doi:10.1006/mthe.2000.0121, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Delivery of a Retroviral Vector Expressing Human β-Glucuronidase to the Liver and Spleen Decreases Lysosomal Storage in Mucopolysaccharidosis VII Mice

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Received for publication May 9, 2000, and accepted in revised form July 25, 2000

Mucopolysaccharidosis VII (MPS VII) is caused by β-glucuronidase (β-gluc) deficiency and results in lysosomal storage due to the inability to degrade glycosaminoglycans. Transfer of a β-gluc gene into the liver reduces hepatic pathology as well as storage in other organs via uptake of secreted protein. A Moloney murine leukemia-based retroviral vector expressing the human β-gluc cDNA was injected intravascularly into MPS VII mice during hepatocyte replication, which was induced with im injection of an adenoviral vector that transiently expressed hepatocyte growth factor (Ad.CMV.HGF). This procedure resulted in transduction of ~1% of hepatocytes, 1% of normal liver enzyme activity, and a reduction in lysosomal storage in the liver at 3.5 months. Surprisingly, controls that received retroviral vector without HGF had transduction of non-parenchymal cells in the liver, significant levels of enzyme and RNA in the liver at 2 but not 3.5 months, and reduced lysosomal storage at 3.5 months. Transduction was also achieved in the replicating cells of the spleen, where lysosomal storage was reduced. An approach using a retroviral vector without a growth factor might temporarily reduce lysosomal storage in the liver and spleen in humans. Addition of HGF might be used to augment and prolong gene transfer.

Key Words: **gene therapy; retroviral vector; β-glucuronidase; lysosomal storage; glycosaminoglycans; liver; spleen; hepatocyte growth factor.**

INTRODUCTION

Lysosomal storage diseases have an overall incidence of approximately one in 7700 live births (1) and are caused by the deficiency of an enzyme that degrades various intracellular compounds. Most lysosomal storage diseases cause bone and joint abnormalities, hepatosplenomegaly, cardiovascular abnormalities, and neurological symptoms. Mucopolysaccharidoses (MPS) are a subset of lysosomal storage diseases that involve the inability to degrade glycosaminoglycans (GAGs) (1). MPS VII is due to a deficiency of β-glucuronidase (β-gluc; EC 3.2.1.31). Although one of the rarest of the MPS syndromes, the availability of mouse (2), dog (3), and cat (4) models have made this an attractive system for using gene therapy to correct this class of disorders. The MPS VII mouse has a single base pair deletion in exon 10 of the β-gluc gene, which causes a frameshift at codon 497 (5). Many lysosomal enzymes, including β-gluc, are modified posttranslationally with mannose 6-phosphates (M6P) that target the protein to the lysosome. A small fraction of the protein produced by a cell is secreted, which can then be taken up by other cells via the M6P-receptor present on the cell surface. This pathway makes it possible for intravenous (iv) injection of enzyme to reduce lysosomal storage in affected animals (6–9).

Cellular or gene therapy could result in the continuous production of β-gluc *in vivo* in affected patients, and correction of disease in the transplanted or transduced cells and possibly at distant sites (10). Bone marrow transplantation has resulted in long-term amelioration of lysosomal storage and clinical symptoms in humans (11) and animals with MPS VII (6, 12, 13), while monocyte transplantation has resulted in only transient effects in mice due to the short half-life of the cells *in vivo* (14). Gene therapy with different viral vectors into a variety of

This work was supported by the National Institutes of Health (R01 DK48028, R01 DK52092, and K02 DK02575 awarded to K.P.P., RO1 HD35671 and R21 DK53920 awarded to M.S.S., and DK 54481awarded to M.E.H.).

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1525-0016/00 $35.00

sites has been used to attempt to correct the systemic manifestations of lysosomal storage in mice. A β-gluc gene has been delivered to the bone marrow with a retroviral vector (RV) (15, 16), to the liver with an adenoviral vector (17, 18), to transplanted autologous fibroblasts with an RV (19, 20), to the muscle with an AAV vector (21, 22), or systemically with an AAV vector (21, 23). These gene therapy procedures have resulted in transient or long-term reduction in lysosomal storage in transduced cells and, in some cases, in distant organs.

RV have been used to deliver genes to the liver (24–26), an organ that has direct contact with the blood, and synthesizes most blood proteins. Although the standard Moloney-murine leukemia virus (MoMLV)-based vectors only transduce dividing cells, hepatocyte growth factor (HGF) or other growth factors can stimulate hepatocyte replication and promote RV transduction *in vivo* with no apparent toxicity (27–31). In this study, we transferred a RV expressing human β-gluc into the liver and spleen after transient hepatocyte replication was induced with the im injection of an adenoviral vector that expressed HGF. Relatively high enzyme activity was observed in liver and spleen, which was associated with a marked reduction in lysosomal storage in these organs. An approach in which purified HGF protein with or without inducers of splenocyte replication is combined with a RV expressing human β-gluc might be useful in humans with MPS VII if they can be demonstrated to have sufficiently low toxicity.

MATERIALS AND METHODS

Construction of the RV designated hAAT-β-gluc. The RV plasmid hFX-514 (24) was digested with *Not*I, filled in with Klenow, and ligated with a 2.2-kb human β-gluc cDNA (32) with filled-in *Eco*RI ends to create hAAT-β-gluc-745. Murine ecotropic GP+E86 (33) and amphotropic GP+*env*AM12 packaging cells (34) and murine β-gluc-deficient 3521 cells (35) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% supplemented calf serum (Hyclone Laboratories, Logan, UT), penicillin, and streptomycin. hAAT-β-gluc was cotransfected with PGK-neo into GP+E86 cells and the supernatant from a pool of G418-resistant cells (26) was used to infect GP+*env*AM12 cells at a multiplicity of infection (m.o.i.) of ~2. Individual methotrexate-resistant colonies (25) underwent three rounds of screening for the number of red forming units (rfu)/ml in their conditioned medium. This titer was determined after infection of 3521 cells and β-gluc staining (36). For *in vivo* delivery, RV was concentrated by ultrafiltration as described (37) and polybrene was added to the injectate at a final concentration of 8 μg/ml. The injectate contained 13,000 U of β-gluc activity per milliliter, and each animal received ~20,000 units.

Animal procedures. MPS VII mice in a B6.C-H-2^{bm1} background were maintained in a pathogen-free environment. For all procedures, mice were anesthetized with metophane. Adenoviral vectors Ad.RR5 (38) and Ad.CMV.HGF (27) were grown and purified as described previously (27). Mice were injected im with 100 μl of PBS, or with 2×10^{12} particles of adenoviral vector diluted in PBS as a single injection into the quadriceps muscle. BrdU was injected IP at 100 mg/kg/dose at 76, 96, 100, 120, and 122 h after the im injection, and animals were sacrificed at 124 h. BrdU staining was performed as described previously (27, 39). For RV transduction, 300 μl of hAAT-β-gluc was injected into the tail vein of 5- to 7-week-old MPS-VII mice at 78, 96, 102, 120, and 126 h after the injection of PBS or adenoviral vector. The total dose was ~$1–2 \times 10^7$ rfu, resulting in an m.o.i. of retroviral vector:hepatocytes of ~0.2.

Analysis of organ sections, organ homogenates, or serum for β-gluc activity or GAG levels. Eight-micrometer frozen sections were fixed and stained for β-gluc activity as noted above (36). To obtain the average percentage of transduced hepatocytes, the number of large intensely red cells in 20 high power fields was counted, and divided by the total number of hepatocytes in 20 fields, as detailed previously (27). Five microliters of serum or ~25 μg of organ homogenate was incubated with 4-methylumbelliferyl β-D-glucuronide (Sigma Chemical, St. Louis, MO) for 2 to 16 h, and the fluorescence measured as described (36, 40). For organs, β-gluc activity was normalized to total protein as determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA). One unit of enzyme activity releases 1 nmol of 4-methylumbelliferone per milligram of protein per hour. For pathological analysis, samples were fixed with freshly prepared 2% glutaraldehyde/4% paraformaldehyde in PBS, embedded in Spurr's resin, and 0.5-μm sections were stained with toluidine blue (2). GAGs were measured by the Alcian blue method as described (41) and normalized to milligrams of protein present in the sample.

