = 5$) $p < 0.05$	2.5 ± 1.7 ($n = 7$) NS[b]	1.0 ± 1.0 ($n = 4$) NS	ND
	Frequency of aortic insufficiency[h]	1 of 5 (20%) $p = 0.01$	7 of 7 (100%) NS[b]	3 of 4 (75%) NS	ND

Adult MPS I mice (Fig. 2) were treated with HGF and i.v. injection of the medium (10^{10} TU/kg) or high (1.7×10^{10} TU/kg) dose of reverse-hAAT-cIDUA, and were evaluated at 8 months of age, which was 6.5 months after transduction. Heterozygous normal and untreated MPS I mice were from the same breeding colony. The mean $\pm$ SD values of specialized tests were determined for the given number of animals in each group (n). For some categories, only the medium dose reverse-hAAT-cIDUA vector-treated animals were evaluated. [a]p-value for normal versus untreated MPS I mice. [b]p-value for untreated MPS I versus medium dose reverse-hAAT-cIDUA-treated MPS I mice. [c]p-value for untreated MPS I versus high dose reverse-hAAT-cIDUA-treated MPS I mice. [d]p-value for reverse-hAAT-cIDUA-treated MPS I versus normal mice. [e]The diameter of long bones relative to the average value for normal mice. [f]Auditory-evoked brainstem response. [g]ANOVA with Tukey post-hoc analysis was used to compare values in different groups. [h]Fisher's exact test was used to determine whether the frequency of aortic insufficiency was significantly different between the groups. NS, not significant; ND, not determined.

IDUA activity in organs

We hypothesized that IDUA that was tagged with M6P and secreted into the blood would diffuse into organs and correct the manifestations of disease. To assess this, animals were sacrificed at 8 months of age (6.5 months after transduction), and organs were evaluated for IDUA activity and for the resolution of biochemical manifestations of disease (Figure 3). In addition, the heart, eye, ear and bone were evaluated with specialized testing (Table 1). Animals that received the low dose of reverse-hAAT-cIDUA were not evaluated because only one of two mice had stable expression, and statistical comparisons could not have been performed.

For MPS I mice that received the medium dose of reverse-hAAT-cIDUA, all organs except brain had enzyme activity that was >14% of normal. In addition, most organs had biochemical evidence of correction of disease because the levels of a secondary lysosomal enzyme, β-hex (which degrades the ganglioside GM2), were significantly reduced in liver, spleen, ovary, aorta, heart, kidney and lung, whereas levels of GAGs were significantly reduced in liver, spleen, ovary, thymus, heart, kidney and lung when compared with values in untreated MPS I mice. However, although the aortas of the medium dose mice had 37% of normal enzyme activity and β-hex activity was reduced ($p = 0.002$ versus untreated MPS I mice; not significant versus normal), GAG levels were unchanged compared to untreated MPS I mice. The brain had very low levels of IDUA activity in medium dose reverse-hAAT-cIDUA-treated MPS I mice at 0.4% of normal, which resulted in normalization of β-hex activity, although the effect on GAGs could not be evaluated as they were not elevated in untreated MPS I mice. These results are consistent with our previous findings showing that the aorta and brain are relatively resistant to IDUA diffusion at low serum levels of IDUA, and with the finding that the aorta was dilated in the medium dose reverse-hAAT-cIDUA-treated MPS I mice (Table 1). The testes showed a trend towards normalization in the medium dose group but could not be statistically evaluated as only one male animal was evaluated.

Only the aorta and brain were evaluated in the MPS I mice that received the high dose of reverse-hAAT-cIDUA because the other organs were readily corrected with the medium dose of vector. High dose vector resulted in 27% of normal IDUA activity in aorta, which was sufficient to significantly reduce the levels of β-hex activity and GAG levels. Brain IDUA activity was 19% of normal in the high dose mice, which reduced β-hex activity to normal.

