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Research Paper

Lysosomal Enzyme Replacement of the Brain with Intravenous Non-Viral Gene Transfer

Yun Zhang,[1] Yuntao Wang,[1] Ruben J. Boado,[1] and William M. Pardridge[1,2]

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Purpose. The delivery of non-viral plasmid DNA to brain across the blood-brain barrier (BBB) with intravenous administration of non-viral plasmid DNA encoding a lysosomal enzyme, β-glucuronidase (GUSB), was examined in GUSB null mice, a model of type VII mucopolysaccharidosis.
Methods. The plasmid, designated pCMV-GUSB, is encapsulated in Trojan horse liposomes, which are targeted across the BBB, and the brain cell membrane, with a monoclonal antibody to the mouse transferrin receptor.
Results. The GUSB enzyme activity was increased >50-fold in cell culture of fibroblasts obtained from GUSB null mice, following application of the antibody-targeted liposomes carrying the pCMV–GUSB, and enzyme activity remained high for >2 weeks. Adult GUSB null mice were treated with a single intravenous administration of 0.2 ml of Trojan horse liposomes carrying the pCMV–GUSB at a dose of 10 μg/mouse of plasmid DNA. The GUSB enzyme activity was increased greater than tenfold in brain, liver, spleen, lung, and kidney, but not in heart.
Conclusions. Intravenous Trojan horse liposome administration increased brain GUSB enzyme activity to the therapeutic range of brain GUSB enzyme activity. These studies show it is possible to deliver non-viral plasmid DNA encoding lysosomal enzymes to the brain following intravenous administration of receptor-specific Trojan horse liposomes.

KEY WORDS: blood-brain barrier; liposomes; lysosomal storage disorders; transferrin receptor.

INTRODUCTION

The majority of lysosomal storage disorders adversely affect the central nervous system (1). Treatment strategies for these conditions include either enzyme replacement therapy or gene therapy. However, neither the recombinant lysosomal enzyme (2), or the viral vector for gene therapy (3, 4), cross the brain-capillary endothelial wall, which forms the blood–brain barrier (BBB). Therefore, intravenous administration of either recombinant protein or viral gene therapy is not effective treatment for the brain. The BBB is bypassed with trans-cranial protein or gene delivery strategies, such as intra-cerebroventricular (ICV) or intra-cerebral administration. However, with ICV gene delivery in post-natal animals, the viral vector distributes only to the ependymal surface of the brain (5). A more global pattern of gene distribution is achieved following ICV administration to embryos (6). The intra-cerebral administration of a viral vector results in a focal distribution of the therapeutic at the injection site in brain (7,8). However, since the entire brain is affected in lysosomal storage diseases, it is necessary to treat the entire brain. All cells in the brain can be accessed with a trans-vascular drug or gene delivery strategy, which delivers the therapeutic across the BBB.

There are over 100 billion capillaries in the 1,200 g human brain and every neuron is virtually perfused by its own blood vessel (9). Therefore, if the limiting membrane, the BBB, is traversed, the lysosomal enzyme therapeutic can be distributed to most cells in the brain following intravenous administration. Therapeutic genes, in the form of plasmid DNA, can be delivered across the BBB without viral vectors following intravenous administration of Trojan horse liposomes (THL), which are also called pegylated immunoliposomes (10–12). In this approach, a single plasmid DNA is encapsulated in a 100 nm pegylated liposome. Approximately 1–2% of the polyethylene glycol (PEG) strands on the liposome surface are conjugated with a receptor-specific targeting monoclonal antibody (MAb). THLs have been targeted across the BBB in rodents with an MAb to the mouse or rat transferrin receptor (TfR) (10,11). THLs have been targeted across the Rhesus monkey BBB *in vivo* with an MAb to the human insulin receptor (12). Both the TfR and the insulin receptor are widely expressed on neuronal cell membranes (13,14).Consequently, the targeting MAb distributes the THLs across both the BBB and the neuronal cell membrane following intravenous administration. The global distribution of the gene in brain has been verified in mice, rats, and monkeys with brain histochemistry and confocal microscopy following delivery of an expression plasmid encoding β-galactosidase (10–12).

