Dexamethasone-mediated Upregulation of the Mannose Receptor Improves the Delivery of Recombinant Glucocerebrosidase to Gaucher Macrophages.

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Abbreviations: LSEC, liver sinusoidal endothelial cells; MEM, minimal essential medium; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate buffer saline.

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Abstract

Enzyme replacement therapy for Gaucher disease utilizes a recombinant glucocerebrosidase (Cerezyme) whose oligosaccharide chains have been remodeled to expose the core mannose residues. This modification promotes the uptake of the hydrolase by Gaucher-affected macrophages via mannose receptor-mediated endocytosis. However, studies revealed that amounts of the infused enzyme were also delivered to other mannose receptor-bearing cells such as the liver sinusoidal endothelial cells. To maximize the delivery of Cerezyme to macrophages, agents that increased the cell surface levels of the mannose receptor specifically on macrophages were examined. Treatment with dexamethasone improved the in vitro uptake of Cerezyme by a macrophage but not by liver sinusoidal endothelial or hepatocyte cell lines. The enhanced uptake by the macrophages was due to an increase in surface mannose receptors as the activity could be blocked by the addition of mannans. Pretreatment of rats with the glucocorticoid also preferentially enhanced the delivery of Cerezyme to the Kupffer cells and splenic macrophages. This effect of dexamethasone also applied to substrate-laden macrophages isolated from Niemann-Pick A mice. Together, these data suggest that pretreatment with dexamethasone could specifically enhance the presentation of mannose receptors on Gaucher macrophages with resultant improvement in delivery of the enzyme to the affected cells.
Introduction

Gaucher disease is an inherited lysosomal storage disorder that is caused by a deficiency of the lysosomal hydrolase, glucocerebrosidase (Beutler and Grabowski, 2001). This deficiency results in the progressive accumulation of undegraded glucosylceramide primarily in the macrophages of the liver, spleen, lung, bone marrow and in the most severely affected subjects, the central nervous system. Consequently, affected individuals demonstrate hepatosplenomegaly, hematological abnormalities, bone lesions, pulmonary disease and in severe instances, neurological disease. Type 1 Gaucher disease, the most prevalent, is nonneuropathic while Types 2 (acute) and 3 (chronic) are characterized by neurological disease, an earlier age of onset, and significantly more severe systemic clinical symptoms. This spectrum of disease severity correlates inversely with the level of residual glucocerebrosidase activity, with the Type 2 and 3 variants generally exhibiting lower levels of activity than Type 1.

Presently, Type I Gaucher disease is treated using a recombinant glucocerebrosidase (Cerezyme) that has been remodeled by sequential digestion of the oligosaccharide side chains to expose the core mannose residues (Furbish et al., 1981; Murray, 1987; Brady et al., 1994). This modification enhances the recognition and uptake of the enzyme by the mannose receptor on macrophages, the primary cells affected in Gaucher disease. Additionally, by removing the galactose residues, it also served to minimize the interaction of the enzyme with other carbohydrate receptors such as the asialoglycoprotein receptor present on hepatocytes (Furbish et al., 1981; Lodish, 1991). However, because mannose receptors are also resident on other cell types such as the liver sinusoidal endothelial cells (Magnusson and Berg, 1989; Stang et al., 1990) a significant proportion of the infused mannose-terminated glucocerebrosidase is also internalized by these non-affected cells. Biodistribution studies in mice showed that although remodeling significantly improved the delivery of Cerezyme to the
macrophages, approximately 80-90% of the enzyme was still internalized by the parenchymal and endothelial cells (Bijsterbosch et al., 1996; Xu et al., 1996; Friedman et al., 1999). This uptake of Cerezyme by the unaffected parenchymal and endothelial cells may underlie the need for frequent maintenance infusions of large doses of enzyme for therapeutic effect (Beck et al., 1997; Beutler, 1997; Weinreb et al., 2002). Although increasing the dose of Cerezyme may compensate for the relative inefficiency in delivering Cerezyme to the affected macrophages, higher doses could saturate the receptors available on the cell surface. Indeed, mannose receptor-mediated uptake of Cerezyme has been shown to be saturatable when administered at doses greater than 50 U/kg body weight, at least when tested in mice (Mistry et al., 1996; Friedman et al., 1999).

