Enzyme replacement in a canine model of Hurler syndrome

(α-L-iduronidase/mucopolysaccharidosis I/lysosomal storage/endocytosis)

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ABSTRACT The Hurler syndrome (α-L-iduronidase deficiency disease) is a severe lysosomal storage disorder that is potentially amenable to enzyme-replacement therapy. Availability of a canine model of the disease and a sufficient supply of corrective enzyme have permitted a therapeutic trial lasting 3 mo. Recombinant human α-L-iduronidase, purified to apparent homogeneity from secretions of a stably transfected Chinese hamster ovary cell line, was administered i.v. to homozygous affected animals in doses of ~1 mg. The enzyme rapidly disappeared from the circulation in a biphasic manner, with $t_{1/2}$ of 0.9 and 19 min, respectively, and was taken up primarily by the liver. Biopsy of the liver before and after a very short trial (seven doses administered over 12 days) showed remarkable resolution of lysosomal storage in both hepatocytes and Kupffer cells. After weekly administration of enzyme to three affected animals over a period of 3 mo, the level of enzyme was about normal in liver and spleen, lower but significant in kidney and lung, and barely detectable (0–5% of normal) in brain, heart valves, myocardium, cartilage, and cornea. Light and electron microscopic examination of numerous tissues showed normalization of lysosomal storage in liver, spleen, and kidney glomeruli, but there was no improvement in brain, heart valves, or cornea. Even though the treated dogs developed complement-activating antibodies against α-L-iduronidase, clinical symptoms could be prevented by slow infusion of enzyme and premedication.

The Hurler syndrome is the best known as well as the most severe of the α-L-iduronidase deficiency diseases [known collectively as mucopolysaccharidosis I (MPS I)] (1). α-L-Iduronidase participates in the stepwise degradation of the glycosaminoglycans, dermatan sulfate and hepan sulfate, within lysosomes; its absence interrupts the degradative pathway and causes lysosomal storage of undegraded substrate, with resulting pathology of cells, tissues, and organs. Clinical manifestations of the Hurler syndrome include progressive mental retardation, corneal clouding, facial dysmorphism, dysostosis multiplex, cardiac and respiratory compromise, and death in childhood. A milder form of α-L-iduronidase deficiency, the Hurler/Scheie syndrome, is compatible with normal intelligence and survival to adulthood although with severe physical handicaps, whereas the Scheie syndrome is milder yet and may permit a normal life. These disorders are caused by recessive mutations in the α-L-iduronidase gene (IDUA), with genetic heterogeneity both within and within the three clinical groups (2–12).

Lysosomal storage diseases in general (13), and the Hurler syndrome in particular (14), have long been considered amenable to treatment by exogenous enzyme that would enter the deficient cells by endocytosis. Early studies in cell culture had shown that a “corrective factor” could eliminate the abnormal accumulation of glycosaminoglycans by fibroblasts derived from Hurler patients (15). The factor proved to be α-L-iduronidase with a mannose 6-phosphate targeting signal for high-efficiency uptake (16, 17). There are two widely distributed mannose 6-phosphate receptors, which target endogenous as well as endocytosed enzyme to lysosomes (18, 19); other carbohydrate receptors, which are specific for endocytosis, include the galactose (asialoglycoprotein) receptor of hepatocytes (20) and the mannose receptor of macrophages (21). The latter is used in enzyme-replacement therapy of type I Gaucher disease; glucocerebrosidase, purified from placentas and modified for targeting to the macrophage mannose receptor, is now a clinically effective pharmaceutical (22, 23).

The low abundance of α-L-iduronidase in tissues has required the use of recombinant technology to obtain significant quantities of the enzyme. A Chinese hamster ovary (CHO) cell line was engineered to produce human α-L-iduronidase (24). The recombinant enzyme was shown to be efficiently endocytosed by the mannose 6-phosphate system (half-maximal uptake at 0.7 nM) and to correct the abnormal glycosaminoglycan accumulation in Hurler fibroblasts. After uptake, the enzyme had a half-life of ~5 days in the recipient fibroblasts. About half the enzyme produced by CHO cells was secreted and could be purified from the medium in quantities sufficient for a trial of enzyme replacement in the MPS I dog.

