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Cellular and Tissue Distribution of Intravenously Administered Agalsidase Alfa

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Abstract

α -Galactosidase A is the lysosomal hydrolase that is deficient in patients with Fabry disease. Intravenous infusion of agalsidase alfa, a preparation of α -galactosidase A, is used for enzyme replacement therapy (ERT) in patients with Fabry disease. Although ERT appears to show some beneficial effects, most patients show only a modest response. We investigated using immunohistochemistry the relative tissue and cellular distribution of agalsidase alfa after a single intravenous injection in a mouse knockout model of Fabry disease. Specific immunostaining for agalsidase alfa was found only in liver, kidney, heart, testes, adrenal gland, spleen and bone marrow. There was no difference in distribution of the infused enzyme distribution among tissues sampled 4, 24, and 48 hours post-injection. The intracellular localization of immunopositivity varied considerably between organs with vascular endothelium being the most commonly positive site. α -Galactosidase A specific activity in tissue homogenates matched the relative extent of agalsidase alfa immunostaining distribution in the same organs. We conclude that intravenously injected agalsidase alfa has a very heterogeneous systemic distribution using an immunostaining technique.

Introduction

Fabry disease is an X-linked disorder caused by a deficiency of the enzyme α -galactosidase A, resulting in reduced catabolism of D-galactosyl-containing compounds [1]. This causes accumulation of glycosphingolipids, particularly globotriaosylceramide (Gb3) in most cell types [2]. Clinical onset of the disease typically occurs during childhood or adolescence with recurrent episodes of severe acroparesthesias associated with a small-fiber neuropathy [3], characteristic cutaneous lesions known as angiokeratomas and a distinctive, but asymptomatic, corneal dystrophy [3]. Vital organs are affected with increasing age by a systemic vasculopathy that is associated with progressive cerebrovascular, cardiac or renal complications and early death [4].

Enzyme replacement therapy (ERT) has been developed over the past 7 years and currently there are two α -galactosidase A preparations commercially available for infusions [5,6]. Although ERT appears to show some beneficial effects, overall, the effects of ERT have been modest. Studies have shown small reductions in neuropathic pain, gastrointestinal symptoms

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and perception threshold for warm and cold sensation. Increased sweating and probably slowing of the progression of renal disease in adult male Fabry patients. [7–9]. While non-controlled studies suggest reduction in left ventricular mass, the clinical impression thus far is that there has been no reduction in the incidence of stroke [10,11]. Because of the modest effect of ERT, we hypothesized that although the intravenously infused α -galactosidase A is distributed to some extent to all relevant organs [12], the enzyme has limited intra-organ access. In order to assess the extent of relative uptake of the infused enzyme in virtually all organs and cell types, we injected intravenously agalsidase alfa into the α -galactosidase A knockout mouse followed by comprehensive immuno-localization of the infused enzyme.

Material and Methods

Mice

This work was done under an approved protocol. The National Institute of Neurological Disorders and Stroke follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals” (National Research Council; 1996; National Academy Press; Washington, D.C.). All mice were males aged 126–149 days. This animal model was previously described in detail [13–15]. A total of 12 *GLA* $-/-$ mice were injected intravenously via the tail vein. Six mice were injected with 0.5 mg/kg of agalsidase alfa (Shire Human Genetic Therapies, Cambridge, MA) and 6 received the vehicle only. Additional uninjected mice were studied. Two enzyme and vehicle injected mice were sacrificed at 4, 24 hours and 48 hours post-injection for immunohistochemistry and enzyme assays, and additional agalsidase alfa injected mice were sacrificed at 72 and 98 hours for enzyme assays only.

Pathology

Mice were euthanized with CO₂ and a comprehensive necropsy examination was performed. Portions of liver, kidney, spleen, heart and lung were snap frozen in liquid nitrogen for subsequent biochemical analysis. The following tissues were collected and fixed in 10% neutral buffered formalin: brain, pancreas, salivary gland, mandibular lymph node, esophagus, parathyroid, thyroid, trachea, adrenal, pituitary, heart, aorta, (thoracic and abdominal), kidney, thymus, gall bladder, liver, spleen, lung, duodenum, ileum, rectum, stomach (glandular), stomach (nonglandular), cecum, colon, jejunum, mesenteric lymph node, epididymis, prostate, seminal vesicles, testes, urinary bladder, eyes, harderian glands, nasal sections, femur with marrow, vertebra, spinal cord, inguinal mammary gland, skin/subcutis, tongue and foot pads from front feet.

Immunohistochemistry

Tissues were trimmed, embedded in paraffin and sectioned at 5 μ for hematoxylin and eosin (H&E) staining and immunohistochemistry for agalsidase alfa using a rabbit polyclonal TK8 antibody (Shire Human Genetic Therapies, Cambridge, MA) [16].