To enhance the delivery of Cerezyme to the target macrophage cells and thereby the efficacy of enzyme replacement therapy, we sought to determine the utility of increasing the abundance of mannose receptors specifically on the surface of these cells. Several agents are reportedly capable of modulating the density of the mannose receptor on macrophages, some of which have been approved for human clinical use (Mokoena and Gordon, 1985; Shepherd et al., 1985; Schreiber et al., 1990; Harris et al., 1992; Stein et al., 1992; Shepherd et al., 1994; Montaner et al., 1999; Piemonti et al., 1999). In this report, we examined the ability of one of these agents, the glucocorticoid steroid dexamethasone, to increase the expression of the mannose receptor specifically on macrophages. We showed that pretreatment with dexamethasone resulted in a greater uptake of Cerezyme by macrophages both in vitro and in vivo. Consequently, it may be possible to enhance the efficacy of enzyme replacement therapy for Gaucher disease or reduce the dose needed for treatment through the use of this strategy.
Materials and methods

Reagents and chemicals

Dexamethasone for the in vitro studies was purchased from CalBiochem Inc., (San Diego, CA, USA) while that for the in vivo studies (water-soluble dexamethasone sodium phosphate) was from American Reagent Laboratories Inc. Bovine serum albumin, yeast mannan and other common chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. The macrophage-specific monoclonal antibody CD11b and 0.5 µm red fluoresbrite microbeads were from Serotech (Raleigh, NC, USA) and PolyScience Inc., (Warrington, PA, USA) respectively. Preclinical grade unmodified and remodeled recombinant glucocerebrosidase (Cerezyme) was from Genzyme Corp (Boston, MA, USA). All cell culture reagents were from Gibco-BRL Life Science Technologies, Inc. (Carlsbad, CA, USA). The anti-von Willebrand factor antibody was purchased from Cell Systems, Inc. (Kirkland, WA, USA).

Cell culture conditions

The rat alveolar macrophage cell line NR8383 (CRL-2192) obtained from the American Type Tissue Culture Collection (ATCC) (Manassas, VA, USA) was cultured in Kaighn’s modified F12K nutrient mixture supplemented with 2 mM L-glutamine, 15% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin. Hep3B cells were grown in MEM with Earles salts, 10% FBS, non-essential amino acids, sodium pyruvate, penicillin and streptomycin. Human liver sinusoidal endothelial cells (LSEC) were obtained from Applied Cell Biology Research Institute through Cell Systems, Inc. LSEC were cultured in CS-C Media containing 10% FBS. Culture dishes or flasks were routinely pretreated with attachment factor solution prior to seeding with the cells.
Cerezyme uptake in vitro

Macrophages, hepatocytes and LSEC were grown under the conditions described above and were treated with dexamethasone or other drugs as indicated in the figure legends. When treatment was for greater than 2 days, fresh drug was added to the culture every 24 h. For the Cerezyme uptake studies using the macrophage cell line NR8383, the cells were first harvested from the culture flasks using phosphate-buffered saline (PBS). After washing with Kaghan’s media, the cells were resuspended in uptake media (Kaghan’s media containing 4 mg/ml bovine serum albumin (BSA) and 25 mM Hepes, pH 6.8) to a final concentration of $10^6$ cells/ml. One ml of the cells was then distributed into 1.5 ml microcentrifuge tubes in duplicate. Where appropriate, yeast mannan (dissolved in deionized water) was added to a final concentration of 2 mg/ml to inhibit mannose receptor-mediated uptake of the enzyme. Following the addition of Cerezyme (1 U/ml) the cells were incubated at 37°C for 2 h with periodic mixing every 15 min. The cells were then pelleted by centrifugation at 1000 g for 3 min in a microcentrifuge, washed twice with 1 ml of ice-cold PBS containing 1 mg/ml of yeast mannan and twice more with PBS alone. Cell pellets were dissolved in 1 ml of 50 mM potassium phosphate, 0.25% Triton X-100, pH 6.5 (KP buffer) containing complete protease inhibitors (Roche, Indianapolis, IL, USA). For the Cerezyme uptake studies with Hep3B cells and LSECs, the procedure used was similar to that described above for macrophages except that these studies were performed with adherent cells. The uptake media used with these cells was DMEM containing 4 mg/ml BSA and 25 mM Hepes, pH 6.8. Following incubation with Cerezyme, the wells were washed twice with PBS containing 1 mg/ml yeast mannan and twice more with PBS and then lysed in 1 ml KP buffer as for the macrophages. Lysates were assayed for glucocerebrosidase activity using the artificial fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside as described previously (Marshall et al., 2002). Protein levels were quantified using the MicroBCA protein assay kit (Pierce, Rockford, IL, USA).
Cerezyme uptake in vivo