Assay for anti-human-β-gluc antibodies. An ELISA plate was coated with 1 μg/ml of human β-gluc. It was incubated with plasma at a ⩾1:100 dilution, followed by a 1:120 dilution of affinity purified goat anti-mouse IgG antibody coupled to HRP (Sigma Chemical Co.) and was developed as described previously (24). The titer was reported at the highest dilution at which the OD was at least twice as high as that observed in the 1:100 dilution of the pretreatment sample for that mouse. If the assay was negative at a 1:100 dilution, the titer was reported as 1:50.

PCRs for detection of RV DNA and RNA sequences. For analysis of nucleic acids from organs, DNA or RNA was isolated after homogenization in guanidinium (42). DNA from nontransduced MPS VII mice was isolated and amplified at the same time as the experimental mice to demonstrate the lack of PCR contamination. 1 μg of DNA underwent 30 cycles of PCR at 92°C for 1.5 min., 61°C for 1 min., and 72°C for 1 min. in a volume of 25 μl using a buffer containing 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Triton X-100, 0.45% Tween 20, 100 μM of each dNTP, and 0.5 units of *Taq* polymerase (Perkin–Elmer, Norwalk, CT). Primers specific for exon 6 (E6) at nt 1009 to 1028, and E7 at nt 1249 to 1230, of the human β-gluc cDNA (22) were added to a final concentration of 1 μM. Samples were electrophoresed on a 1.6% agarose gel, transferred to an Optitran reinforced nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and Southern blot was performed with a *Cla*I fragment containing nt 954 to 1480 of the human β-gluc cDNA. The final wash was performed at 65°C with 0.2× SSC (300 mM NaCl, 3 mM sodium citrate) with 0.1% sodium dodecyl sulfate. For the standards, the signal for the 240 bp human band was determined with a phosphorimager and divided by that in the 454-bp mouse product to create a standard curve. Values in experimental samples were compared with this standard curve to determine the copy number. For reverse transcriptase PCR (RT-PCR), 2 μg of DNase I-treated total RNA was amplified using Ready-To-Go RT PCR beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the instructions of the manufacturer. Primers specific for human β-gluc cDNA at nt 1928 to 1952 (5′-GTATCCCCACTCAGTAGCCAAGTCA-3′) and at nt 2125 to 2101 (5′-TTCCCTGC-

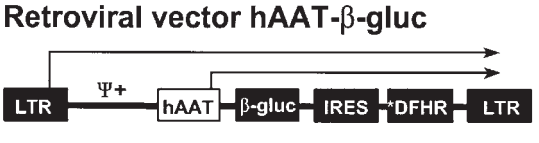


FIG. 1. Retroviral vector (RV) hAAT-β-gluc. The RV designated hAAT-β-gluc contains complete long terminal repeats (LTRs) at both ends, an extended packaging signal (ψ^+), the human α1-antitrypsin promoter (hAAT) from nt –347 to +56, and the human β-glucuronidase cDNA (β-gluc). Transcription from either the LTR or the hAAT promoter results in an RNA that can be translated into protein. hAAT-β-gluc also contains an internal ribosome entry site (IRES) sequence from the encephalomyocarditis virus, which allows translation of the downstream sequence of a dicistronic mRNA, and a mutant dihydrofolate reductase (DHFR) cDNA that confers methotrexate resistance.

TAGAATAGATGACCACA-3′) of the human β-gluc cDNA, which are poorly conserved between mice and human, were used. Southern blot was performed with a *Sac*I–*Xba*I fragment of hAAT-β-gluc-745, which contains nt 1780 to 2191 of the human β-gluc cDNA. Hybridization and wash conditions were as noted above. To demonstrate that the RNA was of good quality, RT-PCR with primers specific for mouse β-actin (43) followed by agarose gel electrophoresis and staining with ethidium bromide was performed.

Statistical analysis. For comparison of levels between two groups, the Student's *t* test was performed using the program Instat from GraphPAD Software (San Diego, CA).

RESULTS

Generation of an RV That Expresses Human β-gluc (hAAT-β-gluc)

An RV designated hAAT-β-gluc was constructed in which the human α_1-antitrypsin (hAAT) promoter directed expression of human β-gluc, as diagramed in Fig. 1. This promoter has been successfully used to direct long-term (>1 year) expression of three different cDNAs in the liver from an RV (24–26). An amphotropic packaging cell line was generated with a titer of 1×10^6 rfu/ml. This was further concentrated to 1×10^7 rfu/ml by ultrafiltration.

Hepatocytes from MPS-VII Mice Replicate in Response to HGF

Hepatocyte replication is necessary for transduction with an MoMLV-based RV. We previously demonstrated that im injection of an E1/E3-deleted adenoviral vector that expressed HGF from the CMV promoter (Ad.CMV.HGF) resulted in transient replication of hepatocytes in normal mice (27). We tested if hepatocytes from MPS VII mice would behave similarly. Mice were injected into the muscle with PBS, a control adenoviral vector that does not encode a transgene (Ad.RR5), or Ad.CMV.HGF. Serum human HGF peaked at 4 ng/ml at days 4 and 5 and was undetectable at 10 days or later after injection for the Ad.CMV.HGF-treated mice. This was similar to the response observed in normal mice (27). Serum human HGF was undetectable for the control groups, as expected.

The percentage of replicating cells was determined after IP injection of BrdU at multiple times between 3 and 5 days after the im injection, and performing anti-BrdU immunocytochemistry on liver sections. BrdU is a thymidine analog that is only incorporated into newly synthesized DNA. Representative examples of BrdU staining are shown in Fig. 2, and quantitation of the percentage of replicating cells is shown in Fig. 3. The hepatocyte labeling index in mice that received Ad.CMV.HGF was ~20-fold higher than that observed after PBS (P = 0.003), and was ~3-fold higher than that observed after injection of Ad.RR5 (P = 0.01). Replication in mice that received Ad.RR5 was 7-fold higher than in controls that received PBS (P = 0.04). We conclude that lysosomal storage does not preclude MPS VII hepatocytes from replicating in response to HGF. We also detected replication in ~30% of the non-parenchymal cells from Ad.CMV.HGF- or Ad.RR5-treated mice. This was higher than, but not statistically different from, that observed in mice that received PBS (8.9 ± 6%). Replication of non-parenchymal cells may be a non-specific response to an adenoviral vector (27). There were also large numbers of replicating cells in the red pulp of the spleen for all groups (Figs. 2D–2F). Although the white pulp from the PBS-treated mice was largely devoid of replicating cells, the administration of either the control or the HGF-expressing adenoviral vector resulted in induction of replication of these cells.