[1] Department of Medicine, UCLA, Warren Hall 13-164, 900 Veteran Ave., Los Angeles, CA 90024, USA.
[2] To whom correspondence should be addressed. (e-mail: wpardridge@mednet.ucla.edu)

Table I. Oligodeoxynucleotide Primers for RT-PCR Cloning of hGUSB

Oligodeoxynucleotide Primers	Sequence
GUSB–PCR FWD	ATC*GAATTC*ATGGCCCGGGGGTC
GUSB–PCR REV	GAT*AAGCTT*TCAAGTAAACGGGCTGT
GUSB–Kozak–SDM–FWD	GTGCTGGATATCTGCCGCCACCATGGCCCGGGGGTC
GUSB–Kozak–SDM–REV	GACCCCCGGGCCATGGTGGCGGCAGATATCCAGCAC

The GUSB–PCR forward (FWD) and reverse (REV) PCR primers introduce EcoRI and HindIII sites (italics), respectively.

The present studies test the feasibility of treating mice that are null for the β-glucuronidase (GUSB) lysosomal enzyme, which is a model for type VII mucopolysaccharidosis (MPS; 15,16). A human GUSB expression plasmid, designated pCMV–GUSB, was genetically engineered for these studies and was encapsulated in THLs targeted with the rat 8D3 MAb to the mouse TfR. GUSB enzyme activity was initially measured in fibroblasts derived from GUSB null mice following delivery of expression plasmid to cells in tissue culture with the TfRMAb-targeted THLs. Subsequently, GUSB enzyme activity was measured in brain, and other organs, in GUSB null mice following intravenous administration of TfRMAb-targeted THLs carrying the GUSB expression plasmid.

MATERIALS AND METHODS

Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), DDAB (dimethyldioctadecylammonium bromide), distearoylphosphatidylethanolamine (DSPE)–PEG^{2000} and DSPE–PEG^{2000}–maleimide were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL).

Genetic Engineering of pCMV–GUSB Expression Plasmid

Human liver polyA+ RNA was reverse transcribed (RT) with oligo d(T)$_{18}$ and SuperScript reverse transcriptase (RT) as previously described (17). The hGUSB open reading frame cDNA was cloned by polymerase chain reaction (PCR) using the ODN primers described in Table I. The hGUSB–PCR forward and reverse primers introduce *Eco*RI and *Hin*dIII sites, respectively (Table I), for directional insertion into the pcDNA3.1 expression vector. The PCR cloning of the hGUSB cDNA was performed in an Eppendorf Mastercycler personal temperature cycler (Brinkmann, Westbury, NY) using the following temperature cycles: (a) 1×95°C for 2 min, (b) 30×95°C for 30 s+55°C for 30 s+72°C for 2 min, and (c) 1×72°C for 10 min. PCR products were resolved by agarose gel electrophoresis and the expected major band of ~2 kb corresponding to hGUSB cDNA is shown in Fig. 1a. Both the PCR generated hGUSB cDNA and the pcDNA3.1 vector were double digested with *Eco*RI and *Hin*dIII, and the DNAs were purified from agarose gels following electrophoresis with the Qiagen gel extraction kit. The ~2.0 kb hGUSB cDNA was inserted into the pcDNA3.1 vector with T4 DNA ligase to form the pCMV–GUSB expression plasmid. A full Kozak site (i.e. GCCGCCACCA) was later introduced by site directed mutagenesis (SDM) using the SDM–ODNs shown in Table I and the QuickChange II XL SDM kit as previously described (18). The SDM was performed in using the following temperature cycles: (a) 1×95°C for 1 min, (b) 18×95°C for 50 s+60°C for 50 s+68°C for 9 min, and (c) 1×68°C for 10 min. Positive clones encoding the full Kozak site were identified by DNA sequencing using the T7 ODN primer. Confirmation of the complete hGUSB expression cassette between the CMV promoter and the BGH polyA site was performed with DNA sequencing as previously described (18).

