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***In utero* transplantation of monocytic cells in cats with alpha-mannosidosis**

Janis L. Abkowitz¹, Kathleen M. Sabo¹, Zhantao Yang¹, Charles H. Vite², Laurence E. Shields³, and Mark E. Haskins²

¹ Division of Hematology, Department of Medicine, Box 357710, University of, Washington, Seattle, Washington 98195-7710 USA

² Departments of Clinical Studies and Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6051 USA

³ Obstetrix of the Central Coast /100 Casa St D3/ San Luis Obispo, CA 93405 USA

Abstract

Background—Lysosomal storage diseases are devastating illnesses, in large part because of their neurological consequences. Since significant morbidity occurs prenatally, *in utero* therapy is an attractive therapeutic approach.

Methods—We studied the feasibility and efficacy of *in utero* (IU) injections of monocytic cells (derived from normal marrow) in feline alpha-mannosidosis. Heterozygous cats were interbred to produce affected (homozygous) and control (heterozygous and wild-type) offspring. Thirty-seven pregnancies were studied in which fetuses were transplanted intraperitoneally (1×10^8 cells/kg recipient) at gestational day 27–33 and then each week for two weeks (term=63 days). After birth, affected kittens were evaluated clinically and pathologically, tissue alpha-mannosidase (MANB) levels were assayed, and in many studies, the numbers of MANB-containing cells were enumerated. When male donor cells were transplanted into female recipients, engraftment was also quantified using PCR to amplify a Y-chromosome-specific sequence.

Results—We establish methods to transplant cats intraperitoneally while IU using ultrasound guidance, thus, describing a new large animal model for prenatal therapy. We show that the donor monocytic cells engraft and persist (for up to 125 days) in brain, liver, and spleen, albeit at levels below those needed to alter the clinical or pathological progression of the alpha-mannosidosis.

Conclusions—This is the first study of monocyte transplantation in a large animal model of a lysosomal storage disorder and demonstrates its feasibility, safety, and promise. Delivering cells IU may be a useful strategy to prevent morbidities before a definitive therapy, such as hematopoietic stem cell transplantation, can be administered after birth.

Keywords

in utero transplantation; lysosomal storage disease; alpha mannosidosis; monocytes

Introduction

Lysosomal storage disorders are diverse diseases linked by overlapping phenotypes. Because of a defect in the function of a lysosomal enzyme, precursor substrates accumulate in lysosomes

resulting in serious toxicities, including death (1,2). Over 45 lysosomal storage disorders are described in humans (2).

In some disorders, such as type 1 Gaucher disease, enzyme replacement therapy is efficacious. However, assuring the adequate distribution of the drug to brain and other tissues and its uptake by relevant cells can be problematic (reviewed in 3). Marrow or peripheral blood stem cell transplantation is another therapeutic option. By replacing hematopoietic stem cells, one can replace tissue macrophages, such as Kupffer cells in liver and microglia in brain (4–12). Transplantation, however, requires a matched sibling or unrelated donor, which is not available for many patients. Also, in many lysosomal storage disorders, irreversible damage occurs in the central nervous system before birth (2). In addition, tissue macrophages, other than in the spleen, turn over slowly, so that macrophages derived from hematopoietic stem cells (HSCs) do not repopulate these sites for long periods of time. In the mouse, for example, only 51% of Kupffer cells and 30% of microglia are of donor origin 1 year after marrow transplantation (6), implying that the average lifespan of tissue macrophages in liver and brain is ≥ 1 year, even after exposure to radiation (1050 cgy) during the preparative regimen. In man, the kinetics of tissue macrophage turnover is unknown but is likely slower. Therefore, other approaches are needed.

IU transplantation for storage diseases is an attractive possibility as corrective donor cells can be provided before there is a permanent deficit. The IU transplantation of HSCs in immunocompetent mice and primates has resulted in low and often transient chimerism (13, 14).