Histochemical Analysis of Liver, Spleen, and Other Organs

We tested if hAAT-β-gluc could transduce liver and express enzyme in MPS VII mice. hAAT-β-gluc was injected iv at 3–5 days after im injection of PBS, Ad.RR5, or Ad.CMV.HGF, and livers were analyzed histochemically

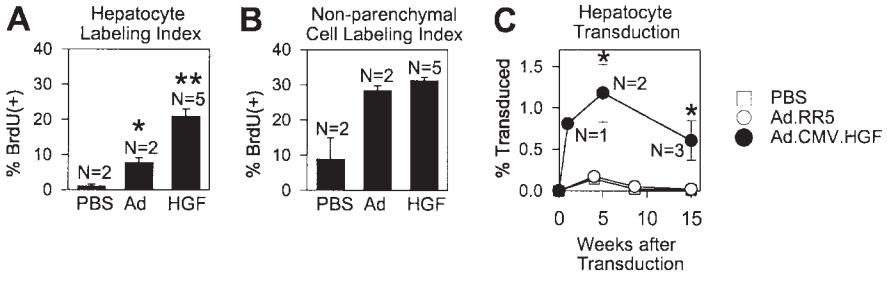


FIG. 3. Quantitation of the percentage of replicating cells in the liver. A (hepatocyte) and B (nonparenchymal cell) labeling index. BrdU staining was performed as shown in Fig. 2, and the percentage of replicating cells is reported as the average ± standard error of the mean (SEM) for the indicated number (*N*) of animals. Values in the experimental groups were compared with those in normal mice that did not receive any injections. * represents a *P* value between 0.05 and 0.005; ** represents a *P* value between 0.005 and 0.0005, and *** represents a *P* value <0.0005 in this and subsequent figures. (C) Percentage of transduced hepatocytes. MPS VII mice were treated with im injection of PBS, Ad.RR5, or Ad.CMV.HGF as described in the legend to Fig. 2, followed by injection of hAAT-β-gluc. Transduced hepatocytes were identified on liver sections as large intensely red cells after staining for β-gluc activity, as shown in Fig. 4. Averages ± SEM are shown for the indicated number of animals for Ad.CMV.HGF-treated mice. For mice that received PBS or Ad.RR5, only one animal was analyzed at each time point. Statistical comparisons were between mice that received Ad.CMV.HGF prior to RV, and mice that received PBS or Ad.RR5 prior to RV.

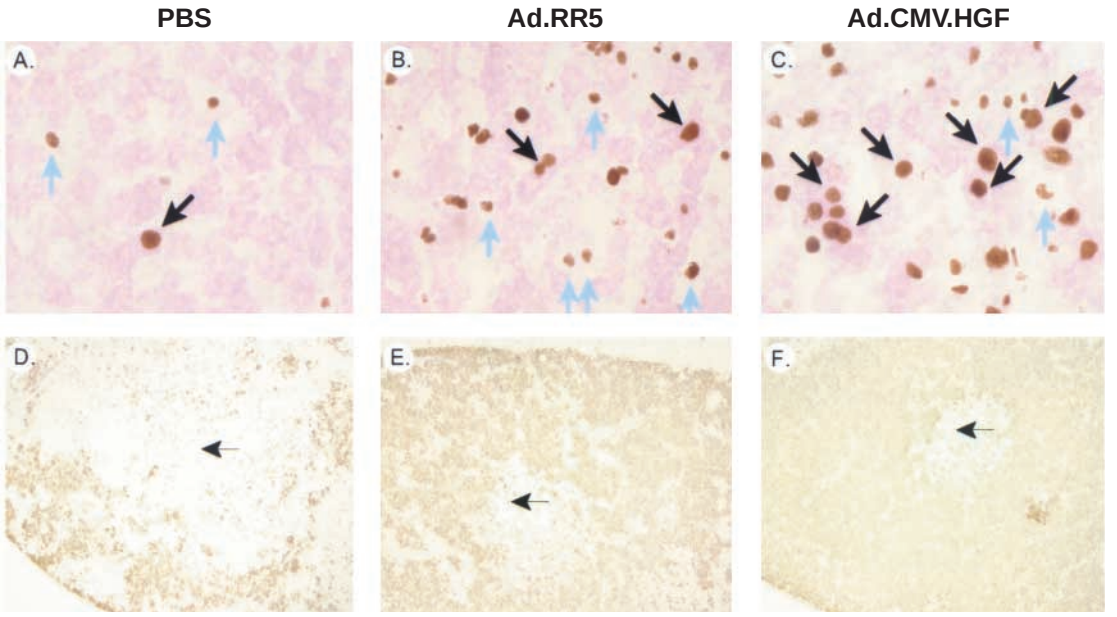


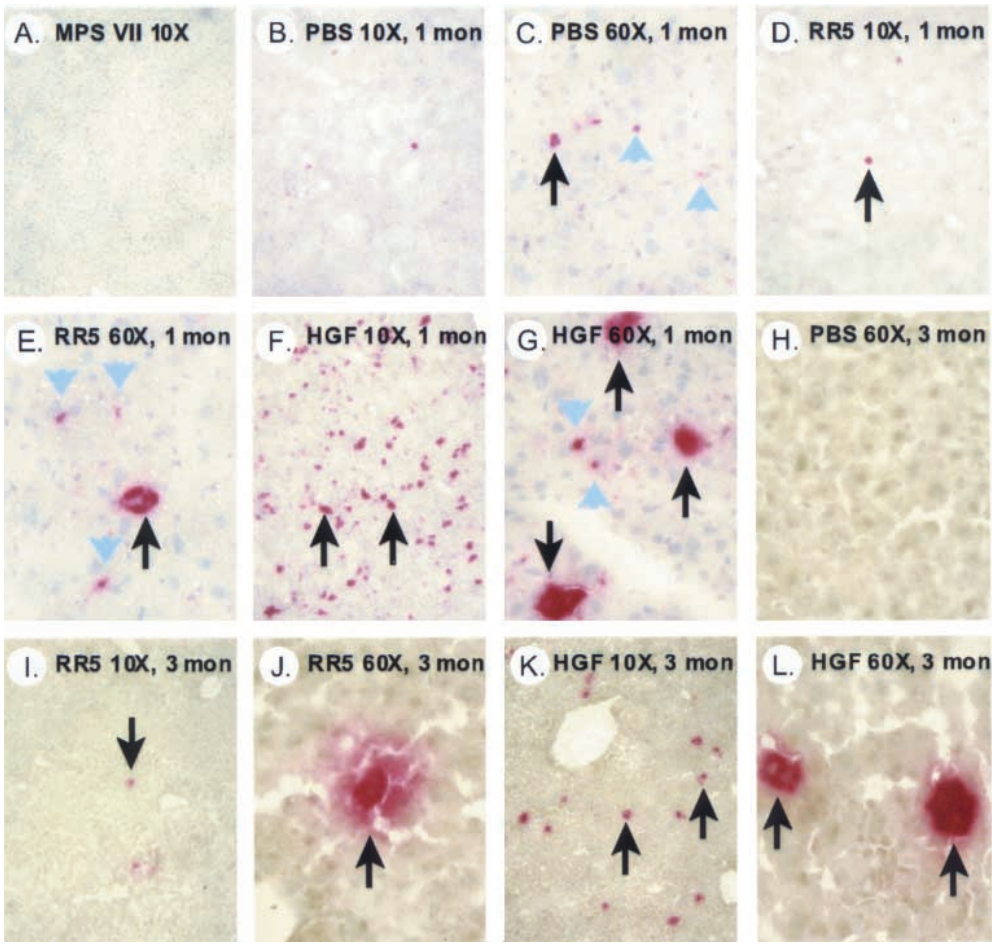
FIG. 2. Determination of the percentage of replicating cells in liver and spleen. Four- to 5-week-old MPS VII mice were injected im with PBS, or 2×10^{12} particles of the control adenoviral vector Ad.RR5 or Ad.CMV.HGF. Bromodeoxyuridine (BrdU) was injected ip multiple times 3 to 5 days later. A (PBS), B (Ad.RR5), and C (Ad.CMV.HGF). Liver. Frozen liver sections were stained for BrdU-labeled cells using immunocytochemistry, and counterstained with eosin. Black arrows identify large cells with eosin-staining cytoplasm and dark nuclei, which represent hepatocytes that replicated between 3 and 5 days. Blue arrows identify BrdU-labeled small nonparenchymal cells of the liver, whose cytoplasm does not stain with eosin. Nonparenchymal cells consist primarily of Küpffer cells and endothelial cells. 60× original magnification. D (PBS), E (Ad.RR5), and F (Ad.CMV.HGF). Spleen. Frozen sections of the spleen were stained for BrdU-labeled cells. Since no counterstain was applied, only the dark nuclei from BrdU-labeled cells are visible. Black arrows identify labeled nuclei. 10×. The unlabeled regions in D represent the white pulp of the spleen.

for the presence of β-gluc enzyme. The percentage of hepatocytes with high levels of enzyme activity, which presumably represent transduced cells, is shown in Fig. 3C. Representative examples of β-gluc staining are shown in Fig. 4. MPS VII mice that did not receive any RV or adenoviral vector will be referred to as MPS VII mice hereafter. Livers from MPS VII mice had no cells with enzyme activity (Fig. 4A), while all cells were positive in a normal liver (not shown). For mice that received Ad.CMV.HGF, ~1% of the hepatocytes had high levels of enzyme activity at both times (Figs. 4F, 4G, 4K, and 4L), which was >7-fold higher than in the control groups at both times ($P < 0.04$). We conclude that the administration of HGF prior to delivery of the RV increased the number of transduced hepatocytes. All groups had large numbers of small non-parenchymal cells with enzyme activity at 1 month (Figs. 4B–4G), but the number of these cells was much lower at 3.5 months (Figs. 4H–4L). Data presented below suggest that these probably represent transduced non-parenchymal cells.