Trojan Horse Liposome Production

POPC (18.8 μmol), DDAB (0.4 μmol), DSPE–PEG^{2000} (0.6 μmol), and DSPE–PEG^{2000}–maleimide (0.2 μmol) were dissolved in chloroform followed by evaporation (19). The lipids were dispersed in 0.2 ml 0.05 M Tris–HCl buffer (pH=7.0) and vortexed 1 min followed by 2 min of bath sonication. Supercoiled DNA was ^{32}P-labeled with [α-^{32}P]dCTP by the nick translation system as described previously (19). pCMV–GUSB plasmid DNA, produced by Endofree maxiprep (Qiagen; 200 μg), and 1 μCi [^{32}P]DNA were added to the lipids. The dispersion was frozen in liquid nitrogen for 5 min, thawed at 37C for 2 min and then room temperature for 4 min. This freeze–thaw cycle was repeated five times. The liposome dispersion was diluted to a lipid concentration of 40 mM, followed by extrusion five times each through two stacks each of 400-, 200- and 100-nm pore size polycarbonate membranes with a hand held LipoFast™–Basic extruder (Avestin, Ottawa, Canada), as described previously (19). The mean vesicle diameters were

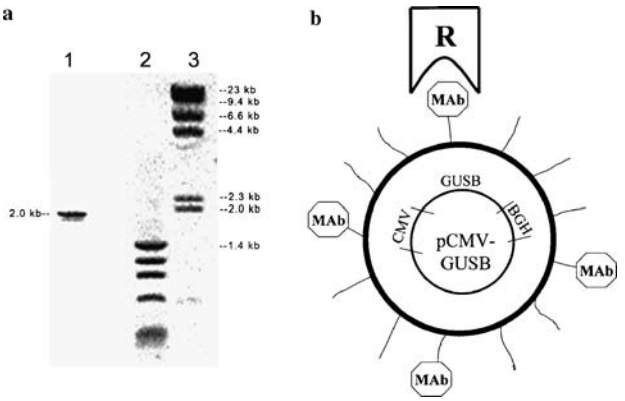

Fig. 1. a Ethidium bromide stain of agarose gel of PCR reaction showing amplification of the 2.0 kb human GUSB cDNA (*lane 1*). **b** Schematic diagram of a THL encapsulated with pCMV–GUSB. The human GUSB open reading frame is under the influence of the CMV promoter and the BGH transcription termination sequence. The surface of THL is conjugated with several thousand strands of 2,000 Da polyethyleneglycol (PEG), and the tips of 1–2% of the PEG strands is conjugated with a receptor (R)-specific targeting monoclonal antibody (MAb).

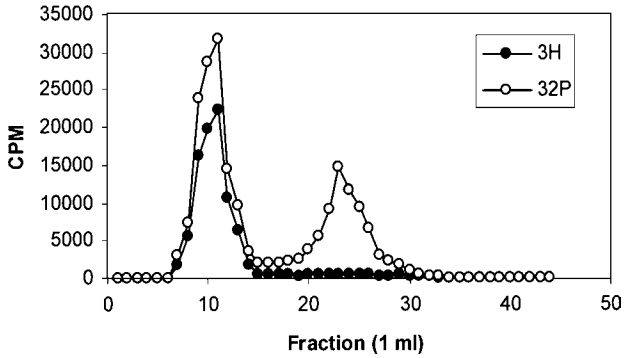

Fig. 2. Radioactivity profile of THL elution from a sepharose CL-4B gel filtration column. The MAb is labeled with [^{3}H] and the plasmid DNA is labeled with [^{32}P]. The THLs migrate on the column in the void volume at fraction 10 and the co-elution of the plasmid DNA and the MAb demonstrate the formation of the DNA encapsulated THL. The ^{32}P peak migrating at 25 ml represents nuclease degraded exteriorized plasmid DNA.

determined with a Submicron Particle Sizer NICOMP 380 (Santa Barbara, CA). The plasmid absorbed to the exterior of the liposomes was removed by nuclease digestion (19). For digestion of the unencapsulated DNA, 5 U of pancreatic endonuclease I and 5 U of exonuclease III were added in 5 mM $MgCl_2$ to the liposome/DNA mixture after extrusion. After incubation at 37°C for 1 h, the reaction was stopped by adding 20 mM EDTA. The rat 8D3 MAb against the mouse TfR was purified by protein G affinity chromatography from ascites produced in nude mice. The 8D3 MAb was radiolabeled with ^{3}H-*N*-succinimidyl propionate. The [^{3}H]MAb had a specific activity of >0.11 μCi/μg and a trichloroacetic acid precipitability of >97%. The MAb (3.0 mg, 20 nmol) was thiolated with a 40:1 molar excess of 2-iminothiolane (Traut's reagent), as described previously (19). The thiolated MAb, which contained a trace amount of [^{3}H] labeled MAb, was conjugated to the pegylated liposome overnight at RT. The next day, the unconjugated MAb, and the nuclease degraded external plasmid DNA was separated from the THLs by Sepharose CL-4B column chromatography (Fig. 2). The number of MAb molecules conjugated per liposome was calculated from the specific activity of the [^{3}H]–MAb (19). The THL solution was sterilized for use in tissue culture with a 0.22 ìm filter (Millipore Co., Bedford, MA) as described previously (20).

