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Effect of neonatal administration of a retroviral vector expressing α-L-iduronidase upon lysosomal storage in brain and other organs in mucopolysaccharidosis I mice

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Abstract

Mucopolysaccharidosis I (MPS I) due to deficient α-L-iduronidase (IDUA) activity results in accumulation of glycosaminoglycans in many cells. Gene therapy could program cells to secrete IDUA modified with mannose 6-phosphate (M6P), and enzyme could be taken up by other cells via the M6P receptor. We previously reported that newborn MPS I mice that were injected intravenously with 10^9 (high-dose) or 10^8 (low-dose) transducing units/kg of a retroviral vector (RV) expressing canine IDUA achieved stable levels of IDUA activity in serum and had reduced disease in heart, eye, ear, and bone in a dose-dependent fashion. However, the dose required for improvement in manifestations of disease in other organs was not reported. High-dose and low-dose RV mice with an average serum IDUA activity of 1037 ± 90 U/ml (471-fold normal) and 43 ± 12 U/ml (20-fold normal), respectively, had complete correction of biochemical and pathological evidence of disease in the liver, spleen, kidney, and small intestines. Although mice that received high-dose RV had complete correction of lysosomal storage in thymus, ovary, lung, and testis, correction in these organs was only partial for those that received low-dose RV. Storage in brain was almost completely corrected with high-dose RV, but was not improved with low-dose RV. The correction of disease in brain may be due to diffusion of enzyme from blood. We conclude that high-dose RV prevents biochemical and pathological manifestations of disease in all organs in MPS I mice including brain.

Keywords: Gene therapy; Lysosomal storage disease; Retroviral vector; Mucopolysaccharidosis; Glycosaminoglycan; Neonatal; Liver; Iduronidase

Introduction

The mucopolysaccharidoses (MPS) involve the inability to degrade glycosaminoglycans (GAGs) [1]. Mucopolysaccharidosis I (MPS I) is an autosomal recessive disease due to deficiency of α-L-iduronidase (IDUA; EC 3.2.1.76). It results in the accumulation of heparan and dermatan sulfate [1], and has an incidence of 1:100,000 live births [2]. The phenotype varies from severe (Hurler syndrome; OMIM #607014) to mild (Scheie syndrome; OMIM #607016). Although cat [3] and dog [4,5] models of MPS I have been extensively studied, mouse models were only developed recently [6,7] and some aspects of disease have not been fully evaluated. Defining parameters that are abnormal in mice will be important for studies that test novel therapies for their effect on disease.

MPS I is currently treated with hematopoietic stem cell transplantation (HSCT) or enzyme replacement therapy (ERT). HSCT has reduced the manifestations of disease in mice [8], dogs [9], and humans [10] with MPS I. One possible mechanism is migration of blood cells into organs, where they secrete enzyme modified with mannose 6-phosphate (M6P) that can be taken up by adjacent cells via the M6P receptor (M6PR) present on the surface [11]. HSCT is limited by the need for a compatible donor, and the risks and costs of the procedure. ERT involves the intravenous

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(IV) injection of enzyme that has M6P, which can diffuse into organs and be internalized via the M6PR [12]. ERT has been therapeutic in cats [13], dogs [14,15], and humans [16] with MPS I. Difficulties with ERT include the need for infusion once a week and the high cost of over $10,000 per kg body weight per year.

Gene therapy has been successfully used to treat MPS in animal models [17]. This could involve transduction of a patient's HSCs, whose progeny migrate into other organs and correct disease in a fashion analogous to HSCT. HSC-directed gene therapy has reduced the manifestations of MPS I in liver, spleen, kidney, bladder, and brain in mice [8]. Correction in brain was attributed to migration of transduced cells into the brain. However, HSC-directed gene therapy was not successful in dogs, which was due at least in part to an immune response [18].

Alternatively, other organs or cells could be modified to secrete enzyme with M6P into blood, and enzyme in blood could be taken up by cells in other organs via the M6PR. Indeed, neonatal administration of an AAV vector reduced disease in liver, heart, lung, and bone. In addition, it reduced storage in the cerebellum and improved neurological function [19], which was attributed to secretion of enzyme from liver into blood, as no vector copies were observed in brain. Similarly, neonatal administration of lentiviral vector improved bone disease and reduced storage in liver, spleen, heart, and kidney [20]. It also reduced storage in neurons, although in this case improvement was attributed to transduction of neurons with the lentiviral vector. We previously reported that neonatal gene therapy with a gamma retroviral vector (RV) reduced disease in heart, eye, ear, and bone [21]. Secretion of enzyme from liver into blood was likely the mechanism of correction, as RV DNA and RNA levels were >10-fold and >100-fold higher, respectively, in liver than in other organs at 6 months after transduction with a similar RV [22]. In contrast to the success that has been achieved with gene therapy in newborns, there was little, if any, long-term effect on disease after transfer of lentiviral or gamma RV into non-hematopoietic cells in adults [20,21,23]. Expression from plasmid vectors in adults has been low [24].

The goals of this study were to: (1) evaluate organs that have not been examined previously for evidence of disease in MPS I mice; (2) further evaluate the effect of our previously reported neonatal gamma RV-mediated transduction [21] upon biochemical and pathological disease manifestations. We report herein that thymus, ovary, and testis have substantial amounts of lysosomal storage in untreated MPS I mice. We define the dose of RV necessary to prevent manifestations of disease in these and other organs.

Materials and methods

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Animals

MPS I mice [7] in a C57BL/6 background were injected with hAAT-cIDUA-WPRE via the temporal vein at 2–3 days after birth as described previously [21]. This LNL-6-based RV contains long-terminal repeats (LTR) at both ends, the human α1-antitrypsin promoter, the canine IDUA cDNA, and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Other MPS I or heterozygous normal littermates did not receive any treatment at birth. Animals received transcardial perfusion with 20 ml of normal saline prior to sacrifice.

IDUA activity, β-hexosaminidase activity, and GAG levels

Cells or organs were homogenized in lysis buffer as described [7] and the same homogenate was used for enzyme and GAG assays. The total protein concentration was determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA). IDUA and total β-hexosaminidase (β-hex) assays were performed using 4-methylumbelliferyl-α-L-iduronide (Calbiochem, San Diego, CA) and 4-methylumbelliferyl-2-acetamido-2deoxy-β-glucopyranoside as substrates, respectively, as described [7]. One unit (U) of enzyme releases 1 nmol of 4-methylumbelliferone per hour at 37 °C. IDUA activity in organs from homozygous normal mice was assumed to be twice the level determined for heterozygous normal mice. GAGs were determined using a sulfated glycosaminoglycan kit from Blyscan (Newtownabbey, N. Ireland) as described [25] in which the dye 1,9-dimethyl-methylene blue in an inorganic buffer binds to GAGs at an acid pH. Chondroitin 4-sulfate was used as the standard. The dye-GAG complex was dissociated by the Blyscan reagent, and the sample OD determined at 655 nm.