Histochemical staining was also performed on spleen sections at various times after transduction, as shown in Fig. 5. Two weeks after injection of hAAT-β-gluc into mice that received Ad.CMV.HGF, the majority of the spleen was faintly red, and there were numerous intensely red cells with very high levels of enzyme activity (Fig. 5A). These intense-staining cells were still present at 1 (Fig. 5B) and 3.5 (Figs. 5C and 5D) months after transduction, while the faintly red cells were no longer identified. Mice that received PBS (Figs. 5E and 5F) or Ad.RR5

FIG. 4. Histochemical analysis of β-gluc enzyme activity in livers of untreated or hAAT-β-gluc-injected MPS VII mice. Sections of liver were stained for β-gluc enzyme activity (red), and counterstained with hematoxylin (blue) to identify nuclei. (A) Untreated MPS VII mouse. No enzyme activity is present (10×). (B–L) hAAT-β-gluc injected mice. Five- to 7-week old MPS VII mice were injected im with PBS (PBS), or 2×10^{12} particles of Ad.RR5 (RR5) or Ad.CMV.HGF (HGF), as indicated on the panel. Multiple doses of RV were injected via tail vein 3 to 5 days later. The original magnification was at 10× (10×) or 60× (60×), as indicated on the panel. Animals were sacrificed at 1 (1 mon) or 3.5 (3 mon) months after injection of RV. Black arrows identify the large hepatocytes with enzyme activity, and blue arrows identify the small nonparenchymal cells with enzyme activity.

FIG. 5. Histochemical analysis of β-gluc enzyme activity in spleens of hAAT-β-gluc injected MPS VII mice. Mice were treated as described in the legend to Fig. 4. Frozen sections of spleen were stained for β-gluc enzyme activity (red), and counterstained with hematoxylin (blue). Arrows in A and B identify the white pulp, which was generally devoid of enzyme activity. The reagent that was injection prior to the RV, the original magnification of the photograph, and the time of sacrifice after the injection of RV, are indicated as detailed in the legend to Fig. 4.



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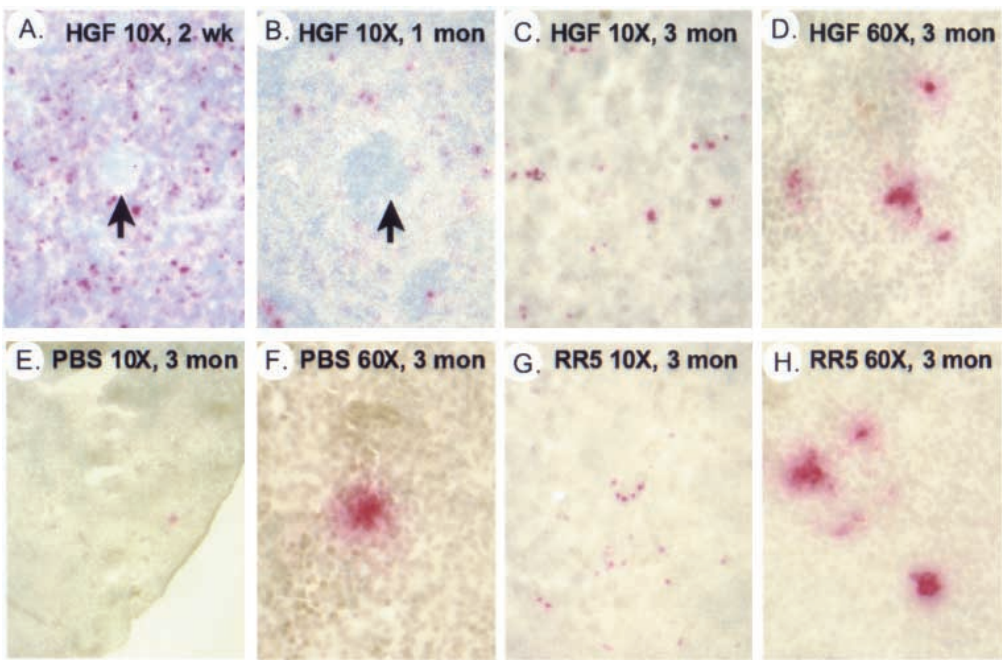

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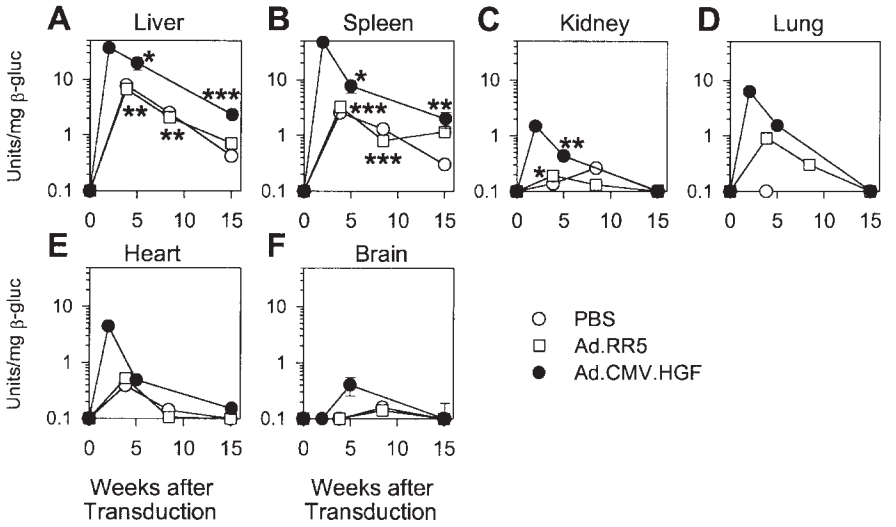

FIG. 6. Quantitation of β-gluc enzyme activity in organs after injection of hAAT-β-gluc. Organs from all mice whose histochemical analysis in the liver is shown in Fig. 3C were homogenized, and the β-gluc activity was determined and normalized to the total protein concentration. For mice that received Ad.CMV.HGF prior to hAAT-β-gluc, values in individual animals were averaged and reported as the units/mg ± SEM. Statistical comparisons were between samples from these mice vs samples from MPS VII mice. For mice that received PBS or Ad.RR5 prior to RV, data from a single animal is shown. The results in mice that received PBS or Ad.RR5 were averaged and compared with the results for MPS VII mice. The time refers to the weeks after the administration of hAAT-β-gluc. Samples with activity that was ≤0.1 U/mg were shown as having 0.1 U/mg on this semilog scale. (A) Liver. Activity in homozygous normal mouse liver was 255 ± 53 U/mg (standard deviation), and 0.06 ± 0.04 U/mg in untreated MPS VII mice. (B) Spleen. Homozygous normal and MPS VII mice had 470 ± 58 U/mg and 0.04 ± 0.01 U/mg, respectively, in spleen. (C) Kidney. Normal and MPS VII mice had 156 ± 43 U/mg and 0.05 ± 0.02 U/mg in kidney, respectively. (D) Lung. Normal and MPS VII mice had 166 ± 13 U/mg and 0.2 ± 0.1 U/mg, respectively, in lung. (E) Heart. Normal and MPS VII mice had 12 ± 0.3 U/mg and 0.05 ± 0.01 U/mg, respectively, in heart. (F) Brain. Normal and MPS VII mice had 31 ± 4.6 U/mg and 0.1 ± 0.05 U/mg, respectively, in brain.