In these studies, we consider an alternative IU approach, the transplantation of monocytic cells in cats with alpha-mannosidosis. We reasoned that these cells might quickly engraft in tissues since mature monocytes are the immediate precursor cell of tissue macrophages (15). As the fetus grows, monocytes are continually re-populating brain, liver, and other locations, so that the slow turnover of existing tissue macrophages should not be an obstacle to engraftment. In addition, before birth, the blood-brain interface may be more permeable which should facilitate cellular trafficking. The transplantation of peritoneal or marrow-derived monocytes transiently corrects beta-glucuronidase levels in mice with mucopolysaccharidosis type VII (16,17), and this was more efficacious for neonatal than adult animals (16). Finally, exposure to unmatched monocytes IU may confer tolerance for postnatal administration of HSCs from the same donor.

Human alpha-mannosidosis (Online Mendelian Inheritance in Man #248500) is an autosomal recessive disease due to deficient activity of lysosomal alpha-mannosidase (MANB). Mannose-rich oligosaccharides accumulate in cells before birth as well as through early life, resulting in mental retardation and progressive skeletal abnormalities (1,18). Hepatosplenomegaly, recurrent bacterial infections, hearing loss, gingival hyperplasia, synovitis, hydrocephalus, paraplegia, and corneal and lenticular opacities are described. Neurons, glial cells, and endothelial cells are vacuolated. Transplantation is an attractive option since MANB is efficiently secreted from cells and, because it contains mannose-6-phosphate residues, can be endocytosed via specific cell surface receptors and trafficked to the lysosome. Therefore, if tissue macrophages were corrected, Kupffer cells and microglia would secrete MANB leading to the “cross correction” of nearby hepatocytes and brain cells, respectively, amplifying the therapeutic potential. Transplantation of HSCs ameliorates disease progression, although neurologic signs respond poorly (19–22).

The clinical, pathological, and biological manifestations of alpha-mannosidosis in cats are similar to man. Affected cats have gross neurological deficits, skeletal deformities, hepatomegaly, growth retardation, gingival hyperplasia, and corneal and lenticular opacities (23–28), and without treatment, must be euthanized before six months of age. After HSC

transplantation, systemic and neurologic signs stabilize (29, and Haskins, unpublished observations).

In this report, we develop methods for the IU transplantation of monocytic cells in cats with alpha-mannosidosis, study cell dose and scheduling, and track outcomes.

Materials and Methods

IU Transplantation

Cats were bred at the School of Veterinary Medicine, University of Pennsylvania, under NIH and USDA guidelines. At 20–27 days of gestation (term = 63 days), females were transported to the University of Washington. Isoflurane (5%) was used to induce anesthesia, atropine sulfate (0.05 mg/kg) was given prior to intubation, and 1.5–2% isoflurane was used as maintenance. All studies were approved by the IACUC at the Universities of Pennsylvania and Washington (protocol #s 212900 and 2001–09, respectively).

In our initial studies, in collaboration with Grady Shelton DVM (Pacific Northwest Research Foundation, Seattle, WA) and E. B. Okrasinski DVM (veterinary surgeon, Seattle, WA), at 30–35 days of gestation, the tricornate uterus was visualized by laparotomy, and a ~ 2 cm uterine incision was made over each fetal sac, directly adjacent to the placental band. The incision was extended to the intermedium, fibers of the allantochorion were separated by blunt dissection to expose the transparent amniotic membrane, and monocytic cells (see below) were injected intraperitoneally via a 30-gauge needle, under direct vision. The uterine incision was closed in two layers (endometrial and myometrial) with 4.0 absorbable sutures and the abdominal wall surgically closed.

For later studies, ultrasound (ATL Ultramark V, 10 MHZ transducer, Bothell, WA) was used to confirm pregnancies, enumerate fetuses, assess gestational age by biometry calculations, transplant cells, and assess morbidity.

Monocytic cells were injected transcutaneously into the peritoneal cavity of fetuses with a 22-gauge spinal needle. The appearance of an air bubble confirmed appropriate targeting. Cells were infused each 7-days beginning at gestational day 27–33 for a total of 3 injections. In experiments involving 6 of 31 affected offspring, the transplantations were continued at postnatal weeks 2, 5, and 7. 10^8 cells/kg were infused at all time points. In cats, monocytes are first identified within the fetal liver at gestational day 25 (30).

The optimal volumes for transplantation were derived from preliminary studies where blue dye was infused intraperitoneally into 5 fetuses at day 32. Dye leaked from the injection site when >100 μ l was infused so subsequent experiments used <100 μ l at gestational days 28–33 and 20 μ l/g estimated fetus weight at other gestational times. The relationship of fetal weight to crown–rump length was also determined and corresponded to published data (31).