Tissue sections were pretreated by microwaving in 10mM citrate buffer, pH6.0. Primary antibody TK8 at a dilution of 1:900 or an equivalent concentration of normal rabbit IgG (Zymed, San Francisco, CA) was applied to tissue sections which then were incubated overnight at 40C. The avidin-biotin complex (ABC) technique was used with goat anti-rabbit IgG biotinylated secondary antibody (1:200 in antibody diluent) and avidin/biotinylated horseradish peroxidase from the Elite Rabbit IgG Vectastain ABC kit (PK-6101 (Vector Laboratories, Burlingame, CA). 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen and hematoxylin as counterstain. Tissues from mice injected with enzyme or vehicle control or uninjected were stained with TK8. In addition, all tissues from two mice,

one injected with enzyme and one vehicle control were stained with normal rabbit IgG. The study pathologist (M.R.A.) compared immunohistochemical reactivity in all sets of mice in order to identify immunostaining specific to enzyme injection and its cellular localization.

α -Galactosidase A Assay

The standard fluorimetric assay for alpha-galactosidase A was performed on tissue extracts as described[15,17] using 5 mM 4-methylumbelliferyl-alpha-D-galacto-pyranoside (Research Products International, M65400) at pH 4.4 in the presence of 0.1 M N-acetylgalactosamine, a specific inhibitor of alpha-galactosidase B[18].

Results

Specific staining was seen in the liver, kidney, heart, testes, adrenal gland, spleen and bone marrow in all enzyme injected mice. Specific enzyme reactivity occurred in a granular pattern in endothelium and thus, was distinguishable from lighter and more uniform nonspecific endothelial staining in other parenchymal organs. There was no difference in tissue or cellular distribution between the various time points.

In the liver, specific staining was seen in sinusoidal lining cells (endothelium and/or Kupffer cells) and hepatocytes (Figure 1). In the kidney, cytoplasmic staining occurred in cortical tubular cells but not in the glomeruli or in podocytes (Figure 2). On the other hand, in the heart only the vascular endothelial cells were stained but not myocytes (Figure 3). In the adrenal gland, staining for agalsidase alfa was present in the cytoplasm of the adrenal cortical zona fasciculata and endothelium in the cortex and medulla (Figure 4). Immunostaining in the testis was present in endothelial cells but not in seminiferous tubules. Interstitial (Leydig) cells contain endogenous biotin so this reaction is not specific (Figure 5). In the bone marrow only the capillaries showed specific agalsidase alfa staining (Figure 6). The spleen had extensive granular staining in the red pulp, likely representing endothelium and/or reticuloendothelial cells (Figure 7). Staining was seen in lymph nodes near the injection site (not shown) but none in the brain (Figure 8) and or in any of the other mouse organs (not shown). In the lungs, background staining complicated interpretation but granular staining was not obvious.

Immunoreactivity that may represent cross-reaction with another epitope occurred in a similar pattern in agalsidase alfa, vehicle control and untreated mice in a number of organs. The most common reaction was the presence of positive hematopoietic/lymphoid cells in the bone marrow and spleen. Positive cells (likely lymphoid) were uniformly present in the medulla of lymph nodes, the cortex and medulla of the thymus and in the lamina propria of the small and large bowel (not shown).

α -Galactosidase A specific activity from liver, spleen, kidney, heart, and lung homogenates at the different time points after a single injection of agalsidase alfa is shown in Figure 9. The amount of enzyme activity in organ homogenates corresponded in relative terms to the extent of immuno-staining for agalsidase alfa. However, note presence of enzyme activity in the lungs despite negative immunostaining in this organ. The calculated apparent half-life (in hours) of agalsidase alfa in these organs was: liver 47, spleen 73, kidney 36, heart 36 and lung 29.

Discussion

In this study we describe for the first time detailed tissue and cellular distribution of agalsidase alfa a form of infusible α -galactosidase A that is approved for the treatment of patients with Fabry disease. It should be emphasized that our findings indicate only the relative distribution of enzyme after a single intravenous injection rather than the absolute values. α -Galactosidase A activity in whole organs corresponded to the relative extent of tissue distribution seen on

immunohistochemistry in the same organs. As evidenced by the presence of significant enzyme activity in the lung tissue homogenates, enzyme may be present in cells even in the absence of visible immunostaining. Specific staining was consistently found in only 7 out of the 41 organs examined. Among the positively stained organs, there was a range of patterns of cellular uptake; from enzyme presence in parenchymal as well as endothelial cells in the liver and the adrenal glands, to selective uptake into certain cell types such as in the kidney, and spleen, to the heart and bone marrow where immunostaining was observed only in the vascular endothelial cells. In addition, we can assume that a lesser amount of enzyme (if any) was present in organs such as the brain that showed no visible immunostaining. The relative distribution of agalsidase alfa mirrors to some degree the clinical efficacy of ERT in patients with Fabry disease. The predominant evidence is that ERT slows the progression of the kidney disease, has a small effect on the cardiomyopathy but does not prevent cerebrovascular strokes [19,20]. It is theoretically possible that repeated administration of agalsidase alfa, as occurs in the clinical setting, is associated with a different tissue or cellular distribution of this enzyme preparation.