Pathological evaluation

Pieces of organs were fixed, embedded in plastic, and 1 μm sections were stained with toluidine blue as previously described [21]. Pathology was evaluated without knowledge of the genotype or treatment status.

Nucleic acid analysis

Liver and brains were homogenized in guanidinium for DNA isolation as described previously [22], or in TRIzol Reagent (Invitrogen Corporation, Carlsbad, California 92008) for RNA isolation according to the manufacturers instructions. DNA was isolated from bone marrow (BM) using the QIAamp DNA Blood Mini Kit from Qiagen Inc. (Valencia, CA 91355), and RNA was isolated from BM and blood cells using the QIAamp RNA Blood Mini Kit from Qiagen Inc. Real-time polymerase chain reaction (PCR) was used to determine the amount of RV DNA using 100 ng DNA and Taqman technology with reagents from Applied Biosystems (Foster City CA 94404) and primers and probes specific for the WPRE of the RV with

normalization to the β-actin signal. Standards were mixtures of genomic DNA isolated from murine cells with one copy of a WPRE-containing RV per cell [22] and genomic DNA from a non-transduced mouse. For analysis of RNA levels, reverse transcription (RT) was performed on approximately 100 ng DNaseI-treated RNA and the reverse primers for the WPRE and β-actin, followed by real-time PCR with Taqman technology. No signal was observed using samples that did not receive RT.

Statistics

Statistical comparisons were performed using Sigma Stat (Sigma Chemical). ANOVA with Tukey post hoc analysis was used to compare values for more than two groups, while the Student's *t*-test was used to compare values in two groups.

Results

We have previously demonstrated that neonatal IV injection of MPS I mice with 10^9 transducing units (TU)/kg (high-dose RV) or 10^8 TU/kg (low-dose RV) of hAAT-cIDUA-WPRE resulted in stable expression of IDUA in serum for 8 months, with average levels of ~1000 and ~100 U/ml, respectively [21]. For this study, these previously reported mice were tested for biochemical and pathological manifestations of disease at 8 months of age in organs that were not evaluated previously. For the high-dose RV mice, the individuals analyzed had an average IDUA activity of 1037 ± 90 [standard error of the mean (SEM)] U/ml, which is representative of the entire group. For the low-dose RV mice, the individuals analyzed had an average IDUA activity of 43 ± 12 U/ml, which is lower than the average for this group.

IDUA activity in organs

It is important to determine the organ IDUA activity, and compare that with the level expected to improve disease. All organs from untreated MPS I mice had IDUA activity that was <1% of that found in homozygous normal mice, as shown in Fig. 1A. For mice that received the high-dose RV, IDUA activity at 8 months was highest in liver and spleen at 179 ± 24 U/mg (74-fold that in homozygous normal) and 235 ± 105 U/mg (18-fold normal), respectively. Activity in other organs is organized in Fig. 1A from left (highest) to right (lowest) according to the relative levels of IDUA in U/mg in high-dose RV mice. IDUA activity in thymus, kidney cortex, small intestine, ovary, lung, testis, large intestine, and muscle was >50% of the values found in homozygous normal mice. IDUA activity in brain of high-dose RV mice was lower at 0.23 ± 0.07 U/mg (7% of normal). These levels of activity would be expected to have at least a partial therapeutic effect, as the enzyme activity in fibroblasts from Scheie patients is usually <5% of normal [26,27].

The IDUA activity in organs of low-dose RV mice was ~3% of that found in high-dose RV mice, which is consistent with the fact that the serum activity in low-dose RV mice was 4% of that in high-dose RV mice. This suggests that organ IDUA activity is directly proportional to serum activity. IDUA activity was 5.8 ± 1 U/mg in liver (240% normal), 2.8 ± 0.7 U/mg in spleen (22% normal), 16.9 ± 5.7 U/mg in thymus (62% normal), 1.1 ± 0.4 U/mg in kidney (31% normal), 0.5 ± 0.1 U/mg in small intestine (7% normal), 0.9 ± 0.3 U/mg in ovary (3% normal), 0.2 ± 0.02 U/mg in lung (2% normal), 0.4 ± 0.03 U/mg in testes (6% normal), 0.2 ± 0.03 U/mg in large intestine (2% normal), 0.03 ± 0.003 U/mg in muscle (3% normal), and 0.03 ± 0.002 U/mg in brain (1% normal).

β-hex activity

MPS I results in an elevation in the activity of other lysosomal enzymes in cells, which may reflect an increased total mass of lysosomes, or alterations in gene expression. Since normalization of this elevation by effective treatment correlates well with improvements in lysosomal storage, organs were tested for β-hex activity, as shown in Fig. 1B. β-hex activity in untreated MPS I mice was 2- to 84-fold that found in normal mice, and values were statistically higher than in normal mice in all organs ($p < 0.01$). Organs that have not been studied previously for β-hex activity in mice with MPS I include thymus with 7814 ± 1024 U/mg (5-fold normal), small intestines with 10,370 ± 652 U/mg (2-fold normal), ovary with 23,138 ± 3387 U/mg (84-fold normal), lung with 14,335 ± 2580 U/mg (12-fold normal), testis with 6769 ± 293 U/mg (68-fold normal), large intestine with 35,737 ± 3765 U/mg (2-fold normal), and muscle with 1334 ± 124 U/mg (7-fold normal).

The high-dose RV mice had normalization of β-hex activity in all organs. Values in high-dose RV mice were statistically lower than in untreated MPS I mice ($p < 0.01$ for all organs), and were not statistically different from values in normal mice. Low-dose RV mice had normalization of the β-hex activity in liver, spleen, kidney, small intestine, and muscle, with values that were statistically lower than in untreated MPS I mice ($p < .01$), and were not statistically different from normal mice. For the following organs, β-hex activity was statistically lower in low-dose RV mice than in untreated MPS I mice, but appeared to have a modest increase as compared with normal mice, although there were no statistically significant differences between the low-dose RV and the normal mice: thymus had 3609 ± 688 U/mg (46% of MPS I, 2-fold normal), ovary had 1168 ± 128 U/mg (5% of MPS I; 4-fold normal), lung had 2720 ± 715 U/mg (19% of MPS I; 2-fold normal), and testis had 915 ± 260 U/mg (14% of MPS I; 9-fold normal). In contrast, β-hex activity remained elevated in brain in low-dose RV mice at 9359 ± 707 U/mg, which was 87% of the value found in untreated MPS I mice (not significant for low-dose RV vs. MPS I), and was 3-fold the value found in normal mice ($p < 0.01$ for low-dose RV vs. normal). In addition,