Monocytic Cell Preparation

Mononuclear marrow cells (32) were obtained from male domestic cats ($n=10$ unrelated donors), depleted of T and B-lymphocytes using murine monoclonal antibodies specific for feline CD5 (FE1.1B11) and canine CD21 (CA2.1D6) (Leukocyte Antigen Biology Laboratory, School of Veterinary Medicine, University of California, Davis, CA), and negative selection with a VarioMacs (Miltenyi). CD5- and CD21-positive cells were not detected by flow cytometry after depletion. The resultant cells were washed and resuspended at $1-2 \times 10^6$ /ml in Iscove's Modified Dulbecco's Medium supplemented with 20% heat inactivated FBS (Summit Biotech, Fort Collins, CO), 1% antibiotic-antimycotic (GIBCO/BRL), 1% BSA, recombinant human M-CSF (1.5 ng/ml, Sigma Chemical Co., St. Louis, MO or R&D Systems,

Minneapolis, MN), and recombinant human flt3 ligand (100 ng/ml, provided by Immunex, Seattle, WA), using methods similar to those optimized for murine studies (6,16). After 5–6 days (at 5% CO₂, 37°C), all cells were removed from the 75 cm² flasks using a disposable cell scraper, washed twice, and resuspended at 5×10^6 /ml for the injection procedure. The cells had monocyte morphology on Wright-Giemsa stain and expressed non-specific esterase. In some cases, cells previously prepared and frozen in liquid nitrogen were thawed and used. All cells for each litter were from a single donor.

Tissue collection

Animals were sacrificed with barbiturates in accordance with the AVMA guidelines. Tissue aliquots were either frozen or immersed in cold fixative (4% paraformaldehyde, 2% glutaraldehyde in PBS) for a total of 3 hours, followed by storage in PBS at 4°C.

Enzyme histochemistry

35 – 45µm tissue sections were cut from fixed tissue using a Vibratome 1000 Classic (EBS Sciences, East Granby, CT) and allowed to dry (2 hr to overnight) onto Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). MANB activity was shown by placing drops of a solution of the substrate 5-bromo-4-chloro-3-indolyl α – mannopyranoside (X-Man, 15 mg/ml; Sigma) solubilized in DMSO (15% final concentration in solution) and diluted with 1.5 mM ZnCl₂, 11 mM K₄Fe(CN)₆, 11 mM K₃Fe(CN)₆, and 1.1 mg/ml MgCl₂ in 50 mM citrate buffer, pH 3.8, on the sections. After overnight incubation at 37°C, the sections were washed x3 in pH 3.8 citrate buffer. Some sections were counterstained with 0.1% Safranin O in distilled water. The enzymatic stain is somewhat erratic (29) and at times, especially when fixed tissue samples were stored prior to analysis, tissues from control (unaffected) littermates lacked enzymatic staining. Thus, our results likely underestimate the numbers of mannosidase-containing cells in tissue. Although no MANB staining was sometimes observed in tissues of normal animals, false positive staining was extremely rare (<1 cell in 1000 fields in tissues from an affected untreated animal).

PCR

Feline SRY (sex-determining region Y) gene and GAPDH gene were amplified from normal male and/or female feline genomic DNA samples, respectively, by PCR with the following primers (all 5' to 3'). For the SRY gene, primers TGAGAGTATTGAGCAGCG and GTTGTGGAGGTCCTGTG amplify a 599 bp product. For the GAPDH gene, primers ATGGTGAAGGTCGGTGTGAAC and TTAATCCTTGGAGGCCATGT amplify a 1002 bp product. The PCR products were cloned into pGEM-T Easy Vector System I (Promega) and sequenced in both directions to confirm fidelity by alignment with the online database for SRY (accession AB099654, GI: 27597196) and GAPDH (accession AB038241, GI: 6983848), then expressed, isolated and purified as standards for quantitative real time PCR using TaqMan chemistry (with probe labeling of 5'/56FAM- and -36TAM/3'). For SRY, primers are AACGACCCATGAACGCATTC and CCCCAGCTGCTTGCTGAT, probe is TCTAGAGAATCCCCAAACGCAAACTCAGA for GAPDH, primers are CAAGGCTGAGAACGGGAACT and GACCCAGTAGACTCCACAACATAC, probe is CCAGGAGCGAGATCCCGCCAAC. The copy number of male cat SRY gene transplanted into female cats was normalized using the copy number of the feline housekeeping gene GAPDH.