Plasmid DNA Delivery with THLs to Cultured Mouse Fibroblasts

Fibroblasts (21,22) from GUSB null mice, 3521 cells, and from control mice, 3522 cells, were plated on 35-mm collagen-treated dishes in DMEM containing 10% fetal bovine serum (FBS). When the cells reached 60–70% confluence, the medium was removed by aspiration, and 2.5 ml of fresh medium with 0.5% FBS was added to the cells, and the GUSB null fibroblasts were treated either with saline or with 4 ug/dish of pCMV–GUSB plasmid DNA encapsulated in 8D3-targeted THLs. The cells were incubated for 2 to 16 days; the medium (without further THL treatment) was replenished every 2–3 days. In the dose response study, all dishes were incubated for 48 h following the application of 0.1–10.0 ug/dish of pCMV–GUSB DNA encapsulated in 8D3-targeted THLs. At the end of the incubation, the cells were washed three times with cold 0.01 M PBS/7.4, and 0.5 ml/well of cell lysis buffer (0.01 M Tris/0.15 M NaCl/7.5/1 mM DTT/0.2% Triton X-100) was added to each dish. The cells were collected with a rubber policeman followed with three freeze–thaw cycles. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant was stored at −20°C for GUSB enzyme activity measurement and protein concentration determination.

GUSB Enzyme Activity Measurement

A 10 mM solution of 4-methylumbelliferyl β-D-glucuronide was prepared in 0.1 M sodium acetate/pH=4.8; 100 ul of this solution was added to 100 ul of media or organ samples and incubated at 37 C for 60 min. At the end of this reaction, 1.8 ml of the stop solution (320 mM glycine in 200 mM sodium carbonate/pH=10.5) was added. The fluorescent intensity was read with an A-1 Filter Fluorometer from Farrand Optical Components & Instruments (Valhalla, NY) with an emission wavelength of 450 nm and an excitation wavelength of 365 nm. A standard curve was established with a final concentration of 0.03–3.0 nmol/ml 4-methylumbelliferone in the stop buffer. The protein concentration was determined with the Pierce protein assay kit per the manufacturer's instruction. The enzyme activity was expressed as nmol 4-methylumbelliferone/hour/mg protein or nmol/hour/ml serum, where 1 U=1 nmol/h (23). The sensitivity of the assay was 10 pmol. The intra-assay coefficient of variation (CV) was 10.8±2.3%.

COS Cell Transfection

COS cells were grown in 6-well dishes and transfected with pCMV–GUSB with Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Following transfection, cells were grown in serum free medium, and medium was collected at 3–4 or 7 days after transfection for measurement of medium GUSB enzyme activity, expressed as nmol h^{-1} ml^{-1}. Transfected cells were washed, and lysed for measurement of GUSB enzyme activity, expressed as nmol h^{-1} mg^{-1} protein.

In Vivo Gene Administration

The animal research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85–23, revised

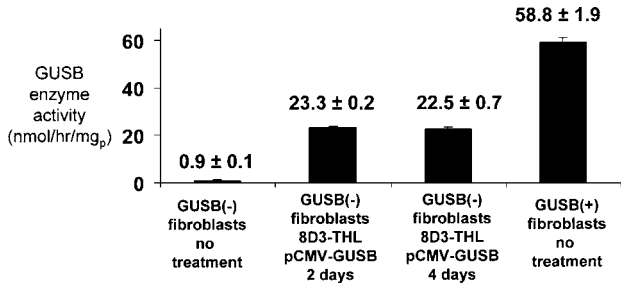

Fig. 3. GUSB enzyme activity in GUSB null (−) fibroblasts and in fibroblasts obtained from wild type (+) mice. Fibroblasts were treated either with saline or with TfRMAb-targeted THLs encapsulated with the pCMV–GUSB expression plasmid. Data are mean±SE (n=4). The difference in GUSB enzyme activity in the THL treated cells is significantly different from the untreated cells from the GUSB null mice (p<0.01).