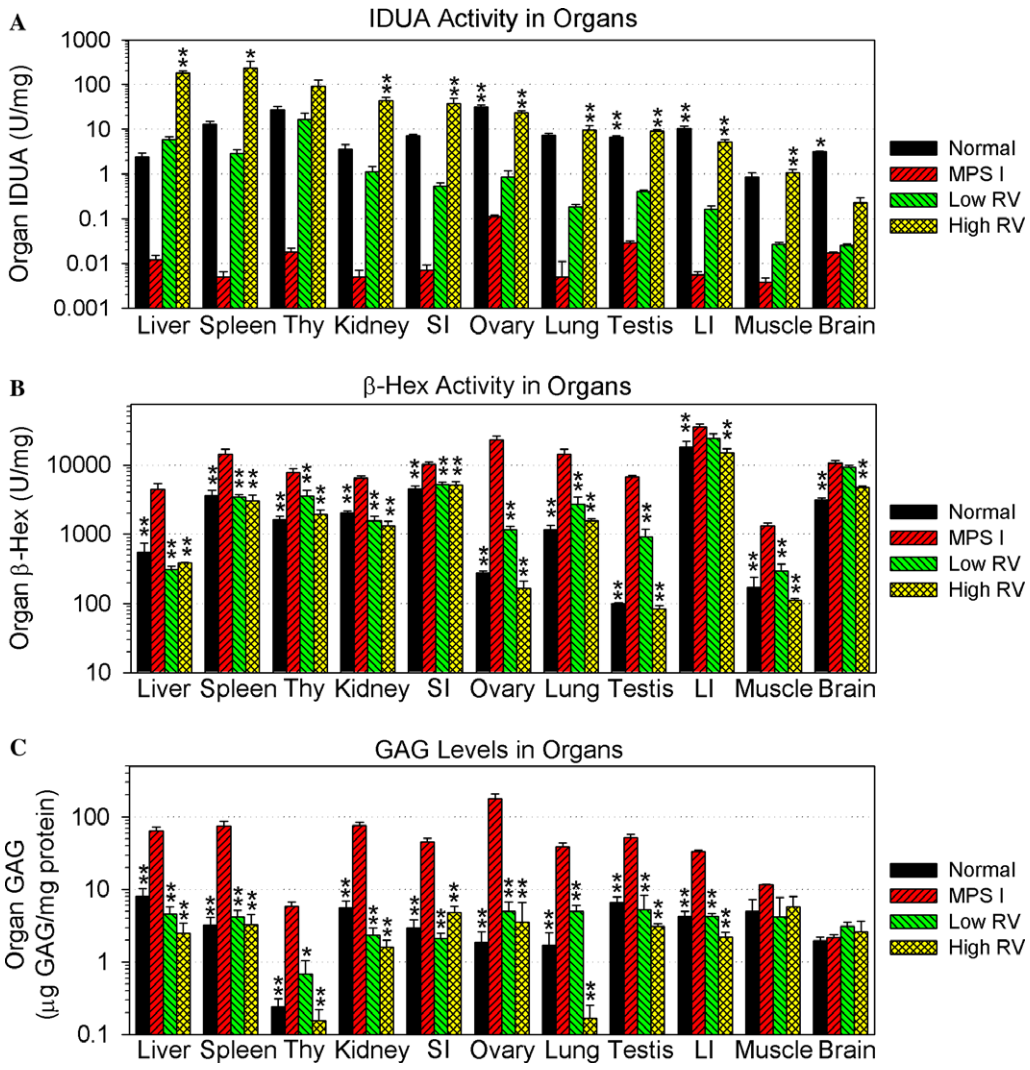

Fig. 1. IDUA activity, β-hexosaminidase (β-hex), and GAG levels in organ extracts. Heterozygous normal mice from the MPS I breeding colony (Normal), and some homozygous-deficient MPS I mice (MPS I) did not receive any treatment. Other homozygous-deficient MPS I mice were injected IV at 2–3 days after birth with the low-dose of RV (10^8 TU/kg; Low RV) or with the high-dose of RV (10^9 TU/kg; High RV). Mice were sacrificed at 8 months of age, and organs from six mice in each group were homogenized. (A) IDUA activity. The IDUA activity was determined and normalized to the protein concentration. For normal animals, the experimental values were multiplied by 2 to correct for the fact that heterozygous mice were actually evaluated. Averages ±SEM were determined, and statistical comparisons between groups were performed using ANOVA with Tukey post hoc analysis. An asterisk above a bar indicates that there was a statistical difference between the values for that group and those of untreated MPS I mice; ∗ indicates a p value of 0.01–0.05, while ∗∗ indicates a p value <0.01. Values for the liver appear on the left, and values for other organs are organized from left (highest) to right (lowest) according to the IDUA activity in U/mg for mice that were treated with high-dose RV. Abbreviations include Thy (thymus), SI (small intestine), and LI (large intestine). (B) β-hex activity. The β-hex activity was determined and averages ±SEM were plotted. Statistical comparisons were performed as described in (A). (C) GAG levels. GAG levels were determined, and plotted at the average μg GAG/mg protein ± SEM. Statistical comparisons were performed as described in (A).

β-hex activity in large intestine of low-dose RV mice was not significantly different from that in untreated MPS I mice.

GAG levels

Deficient IDUA enzyme activity leads to accumulation of dermatan and heparan sulfate, resulting in high levels of sulfated GAGs. Since effective treatments can reduce GAG levels to normal, soluble sulfated GAGs were evaluated in organ extracts, as shown in Fig. 1C. As reported previously, GAG levels were increased in untreated MPS I mice in liver (64 ± 8 μg GAG/mg protein; 8-fold normal), spleen (75 ± 12 μg GAG/mg protein; 23-fold normal), kidney (76 ± 8 μg GAG/mg protein; 14-fold normal), and lung (39 ± 5 μg GAG/mg protein; 23-fold normal). The relatively greater elevation in GAGs in these organs in our study, as compared with previous studies [8,20,23], may relate to a later age of analysis in our study, or to differences in how the assays were performed. The GAG levels in brain of untreated MPS I mice of 2.2 ± 0.2 μg GAG/mg protein were not different from values in normal mice, which is consistent with other reports that GAG levels were not elevated [8,23], or were only marginally elevated [20], in brain in

MPS I mice. This failure to increase total sulfated GAGs in brain of MPS I mice may be due to the relatively small mass of GAGs in cells with storage. It does not indicate that brain is unaffected, as MPS I mice have defects in an open field habituation neurological test [19].

GAGs were also evaluated in several organs that have not been evaluated previously in mice with MPS I. MPS I mice had elevated GAGs in thymus (5.9 ± 0.9 μg GAG/mg protein; 8-fold normal), small intestine (45 ± 5 μg GAG/mg protein; 21-fold normal), ovary (180 ± 27 μg GAG/mg protein; 36-fold normal), testes (52 ± 6 μg GAG/mg protein; 10-fold normal), and large intestine (33 ± 2 μg GAG/mg protein; 8-fold normal). Although the GAG levels of 12 ± 0.4 μg GAG/mg protein in muscle were 2-fold that in normal mice, this difference was not statistically significant. The relative increase in GAG levels is reasonably consistent with the relative increase in β-hex activity in different organs.