(Figs. 5G and 5H) prior to injection of hAAT-β-gluc also had intensely red cells at 1 month (not shown) and at 3.5 months. The localized intense staining suggests that this activity might be due to transduced cells that express the enzyme. Little or no enzyme activity was observed in heart, brain, lung, or kidney (not shown).

Quantitation of β*-gluc Activity in Organs after RV Transduction*

To better quantify the level of β-gluc, organs were homogenized and β-gluc activity was determined, as shown in Fig. 6. Activity in livers at 1 month was 19.8 U/mg (8% of normal) for mice that received Ad.CMV.HGF prior to hAAT-β-gluc. This activity was 330-fold higher than that in MPS VII mice ($P = 0.009$), but was only 2.7-fold higher than that in the control groups that received PBS or Ad.RR5 prior to RV (not significant). Enzyme activity remained high at 3.5 months for Ad.CMV.HGF-treated mice at 2.4 U/mg (1% of normal), which was 40-fold higher than that in MPS-VII mice ($P = 0.0001$), and was 4.2-fold higher than that observed in the control mice that did not receive Ad.CMV.HGF prior to RV at this time ($P = 0.02$). Activity in these controls was 121-fold higher at 1 month ($P = 0.001$), 38-fold higher at 2 months ($P = 0.002$), and 9.1-fold higher at 3.5 months (not significant) relative to MPS VII mice. We conclude that there was considerable enzyme activity in all groups that received RV at 1 month, but that only mice that received Ad.CMV.HGF prior to RV maintained significantly higher enzyme activity than MPS VII mice at 3.5 months.

Spleens from Ad.CMV.HGF-treated mice had enzyme activity that was 7.8 U/mg (1.6% of normal) at 1 month, which was 195-fold higher than that in MPS VII mice ($P = 0.006$). This was 2.7-fold higher than, and not statistically different from, that observed in mice that received PBS or Ad.RR5 prior to the RV. Activity remained detectable at 3.5 months for mice that received Ad.CMV.HGF at 2 U/mg (0.4% of normal) which was 50-fold higher than that in MPS VII mice ($P = 0.003$), but only 2.7-fold higher than, and not significantly different from, the level found in mice that received PBS or Ad.RR5 prior to RV. At 1, 2, and 3.5 months, the enzyme activity in these control groups was 72-, 25-, and 18-fold that found in MPS VII mice ($P < 0.002$ vs MPS VII at 1 and 2 months). Enzyme activity was also analyzed in other organs. There was detectable, but low, enzyme activity in Ad.CMV.HGF-treated mice at 1 month in kidney (0.43 U/mg; 0.3% of normal), lung (1.5 U/mg; 1%), heart (0.5 U/mg; 5%), and brain (0.4 U/mg; 1%) despite the inability to detect red cells on histochemical staining. Enzyme activity fell to much lower or undetectable levels by 3.5 months. The control groups had lower levels of enzyme activity at 1 month, which fell to undetectable levels by 3.5 months.

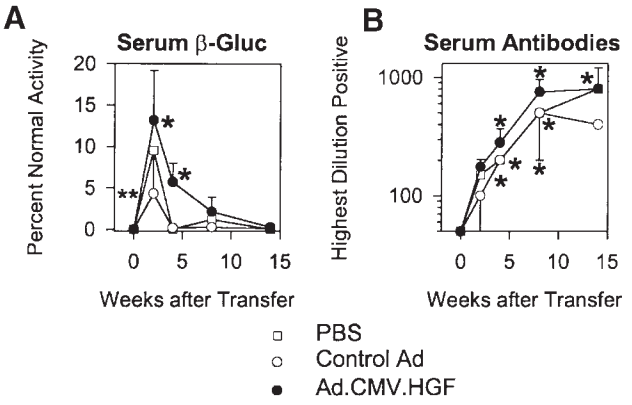

FIG. 7. Serum β-gluc enzyme activity and anti-human β-gluc antibody titer. (A) Serum β-gluc activity. Serum was tested for β-gluc enzyme activity, normalized to the level observed in homozygous normal mice (27.8 ± 2.2 U/ml), and the averages of values from all surviving animals ± SEM are shown at each time point. MPS VII mice had 0.18 ± 0.1% of normal activity. The values in mice that received PBS, Ad.RR5, or Ad.CMV.HGF prior to hAAT-β-gluc were compared with the values from MPS VII mice for statistically significant differences. (B) Anti-human β-gluc antibody titer. Serum was tested for antibodies directed against human β-gluc. Data are plotted as the average of the highest titer positive ± SEM, and statistical comparisons were between experimental groups and MPS VII mice.

Analysis of Serum for β-gluc Activity and Antibodies to Human β-gluc

An hypothesis of these experiments was that the liver could secrete sufficient amounts of enzyme into the blood to reach other organs. Indeed, at 2 weeks after injection of hAAT-β-gluc, mice that received Ad.CMV.HGF had serum β-gluc activity that was 3.7 ± 1.6 U/ml (13.2% of normal), as shown in Fig. 7A. This was almost certainly not due to the enzyme that was injected along with the RV, as the half-life of human β-gluc in blood is 3.5 min in rats (44). This was 74-fold higher than that of MPS VII mice (P = 0.02), but only 1.9-fold that of the controls that received PBS or Ad.RR5 prior to RV (not significant). At 1 month after transduction, serum from mice that received Ad.CMV.HGF had 1.6 U/ml (5.7% of normal) of enzyme activity, which was 32-fold higher than that in MPS VII mice (P = 0.04) but, again, was not statistically higher than in mice that received PBS or Ad.RR5 prior to injection, due to the marked individual variation. Serum activity fell progressively thereafter, however, and antibodies directed against the human β-gluc developed in all groups, as shown in Fig. 7B.

Distribution of RV DNA Sequences

The hypothesis of this project was that this gene therapy approach would primarily result in transduction of hepatocytes, which would serve as a factory to produce enzyme for other organs. Since it was possible that enzyme activity in spleen and other sites was due to the transduction and expression of the RV in that organ, we tested DNA from organs for RV sequences, as shown in Fig. 8. The PCR used primers from different exons that recognized both human and mouse sequences, but resulted in a shorter product for the human cDNA which does not contain an intron. This allowed us to compare the human to the mouse signal for samples, and quantitate the copy number by comparison with the ratio observed with a standard curve. For mice that received Ad.CMV.HGF prior to hAAT-β-gluc and were sacrificed at 2 or 4 weeks, RV DNA sequences were present in 1.21 ± 0.45% of liver cells. Although this was statistically higher than the DNA copy number in MPS-VII mice (P = 0.04), it was not significantly different from the DNA copy number in mice that received hAAT-β-gluc without