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, NIH Publication No 86-23). To assess the effect of dexamethasone on Cerezyme uptake by Kupffer cells in vivo, Fisher rats (female, ~130 g) were divided into four groups (n=3 animals/group). Two groups of rats received vehicle (PBS) without dexamethasone. The other two groups were administered water-soluble dexamethasone sodium phosphate intraperitoneally at a dose of either 10 µg or 100 µg/rat. For the treated groups, 4 doses of dexamethasone were given over two consecutive days, one dose in the early morning and one dose in the late afternoon of each day. On the third day, 2.5 U Cerezyme (~18 U/kg) was administered intravenously (via the tail vein as a bolus injection) to one group of rats that had been mock-treated with PBS and two groups of rats that had been pretreated with dexamethasone. Two hours after the injections, the rats were euthanized and their livers harvested and fixed in 2% paraformaldehyde (Electron Microscopy Sciences (EMS), Fort Washington, PA, USA) and 0.01% glutaraldehyde (EMS) in PBS for 1 h at room temperature. The livers were then placed into an ice-cold 30% sucrose solution and incubated overnight at 4°C. The tissues were embedded in tissue-freezing OCT compound (EMS) and 5 µm sections were prepared. Liver sections were stained for macrophages using the antibody CD11b (Serotech) and for human glucocerebrosidase using the antibody 1B5 and then visualized by immunofluorescence microscopy. A goat anti-mouse IgG conjugated to Alexa568 and a goat anti-rat IgG conjugated to Alexa488 were used to visualize the macrophages and enzyme respectively.

Quantitation of the effects of dexamethasone on Cerezyme uptake in vivo was performed with splenic macrophages isolated using the procedure by Gessani et al. (2000). Fisher rats (female, ~130 g) were treated with dexamethasone and Cerezyme as described above. Following treatment, the spleens were harvested and then cut into
small pieces. The spleen cells were released by gently squeezing the pieces between two frosted cover slides. The released cells were washed with 15 ml of RPMI media containing 10% heat-inactivated FBS and the red blood cells lysed using red blood cell lysis buffer (Sigma). The isolated splenocytes were seeded onto 150-mm tissue culture dishes and incubated at 37°C for 1 h to allow the splenic macrophages to attach. After removing the unattached cells, the attached cells (predominantly macrophages) were washed five times with PBS and then lysed in KP buffer. The lysates were then assayed for glucocerebrosidase activity and protein levels as described above. The purity of the isolated macrophages was confirmed by staining with the macrophage-specific antibody CD11b. The phagocytic activity of the isolated macrophages was also assessed by incubating them with 0.5 µm red fluoresbrite beads (Polyscience) for 1 h at 37°C. The cells were pelleted, washed three times with PBS, treated with 4% paraformaldehyde and stained with a rabbit anti-bovine CI-MPR antibody followed by a goat anti-rabbit IgG antibody conjugated to Alexa 546 (Molecular Probes, Eugene, OR, USA). Confocal microscopy was used to determine if the microbeads were on the cell surface or in the interior of the cells.

The total amount of glucocerebrosidase present in the spleens was also measured by homogenizing the tissues in KP buffer (1 ml KP buffer/100 mg wet tissue). Following centrifugation at 14,000g for 15 min, the supernatants were collected and assayed for glucocerebrosidase activity using the artificial fluorogenic substrate as described above. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls multiple comparison test.