MPS I mice that received high-dose RV had GAG levels that were statistically lower than in untreated MPS I mice in all organs for which GAG levels were elevated in untreated MPS I mice ($p < 0.01$ for high-dose RV vs. MPS I), and values in high-dose RV mice were not statistically different from values in normal mice. MPS I mice that received the low-dose RV has normalization of GAG levels in liver, spleen, kidney, small intestine, testis, and large intestine; values in these organs were statistically lower than in untreated MPS I mice ($p < 0.01$ for low-dose RV vs. untreated MPS I), and were not significantly different from values in normal mice. For the following organs, GAG levels appeared to be modestly elevated in low-dose RV mice, although they were statistically lower than in untreated MPS I mice, and were not significantly different from values in normal mice: thymus had 0.7 ± 0.4 μg GAG/mg protein (3-fold normal; 12% MPS I), ovary had 5 ± 2 μg GAG/mg protein (3-fold normal; 3% MPS I), and lung had 5 ± 1 μg GAG/mg protein (3-fold normal; 12% MPS I). We conclude that both high- and low-dose RV reduce or prevent GAG accumulation in organs where GAG levels were elevated in untreated MPS I mice.

Pathological analysis for lysosomal storage

It is important to evaluate the pathology of organs to determine the cell types that contain lysosomal storage, and if storage can be prevented with neonatal gene therapy. Thin sections of organs for which biochemistry was evaluated in Fig. 1 were stained with toluidine blue, as shown for representative organs in Figs. 2 and 3, and as summarized in Table 1. As noted previously by others, livers of untreated MPS I mice had storage in Kupffer cells and hepatocytes (Fig. 2A), spleens had storage within the red pulp (Fig. 2B), and kidneys had storage within the interstitial region and the glomeruli (Fig. 2C and Table 1). In addition, there was substantial lysosomal storage in the tubules of the kidney, which was not noted previously. Storage was markedly reduced in these organs with both high-dose and low-dose RV.

Thymus, testis, and ovary have not been evaluated previously for evidence of lysosomal storage. Untreated MPS I mice have modest amounts of lysosomal storage in the thymus cortex (Fig. 2D), and large amounts of storage in the thymus medulla (Fig. 2E). High-dose RV completely prevented the accumulation of lysosomal storage in the thymus, although there were modest amounts of storage in the thymus medulla of most low-dose RV mice. Although the seminiferous tubules of the testis did not have storage in untreated MPS I mice, there were large amounts of lysosomal storage within the interstitial regions between tubules (Fig. 2F). This was completely corrected in mice that received high-dose RV, but only partially improved in low-dose RV mice. Untreated MPS I mice have tremendous amounts of lysosomal storage in the stroma of the ovary (Fig. 3A and B) which was completely prevented with high-dose RV, and reduced with low-dose RV. In the lung, storage was prevented in high-dose RV mice and reduced in low-dose RV mice. The pathological evaluation showing some lysosomal storage in the thymus (Fig. 2D–E), testis (Fig. 2F), ovary (Fig. 3A and B), and lung (Table 1) in low-dose RV mice was consistent with the biochemical data suggesting that GAGs and/or β-hex activity were modestly elevated in these organs. Lysosomal storage was present in the small intestine and muscle of untreated MPS I mice, but was reduced with either low-dose or high-dose RV (Table 1). Although GAGs were elevated in the large intestine of untreated MPS I mice, no cells were identified that contained storage that was visible with light microscopy.

The brain is a very important site of disease, as patients with Hurler disease have neurological impairment. Untreated MPS I mice have lysosomal storage within neurons in the cortex, as shown in Fig. 3C. Extensive evaluation of brains from four high-dose RV mice demonstrated that cortical neurons were indistinguishable from those in normal mice. In contrast, a substantial percentage of the cortical neurons from low-dose RV mice had clear evidence of storage. The hippocampus is important for memory, and is a region that has substantial lysosomal storage in MPS VII mice. As shown in Fig. 3D, untreated MPS I mice have large amounts of lysosomal storage in most neurons in the hippocampus. Storage was completely prevented in neurons in all high-dose RV mice, but was not prevented in low-dose RV mice. Untreated MPS I mice have substantial lysosomal storage in microglial cells of the cortex and hippocampus (Table 1), which was reduced but not eliminated by high-dose RV, but was not affected by low-dose RV. Untreated MPS I mice had storage within the majority of the Purkinje cells, as shown in Fig. 3E. Storage in Purkinje cells was completely prevented with high-dose RV, but was not reduced with low-dose RV. The perivascular region (Fig. 3F) and meninges (Table 1) accumulate large amounts of lysosomal storage in untreated MPS I mice, which was reduced or prevented in high-dose RV mice, but was not affected in low-dose RV mice.