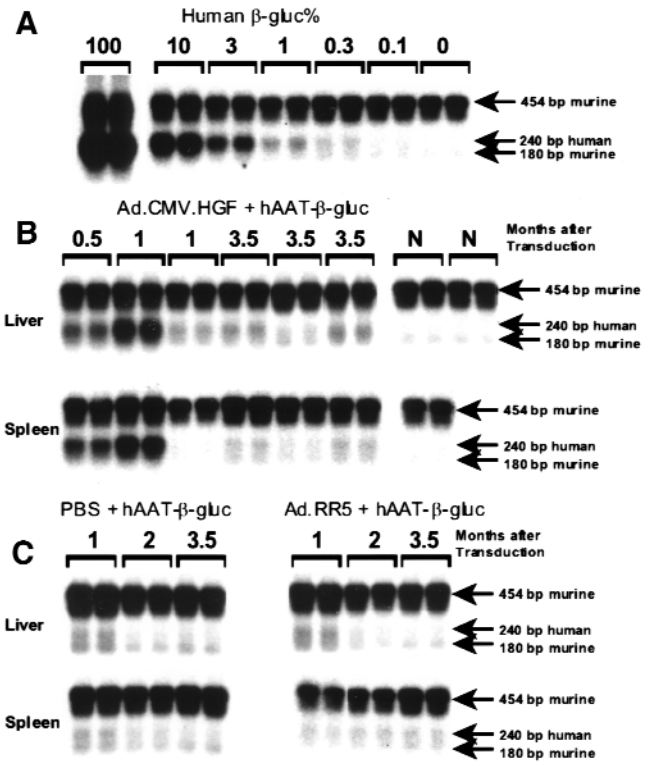

FIG. 8. PCR analysis of DNA for RV sequences. (A) Standard curve to determine the RV DNA copy number. Cellular DNA from cells that contained a single copy of the human β-gluc cDNA was mixed with DNA from MPS VII mice at the ratio shown above each sample. PCR followed by Southern blot was performed. The mouse genomic DNA (454 bp murine) and the human cDNA (240 bp human) products are indicated. The human-derived product was more intense per copy added than the mouse product because the mouse gene has 2 mismatches in the E6 oligo, and a human cDNA probe was used for Southern blot. In some cases, low levels of an additional 180-bp band that presumably represents a non-specific product was identified in samples that did not contain the human β-gluc cDNA (180 bp murine). (B) PCR analysis in mice that received hAAT-β-gluc after Ad.CMV.HGF. DNA derived from liver (top panel) or spleen (bottom panel) was tested for the presence of RV DNA sequences. The time in months after transduction is shown above each lane; each bracket identifies duplicate samples from the same animal. N represents nontransduced MPS VII mice. (C) PCR analysis in mice that received hAAT-β-gluc after PBS (left) or Ad.RR5 (right). Samples are identified as in B.

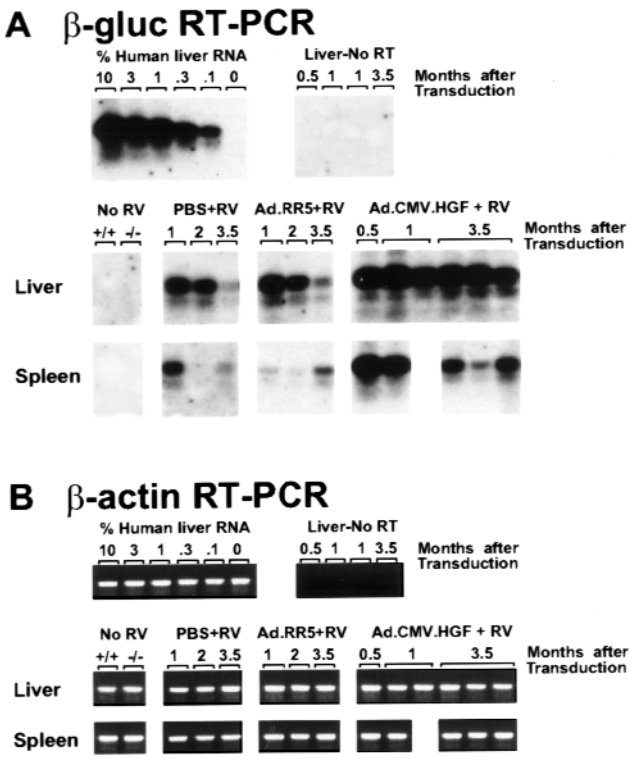

FIG. 9. RT-PCR analysis of RNA for RV sequences. (A) RT-PCR for β-gluc sequences. On the left, a standard curve was generated by mixing RNA from human liver with RNA from an MPS VII mouse liver at the ratio indicated above the lane. RT-PCR followed by Southern blot was performed to identify the 198-bp human β-gluc PCR product. On the right, RNA from livers of mice that received hAAT-β-gluc after Ad.CMV.HGF underwent PCR without RT. The time that each sample was collected after injection of hAAT-β-gluc is indicated. No signal was observed, demonstrating the absence of contaminating DNA in the samples. RT-PCR performed on RNA from nontransduced normal (+/+) and mutant (–/–) mice had no signal, demonstrating that the human-specific primers did not amplify the mouse RNA. Samples from liver and spleen from mice that received im injection of PBS (PBS + RV), Ad.RR5 (Ad.RR5 + RV), or Ad.CMV.HGF (Ad.CMV.HGF + RV) prior to hAAT-β-gluc and were harvested at the indicated time after transduction are indicated. (B) RT-PCR for β-actin mRNA. PCR was performed using primers specific for the mouse β-actin mRNA to document that all samples contained amplifiable RNA. A 2.5% agarose gel that was stained with ethidium bromide is shown.

Ad.CMV.HGF. RV DNA sequences were still present in 0.45 ± 0.16% of liver cells at 3.5 months for mice that received Ad.CMV.HGF prior to RV, which was statistically higher than in MPS-VII mice ($P = 0.05$). For mice that received PBS or Ad.RR5, RV DNA sequences were present in 0.3 ± 0.04% of cells in the liver at 1 month ($P = 0.02$ vs MPS-VII mice), but were not significantly higher than in MPS-VII mice at 2 or 3.5 months. We conclude that the liver was transduced at 1 month in all groups that received RV, but that only mice that received Ad.CMV.HGF prior to RV clearly maintained the DNA at 3.5 months.

In the spleen, mice that had received Ad.CMV.HGF and were sacrificed at 2 or 4 weeks had RV DNA in 2.8 ± 1.3% of cells at 1 month, which was not significantly higher than that in non-transduced mice due to the marked variation between the individual animals analyzed. RV DNA was present in 0.3 ± 0.1% of cells at 3.5 months, which was significantly higher than that in non-transduced mice ($P = 0.05$). For mice that received Ad.RR5, the DNA copy number was 1.3, 0.6, and 0.25 at 1, 2, and 3.5 months, respectively. For mice that received PBS, the copy number was lower at 0.2, 0.15, and 0% at the corresponding times. The heart, kidney, lung, and brain did not contain RV sequences for any groups (not shown). We conclude that spleen cells were also transduced with the RV for all groups, although the efficiency appeared to be higher for those that received the control or the HGF-expressing adenoviral vector than for those that received PBS.

Analysis of RNA for RV Sequences

RNA obtained from organs was analyzed using RT-PCR to determine if transcription of the RV had occurred, as shown in Fig. 9. Although the RV has a liver-specific promoter directly upstream of the β-gluc cDNA, it also contains the LTR in the same orientation. The latter promoter directs expression of an RNA that is translated into β-gluc protein in the fibroblast-derived 3521 cells, and could be used to direct expression in organs other than the liver. Livers from mice that received hAAT-β-gluc after Ad.CMV.HGF clearly expressed β-gluc mRNA for up to 3.5 months. This signal was not due to contaminating DNA, as samples that did not receive RT had no signal. This signal was not due to amplification of the mouse β-gluc mRNA, as RNA from non-transduced mouse liver had no signal. For mice that received PBS or Ad.RR5 prior to hAAT-β-gluc, β-gluc mRNA was clearly detectable in liver at 1 and 2 months, but had fallen to lower levels by 3.5 months. The spleen also clearly expressed human β-gluc mRNA for all mice that received Ad.CMV.HGF prior to hAAT-β-gluc. Human β-gluc mRNA was also present in the spleen of some mice that received PBS or Ad.RR5 prior to RV. All samples had intact RNA, as demonstrated by the RT-PCR amplification of β-actin. No human β-gluc mRNA was identified by RT-PCR in any animals using RNA from lung, kidney, heart, or brain (not shown).