Percent M6P modification of serum IDUA

As discussed below, the organ activity found in medium dose reverse-hAAT-cIDUA-treated MPS I mice was higher than expected for the level of serum IDUA activity that was achieved when a comparison was made with the values previously observed in adult forward-hAAT-cIDUA-treated mice [9]. A possible explanation for this discrepancy is that the IDUA that was secreted into blood in the reverse-hAAT-IDUA-treated mice was more efficiently modified with M6P than was the enzyme that was secreted in forward-hAAT-cIDUA-WPRE-transduced mice. Because M6P-modified enzyme is taken up more efficiently by most organs than non-modified enzyme [38], this could result in greater delivery to organs and thus a shorter half life, which would lower serum activity.

Serum was therefore evaluated to determine the percentage of IDUA that contained M6P (Figure 4). For normal mice, $57 \pm 6\%$ of the enzyme in serum contained M6P as determined by testing the amount of enzyme that was retained on a M6P receptor column and eluted with M6P. By contrast, only $13 \pm 6\%$ of the IDUA contained M6P for mice that were treated with the forward-hAAT-cIDUA-WPRE vector ($p < 0.01$ versus normal). The percentage of serum IDUA with M6P was intermediate at $35 \pm 9\%$ for mice that received the highest dose of the reverse-hAAT-cIDUA vector, which was three-fold greater than the value in mice receiving the forward vector ($p < 0.01$), and 62% of the value in normal mice ($p < 0.01$). These data suggest that IDUA in the serum of reverse-hAAT-cIDUA-transduced mice was more efficiently modified with M6P than was the enzyme from the forward-hAAT-cIDUA-WPRE-transduced mice, which could explain why the relative delivery to organs for a particular level of serum IDUA activity was higher for the reverse-hAAT-cIDUA vector than for the forward-hAAT-cIDUA-WPRE vector. Serum from high-dose reverse-hAAT-cIDUA-treated mice had an average of 20.1 ± 2.0 U/ml of total IDUA activity and 7.0 ± 2.8 U/ml of M6P-modified IDUA activity. The percentage of IDUA that was modified with M6P was not determined for the mice that received the medium dose of reverse-hAAT-cIDUA because insufficient amounts of serum were collected at the time of death. In a previous study in neonatal mice that received the forward-hAAT-cIDUA-WPRE vector, the percentage of IDUA activity with M6P modification did not vary when different doses were used.

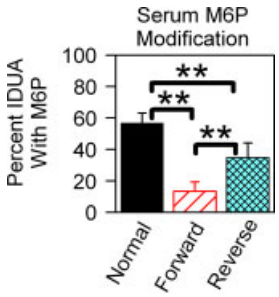

Figure 4. Determination of the percentage of IDUA with M6P in serum. M6P-modified lysosomal enzymes in serum from transduced mice at 6–8 months of age were captured by a M6P receptor column, and the percentage of enzyme that was eluted with M6P was determined for four mice in each group. Samples were from heterozygous normal mice, MPS I mice that received 10^{10} TU/kg of the forward-hAAT-cIDUA-WPRE vector as newborns (Forward), or MPS I mice that received 1.7×10^{10} TU/kg of the reverse-hAAT-cIDUA vector as adults (Reverse). Statistical comparisons were performed as described in Figure 3. All values shown represent the mean ± SD. Two asterisks (**) denote $p < 0.01$ for the statistical comparison of the two groups were are connected with a bracket

Evaluation of bone, heart and aorta, vision and hearing

Untreated MPS I mice develop abnormalities in bone, hearing, vision, aorta and heart, and effective treatments can reduce these abnormalities. Table 1 summarizes the results of evaluation of these sites at 8 months of age in MPS I mice that received the medium (10^{10} TU/kg) or high (1.7×10^{10} TU/kg) dose of reverse-hAAT-cIDUA, and shows statistical comparisons with values in age-matched heterozygous normal and untreated MPS I mice. Untreated MPS I mice had femur diameters that were $127 \pm 8\%$ of normal and BMD that were $117 \pm 3\%$ of normal. The medium-dose, reverse-hAAT-cIDUA-treated mice had an improvement, but not normalization, of femur width to $107 \pm 4\%$ of normal, and normalization of BMD to $100 \pm 6\%$ of normal.

