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Glycosaminoglycan storage in neuroanatomical regions of mucopolysaccharidosis I dogs following intrathecal recombinant human iduronidase

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Abstract

Intrathecal (IT) recombinant human α -L-iduronidase (rhIDU) has been shown to reduce mean brain glycosaminoglycans (GAGs) to normal levels in MPS I dogs. In this study, we examined storage in neuroanatomical regions of the MPS I dog brain, including frontal lobe, cerebellum, basal ganglia, thalamus, hippocampal formation, and brainstem, to determine the response of these functional regions to treatment with IT rhIDU. GAG storage in untreated MPS I dogs was significantly different from normal dogs in all examined sections. GAG levels in normal dogs varied by region: frontal lobe (mean 2.36 ± 0.54 μ g/mg protein), cerebellum (2.67 ± 0.33), basal ganglia and thalamus (3.51 ± 0.60), hippocampus (3.30 ± 0.40), and brainstem (3.73 ± 1.10). Following intrathecal treatment, there was a reduction in GAG storage in each region in all treatment groups, except for the brainstem. Percent reduction in GAG levels from untreated to treated MPS I dogs in the deeper regions of the brain was 30% for basal ganglia and thalamus and 30% for hippocampus, and storage reduction was greater in superficial regions, with 61% reduction in the frontal lobe and 54% in the cerebellum compared to untreated MPS I dogs. Secondary lipid storage in neurons was also reduced in frontal lobe, but not in the other brain regions examined. Response to therapy appeared to be greater in more superficial regions of the brain, particularly in the frontal lobe cortex.

Keywords

mucopolysaccharidosis; intrathecal enzyme replacement therapy; lysosomal storage disease; glycosaminoglycans; iduronidase

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Introduction

Central nervous system disease is a feature of many of the mucopolysaccharidoses (MPS), which are a group of lysosomal storage diseases. Neurological dysfunction is seen in MPS type I (Hurler syndrome), including retinal degeneration, sensorineural hearing loss, hydrocephalus, spinal cord compression, and cognitive impairment. However, the etiology of cognitive impairment in MPS is not completely clear. Glycosaminoglycans (GAG) accumulate in MPS disease, but these substances do not appear to be directly neurotoxic. Secondary accumulation of GM2 and GM3 gangliosides occurs, and these are the major stored material in brain neurons (1;2). Gangliosides may cause dendritic sprouting (3). Other downstream effects of lysosomal storage on cells include dysregulated autophagy, oxidative stress, and inflammation (4-6). Cerebral atrophy is seen on brain imaging studies of MPS I patients and may correlate with cognitive impairment, supporting the hypothesis that neuronal cell loss is at least partly responsible for intellectual decline (7).

To address the neurological disease in MPS I, we have employed an approach in which recombinant enzyme is injected into the cerebrospinal fluid, providing the missing enzyme to the brain. Intrathecal (IT) enzyme replacement with recombinant human iduronidase (rhIDU) has been shown to be effective in reducing lysosomal storage in the brain of the MPS I dog (8;9), which is a naturally-occurring animal model of the disease (10). Repeated rhIDU doses administered into the cisterna magna of MPS I dogs achieved high levels in spinal meninges and brain (8;9). As expected, superficial areas of the brain including the cortical surface (to a depth of 3 mm) reached higher enzyme levels than deeper regions (9;11). However, even deep regions show enzyme levels that were above the mean for carrier animals, and thus were able to effect reduction in GAG storage. Enzyme activity levels of alpha-L-iduronidase in the dog brain on average reached 23-fold normal levels (8). Averaged over the entire brain, GAG storage became normal in all IT-treated MPS I dogs regardless of the age at which treatment was initiated (8;9).

Based on these previous data, we performed GAG storage assays and pathological evaluations in functional neuroanatomical regions of the brain in normal dogs, untreated MPS I dogs, and treated MPS I dogs. Our goals were to evaluate brain regions in the MPS I dog that had not previously been examined separately, and to assess the effects of treatment on these regions. We examined storage in the hippocampal formation, in addition to other functionally important neuroanatomical regions--the frontal lobes, the cerebellum, the brainstem, and the deep nuclear structures--basal ganglia and thalamus.

Materials and methods

The canine MPS I colony is derived from a beagle/Plott hound mix. The Los Angeles Biomedical Institute at Harbor-UCLA (formerly the Harbor-UCLA Research and Education Institute) and the Iowa State University Department of Animal Science are AALAC accredited facilities. The animals were studied under protocols approved by the Animal Care and Use Review Committee at both institutions.