Alpha-mannosidase and s-hexosaminidase activity

Enzyme activities were measured in tissue using a fluorescence assay (33). Tissue sections (2×2×3mm) were ground in 0.5ml 0.2% Triton X-100, 0.9% saline, freeze-thawed, and cellular debris was pelleted in a microcentrifuge (12,000 x g, 2 min.). A standard curve was prepared

from 4-methylumbelliferone (4-MU). The substrates were 3mM 4-MU α -D-Mannopyranoside (Sigma) (for α -mannosidase) and 1.0mM 4-MU N-acetyl- β -D-glucosaminide (Glycosynth, England) (for β -hexosaminidase), both in 0.2M citrate-phosphate buffer, pH 4.4. Enzyme assay mixtures contained 100 μ l of substrate with 12.5 μ l of sample, final volume 0.2ml. The reaction was incubated at 37°C for 30 min, and then stopped by adding 2ml of 0.32M glycine, 0.2M sodium carbonate, pH 10.5. Fluorescence was measured on a Bio-Rad model VersaFluor™ (excitation wavelength 365 nm, emission wavelength 450nm). Protein concentrations were determined with the Bio-Rad protein assay. Activity was expressed as nanomoles of 4-MU released per milligram protein/hour.

Results

The transplantation of monocytic cells by a surgical procedure results in unacceptable fetal mortality

Cats heterozygous for α -mannosidosis were interbred to produce affected (homozygous) and control (heterozygous and wild-type) offspring. In our initial studies (n=6 pregnant cats), at 30–35 days of gestation (term=63 days), the tricornate uterus was surgically visualized, and 1×10^8 monocytic cells per kg estimated fetal body weight were injected under direct vision. Although there were no maternal complications, the procedure-related fetal deaths were frequent (11/23 fetuses, 48%) and this approach was abandoned.

The transplantation of monocytic cells with ultrasound guidance is relatively safe and feasible

Ultrasound-directed transcutaneous injections were explored as an alternative approach (n=37 pregnancies). Fetuses were transplanted with 1.0×10^8 monocytic cells/kg estimated body weight each 7 days beginning at gestational day 27–33 for total of 3 injections. These pregnancies resulted in 126 kittens born, a birth rate of 3.4 kittens per litter, and 3 (2%) dead at birth. The finding of 31 affected kittens (24.6%) is consistent with Mendelian genetics. In the subset of 10 pregnancies that were studied more completely by serial ultrasound examinations throughout the pregnancy, fetal loss was 15% (Table A). As a control and for comparative analysis, we observed the outcomes of 23 untreated MANB+/- $\times$ MANB+/- pregnancies. These mothers and fetuses were not subjected to serial ultrasound examination or repeated anesthesia. The control pregnancies resulted in 99 kittens (4.3 kittens/litter) and two (2%) dead at birth. As the treated queens gave birth to 3.4 kittens/litter, the excess fetal loss associated with the IU therapy can be estimated as 0.9/4.3 or 21%.

In two additional pregnancies, 1.0×10^9 monocytic cells/kg were injected into a total of 9 fetuses at gestational day 28. There was no procedural morbidity per ultrasound. However, when ultrasounds were repeated 1 week later (d 35), 5 fetuses had massive ascites (hydrops fetalis), two had omphaloceles, and there were two fetal deaths. At day 42, the two fetuses with persistent ascites were removed by Caesarian section. The pathology demonstrated some inflammatory cells, no infection (per cultures), no organ system abnormalities, and no evidence of graft-versus-host disease. We suspect that inflammatory cytokines released from the high quantities of monocytic cells led to this mortality.