Table II. Time Course of GUSB Gene Expression in GUSB Null Fibroblasts Following Plasmid DNA Transfer with THLs

Day of Incubation	GUSB Enzyme Activity (nmol h^{-1} mg^{-1} Protein)	
	Saline Treatment	THL Treatment
2	0.8±0.04	27.4±0.9
5	0.8±0.03	38.4±1.5
9	0.9±0.04	47.3±1.6
13	0.8±0.06	43.6±1.6
16	0.8±0.02	47.7±1.1

Mean±SE (n=4 dishes/point). The pCMV–GUSB plasmid DNA was encapsulated in TfRMAb-targeted THLs and added at zero time at a dose of 4 μg/dish of plasmid DNA. GUSB enzyme activity in the cells was measured 2–16 days after the single application of THLs. The difference in GUSB enzyme activity in the THL treated cells as compared to the saline treated cells is significant (p<0.0005).

in 1985). Male MPS type VII (B6.C–*H2*^{bm1}/byBir–*Gusb*^{mps}/J) GUSB null mice, 4–6 months of age, weighing 20–22 g, were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal studies were approved by the UCLA Animal Research Committee. Mice were treated intravenously with 0.2 ml of either (a) 10 μg/mouse of pCMV–GUSB plasmid DNA encapsulated in of 8D3-targeted THLs via the jugular vein, or (b) 0.05 M Hepes/7.0. At 48 h after injection, mice were euthanized under ketamine/xylazine anesthesia. Blood was collected, and after centrifugation at 12,000 g for 5 min, the serum was saved and stored at −70°C. Brain (cerebral hemisphere), liver, spleen, heart, lung, and kidney were removed, weighed and homogenates were prepared for GUSB enzyme activity measurements. To each tissue, 4 volumes of lysis buffer (0.025 M Tris/7.2/0.14 M NaCl) was added and the sample was homogenized with a Brinkmann Polytron PT3000 (Kinematica, AG) followed by 10 s sonication on ice. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant was stored at −70°C for GUSB enzyme activity measurement and protein concentration determination.

STATISTICAL ANALYSIS

Statistical significance at the p<0.05 level was determined with analysis of variance and Dunnett's test using Program 7D of the BMDP Statistical software package of the UCLA Biomedical computing facility when more than two comparisons are made.

RESULTS

The GUSB expression cassette within the pCMV–GUSB expression plasmid was comprised of 3,029 nucleotides (nt): (1) nt 1–689 CMV promoter, 100% identical to nt 259–947 of AY862402; (2) 30 nt multiple cloning region, 100% identical to nt 1,819–1,790 of AY899303; (3) 9 nt Kozak sequence, GCCGCCACC; (4) 1,956 nt human GUSB open reading frame, 100% identical to nt 27–1,982 of NM_000181, and (5) 345 nt BGH termination sequence, 100% identical to nt 2,413–2,757 of AY029367. COS cells were transfected with the pCMV–GUSB expression plasmid and Lipofectamine 2000, and medium GUSB enzyme activity increased to 2,422±49 and 6,578±1,132 U/ml at 3 and 7 days after transfection, respectively, whereas GUSB enzyme activity in media from COS cells exposed to Lipofectamine 2000 alone was 32±5 U/ml (data are mean±SE, n=3). COS cellular GUSB enzyme activity increased to 4,410±295 U/mg protein at 4 days after transfection.