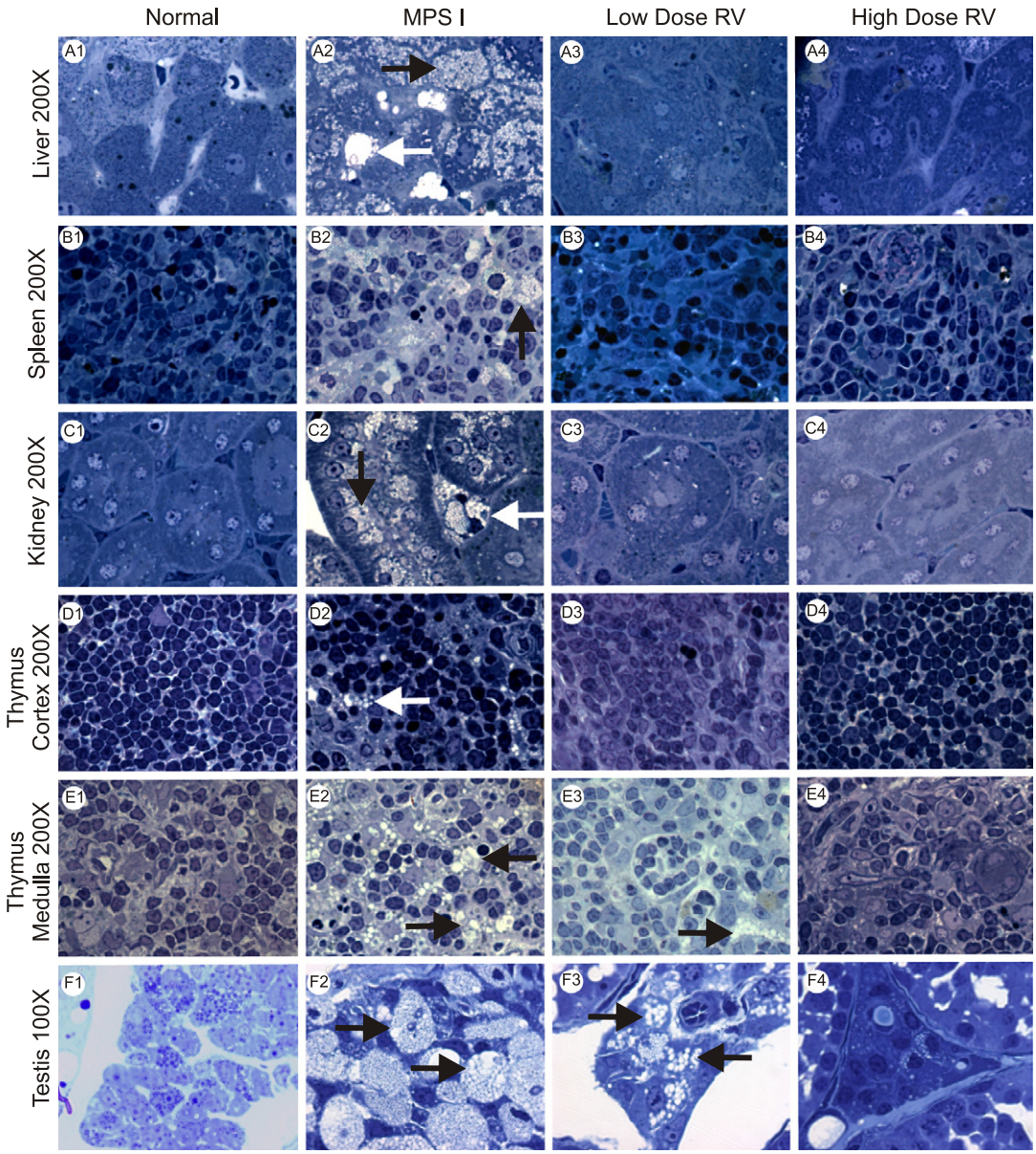

Fig. 2. Pathological evaluation of liver, spleen, kidney, thymus, and testis. Mice were treated as described in Fig. 1 and sacrificed at 8 months after birth. Thin (1 μm) sections of fixed samples were stained with toluidine blue. Organs were from normal mice (Normal; labeled with 1), untreated MPS I mice (MPS I; labeled with 2), MPS I mice that received low-dose RV (low-dose RV; labeled with 3), or MPS I mice that received high-dose RV (high-dose RV; labeled with 4). The organ and the original magnification are identified at the left. (A) Liver. The black and white arrows in the untreated MPS I mouse identify lysosomal storage in hepatocytes and Kupffer cells, respectively. (B) Spleen. The black arrow in the untreated MPS I mouse identifies lysosomal storage in the red pulp of the spleen. (C) Kidney cortex. The black arrow identifies storage in the tubules, and the white arrow identifies storage in an interstitial region. (D) Thymus cortex. The white arrow identifies storage within the thymus cortex. (E) Thymus medulla. The black arrows identify storage within the thymus medulla. (F) Testis. The black arrows identify storage within the interstitial region of the testis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Percentage of neurons with lysosomal storage in the cortex

To better evaluate the effect of gene therapy upon lysosomal storage in the brain, the percentage of neurons with storage was quantified. The cortex was analyzed, as it is technically easier to obtain a similar region of the parietal cortex from different mice than to obtain similar regions of the hippocampus. Cells were scored as positive if the cytoplasm contained two or more white bubbles consistent with lysosomal storage (see Fig. 3C). For untreated MPS I mice, $55 \pm 4\%$ of the cortex neurons were scored positive, which was statistically higher than the value of $1.5 \pm 0.5\%$ in normal mice ($p = 0.002$ with the Student's t-test), as shown in Fig. 4. Only $3.5 \pm 1.3\%$ of neurons from high-dose RV mice had histopathological evidence of lysosomal storage, which was significantly lower than the values in untreated MPS I mice ($p = 0.03$), but was not statistically different from the values in normal mice. For the low-dose RV mice, $52 \pm 18\%$ of the