Evaluation of Lysosomal Storage

All animals had a marked to nearly complete correction in lysosomal storage in the liver at 1 and 2 months after transduction (not shown). At 3.5 months after transduction, livers from mice that were treated with PBS or Ad.RR5 prior to the delivery of hAAT-β-gluc had moderate improvement in lysosomal storage compared with an untreated age-matched MPS VII mouse, but had considerable storage within most of the sinusoids as well as in hepatocytes (Fig. 10). Two mice that had received Ad.CMV.HGF prior to the hAAT-β-gluc had a more marked improvement in storage, although a third mouse had only a moderate improvement. Although the lysosomal storage was markedly reduced in the sinusoids

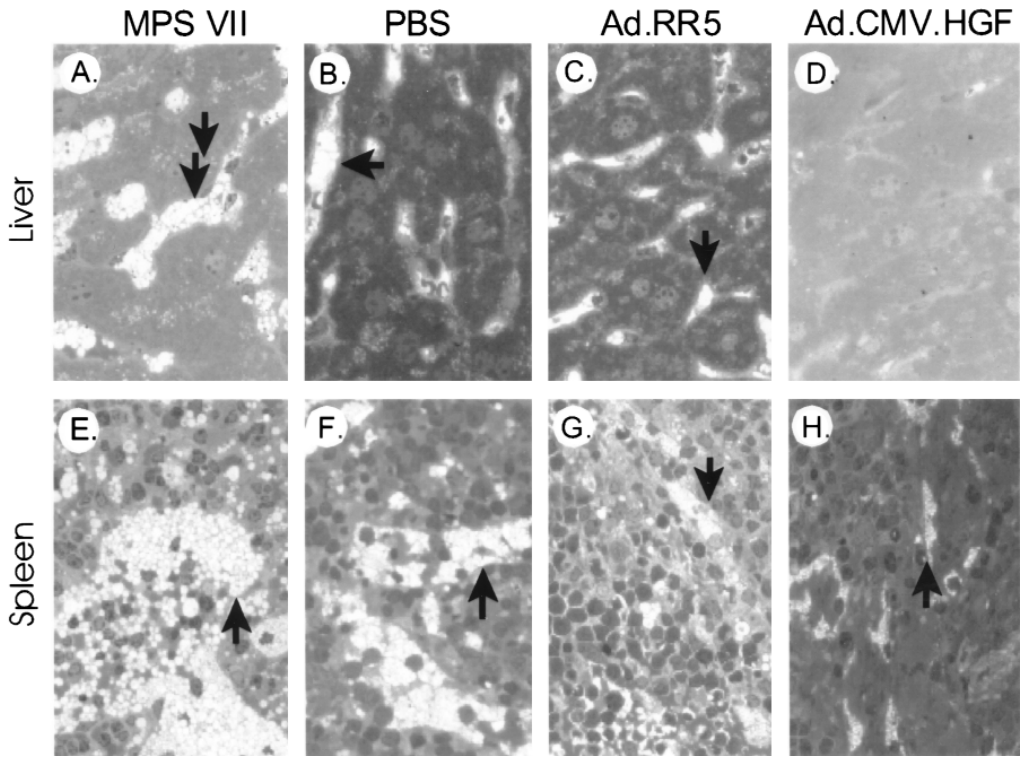

FIG. 10. Histopathological analysis of liver and spleen after injection of hAAT-β-gluc. MPS VII mice were untreated (MPS VII, A and E) or were treated 3.5 months previously with hAAT-β-gluc after im injection of PBS (lanes B and F), Ad.RR5 (lanes C and G), or Ad.CMV.HGF (lanes D and H). All mice were 5 months old at the time of sacrifice. Thin sections from fixed livers were stained with toluidine blue. Arrows identify lysosomal storage, which appears clear. (A–D) Liver samples at 100× original magnification. (E–H) Spleen samples at 100×.

throughout the liver, it was only corrected in a portion of the hepatocytes.

Histopathological analysis showed that all groups had a marked to nearly complete correction in lysosomal storage in the spleen at 1 month after transduction. At 3.5 months after transduction, storage was moderately reduced in a mouse that received PBS prior to hAAT-β-gluc compared with an untreated age-matched MPS VII mouse. Storage was markedly reduced at 3.5 months for the Ad.RR5-treated mouse and for two of the three Ad.CMV.HGF-treated mice. The third Ad.CMV.HGF-treated mouse had a moderate reduction in storage at 3.5 months.

Other organs were also evaluated for lysosomal storage. At 1 to 3.5 months after transduction, all animals had moderate to marked improvement in lysosomal storage in the sinusoidal cells of the bone marrow. However, there was no improvement in storage in kidney, heart valves, cartilage, retinal pigment epithelium, cornea, meninges, or neurons for most animals. One animal that received hAAT-β-gluc after Ad.CMV.HGF at a younger age than the others (5 weeks), and had the highest transduction efficiency and serum levels of β-gluc, also had a marked decrease in lysosomal storage in kidney and brain (not shown).

Quantitative GAG analysis confirmed the results of histopathological analysis, as shown in Fig. 11. Five-month-old MPS VII mice had GAG levels that were 6.5-fold higher than that of age-matched normal mice. Mice that received Ad.CMV.HGF prior to hAAT-β-gluc had a 77% reduction in excess GAG levels ($P = 0.01$ vs MPS VII), while mice that received PBS or Ad.RR5 prior to RV vector injection had a 75% reduction in excess GAG levels ($P = 0.04$ vs MPS VII). However, both groups had 2.3-fold as much GAGs as did normal mice ($P \leq 0.005$), and there were no differences in GAG levels between the Ad.CMV.HGF-treated and the control groups. There were not sufficient amounts of material to perform quantitative GAG analysis on the spleens.

DISCUSSION

Injection of RV during Hepatocyte Replication Results in Transduction of Hepatocytes and Reduction in Lysosomal Storage in the Liver

Gene therapy could permanently correct the clinical manifestations of MPS syndromes. The liver is a reasonable organ to target, as it accumulates lysosomal storage in patients, and could serve as a factory to produce enzyme for other organs because of its direct contact with the blood. We report here that iv injection of an RV

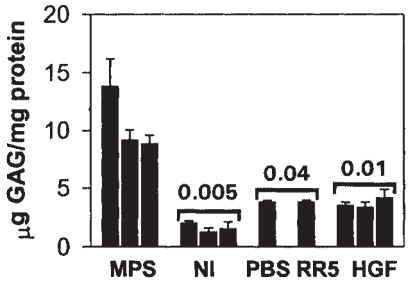

FIG. 11. Quantitation of GAGs in liver after injection of hAAT-β-gluc. Mice were treated and sacrificed as described in the legend to Fig. 10. For each animal, the average μg of GAG/mg of protein ± SEM for two different pieces from the same liver is shown. Values in each group were compared with values in untreated MPS-VII mice for statistically significant differences, as indicated above the groups.

during a period of transient hepatocyte replication induced by HGF resulted in transduction of ~1% of hepatocytes in young adult mice, and markedly decreased lysosomal storage in the liver for at least 3.5 months. The apparent fall in enzyme levels between 1 and 3.5 months after transduction may have been due to variation in transduction efficiency among individual animals and the small number of animals analyzed. Alternatively, it may reflect the fact that transduction of non-parenchymal cells also contributed to the enzyme levels, and these transduced non-parenchymal cells disappeared due to their shorter half-life. Since expression from an RV in the hepatocytes is maintained indefinitely in rodents (24–26), we expect that some level of expression should be maintained long term.