The GUSB expression plasmid was encapsulated in THLs targeted with the 8D3 TfRMAb, as depicted in Fig. 1b. THLs carrying the GUSB expression plasmid were isolated following Sepharose CL-4B gel filtration chromatography (Fig. 2). The unconjugated MAb migrates at 20 ml, and the absence of any peak at this elution volume indicates all of the MAb was conjugated in the THL preparation. The average DNA encapsulation of THLs was 35±3% and the average number of TfRMAb molecules conjugated per individual THL was 65±9. Fibroblasts isolated from GUSB null mice were grown in tissue culture in parallel with fibroblasts obtained from wild type mice. The GUSB enzyme activity in the GUSB null fibroblasts was 0.9±0.1 U/mg protein and the GUSB activity in fibroblasts from the wild type mice was 58.8±1.9 U/mg protein (Fig. 3). The GUSB enzyme activity in fibroblasts from GUSB null mice was increased to values nearly 40% of the control values at 2 or 4 days following delivery of the GUSB expression plasmid with the 8D3 targeted THLs. A time course study was performed wherein GUSB enzyme activity in fibroblasts obtained from GUSB null mice was measured at 2, 5, 9, 13, and 16 days after a single application on day 0 of the GUSB expression plasmid encapsulated in TfRMAb-targeted THLs (Table II). These data show that therapeutic levels of GUSB enzyme activity are elevated for over 2 weeks following a single application of THLs, and maximal values of enzyme activity are >80% of control values (Table II, Fig. 3). The level of GUSB enzyme activity produced in the fibroblasts demonstrated a dose response relative to the amount of plasmid DNA added per dish (Table III). The GUSB gene expression was saturated by the addition of unconjugated 8D3 MAb, as the level of GUSB enzyme activity at 2 days following the application of 4 μg plasmid DNA/dish was decreased from 22.4±0.8 to 10.6±0.7 nmol h^{-1} per mg_p, by the co-incubation of the THLs with 2.6 uM unconjugated 8D3 TfRMAb during the 48 h incubation period (p<0.0005).

Table III. Dose Response of GUSB Gene Expression in GUSB Null Fibroblasts Following Plasmid DNA Transfer with THLs

Plasmid DNA (μg/dish)	GUSB Enzyme Activity (nmol h^{-1} mg^{-1} Protein)
0	0.70±0.04
0.1	0.80±0.02
0.4	2.9±0.1
1.0	6.7±0.4
4.0	22.4±0.5
10.0	37.2±1.4

Mean±SE (n=3 dishes/point). The pCMV–GUSB plasmid DNA was encapsulated in TfRMAb-targeted THLs and added at zero time in varying doses per dish. GUSB enzyme activity in the cells was measured 48 h later. The difference in GUSB enzyme activity in the THL treated cells, as compared to the untreated cells, is significant at the doses of 0.4–10 ug/dish (p<0.01).

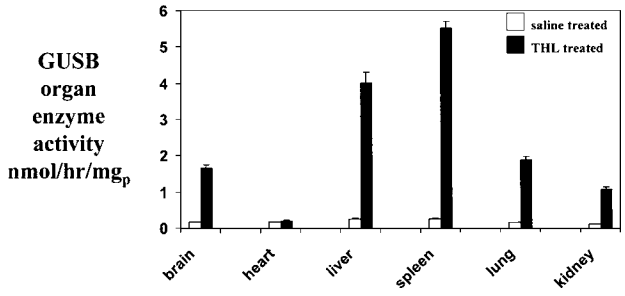

Fig. 4. GUSB enzyme activity in brain and five other organs of GUSB null mice removed at 48 h after single intravenous administration of either saline or 10 μg/mouse of pCMV–GUSB plasmid DNA encapsulated in TfRMAb-targeted THLs. Mean±SE (n=4–5 mice/group). The difference in GUSB enzyme activity in the THL treated mice, as compared to the saline treated mice is significant (p<0.0005), in all organs, except the heart.

GUSB null mice were separated into two treatment groups and treated with either saline or with 10 μg/mouse of pCMV–GUSB expression plasmid encapsulated in 8D3-targeted THLs. Mice were sacrificed at 48 h after the single intravenous administration and brain and other organs were removed for measurement of GUSB enzymatic activity (Fig. 4). The GUSB enzyme activity in the saline treated animals was <0.2 U/mg protein in brain and all other organs (Fig. 4). The GUSB enzyme activity in brain, liver, spleen, lung, and kidney was increased more than tenfold at 2 days following a single intravenous injection of the THLs carrying the GUSB expression plasmid (Fig. 4). There was no increase in GUSB enzyme activity in the heart (Fig. 4). GUSB enzyme activity in serum in the mice was <2 and 10.3±0.1 U/ml in the saline and THL treated mice, respectively, at 48 h after THL injection. The GUSB enzyme activity in the brain of adult BALB/c mice was 13.3±0.9 nmol h^{-1} per mg_p (mean±SE, n=3).

DISCUSSION

These results demonstrate that an increase in brain GUSB enzyme activity in GUSB null mice following a single intravenous administration of THLs carrying the pCMV–GUSB expression plasmid DNA (Fig. 4). In cell culture, GUSB enzyme activity is increased for over 2 weeks following a single treatment of GUSB null cells with TfRMAb-targeted THLs encapsulated with a GUSB expression plasmid (Fig. 3, Table II).