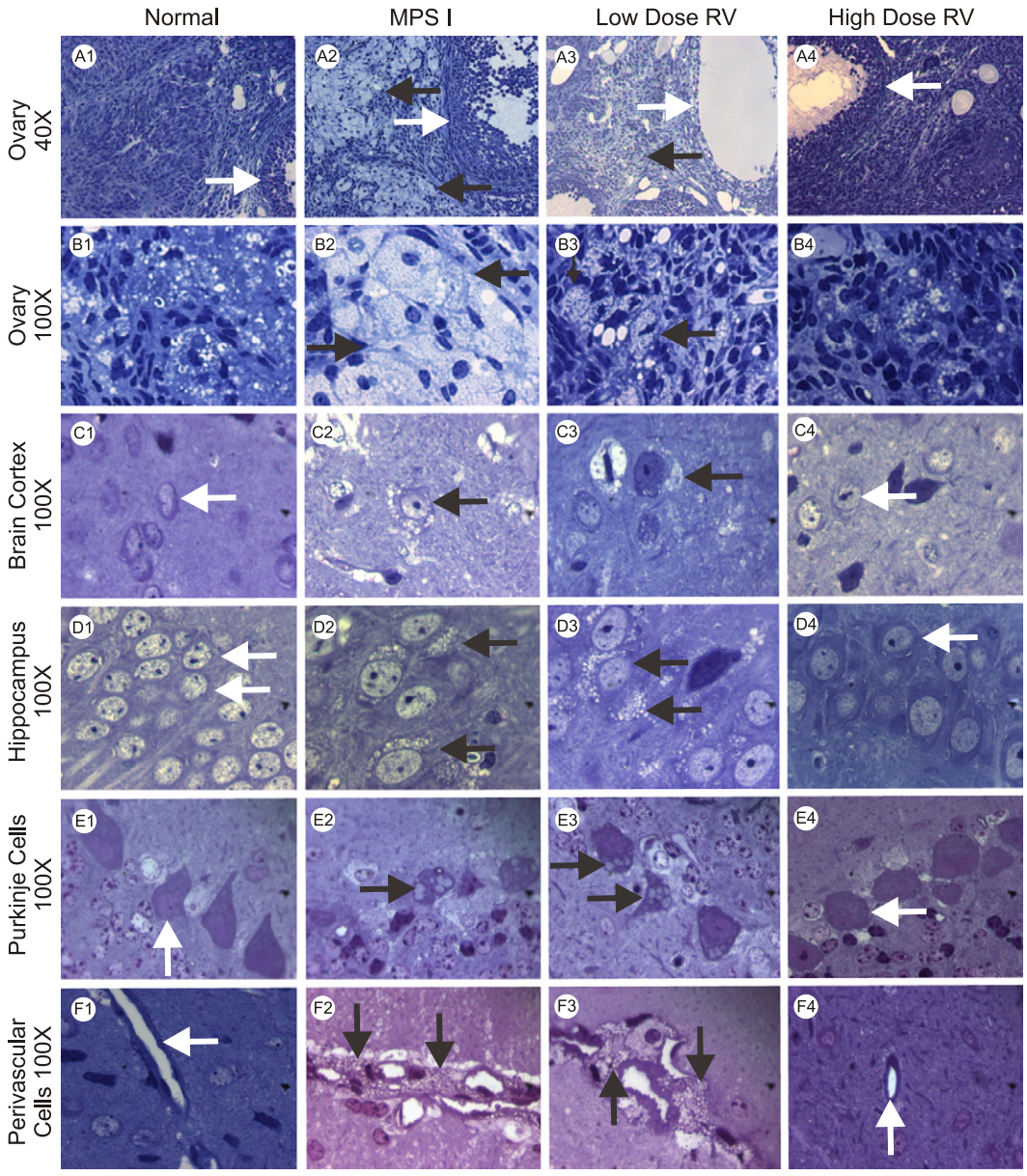

Fig. 3. Pathological evaluation of ovary and brain. Mice were treated as described in Fig. 1, and pathological analyses performed at 8 months after birth as described in Fig. 2. (A and B) Ovary. Low and high power views of the ovary are shown. The black arrows indicate storage in the stroma of the ovary, while the white arrows indicate the edge of a follicle. (C) Brain cortex. Black arrows identify neurons with lysosomal storage, while white arrows identify neurons without storage. (D) Hippocampus. Black arrows identify neurons in the hippocampus with lysosomal storage, while white arrows identify neurons without storage. (E) Purkinje cells. Black arrows identify Purkinje cells with storage, while white arrows identify Purkinje cells without storage. (F) Perivascular cells. Black arrows identify blood vessels with perivascular storage, while white arrows identify blood vessels without perivascular storage.

neurons had evidence of lysosomal storage; this was not statistically significant after comparison with either untreated MPS I or normal mice due to the small number of animals evaluated and the variation in individual mice. These data further support the hypothesis that high-dose RV can reduce storage in neurons, but low-dose RV dose is not effective.

Time course of serum, liver, and brain IDUA activity in RV-treated MPS I mice

There are three potential mechanisms by which enzyme could reach the brain and reduce storage in neurons. If correction in brain was due to transduced blood cells that migrated into brain, differentiated into microglial cells, and released enzyme locally, activity should increase slowly, as this process is slow [28,29]. If correction in brain was due to transduction of brain cells, activity in brain should appear rapidly, and copies of RV DNA and RNA in brain should be readily detected. If correction in brain was due to enzyme in blood that crossed the blood–brain barrier [30], activity in brain should parallel serum activity, and copies of RV DNA and RNA in brain would not need to be high. We therefore evaluated the time course of appearance of IDUA

Table 1
Summary of pathological evaluation for lysosomal storage in mice

Organ	Area or cell type	Normal $N=2$	Untreated MPS I $N=2$ or 3	Low-dose RV $N=4$	High-dose RV $N=4$
Liver	Hepatocytes	0	++, +++	0	0
	Kupffer cells	0	++	0	0
Spleen	Red pulp	0	++,+++	0	0
Thymus	Cortex	0	++	0	0
	Medulla	0	+++	0, +, +, +	0
Kidney	Tubules	0	++,+++	0	0
	Interstitial cells	0	+++	0	0
	Glomeruli	0	+++	0, 0, 0, ++	0
Small intestine	Lamina propria	0	++	0	0
	Submucosa	0	+++	0	0
Ovary	Stroma	0	+++	++	0
Lung	Parenchyma	0	+++	0, 0, +, ++	0
Testis	Interstitial region	0	+++	++	0
Large intestine		0	0	NE	NE
Muscle	Interstitial region	0	++	0	0
Hippocampus	Neurons	0	++, +++, +++	+++	0
	Microglial Cells	0	++, +++, +++	++, +++, +++, +++	0, 0, +, +
Brain cortex	Neurons	0	++, +++, +++	++, +++, +++,+++	0
	Microglial cells	0	+++	++, +++, +++, +++	0, 0, +, +
Cerebellum	Purkinje cells	0	+++	++, +++, +++, +++	0
Brain	Perivascular region	0	+++	+++	0, 0, 0, ++
	Meninges	0	+++	++, +++, ++++, ++++	0

Mice were treated as described in Fig. 1 and sacrificed at 8 months after birth. Pathological analyses of thin sections stained with toluidine blue were performed as shown for representative examples in Figs. 2 and 3 for the indicated number (N) of animals. For untreated MPS I mice, 2 animals were evaluated for most organs, and 3 animals were evaluated for the brain. Only 2 animals were evaluated for the ovary, and only 2 animals were evaluated for the testis of the RV-treated mice. Lysosomal storage was scored as 0 (none detected), + (low amounts of storage in some cells), ++ (moderate storage in many cells), or +++ (severe storage in most cells). If all animals were concordant, one value is shown. If values for the group were discordant, values for each animal evaluated are shown.

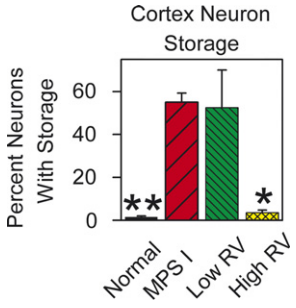

Fig. 4. Evaluation of the percentage of neurons in the cortex with lysosomal storage. Mice were treated as described in Fig. 1, and pathological evaluation was performed at 8 months after birth in the cortex of the brain as described in Fig. 3. The percentage of neurons with small white bubbles that were consistent with lysosomal storage was determined, and plotted as the average ± SEM. Values in normal ($N=2$) and RV-treated mice ($N=4$ for each group) were compared with those in untreated MPS I mice ($N=3$) using Student's t-test. $*$ indicates a p value of 0.005–0.05 and $**$ indicates a p value <0.005 when values in other groups were compared with those in untreated MPS I mice.

activity in serum, liver, and brain, and tested selected organs for RV nucleic acid levels.

Fig. 5A demonstrates that serum IDUA activity increases very rapidly in MPS I mice that received neonatal injection of 10^9 TU/kg of hAAT-cIDUA-WPRE, as the average serum IDUA activity at 2 weeks (564 ± 60 U/ml) was 90% of the value at 6 weeks (625 ± 89 U/ml). Although serum IDUA activity was not evaluated at late times in these specific mice, our previous study demonstrated that serum IDUA activity was stable from 6 weeks until 8 months after birth [21]. Organ activity was determined at two time points for animals that were perfused with 20 ml of normal saline prior to organ collection. In RV-treated mice, liver IDUA activity (Fig. 5B) was stable from 6 weeks at 114 ± 16 U/mg (127-fold the value in age-matched normal mice) until 8 months at 179 ± 24 U/mg (75-fold the value in age-matched normal mice), while brain IDUA activity (Fig. 5C) was relatively stable from 6 weeks at 0.46 ± 0.2 U/mg (13% normal) until 8 months at 0.23 ± 0.07 U/mg (7% normal). Thus, serum IDUA activity increases rapidly after neonatal RV administration, and is directly proportional to liver and brain activity at both early and late times of analysis.