Hearing was assessed with ABR, which measures the minimum threshold of sound that evokes a brainstem-response. Untreated MPS I mice had a marked reduction in hearing at all frequencies that were evaluated. Medium and high-dose reverse-hAAT-cIDUA-treated mice had a statistically significant improvement in hearing compared to untreated MPS I mice. Furthermore, high-dose reverse-hAAT-cIDUA-treated mice had hearing that was not statistically different from normal mice (Table 1).

MPS I mice have many visual manifestations of disease, which include corneal clouding and retinal dysfunction. The dark-adapted ERG a-wave primarily reflects rod photoreceptor function in response to light, whereas the b-wave primarily reflects bipolar cell function. MPS I mice had dark-adapted ERG a- and b-wave amplitudes that were $20 \pm 8\%$ and $36 \pm 11\%$ of normal, respectively. Medium-dose reverse-hAAT-cIDUA-treated mice had marked improvement compared to untreated MPS I mice in the dark-adapted ERG, with values that were $112 \pm 23\%$ and $100 \pm 55\%$ of normal, respectively. Similarly, high-dose reverse-hAAT-cIDUA-treated mice had amplitudes of $122 \pm 47\%$ and $98 \pm 43\%$ of normal, respectively. These values for medium and high-dose RV-treated mice were not statistically different from normal.

Untreated MPS I mice had aortic diameters that were markedly dilated at $147 \pm 32\%$ of normal. In addition, they had a maximum aortic insufficiency (AI) jet of 2.5 ± 1.7 mm, which was eight-fold greater than the

value of 0.3 ± 0.4 in normal mice. Furthermore, 100% of untreated MPS I mice had AI as defined by a maximal AI jet of >0.5 mm by pulse-wave Doppler mapping, whereas only 20% of normal mice had AI by this criterion. Aortic disease was not prevented in the medium dose (10^{10} TU/kg) reverse-hAAT-cIDUA-treated mice because the aortas remained markedly dilated, and 75% of mice had AI. Although the magnitude of the AI jet appeared to be reduced at 1.0 ± 1.0 mm in the medium dose RV-treated mice, this was not statistically different from the values in untreated MPS I mice.

DNA and RNA levels in liver and spleen

We previously determined the spleen to be the most significant source of extrahepatic IDUA expression with the forward-hAAT-cIDUA-WPRE vector, producing 10% as much canine IDUA RNA as the liver at 1 week after transduction of adults [9]. To determine whether the reverse-hAAT-WPRE vector was liver-specific, MPS I mice were transduced at 6 weeks with 10^{10} TU/kg of either the forward-hAAT-cIDUA-WPRE or the reverse-hAAT-cIDUA vector. Mice were sacrificed 1 week after transduction, and nucleic acids were analysed for retroviral DNA copies and cIDUA-containing RNA. After transduction with the forward-hAAT-cIDUA-WPRE vector, there were 63.3 ± 32.4 IDUA DNA copies per 100 cells in the liver and 3.0 ± 0.6 IDUA DNA copies per 100 cells in the spleen (Figure 5). Furthermore, there was substantial canine IDUA expression in both organs, which was approximately 10% of the level of β-actin RNA in the liver and approximately 1% of the level of β-actin RNA in the spleen.