When 2×10^8 monocytic cells/kg were injected per fetus (n=2 pregnancies, 9 fetuses) at days 28, 35, and 42 of gestation, mild ascites was sometimes noted on ultrasound examination on day 35 and subsequently. Eight kittens were born live with ascites, one of which was homozygous for α -mannosidosis (#4471). These observations prompted the use of 1×10^8 monocytic cells /kg in the above studies. None of the 126 animals born after repeated injections of 1×10^8 monocytic cells/kg had congenital malformations and only two had ascites.

The one healthy affected animal (kitten #4471) was clinically well until 10 weeks of age when it developed a head tilt, which improved over 1 week. By 14 weeks, however, the kitten had typical symptoms of alpha-mannosidosis, including head tremor, cataract, and ataxia, and was sacrificed at 15 weeks. Liver and brain cells had extensive cytoplasmic vacuolization. With X-Man staining, rare positive cells, including doublets, were detected in brain (Figure 1), but not liver. We saw this as an encouraging result, as this animal was sacrificed long (15 weeks) after birth (18 weeks (125 days) after the last injection of donor monocytic cells).

Outcomes

Since IU transplantation in cats has not been previously reported, we systematically adapted our protocol to optimize efficacy. Given the evolving study design, our data are presented both as case studies and as composite results. **Case Study 1.** Female kitten #4738 received 3 IU injections of monocytic cells and was sacrificed at birth. MANB to HEXA ratios (brain 0.329, liver 4.64, lung 0.11, spleen 0.611), were, respectively, 3.6-, 21-, 2-, and 6.94-fold higher than the mean value for untreated affected animals, representing 4.7, 91, 1.5, and 16% of wild-type values. Y chromosome copy numbers (brain 1.3, liver 151.91, lung 49.99, and spleen 427.41) were, respectively, 0.02-, 3-, 2-, and 10-fold higher than the mean value for untreated affected animals, representing 0.002, 0.10, 0.064, and 0.48% of male kitten levels. Histologically, the brain had storage in the choroid plexus with some cytoplasmic vacuolation of cortical neuronal. Liver was not examined. Staining for MANB enzyme activity was not done. Significant, consistent levels of donor monocyte engraftment were seen in all tissues. **Case Study 2.** Female kitten #6357 received 3 IU and 3 postnatal infusions of cells and was sacrificed at 57 days, 1 week after the last infusion. The kitten had a mild head tremor, consistent with untreated affected cats of similar age. Histologically, hepatocytes contained large eosinophilic inclusions atypical for oligosaccharide storage. The brain had typical but not extensive cytoplasmic vacuolation of cerebellar Purkinji cells. MANB to HEXA ratios were similar to untreated affected animals in all tissues. Y-chromosomes were not detected by PCR in any tissue, which suggests that engraftment was patchy when at a very low level. Still, MANB-containing cells were detected in brain (0.58/field, 2.5% of normal) and spleen (0.1/field, 0.24% of normal). As the assay is specific (positive cells were extremely rare in studies of tissue from untreated affected animals), but not sensitive (29, methods and Figure 3), it nicely complements the other methods, confirming the engraftment of donor cells. Post-natal infusions of monocytic cells did not appear to increase the engraftment or ameliorate disease progression in this or other animals. The relative persistence of MANB-positive cells in the brain of this animal and kitten #4471 (Figure 1) raise the possibility that immunological rejection contributed to the low engraftment and that the brain was a protected site. **Case Study 3.** Male kitten #6358 received 3 IU and two postnatal infusions and was sacrificed at 133 days, 101 days after the last infusion of monocytic cells. His clinical signs were typical for affected kittens his age with a significant head tremor, hypermetria, and ataxia, and liver and brain were typical histologically for an untreated cat at four months of age. MANB to HEXA ratios were not different from the mean of untreated affected animals except in lung (0.072 or 0.95% of normal); however, MANB-positive cells were observed in brain (0.033/field, 0.14% of normal), liver (0.02/field, 0.0008% of normal), and spleen (0.068/field, 0.16% of normal). These data confirm that donor cells can persist at all sites for long periods of time.

Cumulative Results

The ratios of MANB to HEXA in the affected offspring are presented in Figure 2. Mean ratios in affected treated animals overlap values from affected untreated cats. However, some affected animals that received IU transplantation of monocytic cells had enzyme activities in brain, liver, and spleen over 100-fold higher than enzymatic activities seen in affected untreated animals, and in 8 cats (31% of animals assayed) one or more tissues had MANB activities greater than or equal to 10-fold the mean value of affected untreated animals. The mean values

of affected treated animals were 1.3, 4.3, 0.72, and 2.3% of wild-type values in brain, liver, lung, and spleen, respectively, close to but less than 5% which is thought to be required for clinical efficacy.