RV nucleic acid levels in liver, bone marrow, blood, and brain

RV nucleic acid levels were determined at 1.5 and 8 months after gene transfer by real-time PCR for the WPRE of the RV, with normalization to the β-actin sequence. At 1.5 months after transduction, the liver contained 20.6 ± 5.9

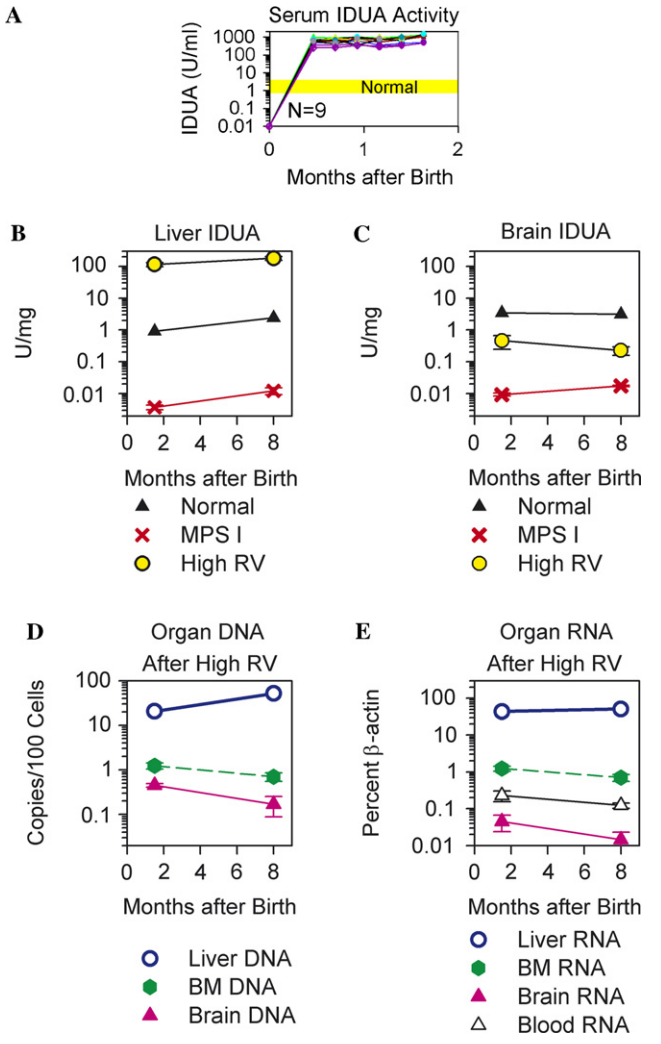

Fig. 5. Time course of serum IDUA activity, liver and brain IDUA activity, and organ nucleic acid levels after neonatal transduction. Some MPS I mice were injected IV with high-dose RV (1×10^9 TU/kg) at 2–3 days after birth, as described in Fig. 1. (A) Serum IDUA activity. Each line represents serum IDUA activity for an individual high-dose RV-transduced mouse at the indicated months after birth. The values of 0.01 U/ml at 0 months represent the level present in adult untreated MPS I mice, which was undetectable. The average values for heterozygous normal mice were multiplied by 2 to give the values in homozygous normal mice of 2.2 ± 1.6 U/ml (±2 standard deviations), as indicated by the shaded area. (B and C) Time course of liver and brain IDUA activity. Organs from normal, untreated MPS I, or high-dose RV-treated MPS I (High RV) mice were evaluated at 1.5 or 8 months after birth for liver and brain IDUA activity. The experimental values in heterozygous normal mice were multiplied by 2 to give values for homozygous normal mice. There were 4–6 mice in each group, and averages ± SEM are shown. (D) RV DNA levels in RV-treated mice. Nucleic acids were isolated at 1.5 or 8 months after birth from high-dose RV-treated MPS I mice. Real-time PCR of DNA was performed for the WPRE of the RV, with normalization to the β-actin sequence. There were four mice in each group, and averages ± SEM are shown. (E) RV RNA levels in RV-treated mice. RNA was reverse transcribed, then real-time PCR was performed for the WPRE and β-actin sequences.

copies of RV DNA per 100 cells, as shown in Fig. 5D. RV DNA was also detected in bone marrow at 1.2 ± 0.2 copies/100 cells (6% liver), and in brain at 0.5 ± 0.05 copies/100 cells (2% liver). Although the signal in liver of RV-transduced mice was well above the background in non-transduced controls of 0.01 RV copies/100 cells, the signals in BM and brain of RV-treated mice were only marginally above the background found in non-transduced BM and brain of 0.6 ± 0.4 and 0.1 ± 0.1 copies of RV DNA per 100 cells, respectively. DNA copies in liver, BM, and brain were similar at 8 months to the values found at 6 weeks.

Since the LTR of the RV can direct expression in non-hepatic cells, organs were also evaluated for RV RNA to determine if the DNA was expressed. At 6 weeks after transduction, liver RV RNA levels were $44 \pm 18\%$ of the β-actin level if one assumes that the WPRE and β-actin primer sets amplify equally well. Bone marrow, blood, and brain contained $1.2 \pm 0.1\%$, $0.23 \pm 0.074\%$, and $0.045 \pm 0.02\%$ as much RV RNA as β-actin RNA, respectively, which was 2.7, 0.5, and 0.1% of the value in liver, respectively. At 8 months after transduction, brain RV RNA was very low at 0.1% of the level of β-actin RNA in brain, which was 0.03% of the relative level of RV RNA in liver. RV RNA was <0.0001% the level of β-actin RNA in non-transduced controls (not shown). We conclude that brain contains very low levels of RV RNA, although levels are clearly about the background level in non-transduced controls.

Discussion

Lysosomal storage is markedly reduced in liver, spleen, kidney, and lung with high- or low-dose neonatal RV

The goal of this study was to further evaluate MPS I mice for biochemical and histopathological evidence of lysosomal storage, and to define the dose of RV necessary to prevent manifestations of disease after neonatal gene therapy. We previously calculated that high-dose neonatal RV resulted in secretion of ~539,000 U of mannose 6-phosphorylated IDUA into blood per kg per week [21], which is ~4-fold the amount of IDUA injected per week into humans that receive ERT.

In this study, liver, spleen, kidney, and lung had lysosomal storage in untreated MPS I mice in the regions that were reported to be abnormal previously. There was also substantial lysosomal storage in kidney tubules, which has not been reported previously. This discrepancy may be due to the younger age of analysis in previous studies. Both doses of RV markedly reduced lysosomal storage in these organs. In contrast, kidney tubule storage was difficult to correct in MPS VII mice [22]. It is possible that the smaller size of IDUA (70 kDa) as compared to GUSB (340 kDa) facilitates diffusion into the tubules, or that a relatively small amount of IDUA is sufficient to correct disease at this site.

Lysosomal storage in thymus, small intestine, ovary, and testis of untreated MPS I mice can be reduced with neonatal gene therapy

We demonstrate here that thymus, small intestine, ovary, and testis have substantial amounts of lysosomal storage in

MPS I mice, which has not been reported previously. The medulla of the thymus has large amounts of lysosomal storage, which was completely corrected with high-dose RV, and substantially improved with low-dose RV. MPS VII mice [22] and dogs [25] also have lysosomal storage in the thymus, and mice have blunted T lymphocyte proliferative responses and decreased antibody formation after immunization due to an inability to process antigens properly [31]. Further studies will determine if MPS I mice have a similar immunological defect, and if it can be prevented with gene therapy.