The canine model of α-L-iduronidase deficiency provides an excellent in vivo test system. Its biochemical, molecular, pathological, and clinical features are similar to those of human MPS I (25–28). Bone marrow transplantation has been successful in slowing the progression of the canine disease (29, 30) and of the human disease (refs. 31, 32; for review, see ref. 1). This effect is believed to depend, at least in part, on uptake of enzyme produced by cells derived from the donor’s bone marrow into the deficient cells of the host (33–35).

We have examined the effectiveness and safety of administering recombinant α-L-iduronidase to deficient dogs for periods ranging up to 3 mo. A preliminary account of this work has been presented in abstract form (36).

MATERIALS AND METHODS

Enzyme Preparation and Assay. Recombinant human α-L-iduronidase was produced in CHO cells as described; secreted enzyme was purified under aseptic conditions to apparent homogeneity, ~250,000 units per mg of protein (24). The purity of each batch was confirmed by SDS/PAGE and

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Abbreviation: MPS, mucopolysaccharidosis.

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silver staining, and each was shown free of endotoxin by the Limulus lysate assay (E-toxate, Sigma). The enzyme was stored in sterile vials at 4°C at a concentration of 50,000–100,000 units/ml of phosphate-buffered saline (PBS; 150 mM NaCl/10 mM NaPO₄), pH 5.8. Enzyme assays were done as described (24), except where otherwise specified; a unit is defined as the activity catalyzing 1 nmol of 4-methylumbelliferyl α-L-iduronide per hr. Actual incubation time varied from a few minutes to overnight. Protein was measured by the Bio-Rad modification of the Bradford dye-binding assay (37), with bovine serum albumin used as a standard.

**Experimental Animals and Tissues.** Affected animals were born and raised in the Laboratory Animal Facility of the University of Tennessee College of Veterinary Medicine. They are the result of several generations of crosses between the original Plott hounds (25) and beagles. Information on the animals used in this study is provided in Table 1. In addition, three random mixed-breed dogs, all unaffected young adults, were used to obtain control tissues.

Enzyme (250,000 units, ∼1 mg) was administered through catheters placed aseptically in either the cephalic or saphenous vein. For dogs Pi, Zo, and Al, the enzyme was diluted to 10 ml in 10 mM PBS, pH 5.8, and infused over a 10-min period; this procedure was repeated every other day. After the third dog treated in this manner (Al) had an anaphylactoid reaction, the procedure was modified to infuse the enzyme more slowly (over a period of 1 hr) as well as in more dilute form (in 65 ml of PBS, pH 5.8, containing canine serum albumin at 1 mg/ml). In addition, the dogs were fasted overnight and premedicated with diphenhydramine (0.4 mg/kg) to counter any allergic reaction and butorphenol (0.04 mg/kg) for mild sedation.

Plasma samples were anticoagulated with EDTA. Mononuclear leukocytes were separated by density gradient centrifugation (Ficoll/Paque, Pharmacia). Liver samples were obtained by surgical laparotomy or ultrasound-guided needle biopsy. Euthanasia was carried out by i.v. administration of barbiturate overdose. Tissues for EM were collected within 5 min of death, fixed in 2% glutaraldehyde/0.1 M cacodylate buffer, pH 7.3, and processed as described (26). Tissue for light microscopy was fixed in 10% (vol/vol) formalin, pH 7.0, and stained with hematoxylin/eosin. Tissue specimens for enzyme assay were frozen on dry ice and stored at −70°C.

**Antibodies and Complement.** Antibodies to the human recombinant enzyme in the serum of treated animals were assayed by a standard ELISA with 0.1 μg of purified α-L-iduronidase per well, 3% bovine serum albumin as blocking agent, and goat anti-canine IgG conjugated to alkaline phosphatase. Immunoprecipitation of α-L-iduronidase activity by antibodies in serum was assisted by anti-canine IgG and polyethylene glycol; enzyme activity was measured in the precipitate as well as in the supernatant solution.

