Comparative Effects of Recombinant Acid Sphingomyelinase Administration by Different Routes in Niemann-Pick Disease Mice

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Abstract: An inherited deficiency of acid sphingomyelinase (ASM) activity results in the Type A and B forms of Niemann-Pick disease (NPD). The aim of this study was to evaluate the effects of recombinant human ASM (rhASM) replacement therapy on the mouse model, by comparing different routes of administration. Eight NPD mice received rhASM via an intravenous injection (IV) administered at a dose of 1 mg/kg and another group of 8 NPD mice received the same dose by subcutaneous injection (SC). The plasma levels of ASM activity in intravenously administered mice were significantly elevated immediately after injection. In contrast, in the subcutaneously injected mice, the level of ASM activity was maximal 6 h after injection. The levels of ASM activity in both groups had declined substantially by 2 days after injection. It was concluded that rhASM administered by subcutaneous injection is completely absorbed, and offers a similar efficacy to intravenously administered recombinant enzyme.

Key words: Acid sphingomyelinase, enzyme replacement therapy, lysosomal storage disorder

Introduction

Types A and B Niemann-Pick disease are two clinically distinct forms of an inherited lysosomal storage disorder resulting from the deficient activity of acid sphingomyelinase (ASM; sphingomyelin phosphodiesterase; E.C. 3.1.2.14) [1]. ASM is responsible for hydrolyzing the lipid, sphingomyelin (SPM), to ceramide and phosphocholine, and both forms of Niemann-Pick disease are characterized by extensive lipid storage in various cells and tissues.

Enzyme replacement therapy was first suggested as an approach to the treatment of lysosomal storage disorders over thirty years ago [2]. During the past decade the isolation of genes encoding most lysosomal enzymes and the development of expression systems that produce large quantities of recombinant proteins has led to the successful treatment of several lysosomal storage diseases in animal models, providing “proof of principle” for this approach [2, 3]. In addition, enzyme therapy has become available for three human lysosomal storage diseases, Type 1 Gaucher disease, Fabry

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disease, and mucopolysaccharidosis type 1 [4–6], and clinical trials are underway for several other disorders [7–10]. However, enzyme therapy is limited by short enzyme half-life and thus requires continuous intravenous (IV) infusion. Thrombosis, paradoxical embolism, and treatment interruptions due to occlusion and logistical difficulty have all been reported with intravenous administration. Therefore, as an alternative, subcutaneous (SC) administration has been proposed [11].

The goal of the current study was to evaluate the efficacy of rhASM administered either IV or SC directly and to examine the pharmacokinetics of the active metabolite in a NPD mouse model. The data support the efficacy of SC enzyme replacement therapy using rhASM and may present a viable alternative to current enzyme replacement therapy for NPD patients.

Materials and Methods

Animals

The NPD mouse model was created by gene targeting as described previously [12]. The NPD knockout mouse has been used to study the pathogenesis and treatment of type A and B NPD [13]. Affected mice have no detectable ASM activity, but develop normally until ~8 weeks of age, when ataxia and mild tremors become noticeable. The disease then follows a neurodegenerative course that leads to death between 6 and 8 months of age. Characteristic lipid-laden foam cells (NPD cells) are found in most major organs, associated with elevated sphingomyelin levels. Affected mice can be distinguished from normal animals using a PCR-based assay [12]. The animals in our study were maintained on a 12 h light/dark cycle with water *ad libitum* and Purina rodent chow 5001. All animals were used in compliance with the *Guideline for the Care and Use of Laboratory Animals*, Mount Sinai School of Medicine.

Enzyme administration

Two groups of test animals were injected with purified recombinant human acid sphingomyelinase (rhASM) (1.4 mg/ml, GENZYME). The doses of 1 mg rhASM/kg of mouse body weight were administered by IV and SC injection with a Bioinjector (BIOJECT®, Portland, OR) into 5-month-old NPD mice (8 mice each per group). Plasma was taken at 0, 5, 10, 30 min, 1, 2,

4, 6 h and 1, 2, 3, 4, 5 days after injection for biochemical analyses. Mice received injections every other day for 1 week (3 injections) and were sacrificed 48 h after the last injection to assess their tissue ASM activity and SPM content.

ASM activity and protein assays

The ASM activity was determined as previously described [14, 15]. A fluorescence-based, high performance liquid chromatography method was used to measure ASM activity in macrophage lysates. Briefly, equal volumes of cell lysates (prepared by 3 cycles of freeze-thaw in 0.01 M Tris-HCl buffer, pH 7.0, containing 0.25% Triton X-100) and 0.2 M BODIPY®-labeled C₁₂-sphingomyelin (Molecular Probes, Eugene, OR) diluted in assay buffer (0.1 M sodium acetate, pH 5.0, containing 0.1 mM ZnCl₂, and 0.5% Triton X-100), were incubated at 37°C for 60 min. The reaction was stopped by the addition of ethanol, and the hydrolytic product (BODIPY® C₁₂-ceramide) was detected and quantified by chromatographic analysis using a reverse phase column (Aquasil C-18, Keystone Scientific Inc., St. Marys, PA). Total protein was determined using the Bio-Rad protein assay kit according to the manufacturer's instructions.