The small intestines of untreated MPS I mice have lysosomal storage in the lamina propria and the submucosa, which was completely corrected with both high- and low-dose RV. This storage is similar to that in MPS VII mice [22] and dogs [25]. Although the functional significance of storage in small intestines is unclear, gastrointestinal symptoms can occur in patients with MPS I, which are improved with ERT [32].

This study demonstrates that there are very large amounts of lysosomal storage in the ovary of untreated MPS I mice, which is consistent with our finding that untreated MPS I females breed poorly. The improved fertility in high- or low-dose RV-treated MPS I mice (data not shown) is consistent with the fact that lysosomal storage in the ovary is markedly reduced with neonatal gene therapy. Although untreated MPS I males have lysosomal storage in the interstitial region of the testis, the fact that seminiferous tubules appear normal and young males can breed suggests that this storage does not have major clinical significance.

Mechanism of correction of lysosomal storage in brain with high-dose but not low-dose RV may involve diffusion of enzyme from blood

As patients with Hurler disease have neurological impairment, it is important to understand the effect of this gene therapy approach upon lysosomal storage in brain. As previously reported [7,8,20,33], untreated MPS I mice had substantial lysosomal storage in neurons and microglial cells of the cortex and hippocampus, Purkinje cells of the cerebellum, the perivascular region, and the meninges. In this study, high-dose RV markedly reduced lysosomal storage in all of these sites, although low-dose RV was not effective. Thus, these data identify the dose of RV necessary to prevent storage in the brain in mice, which will need to be verified in large animals.

There are three potential mechanisms by which storage in brain could be corrected: (1) transduced hematopoietic cells could migrate into brain; (2) brain cells could be transduced at the time of neonatal gene therapy; or (3) enzyme in blood could diffuse into brain. It is unlikely that migration of blood cells is responsible for correction of storage in brain. Enzyme activity in brain paralleled serum IDUA activity and increased fairly rapidly, as levels in brain at 6 weeks after transduction were similar to the levels found at 8 months. However, migration of blood cells into brain is a slow process [28,29], and neonatal non-myeloablative HSCT to MPS VII mice resulted in very few cells that were positive by histochemical analysis, and an enzyme level that was only 0.3% of normal in brain at 1 year after transplantation [29]. In addition, adult HSCT failed to completely eliminate storage in neurons in MPS I mice [8], although the transduction efficiency of blood cells was higher than what we achieved here, and the LTR of the MND vector used would likely express better in brain than the Moloney murine leukemia virus-derived LTR present in our vector.

The possibility that brain cells were transduced at the time of neonatal IV injection cannot be ruled out at this point. Indeed, in a study by Kobayashi et al. [20], the brain contained substantial numbers of transduced neurons by histochemical staining, and had 1 copy of lentiviral vector per 100 cells at 5 months after neonatal transduction. In addition, the newborn mouse brain contains replicating neurons [34], which would be amenable to gamma RV transduction. Indeed, there were very small numbers of histochemically positive cells in the cortex, hippocampus, and cerebellum of some MPS VII mice that received neonatal IV injection of an RV expressing GUSB that was otherwise similar to the RV used in this study [22], although transduced cells were not detected in brain by histochemical stain after neonatal transduction in dogs [25,35]. Furthermore, at 8 months after neonatal transduction in mice in this study, brain contained RV DNA at 0.2 ± 0.1 copies per 100 cells, and RV RNA at $0.015 \pm 0.008\%$ of the level of brain β-actin RNA. Nevertheless, although these RNA values were above the background signal in non-transduced mice, the brain RV RNA level was still very low at only 1/3500 of the level found in transduced livers. The fact that RV RNA levels are very low reduces the chance that correction in brain is due to transduction of brain cells, although this mechanism cannot be ruled out as the target level of RV RNA is unclear.

We favor the hypothesis that neurons were corrected by enzyme that diffused from blood into brain. Recent studies have refuted the long-standing dogma that lysosomal enzymes cannot cross the blood–brain barrier in adults. The most-compelling is the study by Vogler et al. [30], in which IV administration of very high doses of GUSB to adult MPS VII mice resulted in 2.5% of normal GUSB activity in brain, and reduced storage in neurons. Similarly, adult gene therapy with an AAV vector expressing GUSB reduced storage in neurons of MPS VII mice without detectable vector copies in the brain [36]. It was also recently reported in abstract form that human IDUA can diffuse into the brain of dogs with MPS I during ERT and reduce storage in neurons [37]. Finally, transfer of hAAT-cIDUA-WPRE to adult (6-week-old) MPS I mice resulted in reduced storage in neurons at 8 months of age, and RV DNA and RNA levels were undetectable in the brain (X. Ma, M. Tittiger, and K.P. Ponder, unpublished data).

The failure to correct lysosomal storage in Purkinje cells at 8 months of age with low-dose RV mice that achieved 43 ± 12 U/ml of IDUA activity in serum in our study differs

from the results of Hartung et al. [19], who reported complete or partial reduction in lysosomal storage at 5 months in 3 of 4 animals that achieved plasma IDUA activity of ~10 U/ml after neonatal transduction with an AAV vector expressing human IDUA from the CMV-β-actin promoter. This discrepancy may be due to the difference in the age of analysis or to differences in scoring the degree of pathology, which is somewhat subjective. Alternatively, it is possible that early enzyme activity is critical, and that expression from the AAV vector was higher in the neonatal period than the value of ~10 U/ml of IDUA activity observed in plasma at 1 month or later. Indeed, expression from a similar AAV vector that expressed GUSB was ~10% as high at 1 month after neonatal gene therapy as it was at 1 week [38]. Definitive resolution of the mechanism by which neurons can be corrected will require testing if high-dose ERT can prevent storage in neurons of MPS I mice, and/or if an RV that is completely liver-specific can have the same effect. In addition, further studies will need to determine the effect of this neonatal gene therapy approach on cognitive function, which was not evaluated here.

Implications for patients with MPS I

We demonstrate here that MPS I mice have substantial amounts of lysosomal storage in several regions that have not been evaluated previously. These include the tubules of the kidney cortex, the thymus, the small intestines, and the ovary. It is likely that storage in these organs contributes to the clinical manifestations of disease, and that evaluation of these organs in future studies will be useful to evaluate the efficacy of treatment. We also find that storage is completely or substantially improved in these as well as most other organs with both high- and low-dose gene therapy. The exception is in the brain, where low-dose RV had very little impact upon lysosomal storage, although high-dose RV was quite effective. These data help to define the dose of RV needed to correct different aspects of disease, which will need to be confirmed in large animals prior to using this approach in humans.

Acknowledgments

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