Serum complement activity was assayed by a modified hemolytic assay (38) that used bovine erythrocytes sensitized with rabbit antiserum raised against bovine erythrocyte membranes. The release of hemoglobin was measured by absorbance at 540 nm. The fraction of lysed erythrocytes was determined by comparison with complete lysis of erythrocytes in water.

**RESULTS**

**Plasma Clearance and Tissue Distribution.** α-L-Iduronidase administered by infusion over the span of 10 min disappeared from circulation in two phases, as shown in Fig. 1A. The first phase was a sharp decrease in serum enzyme activity; t½(A) was 0.9 min, probably representing distribution within the vascular space and extracellular fluid. This sharp decrease was followed by a slower decrease with a t½(B) of 18.9 min. By contrast, activity in leukocytes rose for 4 hr after infusion and increased further after additional daily infusions (Fig. 1B).

Administered α-L-Iduronidase appeared at a high level in liver. Four hours after the last of three daily doses, the enzyme activity of biopsied liver tissue from dog Zo was 10-fold the level of normal dogs. Analysis of multiple tissues after a single administration (dogs Pi and Zo) showed that liver had by far the highest enzyme activity, other tissues having low or undetectable activity (data not shown).

After 13 weekly administrations, enzyme activity was found in liver and spleen at about normal level in all three treated animals, at lesser but still significant levels in lung and kidney and at low or undetectable levels in brain, heart valve, myocardium, cartilage, lymph node, cornea, and pancreas (Table 2).

**Tissue Pathology.** Liver tissue obtained by biopsy in the short-term injections had much improved histologic appearance. There was a dramatic decrease in vacuolation in the liver of dog Al after the seventh dose of α-L-iduronidase, as compared with its appearance before treatment (Fig. 2). Electron microscopy showed a nearly complete normalization of both Kupffer cells and hepatocytes (data not shown), similar to that seen in the longer trial (below).

Comparison of the electron microscopic appearance of tissues from dogs treated for 13 weeks with those of untreated dogs showed a nearly complete normalization of Kupffer cells and hepatocytes (data not shown), similar to that seen in the longer trial (below).

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**Table 1.** MPS I dogs and protocols used in therapeutic trials

<table>
<thead>
<tr>
<th>Dog</th>
<th>Sex</th>
<th>Age, yr, mo</th>
<th>Weight, kg</th>
<th>Clinical disease</th>
<th>Enzyme infusions, no. and frequency</th>
<th>Time tissue collected, hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi</td>
<td>F</td>
<td>3.8, 11</td>
<td>4.4</td>
<td>Advanced</td>
<td>3, daily</td>
<td>1</td>
</tr>
<tr>
<td>Zo</td>
<td>M</td>
<td>0.9, 10</td>
<td>1.7</td>
<td>Moderate</td>
<td>3, daily</td>
<td>1</td>
</tr>
<tr>
<td>Al</td>
<td>M</td>
<td>1.0, 12</td>
<td>1.4</td>
<td>Mild/ moderate</td>
<td>7, every other day</td>
<td>48</td>
</tr>
<tr>
<td>De</td>
<td>F</td>
<td>0.8, 12</td>
<td>1.8</td>
<td>Mild</td>
<td>13, weekly</td>
<td>48</td>
</tr>
<tr>
<td>Do</td>
<td>F</td>
<td>0.8, 12</td>
<td>1.5</td>
<td>Mild</td>
<td>13, weekly</td>
<td>48</td>
</tr>
<tr>
<td>Xy</td>
<td>F</td>
<td>1.8, 14</td>
<td>1.8</td>
<td>Moderate</td>
<td>13, weekly</td>
<td>48</td>
</tr>
<tr>
<td>Xe</td>
<td>F</td>
<td>1.8, 14</td>
<td>1.3</td>
<td>Moderate</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>El</td>
<td>F</td>
<td>0.8, 11</td>
<td>1.9</td>
<td>Moderate</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

*Hours after infusion.