Dogs affected with canine MPS I received IT administrations of 1.38 mg of rhIDU (270,000 units, in volumes of 2.3 mL) alone or diluted in the artificial CSF solution, Elliotts B (Ben

Venue Laboratories, Bedford, Ohio) for a total volume of 6.9 mL. RhIDU was donated by BioMarin Pharmaceutical (Novato, California). It consists of 0.6 mg/mL rhIDU (117,000-150,000 units/mL, depending on formulation) in formulation buffer (150mM NaCl; 100 mM sodium phosphate, 0.001% polysorbate 80, pH 5.8). The enzyme is stored at 2-8° and protected from excess heat and light. Dogs receiving low-dose IT rhIDU were given 0.46 mg rhIDU (90,000 units) diluted in Elliotts B for a total volume of 2.3 mL. The IT injections were administered to the cisterna magna of the anesthetized dogs as previously described (9).

Tissue evaluations

Tissue preparation and analysis were performed as previously described (8). Forty-eight hours after the last IT rhIDU injection, the animals were necropsied and their brains were removed. The left hemispheres of the brains were fixed in 4% paraformaldehyde/2% glutaraldehyde in phosphate buffer (pH 7.2) and then cut into approximately 1 cm coronal sections for histopathology. The right hemispheres were sectioned coronally and snap frozen at approximately -80°C to be assayed biochemically for GAG storage.

Sections of 0.5–1 cm³ were dissected from several neuroanatomical regions of the right brain: the rostral forebrain, cerebellum, brainstem, basal ganglia/thalamus, and hippocampal formation. Rostral forebrain and cerebellar samples are labeled as superficial given the proximity to the subarachnoid cerebrospinal fluid. An effort was made to obtain cerebral and cerebellar cortex (gray matter) in each of the rostral forebrain and cerebellar samples respectively; however, white matter was not specifically excluded. Gray and white matter was not differentiated in samples of the brainstem. Samples of the basal ganglia, thalamus, and hippocampal formation are mostly gray matter and are labeled as deep given their relative distance from the subarachnoid cerebrospinal fluid. GAG levels were quantified by an Alcian blue dye binding method as described by Kakkis et al. and quantified within the linear range with dermatan sulfate standards (12).

Histopathologic evaluation was performed on left brain samples of the same neuroanatomical regions as above. The tissues were post-fixed in osmium tetroxide and embedded in Spurr's resin. Brain sections for light microscopy were stained with toluidine blue. Thin sections of neocortex, cerebellum, hippocampus and thalamus from selected cases were evaluated by electron microscopy using a JOEL 100 CX electron microscope. Qualitative morphometric evaluation was performed by a board-certified pathologist (C.V.) who was blinded to the treatment or affected status of the animals.

Data Analysis

Mean GAG was compared between 4 untreated dogs and 5 normal dogs for each of 5 brain regions separately using unequal variance 2-sample t-tests. An identical analysis was performed comparing the means of 13 treated and 4 untreated MPS I dogs. Analysis of variance with post-hoc Dunnett's test was performed using SYSTAT 12 (Systat Software, Chicago, IL) to compare the four dosing regimens among the treated dogs to mean GAG in the 4 untreated MPS I dogs.

Results

The animals used in the study are summarized in Table 1. Brain tissue was evaluated from these subjects, which had received IT rhIDU in previous research (4, 5). MPS I dogs were treated weekly (n = 2), monthly (n = 5), low-dose monthly (n = 2), quarterly (every 3 months, n = 4) with 3-4 doses of 0.46 or 1.38 mg IT rhIDU. Previous studies showed that

the half-life of rhIDU in brain is approximately 7 days (13), and that IT rhIDU dosing as infrequently as once every three months effectively reduces brain GAG in MPS I dogs (9).