**Uptake studies using alveolar macrophages from Niemann-Pick A mice**

Lung alveolar macrophages were isolated according to the protocol of Brain and Frank (1968) from 4-month old acid sphingomyelinase knockout mice (Dhami et al., 2001) that had been pretreated with dexamethasone for 3 consecutive days. Aveolar
macrophages isolated from non-treated Niemann-Pick A mice were used as controls. The isolated macrophages were seeded onto 12-well tissue culture plates and incubated at 37°C for 1 h to allow for attachment. Cerezyme uptake studies and enzyme assays were then performed as described for the liver cells.
Results

Effect of dexamethasone on in vitro uptake of recombinant glucocerebrosidase

In an attempt to further improve the delivery of Cerezyme to macrophages, the target cells affected in Gaucher disease, we evaluated the relative utility of agents capable of upregulating the mannose receptor on these cells. Figure 1 shows the results obtained with one such agent, dexamethasone, on Cerezyme uptake by alveolar macrophage (NR8383), hepatocyte (Hep3B) and liver sinusoidal endothelial (LSEC) cells. The cells were pretreated with dexamethasone for two consecutive days after which the extent of Cerezyme uptake by the different cells was assayed and compared to that of untreated controls. Incubation of the untreated macrophages and LSEC cells with Cerezyme for 2 h showed that these cells were capable of internalizing the enzyme (Figure 1A and B). Uptake of Cerezyme by the macrophages could be completely inhibited, and that by LSEC, partially inhibited by the addition of yeast mannan. This suggests that uptake of the modified enzyme by the macrophages was mediated primarily through the mannose receptor while that by LSEC involved the mannose but also other receptors. Three other members of the mannose receptor family (the M-type phospholipase A(2) receptor, DEC-205 and Endo180) have been identified and their binding to sugars and expression in various cell types characterized (East and Isacke, 2002). Endo 180 has been shown to be expressed on various cell types including fibroblasts, endothelial cells and macrophages, and binding to mannose is not as avid as binding to N-acetylglucosamine (Sheikh et al., 2000). Therefore, receptors on LSEC that bind Cerezyme via these other receptors (Sato and Beutler, 1993) may account for the population of internalized enzyme that was resistant to inhibition by mannan.

Macrophages that had been pretreated with dexamethasone exhibited an approximately 3-fold greater uptake of Cerezyme when compared to the untreated controls (Figure 1A). Although we did not quantify the mannose receptors on
macrophages following dexamethasone treatment, the enhanced uptake of Cerezyme by the dexamethasone-treated macrophages was most likely the result of increased surface expression of the mannose receptor since the uptake could be completely inhibited by the addition of yeast mannan. Moreover, the ability of dexamethasone to enhance the expression of mannose receptors on macrophages has been reported previously by Shepherd et al. (1985). Associated with this increase in the presentation of the mannose receptor on the cell surface of dexamethasone-treated macrophages was an enhancement in the uptake of lysosomal enzymes. This increased uptake of enzymes may explain the observed increase in the levels of endogenous glucocerebrosidase activity (~130% of untreated control) following dexamethasone treatment (compare first and fourth bar in Figure 1A). In addition, the stimulatory effect of dexamethasone on Cerezyme uptake by the macrophages was time dependent. A minimum of two days of pretreatment was necessary before any increase in uptake of the enzyme was observed (data not shown). This timing correlates well with that reported for the de novo transcription and translation of the mannose receptor by macrophages following treatment by dexamethasone (Cowan et al., 1992). On the contrary, this increase in Cerezyme uptake was not observed in the dexamethasone-treated LSEC cells, indicating that dexamethasone had differential effects on the two different cell types (Figure 1B). The hepatocyte cell line internalized Cerezyme poorly irrespective of whether it was pretreated with dexamethasone (Figure 1C). This observation is consistent with previous findings that hepatocytes do not express the mannose receptor (Takahashi et al., 1998). Hence, pretreatment with dexamethasone had the effect of selectively increasing the uptake of Cerezyme by the macrophage but not by the LSEC or hepatocyte cells in vitro.
Pretreating rats with dexamethasone enhanced the uptake of Cerezyme by Kupffer cells