**Fig. 1.** Kinetics of administered α-L-iduronidase activity in the circulation. Enzyme (250,000 units) was administered to one MPS I dog (Pi) every 24 hr on three consecutive days; numbers on abscissa represent time elapsed from the end of the last infusion, which itself lasted 10 min. (A) Disappearance of enzyme activity [in units (U)] from plasma on day 1 (○) and day 2 (△). Data were fitted to a two-component model by the nstrm program (MicroMath Scientific Software, Salt Lake City); the correlation coefficient was 0.999. (B) Appearance of activity in leukocytes (WBC, white blood cells). The standard enzyme assay (24) was modified by incubation at 37°C.
Table 2.  \(\alpha\)-L-IIduronidase activity in tissues of MPS I dogs after 13 weekly injections, compared to untreated MPS I and normal dogs

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Untreated MPS I animals</th>
<th>Treated MPS I animals</th>
<th>Untreated animals*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Do</td>
<td>De</td>
<td>Xy</td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart valve</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Myocardium</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cartilage</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cornea</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pancreas</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Tissues were homogenized in 3 vol of 10 mM phosphate buffer, pH 5.8/0.1 mM dithiothreitol/0.02% NaNO\(_3)/0.1% Triton X-100, using a Dounce homogenizer; cartilage, cornea, heart valves, lung, and myocardium were first frozen in liquid N\(_2\) and ground into a powder. The enzyme assay was as described (24), except that the NaCl concentration was 75 mM. Both reagent and tissue blanks were used; an internal standard was necessary to correct for fluorescence quenching by colored material, presumably hemoglobin. Activity is expressed as units/mg of protein. ND, not done.

*\(n = 3\), except for cerebrum, cerebellum, and myocardium, where \(n = 2\), and heart valve and cartilage, where \(n = 1\).

†Zero in this column means below the limit of reliable detection (fluorescence equivalent to <0.005 nmol of 4-methylumbelliferone in assay sample).

affected dogs showed marked improvement in some but not all organs. Hepatocytes of treated dogs lost the large number of enlarged lysosomes characteristic of MPS I, although they occasionally retained a few large, possibly coalesced lysosomes (Fig. 3 A and B). Kupffer cells likewise lost the enlarged lysosomes, although some had cytoplasmic granules that contained pigment.

Cells of the renal glomeruli appeared normal in the treated dogs, in contrast to the highly vacuolated appearance in the untreated ones (Fig. 3 C and D). Mesangial macrophages showed the greatest improvement, but vascular endothelial cells and glomerular epithelial cells also had reduced vacuolation. On the other hand, cells in the renal interstitium and cells comprising the renal tubules were as vacuolated in treated and untreated dogs, indicating that these cells did not take up enzyme.

In the spleens of treated animals, macrophages were reduced in size and had very few enlarged lysosomes (Fig. 3 E and F). Lymphoid and hematopoietic cells in the spleens, while only slightly affected in the untreated dogs, were normal in appearance in the three enzyme recipients (data not shown). Lung, a tissue that took up a significant amount of enzyme, was not dramatically altered—mainly because it did not show extensive pathology in the untreated animals (data not shown).

However, many severely affected cell types showed no improvement. These cells include neurons and glial cells in the central nervous system, fibroblasts in corneal stroma and heart valves, and chondrocytes in the tracheal ring and articular cartilage (data not shown).

Antigenic Reactions. The third dog to receive enzyme (A) developed clinical signs suggestive of an anaphylactoid reaction after the seventh dose in a 12-day period. Blanched mucous membranes, bradycardia, weak pulse, and involuntary urination were seen midway through the 10-min period of enzyme administration. The infusion was stopped, and the animal was treated with i.v. fluids and antihistamines, resulting in complete recovery. The reaction was shown to be caused by \(\alpha\)-l-iduronidase itself, as it was not induced by infusion of buffer or of likely protein contaminants. Therefore, further enzyme replacement was discontinued in this animal.