Staining for MANB activity on sections of brain, liver, and spleen in representative tissue sections from treated affected and normal control kittens are shown in Figure 3. Both single positive cells and clusters of positive cells are seen. Positive-staining cells were counted per high power field in the affected treated cats in brain, liver, and spleen and were then expressed as the percents of the MANB-positive cells visualized in tissues from normal animals and were 0.224/field (0.97% of normal, 23.2/field), 0.107/field (0.004% of normal, 2631/field) and 1.22/field (2.9% of normal, 42/field), respectively. The percentage of monocytic origin cells in these samples could not be simultaneously assessed with the enzymatic stain, so the percentage of tissue macrophages that were of donor-origin, a better correlate of donor cell engraftment, could not be quantitated. Importantly, false positive staining was extremely rare and MANB-positive cells were a maximum of 0.0009/field in any tissue from affected untreated (negative) control cats. The results of PCR-based quantification of a Y-chromosome genomic marker (male donor cells) in affected females and normal (wild-type or heterozygous) females (Figure 4) prove that monocytic cells engraft in tissue sites. As the confirmatory PCR assays were done several years after the samples were obtained, sample preservation and quality may account for some of the variability in the data. The engraftment of male cells in affected females was better than the engraftment of male cells in wild-type or heterozygous littermates (data not shown), suggesting MANB-positive cells homed normally and were not uniquely rejected in affected kittens.

The neurological assessments (23) and histological evaluations of brain showed disease progression. Affected cats had fine whole body tremors at five weeks of age. By seven weeks, intention tremors and truncal ataxia developed, progressing to loss of balance, coarse whole body tremors, and a short-strided gait.

Histological examination showed a diffuse decrease in myelin in affected cat brains compared to normal in all white matter tracts examined: the gyrus lateralis, centrum semiovale, and cerebellum. Cytoplasmic vacuolation was present in oligodendrocytes, astrocytes, and endothelial cells. The gray matter of the affected brain showed cytoplasmic vacuolation and distension of neurons, astrocytes, and endothelial cells in the cerebrum, brainstem, and cerebellum. A decrease in the number of Purkinje cells and granular neurons was found in the cerebellum. Both cell types were distended and vacuolated. A diffuse gliosis and prominent vasculature were present throughout, and ependymal cells and cells of the choroid plexus and the meninges were swollen and vacuolated. The liver and brain showed cytoplasmic vacuolation, although the hepatocytes from cat # 6357 had atypical large eosinophilic inclusions instead of the usual vacuoles that contained stored oligosaccharides usually lost during processing. Thus, as anticipated from our engraftment data, although donor monocytic cells entered and persisted at tissue sites, their levels were not sufficiently high to be clinically efficacious.

Discussion

This is the first description of IU transplantation of cells other than HSCs in a large animal system and the first description of IU therapy in cats.

Our results demonstrate that ultrasound-guided injections of monocytic cells are a feasible and relatively safe approach for IU transplantation when moderate numbers (1×10^8 /kg recipient) of cells are transplanted, even on multiple occasions. Interestingly, the IU transplantation of $\geq 2 \times 10^8$ monocytic cells resulted in ascites and hydrops fetalis. We hypothesize that this is a

consequence of inflammatory cytokines released by activated monocytes. It is possible that these observations will provide an animal model to study this complication of pregnancy.

Our enzymatic staining and PCR analyses show that donor cells engraft and persist in host brain, lung, liver, and spleen. Mean MANB activities in tissues from affected treated kittens were also higher than in tissues from affected untreated control animals. Although these results are encouraging, the levels of enzyme, and quantities of cells, including cross-corrected cells, did not reach clinical efficacy. Thus, these results are reminiscent of the outcomes of initial gene transfer studies, before technologies were sufficiently optimized, and it is likely that these outcomes will improve as experimental strategies mature.