A human GUSB expression plasmid DNA, pCMV–GUSB, was genetically engineered wherein a cDNA encoding the human GUSB open reading frame was under the influence of the CMV promoter and the BGH transcription termination sequence ("Results"). The level of GUSB enzyme activity in either cell extracts or media following transfection of COS cells with Lipofectamine 2000 and pCMV–GUSB was 4,410±295 U/mg protein and 6,578±1,132 U/ml, respectively, and these values are comparable to that reported previously with other GUSB expression plasmid DNAs (23). The CMV promoter was selected for these investigations because this promoter is widely expressed in neurons in the CNS (24).

The pCMV–GUSB expression plasmid was encapsulated in TfRMAb-targeted THLs, which were applied to fibroblasts from GUSB null mice. GUSB enzyme activity in the fibroblasts increased to nearly normal values, as the GUSB enzyme level at 16 days in culture (Table II) is 81% of the GUSB enzyme level in untreated wild type fibroblasts (Fig. 3). The GUSB enzyme activity in the THL treated fibroblasts is >50-fold above the GUSB enzyme activity in the untreated fibroblasts from the GUSB null mice (Table III). GUSB enzyme activity persisted at high levels for over 2 weeks in cell culture following a single application of THLs to the GUSB null fibroblasts (Table II). The persistence of GUSB enzyme activity for many days following delivery to cells has been demonstrated previously (25). GUSB gene expression in the null fibroblasts showed a dose response, and was proportional to the amount of expression plasmid DNA added per dish (Fig. 3). The delivery of the GUSB expression plasmid to the fibroblasts by the TfRMAb-targeted THLs was saturated by co-incubation of unconjugated TfRMAb ("Results").

GUSB enzyme activity was increased in multiple tissues *in vivo* in the GUSB null mouse following a single intravenous administration of THLs (Fig. 4). Prior work with radiolabeled THLs demonstrated that the THLs distribute to organs with high TfR (26). If the TfRMAb on the THLs is replaced by an isotype control IgG, then no expression of the transgene in brain, or other organs, is observed (11). In the present study, organ GUSB enzyme activity is increased in organs with high microvascular TfR, such as brain, liver, and spleen (Fig. 4). The GUSB enzyme activity in the cerebral hemisphere was increased more than 10-fold above the level observed in saline treated GUSB null mice and reached values of 1–2 nmol h^{-1} mg^{-1} protein at 48 h after the single THL administration (Fig. 4). Comparable levels of GUSB enzyme activity were observed in lung and kidney, whereas GUSB enzyme activity in liver and spleen was 4–5 nmol h^{-1} mg^{-1} protein (Fig. 4). No increase in GUSB enzyme activity in the heart was observed following THL administration (Fig. 4). The tissue pattern of GUSB gene expression in mouse organs *in vivo* is dependant on the local expression of the transferrin receptor in the vascular barriers of these tissues. The liver and spleen are perfused with fenestrated capillaries that are highly porous, such that 100 nm liposomes can freely cross the vascular barrier in liver or spleen (11). Heart, lung, and kidney are perfused with capillaries with continuous endothelial barriers. These endothelial barriers are porous, as compared to brain capillaries, but nevertheless are impermeable to structures the size of 100 nm liposomes. The observation that GUSB enzyme activity is increased in lung and kidney with TfRMAb-targeted THLs *in vivo* is evidence for the expression of the TfR on the vascular barrier in these organs in the mouse, which corroborates previous observations (12). Prior work with reporter genes has shown that TfRMAb targeted THLs are not delivered across the vascular barrier in heart (10–12).