The mechanism of the differential uptake of agalsidase alfa in the different organs is likely complex. Agalsidase alfa is known to be taken up by cells via the ubiquitous mannose 6-phosphate (M6P) receptor [16]. However, our findings cannot be readily explained by the known tissue distribution of the M6P receptor [21,22]. This receptor is known to be present in all cell types including in non-agalsidase alfa stained cells such as brain vascular endothelial cells, cardiac myocytes and various skin cells [21,22]. On the other hand, the adrenal gland is not known to be particularly rich in M6P receptors [23]. Likewise, the staining pattern for injected agalsidase alfa does not follow the relative organ blood flows or the presence or absence of fenestrated capillaries in various organs. Fenestrated capillaries are present in the liver, the adrenal cortex, and renal tubuli that showed marked enzyme uptake, but they are also present in endocrine tissue, pancreas and sweat glands that showed no detectable immunoreactivity [24,25].

Our data confirm the ability of intravenously administered α -galactosidase A to access endothelial cells in critical organs affected by Fabry disease. This finding is supported by our observation that only vascular endothelial cells were cleared of lipid deposits in a patient who was treated for 2.5 years with the other enzyme preparation (agalsidase beta) [26,27]. It would therefore be important to further study the mechanism of uptake of agalsidase alfa into various cell types and tissues. Modification of this enzyme might allow for a better parenchymal distribution of immunostaining throughout the organs of the body. One approach might be to add a protein transduction domain such as the 11-amino acid TAT peptide.[28] Such an approach had led to a widespread tissue distribution of intravenously infused TAT- β -galactosidase [29].

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Figure 1. Liver – **A.** Agalsidase alfa injected mouse. Specific Agalsidase alfa immunoreactivity in sinusoidal lining cells (s) and in the cytoplasm of hepatocytes (arrows). **B.** Vehicle control shows no immunoreactive staining (40X).

Figure 2.

Kidney – **A.** Agalsidase alfa injected mouse shows immunoreactivity in cortical tubule cytoplasm (arrows). A glomerulus (G) shows no immunoreactivity except for focal nonspecific endothelial staining (e). **B.** Vehicle control shows no tubular or glomerular immunoreactive staining except nonspecific endothelial reactivity (e) (40X).

Figure 3.
Heart – **A.** Agalsidase alfa injected mouse shows granular staining in myocardial capillaries (arrow) but not in myocytes. **B.** Vehicle control shows no agalsidase alfa immunoreactivity (40X).

Figure 4. Adrenal gland – **A.** Agalsidase alfa injected mouse. The cortical zona fasciculata cells shows intracytoplasmic immunostaining (arrows). Sinusoidal endothelial cells (e) also are positive. **B.** Vehicle control adrenal cortical cells and endothelium show no immunoreactive staining (40X).

Figure 5. Testis – **A.** Agalsidase alfa injected mouse. Agalsidase alfa immunoreactive staining is seen in endothelial cells (arrow) but not in the seminiferous tubules. **B.** Vehicle control shows no endothelial immunoreactivity (arrow) (40X).

Figure 6.

Bone marrow – **A.** Agalsidase alfa injected mouse. Agalsidase alfa immunoreactivity is seen in vascular endothelium (arrows). Scattered positive staining of hematopoietic cells (h) is observed in agalsidase alfa and vehicle control mice. **B.** Vehicle control shows no endothelial staining (arrows) although scattered non-specific staining of hematopoietic cells (h) is present (40X).

Figure 7.
Spleen – **A.** Agalsidase alfa injected mouse. Extensive granular immunoreactive staining is seen in the red pulp and in endothelium and/or reticuloendothelial cells. **B.** Vehicle control shows no immunoreactivity in the red pulp (40X).

Figure 8.
Brain – **A.** Agalsidase alfa injected mouse, no immunoreactive staining is observed. **B.** Vehicle control, no immunoreactivity (40X).

Figure 9.

Specific α -galactosidase A activity in whole organ homogenates at different time points after a single injection of 0.5 mg/kg agalsidase alfa to α -galactosidase A knockout mouse. Each time point represents the mean of values from two mice.