The transplantation of monocytic cells has been more efficacious in mice. Specifically, Freeman and colleagues (16) infused beta-glucuronidase-positive (wild-type) monocyte/macrophages, generated *in vitro* from marrow or peritoneum, into adult syngeneic recipients with mucopolysaccharidosis (MPS) type VII. Therapeutic levels of enzyme were noted in liver and spleen that transiently improved the lysosomal storage defect. In studies with newborn murine recipients, wild-type cells were observed in the meninges and brain parenchyma. Ohashi et al (17), transduced macrophages cultivated from MPS VII mice with a retroviral vector encoding human beta-glucuronidase, and then transplanted these cells into MPS VII hosts. Thirty-eight days later, beta-glucuronidase-positive cells were observed histochemically and pathologic improvement was noted. In related myeloablative studies, the transplantation of marrow HSCs engineered to over-express cathepsin A once they differentiated to monocytes and macrophages corrected murine galactosialidosis, providing evidence that macrophages alone are a sufficient source of corrective enzyme if the level and extent of expression are high (34).

There are many differences between mouse and man, and most importantly, vast differences in numbers of tissue macrophages. Thus, we do not anticipate that the IU transplantation of monocytes in a large animal (or man) would be a curative approach, despite the permeability of the blood-brain barrier. Even though microglia in brain and Kupffer cells in liver live for long periods of time, their turnover would suggest that this method would only provide temporary support. In mouse, 50% are replaced by 1–3 years (6). Conceptually, IU transplantation of monocytic cells might correct the enzymatic deficiency before birth, and definitive stem cell transplantation, providing a continual source of monocytes for tissue engraftment, could be performed postnatally.

One attractive rationale for this approach is that one could use a parent as a source of monocytes, minimizing immunological concerns (13,35). Potentially, a parent's cells, although heterozygous for MANB, could be genetically engineered to over-express MANB via gene transfer. In part, this was the rationale for using monocytes expanded from marrow, rather than freshly isolated donor cells, for our studies. After birth, the child might be primed (because of the IU exposure) to accept an allograft of parent HSCs. Our current studies demonstrate the difficulties and importance of a large animal as a preclinical model.

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Abbreviations

HEXA	Hexosaminidase A
IU	<i>in utero</i>
MANB	alpha-mannosidase
HSCs	hematopoietic stem cells

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Figure 1. Brain tissue from affected kitten #4471. Two MANB-containing cells can be seen adjacent to the pia. This animal received 2×10^8 monocytic cells/kg IU $\times 3$. The tissue was obtained 15 weeks after birth (18 weeks after the last IU injection). See text for details.

Figure 2.

Tissues of affected animals transplanted IU with normal male monocytic cells were evaluated for MANB activity expressed as the ratio of MANB to HEXA. All values for tissues from affected treated animals [n=22 (brain), 24 (liver), 20 (lung), 22 (spleen)] are shown with means indicated as horizontal black lines; the number of data points equal to zero is noted below the x axis for each tissue. Whiskers on the vertical lines show standard deviations. Mean values for affected untreated animals (n=4) are shown as horizontal dashed lines. Mean values for normal animals (n=4) are indicated across the top of graph.

Figure 3. Brain, liver and spleen of normal (top row, from left to right; animal #6818) and affected treated (bottom row; animal #6357, Case Study 2, see text for details) animals showing positive (blue) cells when stained for MANB activity. Brain and spleen were counterstained with Safranin O. Scale bar is 40 μ m.

Figure 4.

Tissues of 15 females (11 affected, 1 normal, 3 heterozygote) transplanted IU with normal male monocytic cells were analyzed for Y chromosome copy number normalized to GAPDH. All values [(n=14 (brain, liver, spleen), 15 (lung))] are shown with means for each tissue indicated as horizontal lines. The number of animals with zero copies is indicated below the x-axis for each tissue. Whiskers on the colored vertical lines indicate standard deviations. The value for untransplanted females is 0.00.

Table A

Fate of fetuses visualized by serial ultrasounds. In the subset of 10 pregnancies studied by serial ultrasound examination throughout the pregnancy, fetal loss was 6/40 (15%).

Fetuses counted at first imaging	Kittens born
3	3
6	6
4	5
4	5
3	3
4	2
1	1
5	0
6	6
4	3
Total 40	34