Differential reduction in glycosaminoglycan storage, superficial regions versus deep regions

Brain regions were assayed for GAG and compared among normal dogs, untreated MPS I dogs, and IT-treated dogs (Table 2, Figure 1). GAG levels were significantly elevated in all brain regions in untreated MPS I dogs versus normal controls. In the superficial regions, mean GAG storage in untreated MPS I dogs was 8.81 ± 1.59 $\mu\text{g}/\text{mg}$ protein in the frontal lobe and 7.70 ± 1.27 $\mu\text{g}/\text{mg}$ protein in cerebellum. Mean GAG storage levels in normal dogs were 2.36 ± 0.54 in frontal lobe ($p=0.002$ vs. untreated) and 2.67 ± 0.33 in cerebellum ($p=0.003$ vs. untreated). GAG levels of all untreated MPS I dogs were outside the normal range in these regions, and their means were roughly 3-fold normal levels. Untreated MPS I dogs had mean GAG levels of 6.00 ± 0.67 in the basal ganglia/thalamus, 6.02 ± 0.18 in the hippocampal formation, and 6.09 ± 1.39 in the brainstem. These levels were roughly 1.5-fold the GAG levels in normal dogs (basal ganglia and thalamus 3.51 ± 0.60 , $p=0.006$; hippocampus, 3.30 ± 0.40 , $p<0.0001$; brainstem, 3.73 ± 1.10 , $p=0.034$). Interestingly, while storage levels were higher in superficial regions in untreated MPS I dogs, normal dogs had slightly higher storage in deep regions.

Following IT rhIDU treatment, there was a reduction in GAG storage in all treatment groups, except for the brainstem. MPS I dogs were treated with four different regimens of IT rhIDU: weekly, monthly, low-dose monthly (one-third of the standard dose), and quarterly. A 2-sided Dunnett's test was performed comparing each treatment group with the untreated group in each neuroanatomical region. In the frontal lobe and cerebellum, the mean GAG level in every treatment group was significantly different from the untreated mean ($p\leq 0.005$). Pooling the GAG levels for the treatment groups, storage reduction in these superficial regions was 61% for frontal lobe (to mean 3.45 ± 1.11 , $p=0.003$) and 54% for cerebellum (to mean 3.55 ± 0.96 , $p=0.003$), versus untreated MPS I dogs. In the basal ganglia/thalamus, the mean GAG level for all treatment groups, except for the monthly group, was significantly different from the untreated mean ($p\leq 0.029$). The monthly group showed a trend towards significance with $p=0.08$. Similarly in the hippocampal formation, the mean GAG level for all treatment groups, except for the quarterly group, was significantly different from the untreated mean ($p\leq 0.020$). Pooling the GAG levels for these groups, percent reduction in GAG levels from untreated dogs in the deeper regions was 30% for both hippocampus ($p<0.0001$) and basal ganglia/thalamus ($p=0.018$). Because the frontal lobes and cerebellum samples were primarily cortical and close to the subarachnoid cerebrospinal fluid, they were labeled superficial. In contrast, basal ganglia and hippocampal formation samples of brain tissue were further from the subarachnoid cerebrospinal fluid and thus labeled deep. Thus, it appears that GAG reduction after IT enzyme replacement is greater in the superficial regions of the brain (54–61%) compared to the deeper regions of the brain (30%). Of note, there was no significant change in GAG level after treatment in the brainstem ($p=0.928$), despite its relatively proximal location to the injection site.

Differential reduction of lysosomal pathology by functional neuroanatomical region

Lysosomal storage was evaluated morphologically in the same neuroanatomical regions (Figs. 2 and 3). Storage in neurons was generally electron dense complex and lamellar material, morphologically typical of complex lipid storage, and showed little GAG. Untreated MPS I dogs show abundant electron dense storage within the cytoplasm of cortical neurons in the frontal lobe (Figs. 2a) and cerebellar Purkinje cells (Fig. 2c). IT rhIDU resulted in marked reduction in the amount of lysosomal storage in the frontal lobe neurons (Figs. 2b), but no change in cerebellar Purkinje cell storage (Fig. 2d). Neurons in

the hippocampus showed a small amount of storage, in both untreated (Fig. 2e) and IT-treated (Fig. 2f) MPS I dogs. There was a marked accumulation of more electron lucent storage typical of GAG accumulation in perivascular cells in untreated dogs (Fig. 3a). This material was much less abundant in the IT rhIDU group (Fig. 3b), correlating with the reduction in the amount of GAG identified biochemically.