The intravenous administration of THLs carrying the GUSB expression plasmid increases the serum GUSB enzyme activity from <2 to 10.3±0.1 U/ml ("Results"). The blood volume of the mouse brain is 0.01 ml/g (27). Therefore, the maximal brain GUSB enzyme activity due solely to the plasma pool is 0.1 units/gram. Given 100 mg protein per gram brain, the maximal brain GUSB enzyme activity due to the blood pool is 0.001 U/mg protein, which is >1,000-fold lower

than the brain GUSB enzyme activity (Fig. 4). The cellular origin of GUSB enzyme activity in brain following THL administration was not identified, because the levels of GUSB enzyme activity in brain are not sufficiently high enough to detect with GUSB histochemistry. Using naphthol-AS-BI-β-D-glucuronide as substrate, endogenous GUSB enzyme activity in normal mouse spleen was readily detectable, but GUSB enzyme activity in brain was not measurable by histochemistry (unpublished observations). Similar findings have been reported following the ICV injection of an adeno-associated virus encoding GUSB. Enzyme activity was not detectable in mouse brain parenchyma with naphthol-AS-BI-β-D-glucuronide histochemistry, although transgene expression was demonstrated with the fluorometric assay (28). Previous work on the delivery of a β-galactosidase expression plasmid to brain confirmed expression of the transgene in neurons following transvascular delivery with THLs; confocal microscopy showed the co-localization of the transgene with neuronal-specific markers such as neuN (12).

The GUSB enzyme activity in brain and other organs, while greater than tenfold above the enzyme activity in saline treated GUSB null mice, is less than the GUSB enzyme activity in these organs in wild type mice. The GUSB enzyme activity in brain of adult mice ranges from 3.1±0.4 U/mg protein for heterozygote mice (29) to 13.3±0.9 U/mg protein for BALB/c mice ("Results"). By comparison, the brain GUSB enzyme activity observed at 48 h after a single THL administration is 1.7±0.1 U/mg protein (Fig. 4), which is 55% of the brain level in heterozygotes (29). Therefore, the level of GUSB enzyme activity generated in brain in the null mice with a single IV injection of THLs is in the therapeutic range. The replacement of just 1–5% of lysosomal enzyme activity in an organ may be sufficient to cause therapeutic effects and a reversal of lysosomal storage disease (1). The enzyme activity in brain following intravenous administration of TfRMAb-targeted THLs in GUSB null mice is comparable to GUSB enzyme activity produced in brain following transcranial delivery of viral vectors encoding for the GUSB enzyme. GUSB enzyme activity in hippocampus and cortex was approximately 30% of control in mice administered an adeno-associated virus GUSB vector following ICV administration (28). GUSB enzyme activity levels range from 1–11 nmol h^{-1} mg^{-1} protein in various regions of the brain following direct intra-cerebral injection of a herpes simplex virus type 1 vector (30). Very high levels of GUSB enzyme activity are observed in brain in local areas at the intracerebral injection site. In contrast, supra-normal lysosomal enzyme activity is not produced with an intravenous administration of THLs. Instead, the transvascular delivery approach produces a global expression of sub-normal, yet therapeutic, levels of GUSB enzyme activity (Fig. 4).

The GUSB enzyme activity level observed for peripheral tissues such as liver, spleen, lung, and kidney following THL administration (Fig. 4) is comparable to organ GUSB enzyme activity levels following the intravenous administration of recombinant GUSB protein (31). At 5 h after the intravenous administration of 25,000 U of recombinant phosphorylated GUSB, the enzyme activity in the liver or spleen was 75 and 30 nmol h^{-1} mg^{-1} protein, respectively, and was approximately 5 nmol h^{-1} mg protein in kidney, lung, and heart. In contrast, there was no increase in brain GUSB enzyme activity following intravenous administration of the recombinant protein in adult GUSB null mice (31), owing to absence of transport of the enzyme across the BBB in adult animals (32). These levels of organ GUSB enzyme activity, although sub-normal, were sufficient to cause reversal of a lysosomal storage effects in peripheral organs (31). The production of sub-normal, yet therapeutic, levels of GUSB in brain, and other tissues, is considered advantageous. Transgenic mice that over-express the GUSB enzyme demonstrate cell pathology and tumors in parallel with 100-fold increases in enzyme activity (33). THL gene transfer does not produce over-expression of the enzyme (Fig. 4).

In summary, these studies demonstrate that sub-normal, yet therapeutic levels of GUSB enzyme activity can be achieved in brain, and other organs, following a single intravenous administration of THLs encapsulated with a non-viral GUSB expression plasmid DNA. The plasmid DNA is expressed episomally in brain cells without integration into the host genome (34). Therefore, long-term treatment of lysosomal storage disorders with intravenous administration of THLs will require chronic, repeat administration of the therapeutic at intervals dictated by the persistence of transgene expression in brain and other organs.

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