The clinical reaction was subsequently shown to be due to IgG-mediated complement activation. IgG antibodies against \(\alpha\)-l-iduronidase were detected in the serum of dog A by ELISA and by specific immunoprecipitation of \(\alpha\)-l-iduronidase activity. The antibodies could induce complement consumption, as demonstrated by a reduction in hemolytic complement, 20 min after infusion, to 16% of the preinfusion level. Complement returned to 50% of preinfusion level at 2 hr but was still low (60%) even after 24 hr.

IgG-mediated anaphylaxis was probably not involved, as intradermal skin tests of \(\alpha\)-l-iduronidase and likely protein contaminants were nonreactive.

After the acute reaction in this one animal, the protocol was changed to give enzyme more slowly and in a more dilute form, as well as to mediate the animals with antihistamine and sedative (see Materials and Methods). No adverse clinical reaction was observed under these conditions, and enzyme could be administered at weekly intervals for 3 mo or longer. However, antibodies to the administered enzyme could be detected by ELISA in the serum of all the recipients after two weekly injections; the antibody titers peaked after 4–5 weeks and persisted at a nearly constant level during the test period. Complement consumption still occurred, although less dramatically than before modification of the protocol; in two dogs tested, the hemolytic activity fell to 60% of preinfusion levels, the nadir occurring between 30 and 80 min after the infusion.

Clinical Effects. Except for the one immune reaction before a change in protocol, there were no ill effects from enzyme administration. Results of complete blood counts, serum chemistry, and electrolyte panels were seemingly unaffected by enzyme infusions. There was no obvious change in the course of the underlying \(\alpha\)-l-iduronidase deficiency disease.

DISCUSSION

\(\alpha\)-l-Iduronidase administered to deficient dogs was rapidly cleared from plasma and taken up by tissues. The greatest uptake was by liver and spleen, followed by lung and kidney. The enzyme functioned in clearing accumulated glycosaminoglycans, as demonstrated by the striking disappearance of enlarged lysosomes and normalization of morphology. However, key tissues such as brain, cartilage, and heart valves took up little or no enzyme and showed no improvement in abnormal cell morphology. Because the pathology of these tissues contributes significantly to the
clinical manifestations of the disease, methods must be devised to increase their uptake of α-L-iduronidase. Perhaps the dose administered, 0.08 mg (1.1 nmol) per kg, was simply too small. A wide distribution of β-glucuronidase and amelioration of the pathology of the nervous system were found after administration of that enzyme to β-glucuronidase-deficient newborn mice in much larger quantity (39); doses administered in that study started at 7 mg (20 nmol) per kg and went to 2 nmol per kg as the mice grew over a 5-week period. A preliminary trial suggests that it may be possible to increase the uptake of α-L-iduronidase into many tissues, including brain and cartilage, by administration of larger doses of enzyme (E.D.K. and R.M.S., unpublished results).
The immunological response of the MPS I dogs to recombinant human \(\alpha\)-l-iduronidase was not surprising, given that a foreign protein was repeatedly administered; it is therefore reassuring that the immediate clinical manifestations of this response could be easily prevented. It is anticipated that administration of the human enzyme will also evoke antibody production in Hurler patients because the most common Hurler alleles (W402X and Q70X) contain a premature termination codon. Antibodies against administered glucocerebrosidase and complement activation have been observed in patients with type I Gaucher disease, but the immune reactions were mild, easily controlled, and without effect on the therapeutic efficacy of the enzyme (23, 40).

Because MPS I is a chronic disease that progresses slowly, clearly demonstrable clinical changes were neither expected nor observed over a 3-mo period. The disease of one dog, infused weekly for a year, appears to be progressing more slowly than that of an untreated littermate (E.D.K. and R.M.S., unpublished results), but because of the clinical variability of the disease, even within litters, this preliminary result must be validated on a number of animals.

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