Discussion

As in our previous study which measured GAG reduction in whole brain, this study also demonstrates that intrathecally administered rhIDU reduces GAG storage in MPS I dogs. This study examined neuroanatomically distinct brain regions, including the frontal lobe, the cerebellum, brainstem, basal ganglia, thalamus, and hippocampal formation. In examining the brains of intrathecally treated MPS I dogs, we found that there appeared to be more reduction in lysosomal storage in surface regions such as the frontal lobe and the cerebellum (though storage was not reduced in Purkinje cells) as compared to the deeper regions--basal ganglia, thalamus, and the hippocampal formation. Biochemically, all regions showed reduction in GAG storage versus untreated animals. Quantitatively, there was less storage in the deeper regions in untreated MPS I dogs than in superficial regions, which could partially explain the lower percent reduction. Another explanation is that less enzyme reached the deeper regions. We were unable to measure iduronidase levels in this study, because these tissues had been previously thawed and refrozen resulting in overall reduction of enzymatic activity. However, our previous work showed that iduronidase levels were higher on the surface of the brain than in samples taken at least 3 mm below the surface, but that therapeutic levels can be achieved even in deep tissue structures (9;11). Given that the IT rhIDU was infused at the cisterna magna, samples of the frontal lobe and cerebellum, which were primarily cortical, were likely exposed to greater levels of enzyme than the deep nuclear structures or the hippocampal formation.

On histopathological examination, perivascular cells showed lysosomal storage reduction in all areas examined, including deeper regions. The storage material in these cells had the characteristics of GAG, while neuronal storage was principally lipid. This may explain why perivascular cells showed reduction in lysosomal storage even in deeper regions, while neurons in those regions did not.

In the untreated MPS I dogs, we examined whether there was differential GAG storage in important functional regions of the brain. The brainstem, basal ganglia and thalamus, and hippocampus had less storage in untreated MPS I dogs than did the frontal cortex and cerebellum (Table 2). The finding of relatively less storage in brainstem than cortical regions is consistent with our previous finding of low storage in the spinal cord in untreated MPS I dogs (9). Of note, there was no significant change in GAG storage in the brainstem after IT rhIDU treatment in these adult MPS I dogs. Early treatment with IT rhIDU (i.e., in the first month of life) may prevent spinal cord storage (11), though it had no effect on established disease (14). Brainstem samples were taken from both surface tracts and central gray nuclei, representing both superficial and deep regions. It seems likely that white matter tracts constituted the majority of the brainstem samples, and these may not have abundant lysosomal storage. Although the neuropathology of the MPS I dog model has been described (15), examination of specific functional neuroanatomical regions such as the hippocampus has not been performed. This region has a relatively large amount of storage in MPS I and MPS VII mice (16;17). However, in MPS I dogs, quantitative GAG storage in the hippocampus was only approximately 1.5 fold normal. The significance of this differential storage is unknown. The relatively low amounts of GAG in these regions of the brain in untreated MPS I dogs may have limited our ability to see a substantial difference following treatment with IT rhIDU.

Enzyme administration via cerebrospinal fluid has been studied in animals for several of the lysosomal storage diseases (18–21). Differential distribution of enzyme through the brain has been seen in MPS IIIA mice receiving cisternal sulfamidase, in which thalamic storage responded less completely than in cortex, hippocampus, superior or inferior colliculus (22). Intraventricular acid sphingomyelinase distributed widely in the brain of a murine model of Niemann-Pick A, with wild-type enzyme levels achieved in each of five coronal sections in all dosing groups (21). The thalamus was not separately evaluated, however. Immunofluorescence of rhIDU in normal rat brain showed penetration from an intraventricular injection up to 4 mm from the ventricular surface (13). Enzyme levels were detected by biochemical analysis throughout coronal sections but with a gradient away from the injection site. Our findings support Hemsley et al. (22) that deeper functional regions may show less complete treatment with intrathecally applied enzyme. It is not known whether intraventricular administration would alter enzyme distribution to deeper structures.

We are currently studying IT enzyme replacement for the central nervous system in MPS I patients. A single report demonstrates clinical improvement in spinal cord compression symptoms with IT rhIDU treatment (23). The findings here suggest that IT rhIDU will have its greatest effect in cerebral cortex, with lesser effects in hippocampus and basal ganglia and thalamus. Storage in the brainstem may not respond to IT rhIDU, at least in the case of established disease. However, it is unclear whether brainstem storage leads to clinical neurological deficits in MPS I patients, as the majority of their neurological symptoms in the periphery are due to cervical spinal cord compression (24). In the brain, the two imaging findings that appear to correlate with cognitive impairment in MPS disorders are cortical atrophy and patchy white matter lesions of unknown cause (7;25). If neuronal cell loss and atrophy are a main cause of cognitive dysfunction, then targeting brain gray matter is a reasonable approach to address neurological disease due to MPS I. Further research is needed to study the effects of recombinant enzyme on cognitive symptoms and determine whether this is a viable treatment option for MPS I brain disease.