Tissue processing and lysenin immunostaining

At 48 h after the last injection, injected animals and control littermates were anesthetized with avertin and sacrificed by cardiac perfusion. After post-fixed tissues were cryoprotected in 30% sucrose, they were cut into 15 µm coronal sections using a cryostat. Frozen sections were treated with blocking buffer (PBS, pH 7.4, containing 1% (w/v) BSA) for 1 h. The slides were then incubated with 1 µg/ml lysenin, a sphingomyelin-specific binding protein [16] in 1% BSA-PBS for 2 h, washed with 1% BSA-PBS, and then incubated with anti-lysenin antiserum (1:1000 dilution in blocking buffer) for 1 h. The slides were washed in 1% BSA-PBS and incubated with Alexa Fluor 568-conjugated anti-rabbit IgG (Molecular Probe, Eugene, OR) for 1 h. Nuclei were counter-stained by mounting sections under 1.5 mm thick coverslips in Vectashield with DAPI. The sections were then analyzed using a Nikon Eclipse E800 fluorescence microscope.

Fig. 1. ASM activities in the plasma of rhASM treated NPD mice after IV injection. Data are expressed as mean $\pm$ SEM ($n=8$).

Fig. 2. ASM activities in the plasma of rhASM treated NPD mice after SC injection. Data are expressed as mean $\pm$ SEM ($n=8$).

Results

IV or SC administration of rhASM gave rise to comparable ASM activities in plasma of NPD mice

The levels of ASM activity in IV injected mice elevated significantly immediately after injection (Fig. 1). In contrast, the levels of ASM activity in SC injected mice were maximal 6 h after injection (Fig. 2). Thereafter, the level of ASM activity in SC injected mice declined, substantially 2 days after injection.

Effect of the IV or SC administration on ASM activity in various tissues

NPD mice received injections of rhASM (1 mg/kg) every other day for 1 week and were sacrificed 48 h after the last injection to assess their tissue ASM activity. The level of ASM activity was increased in the livers of IV injected NPD mice, whereas it was increased in the spleen, lungs, hearts, and kidneys of SC injected NPD mice (Fig. 3). The level of ASM activity was increased in the cerebellums of SC injected NPD mice (Fig. 3), however, analysis of brain sections from the treated NPD mice revealed massive Purkinje cell drop-out equivalent to that observed in non-treated NPD mice (data not shown), suggesting that the injected rhASM did not cross the blood-brain barrier to a significant degree.

Sphingomyelin levels in the spleen

Representative tissue sections from the spleen of rhASM treated mice and non-treated NPD mice are

Fig. 3. ASM activities in tissues of rhASM treated NPD mice at 2 days after last injection, in the IV injected (open bar) and SC injected (closed bar) NPD mice. Data are expressed as mean $\pm$ SEM ($n=8$).

shown in Fig. 4. We used lysenin, a sphingomyelin-specific binding protein, to assess sphingomyelin storage in the spleen sections from rhASM treated mice. As shown Fig. 4, lysenin staining in IV and SC injected NPD mice were slightly weaker compared with those tissues from non-treated NPD mice.

Discussion

In recent years, significant efforts have been devoted to the evaluation of enzyme replacement treatment for lysosomal storage diseases. ASM-deficient NPD is considered an excellent candidate for enzyme replacement therapy, particularly for patients without primary neu-

Fig. 4. Lysenin staining of spleen sections from the representative rhASM IV injected NPD, rhASM SC injected NPD, non-treated NPD, and normal control mice at 2 days after last injection. Original magnification $\times 40$.

rological involvement [1]. Previous studies have shown that intravenous injection of “complex” type rhASM obtained from Chinese hamster ovary cells into NPD mice reduced sphingomyelin storage in various visceral organs, including the liver, spleen and heart, and to a less degree, in the lung [17, 18]. However, the treatment is limited by the very short half-life of continuous IV infusion and interrupted by serious problems with the methodology; therefore, subcutaneous administration has been proposed.

Figures 1 and 2 show that IV or SC administration of rhASM gives rise to comparable ASM activities in plasma of NPD mice. The levels of ASM activity in IV injected mice were significantly elevated immediately after injection. In contrast, the levels of ASM activity were maximal in SC injected mice 6 h after injection. The levels of ASM activity in both groups decreased, substantially 2 days after injection. NPD mice were successfully administered with rhASM (1 mg/kg) via IV and SC routes every other day for 1 week, and were sacrificed 48 h after the last injection

to assess their tissue ASM activity. Figure 3 shows that levels of ASM activity were increased in the livers of IV injected NPD mice, whereas the levels of ASM activity were increased in the spleen, lungs, cerebellums, hearts, and kidneys of SC injected NPD mice. We used lysenin to assess sphingomyelin storage in the spleen sections from rhASM treated mice. As shown in Fig. 4, the amount of lysenin staining in IV and SC injected NPD mice was slightly decreased compared with those tissues from non-treated NPD mice. The rhASM enzyme replacement therapy for the NPD mice led to improved storage in visceral organs without detectable neurological changes.

The present results have provided important information for the future development of enzyme replacement therapy, and SC infusion offers a similar efficacy of rhASM enzyme replacement therapy as IV infusion. This represents a far superior method of treatment for confused patients and in cases which have difficulty with IV puncture.

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