Having demonstrated that dexamethasone could differentially affect the uptake of Cerezyme by macrophages, hepatocytes and endothelial cells in vitro, we next examined its effect in vivo. Rats were administered 2.5 U Cerezyme (~18 U/kg), an amount determined previously to result only in minimal or no immunostaining of liver sections using the anti-human specific glucocerebrosidase antibody, 1B5. The liver thin sections were also stained with an anti-macrophage antibody (CD11b) to visualize the Kupffer cells. As shown in Figure 2, liver sections from rats pretreated with dexamethasone consistently generated greater staining of Cerezyme in Kupffer cells than in the untreated animals. Moreover, a greater number of Kupffer cells stained positive for Cerezyme when a higher dose of dexamethasone was used. These in vivo data support the observations in vitro that pretreatment with dexamethasone can result in enhanced uptake of Cerezyme by the Kupffer cells.

Pretreatment with dexamethasone also increased the uptake of Cerezyme by splenic macrophages in vivo

To provide a more quantitative assessment of the effect of dexamethasone in vivo, splenic macrophages were purified from rats treated with the drug and the extent of Cerezyme uptake determined. The spleen, another organ that is profoundly affected in Gaucher disease, was selected for these studies because of the greater ease with which the macrophages could be isolated and purified from this tissue. Splenic macrophages were isolated and enriched using the method of Gessani et al. (2000) that is based on the preferential ability of macrophages to attach to culture plates. Immunofluorescence studies using the macrophage-specific antibody CD11b confirmed that this method routinely generated preparations that were greater than 90% macrophages (data not shown). This contrasts with the less than 10% labeling in the total splenocyte fraction,
which is consistent with the reported percentage of macrophages in spleen. Most of the isolated macrophages also retained their phagocytic activity as indicated by their ability to internalize fluorescent 0.5 µm microbeads (data not shown).

Rats that had been pretreated with either dexamethasone or vehicle were injected with Cerezyme and their splenic macrophages then purified as indicated above and assayed for glucocerebrosidase activity. As shown in Figure 3, macrophages isolated from rats that had been pretreated with dexamethasone and administered 18 U/kg Cerezyme had much higher levels (approximately 2-fold after subtracting the endogenous activity level) of activity than the corresponding vehicle-treated animals. These studies were repeated in 3 independent experiments with similar results for a total of 17 rats in each group.

The effect of dexamethasone in animals administered different doses of Cerezyme was also examined (Figure 3). Administration of 72 U/kg Cerezyme, a dose approximating that used to treat Gaucher patients and shown previously to saturate the mannose receptors in humans (Mistry et al., 1996), generated results similar to those observed with 18 U/kg. In all cases, pretreatment with dexamethasone resulted in an approximately 2-3 fold greater uptake of Cerezyme by the rat splenic macrophages when compared to their respective vehicle-treated counterparts. When adjusted for dose, the dexamethasone-mediated increase in Cerezyme uptake was estimated to be 3-4 fold. For example, the extent of Cerezyme internalized by splenic macrophages of animals pretreated with dexamethasone and 18 U/kg enzyme was similar to that of animals administered vehicle and 72 U/kg Cerezyme (Figure 3). Analysis of the total spleen homogenates also revealed higher glucocerebrosidase activities in the animals that had been pretreated with dexamethasone (data not shown). However, the magnitude of the increase was lower than in the purified macrophages. This may be expected if the macrophage population within the total splenic homogenate was primarily responsible for internalizing the infused Cerezyme. Together, these results suggest that
dexamethasone-mediated upregulation of the mannose receptors (Mokoena and Gordon, 1985; Shepherd et al., 1985; Shepherd et al., 1994) improved the delivery of Cerezyme to macrophages in vivo, the cells that are primarily affected in Gaucher disease.

**Dexamethasone pretreatment also increased the uptake of unmodified recombinant glucocerebrosidase by splenic macrophages.**

The effect of dexamethasone on the uptake of unmodified recombinant glucocerebrosidase was also examined. Unlike Cerezyme, which has been specifically remodeled to expose the core mannose residues on its oligosaccharide side chains, unmodified recombinant glucocerebrosidase contains only a few exposed mannose residues by virtue of a single high-mannose oligosaccharide side chain (Friedman et al., 1999). Interestingly, despite having a lower abundance of exposed mannose residues, the uptake of the unmodified enzyme by splenic macrophages in vivo was also enhanced to a similar extent as the remodeled Cerezyme by dexamethasone pretreatment (Figure 4). Hence the effect of dexamethasone would appear to be applicable to both unmodified and carbohydrate-remodeled recombinant glucocerebrosidase.