Somewhat surprisingly, there were also considerable levels of enzyme activity and similar correction of lysosomal storage in the liver for the control groups that received hAAT-β-gluc after im injection of PBS or the control adenoviral vector Ad.RR5. Part of the correction in storage observed at 1 month may be due to the fact that ~20,000 total units of β-gluc were present in the injectate along with the RV (data not shown); it was previously shown that injection of a larger dose of enzyme resulted in marked clearance of lysosomal storage in the livers of MPS VII mice at 1 month after the last dose (7). However, liver enzyme activity was only 9.6 U/mg at 14 days after injection of 28,000 units of enzyme into 6 week old mice (7). and β-gluc has a half-life of ~2 days in the liver of rodents after iv injection (8, 45). Thus, the average enzyme activity that we observed in the liver of these controls at 1 month (7.3 U/mg) was >100-fold higher than expected for this amount of injected enzyme (0.07 U/mg). In addition, the partial clearance of storage observed at 3.5 months after transduction in our study cannot be due to the injected enzyme, as lysosomal storage had completely returned to the level found in untreated MPS VII mice by 85 days after the last dose of enzyme alone (7). Finally, there were RV DNA and RNA sequences in the liver at 1 and 2 months, although these levels had decreased by 3.5 months. We believe that the RV probably transduced the replicating non-parenchymal cells of the liver in these control groups, which resulted in some expression of β-gluc and contributed to amelioration of lysosomal storage. However, enzyme, DNA, and RNA levels were diminished by 3.5 months after transduction, which is presumably due to the shorter half-life of these cells (14). We conclude that iv injection of RV to young adult rodents without HGF or another growth factor would probably not result in stable improvement of lysosomal storage in the liver.

Injection of RV during Splenocyte Replication Results in Transduction of Splenocytes and Reduction of Lysosomal Storage in the Spleen

The spleen is another organ that has significant lysosomal storage in MPS VII patients. Its direct contact with the blood makes it accessible to RV that is injected iv, and it contains some cells such as hematopoietic progenitor cells (46), monocytes (47), and endothelial cells (48) that can replicate in response to HGF, albeit at higher doses than what was achieved here. We believe that the marked reduction in lysosomal storage in the spleen was due, at least in part, to transduction of splenocytes by the RV for three reasons. First, all groups had spleen β-gluc enzyme activity at 1 month after transduction that was at least 0.5% of normal. This was >20-fold higher than expected at that time for the amount of enzyme that was present in the injectate (7, 8, 45). All groups maintained detectable β-gluc enzyme activity at 3.5 months, albeit at lower levels. This enzyme activity at 1 month or later was due to a small number of intensely red cells, suggesting that they were transduced cells. Second, several animals had RV DNA sequences in the spleen. Third, several mice clearly had RV-derived RNA in the spleen. Transduction of splenocytes was not surprising, as BrdU labeling at the time of RV infusion demonstrated that many cells were replicating in the spleen for all groups of mice.

The ability to transfer a gene that encodes the appropriate enzyme into the spleen, a major site of storage, could be an important factor in any gene therapy protocol for lysosomal storage diseases. This is the first report demonstrating expression of the transgene in the spleen after systemic administration of a vector, although the spleen has not been examined carefully in most studies. The efficiency of splenocyte gene transfer and the stability of the effect would need to be carefully assessed in large animal models prior to using this approach in humans with lysosomal storage diseases.

Failure of Gene Therapy to Correct Lysosomal Storage in Other Organs May Be Due to the Development of Anti-Human β-gluc Antibodies

It was hoped that the RV-transduced liver would secrete sufficient amounts of enzyme to reach other organs. Indeed, serum β-gluc levels were high initially in all groups. Uptake from the blood was presumably why most of these organs had some enzyme activity despite

the absence of detectable RNA or DNA sequences at any times for any of the groups. However, β-gluc serum levels fell progressively at 1 month or later after transduction, enzyme activity declined to background levels in these other organs, and there was little or no effect upon lysosomal storage in any organs other than the liver, spleen, and sinus lining cells of the bone marrow for most animals. This may have been due to the development of anti-human β-gluc antibodies which reduced serum levels, although we have no data that directly supports this hypothesis. Antibody formation might have been stimulated by the injection of protein at the time of gene transfer, or by the gene therapy procedure. Other investigators have also reported the development of anti-human β-gluc antibodies after gene therapy in adult MPS VII mice in the same genetic background (17, 19), although no anti-β-gluc antibodies were reported when an AAV vector was given im to newborn MPS VII mice (22), and in one study these antibodies did not prevent enzyme from reaching the spleen or liver from the peritoneum (20). Although the administration of adenoviral vectors can stimulate the production of antibodies directed against human proteins (49), this did not appear to occur here, as the control group that received PBS prior to hAAT-β-gluc had similar levels of antibody. There did not appear to be a cytotoxic T cell response to cells expressing human β-gluc, as transduced cells were maintained in the liver at 3.5 months for the Ad.CMV.HGF-treated mice.

Implications for Gene Therapy for Lysosomal Storage Diseases

These data demonstrate that long-term (3.5 months) amelioration of lysosomal storage can be achieved in MPS VII liver and spleen when an MoMLV-based RV is injected iv while hepatocytes and splenocytes are replicating. The enzyme activity achieved at 3.5 months in this study in liver (~1% of normal) and spleen (~0.4% of normal) was lower than observed short-term with adenoviral vectors (17, 18). The activity here was similar or somewhat lower than that observed long-term after transplantation of genetically modified bone marrow cells (15, 16) or fibroblasts (19, 20) or after im or iv injection of an AAV vector (21–23). Activity in the liver using our approach should be maintained due to the longevity of hepatocytes, although the activity in the spleen might diminish over time. Although the activity achieved without induction of hepatocyte replication by HGF was higher than expected and was able to significantly reduce lysosomal storage, this approach would likely not be effective over longer periods of time due to the short half life of the nonparenchymal cells of the liver. Readministration of RV would likely be limited by the development of antibodies against the envelope protein of the RV.

The optimal approach for systemic gene therapy for MPS syndromes in humans remains to be determined. Adenoviral vectors are plagued by transient expression due to the immune response, and can be very toxic (49, 50). Transplantation of genetically altered fibroblasts or bone marrow cells requires isolation and culture of autologous cells, which would make this approach more difficult to apply to large numbers of patients. The ability to deliver AAV vectors without concomitant administration of a growth factor is clearly an advantage. However, difficulties in producing sufficient quantities of vector, and the presence of neutralizing anti-AAV antibodies in a significant percentage of humans (51) may limit their utility. Although lentiviral vectors are a subset of RV that can clearly transduce non-dividing cells (52) they are inefficient at transducing nondividing hepatocytes in animals for unclear reasons (53, 54) and some safety concerns remain.

An HGF protein-facilitated MoMLV-based approach may be a safe and efficient method to achieve gene transfer into neonatal, juvenile, or adult animals. Indeed, the requirement for replication for transduction with an MoMLV vector could reduce the chance of germline transmission if the vector is delivered when germ cells are not replicating such as prior to puberty in males, although future experiments will need to document an absence of germline transmission. Although use of an adenoviral vector expressing HGF was convenient for this study, it is clearly inappropriate in humans because of the toxicity of adenoviral vectors and the fact that Ad.CMV.HGF encodes an oncogene. Transient administration of HGF protein has no apparent adverse effects in normal rodents, and has actually been beneficial in models of liver failure (55, 56). Trials using HGF in humans with liver disease should provide information regarding its toxicity. It is also possible that injection of MoMLV-based RV into neonates will be effective without concomitant growth factor administration due to the higher rate of hepatocyte replication, a hypothesis that we are currently testing. It is therefore possible that MoMLV-based vectors may be a safe and effective treatment for the systemic manifestations of MPS syndromes. Since this approach does not result in gene or enzyme transfer into the brain, it would need to be combined with another method for gene transfer into the central nervous system (10) in order to correct the neurological manifestations of these disorders.

ACKNOWLEDGMENTS

We thank William Sly for providing us with recombinant human β-gluc, Carole Vogler for assistance with pathological analysis, Marie Roberts for breeding of MPS VII mice, Neelam Srivastava and Susan Kennedy for cloning and preparing RV, and Chris Hasson for performing GAG analysis.

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