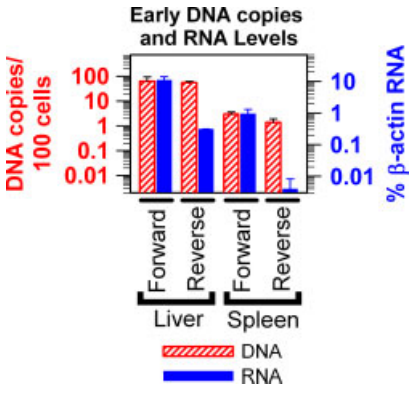

Figure 5. DNA copies and RNA levels in liver and spleen at one week after transduction. MPS I mice received 10^{10} TU/kg of either the forward-hAAT-cIDUA-WPRE vector or the reverse-hAAT-cIDUA vector after HGF was given to induce hepatocyte replication (for details, see Figure 2) and organs were collected 1 week later. Two animals were evaluated in each group, and the mean ± SD is shown. For DNA, real-time PCR with Taqman technology was performed with canine IDUA and β-actin primers and RV DNA copies per 100 cells (striped bars) was determined. For RNA, reverse transcription from liver or spleen samples was performed with canine IDUA and β-actin primers, followed by real-time PCR with Taqman technology to determine the level of RNA relative to β-actin (solid bars)

The medium dose reverse-hAAT-cIDUA vector-treated mice had 55.9 ± 7.0 IDUA DNA copies per 100 cells in the liver and 1.4 ± 0.5 IDUA DNA copies per 100 cells in the spleen at 1 week after transduction, which was similar to the values in the forward-hAAT-cIDUA-WPRE vector-treated mice. The medium dose reverse-hAAT-cIDUA vector-treated liver had a canine IDUA RNA level of 0.3% of the level of β-actin RNA. In the spleen, the canine IDUA RNA level was extremely low, albeit detectable, at 0.004 ± 0.004% of the level of β-actin. These data suggest that a reasonable level of liver specificity was achieved. Although it is likely that APCs in other organs, such as the Kupffer cells of the liver, also failed to express IDUA and this may have contributed to the absence of an immune response, this possibility was not evaluated in the present study.

DNA copies were somewhat lower at 6.5 months after transduction in the liver of reverse-hAAT-cIDUA-transduced mice because there were 10.6 ± 6.4 and 29.4 ± 3.5 IDUA DNA copies per 100 cells for the medium and high dose mice, respectively (data not shown; $p = 0.002$ for t-test comparison between medium and high dose). The DNA copies in medium dose mice at 8 months were 20% of the value seen at 1 week after transduction, and this decrease is similar to that observed previously [9]. The ratio of DNA copies in the liver to dose of RV was higher for the high dose reverse-hAAT-cIDUA vector-treated mice than for the medium dose reverse-hAAT-cIDUA vector-treated mice. This probably reflects the fact that the former group received some injections of RV in middle of the night, which is a time when hepatocyte replication is very high after initiation of HGF injections in the morning (B. Wang, K. P. Ponder, unpublished data).

Discussion

Gene therapy with reverse-hAAT-cIDUA reduced manifestations of MPS I

The present study demonstrated that the reverse-hAAT-cIDUA vector that was modified to reduce an immune response resulted in sufficient expression to correct the disease manifestations of MPS I without immunosuppression. Mice transduced with the reverse-hAAT-cIDUA vector had high levels of IDUA activity in most organs (Figure 3), which prevented the elevation of the levels of GAGs and the secondary lysosomal enzyme β-hex that was seen in untreated MPS I mice. The degree of correction of long bone width and BMD was similar to that reported previously after transduction of adults with the forward-hAAT-cIDUA-WPRE vector in conjunction with immunosuppression to prevent an immune response [9]. Remarkably, hearing and vision were corrected to normal levels with the reverse-hAAT-cIDUA vector, a finding not previously observed in the adult mice who received the forward-hAAT-cIDUA-WPRE vector as adults despite their apparently higher serum IDUA levels.

Although the serum IDUA activity of reverse-hAAT-cIDUA-transduced mice was lower than what was observed in immunosuppressed forward-hAAT-cIDUA-WPRE-transduced mice [9], organ IDUA activity was similar for the two groups. It is likely that this apparent discrepancy is in part due to the fact that the percentage of serum IDUA with M6P in reverse-hAAT-cIDUA-treated mice of $35 \pm 9\%$ was three-fold greater than the percentage of IDUA with M6P in forward-hAAT-cIDUA-WPRE-transduced mice. Higher M6P modification should result in more efficient uptake by organs [38]. The half-life and steady-state serum level of the enzyme would be expected to be lower because the modified enzyme is taken out of circulation more rapidly. It is likely that the liver cells are more efficient at performing M6P modification than other cells such as those in the spleen, which would explain why liver-specific expression results in a higher percentage of enzyme in serum with M6P. These factors illustrate the importance of determining the amount of enzyme that is appropriately modified.