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Fig. 1. Mean GAG levels in different neuroanatomical regions. Error bars reflect standard deviations. FL = frontal lobe, CB = cerebellum, BGT = Basal ganglia and thalamus, HF = hippocampal formation, BS = brainstem.

Fig. 2.

Lysosomal storage in untreated and IT-treated MPS I dogs. Cortical neuron in the frontal lobe of an untreated MPS I dog (a) and an IT-treated MPS I dog (b) 6500x. Purkinje cells of cerebellum from an untreated (c) and treated (d) MPS I dog, 4000x. Neurons in the hippocampus in untreated (e) and treated (f) MPS I dog, 6500x. Neuronal storage (arrows) had the morphological characteristics of complex lipid storage (inset, a). Perivascular storage (arrowhead) had the characteristics of GAG storage. N: neuron, PV: perivascular cell, uranyl acetate-lead citrate.

Fig. 3. Perivascular cells in the cerebellum of an untreated (a) and treated (b) MPS I dog. Perivascular storage (arrowhead) had the characteristics of GAG storage, 6500x, uranyl acetate-lead citrate.

Table 1

Normal and MPS I dogs used in intrathecal (IT) rhIDU studies

MPS I status	Age (months) at sacrifice	Sex	Regimen	IT rhIDU dose	Number of IT rhIDU doses
MPS I					
Ta	19	F	Weekly	1.38 mg	4
Vk	14	M	Weekly	1.38 mg	4
Ad	25	M	Monthly	1.38 mg	4
Ni	40	M	Monthly	1.38 mg	4
Um	33	F	Monthly	1.38 mg	4
Ur	24	M	Monthly	1.38 mg	4
Ye	19	M	Monthly	1.38 mg	3
Bd	18	M	Low Dose Monthly	0.46 mg	4
Cy	15	M	Low Dose Monthly	0.46 mg	4
Ub	35	M	Quarterly	1.38 mg	3
Ui	35	M	Quarterly	1.38 mg	3
Xb	31	M	Quarterly	1.38 mg	3
Xy	31	F	Quarterly	1.38 mg	3
UNTREATED MPS I					
Me	13	F	None	None	None
Wa	17	M	None	None	None
I-121	19	M	None	None	None
I-134	18	M	None	None	None
NORMALS					
Co	19	M	None	None	None
Ev	14	F	None	None	None
Or	42	F	None	None	None

MPS I Status	Age (months) at sacrifice	Sex	Regimen	IT rhIDU dose	Number of IT rhIDU doses
Tr	16	F	None	None	None
Wi	22	M	None	None	None

Table 2

Glycosaminoglycan levels by neuroanatomical region in normal, IT-treated MPS I, and untreated MPS I dogs.

	Normal N=5	IT-treated MPS I dogs					Untreated MPS I dogs N=4	% reduction in GAG Untreated vs. mean IT-treated
		Weekly N=2	Monthly N=5	Low-dose N=2	Quarterly N=4	Mean IT-treated N= 13		
SUPERFICIAL REGIONS								
Frontal lobe	2.36* (1.74–3.04)	2.56* (2.53–2.59)	3.51* (2.43–4.06)	3.22* (2.95-3.48)	3.95* (2.43–6.53)	3.45*	8.81 (7.29–11.0)	61%
Cerebellum	2.67* (2.32–3.16)	2.99* (2.95–3.02)	3.95* (3.04–5.00)	3.94* (2.49–5.39)	3.15* (2.35–3.80)	3.55*	7.70 (6.35–9.29)	54%
DEEP REGIONS								
Basal Ganglia/Thalamus	3.51* (2.79–4.23)	4.09* (3.98–4.20)	4.76 (3.56–5.63)	2.64* (2.43–2.84)	4.38* (3.94–4.89)	4.21*	6.00 (5.40–6.72)	30%
Hippocampal formation	3.30* (2.69–3.63)	3.65* (3.65–3.65)	3.91* (3.43–4.28)	3.73* (2.74–4.71)	5.21 (4.14–6.74)	4.24*	6.02 (5.81–6.24)	30%
BRAINSTEM	3.73* (2.29–4.84)	3.56 (2.70–4.41)	5.36 (4.33–6.10)	7.76 (7.21–8.30)	7.71 (5.02–9.23)	6.17	6.09 (4.97–8.00)	–1%

GAG levels are expressed in µg/mg dry weight. Mean (range) shown.

*
p<0.05, when compared to untreated MPS.