However, not all disease manifestations were corrected in reverse-hAAT-cIDUA-treated mice. The aorta still had marked elevations in β-hex and GAG levels (Figures 4B and 4C) and was markedly dilated on echocardiogram (Table 1). Furthermore, pathological evaluation demonstrated that there were large amounts of lysosomal storage material in smooth muscle cells and fragmentation of the elastic fibers of the aorta, although they were partially reduced compared to untreated MPS I mice (data not shown). Thus, as previously reported, the aorta is very refractory to treatment at low levels of serum IDUA.

Reducing extrahepatic expression results in stable IDUA expression in serum

An important result of this study was the development of an RV that would result in stable expression of canine IDUA *in vivo* without the need for immunosuppression. Previous studies have shown that limiting expression to the liver can be an effective means to avoid an immune response [25,27]. However, it was unknown whether this approach would work for a lysosomal enzyme because secreted enzyme that is modified with M6P can be taken up by other cells via the M6P receptor and translocated to the lysosome via endosomes. Because peptides that derive from proteins that traffic through endosomal pathways can be presented on class I MHC molecules [39,40], this pathway could allow APCs to prime an immune response via cross-presentation. In the present study, 90% of mice that received gene therapy with the reverse-hAAT-cIDUA vector as adults maintained stable expression long-term. By contrast, no adult mice that received gene therapy with the forward-hAAT-cIDUA-WPRE vector had stable expression, which is consistent with our previous results [8,9]. These results demonstrate that restricting IDUA expression to the liver is usually effective at achieving stable expression. It is likely that expression in hepatic nonparenchymal cells such as Kupffer cells was also reduced with the reverse-hAAT-cIDUA vector and could have contributed to the reduced immune response, although this possibility was not addressed in the present study.

Reducing extrahepatic expression of IDUA was also somewhat effective from a lentiviral vector because the use of an albumin promoter in the same orientation as a self-inactivating LTR resulted in higher DNA copies and expression of human IDUA at later times than was observed with a similar vector containing the cytomegalovirus promoter after gene therapy to adult MPS I mice [26]. However, organs including the liver had $<1\%$ of normal IDUA activity after transfer of the vector with the albumin promoter, and GAG levels were at most only partially reduced. It is possible that expression was low because the albumin promoter was relatively weak, or that this vector resulted in sufficient expression to induce a relatively weak CTL response that destroyed cells with relatively high levels of expression. Indeed, a similar lentiviral vector that expressed green fluorescent protein from the albumin promoter resulted in a loss of expression due to a CTL response [27]. It is likely that lentiviral vectors will also result in a sustained therapeutic effect after delivery to adult animals if high expression without an immune response can be achieved.

Implications for future gene therapy studies

The present study demonstrates that modification of a RV to restrict extrahepatic expression can result in stable IDUA expression after performing gene therapy in adult MPS I mice. We are currently testing whether this approach will be successful for MPS I cats, who mounted a CTL response when gene therapy was performed in newborns with the forward-hAAT-cIDUA-WPRE vector [16]. Because humans are expected to have a sufficiently mature immune system at birth to develop a CTL response [20], the cat may be a very important model in which to further test the efficacy of this approach. Antibodies are known to develop in human patients that receive ERT [41], and CTL responses will likely develop after gene therapy in patients with premature stop codons. Future studies will also focus on designing the vector to contain a self-inactivating LTR to reduce the risk of insertional mutagenesis. If necessary, the use of a sequence that is recognized by a microRNA that is expressed in spleen could be used to reduce expression in APCs [25].

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Supplementary Material

The supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1099-498X/suppmat/.

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