**Substrate-laden alveolar macrophages are also responsive to dexamethasone treatment.**

Since Gaucher macrophages, unlike the normal macrophages used in the above studies are laden with accumulated glucosylceramide, the effect of dexamethasone on substrate-burdened macrophages was also examined. In the absence of a viable animal model of Gaucher disease that accumulated extensive amounts of glucosylceramide in the macrophages, we elected to investigate the effects of dexamethasone on macrophages from the Niemann-Pick A mouse. The Niemann-Pick A mouse lacks acid
sphingomyelinase activity and shows extensive lysosomal accumulation of sphingomyelin in the reticuloendothelial system of liver, spleen, bone marrow and lung (Horinouchi et al., 1995; Dhami et al., 2001). Although the substrate that accumulates in the macrophages of Gaucher disease and Niemann-Pick A disease is different, the similarity in the pathology suggests that the macrophages from the Niemann-Pick A mouse may be an appropriate surrogate for those from Gaucher disease. The alveolar macrophages isolated from the Niemann-Pick A mouse reportedly also exhibit altered functions, suggesting that the diseased macrophages may respond differently to dexamethasone (Dhami et al., 2001). To test this, alveolar macrophages were lavaged from the lungs of Niemann-Pick A mice that were either pretreated with dexamethasone or vehicle for three days. The harvested alveolar macrophages were then plated onto culture plates and their ability to internalize Cerezyme assayed. Despite having extensive lysosomal storage, the alveolar macrophages that had been pretreated with dexamethasone showed significantly higher levels (approximately 3-fold) of Cerezyme than the vehicle-treated controls (Figure 5). The uptake of Cerezyme was inhibited by mannans indicating that internalization was primarily mediated via the mannose receptor. Hence, lysosomal storage of sphingomyelin in the alveolar macrophages did not affect the ability of dexamethasone to upregulate the mannose receptors in vivo. This observation supports the possible use of dexamethasone to enhance the delivery of Cerezyme to Gaucher-affected macrophages.
Discussion

Treatment with dexamethasone specifically enhanced the uptake of remodeled recombinant glucocerebrosidase by macrophages in vitro and in vivo

The target cells for treatment by enzyme replacement therapy in Gaucher Type I disease are the tissue macrophages of the liver, spleen, lung and bone marrow. The finding of the mannose receptor on macrophage cells led to the development of a macrophage-targeted glucocerebrosidase (Cerezyme) through remodeling of the enzyme’s carbohydrate moieties (Furbish et al., 1981; Brady et al., 1994). This modification proved to be essential for efficient delivery of the infused recombinant enzyme to the macrophages through mannose receptor-mediated endocytosis. However, biodistribution studies indicated that although more of the remodeled enzyme was delivered to the macrophages compared to the unmodified enzyme, the liver sinusoidal endothelial cells, which also bear the mannose receptor, internalized a significant amount of the infused enzyme (Bijsterbosch et al., 1996; Xu et al., 1996; Friedman et al., 1999). To further improve the delivery of the enzyme to the macrophages, approaches that specifically upregulated the mannose receptor on this cell type were evaluated. It was hypothesized that increasing the presentation of mannose receptors on the macrophages would lead to greater receptor-mediated uptake of the enzyme and thereby the therapeutic efficacy of Cerezyme.

The levels of the mannose receptor on macrophages have been shown to be susceptible to modulation by a variety of inflammatory and anti-inflammatory agents. Addition of the cytokines IL-10 and interferon-γ to macrophages results in down-regulation of the receptor, while treatment with dexamethasone, IL-4 and prostaglandin E up-regulates its expression and in some instances, its activity as well (Shepherd et al., 1985; Cowan et al., 1992; Stein et al., 1992; Schreiber et al., 1993; Montaner et al., 1999). In the case of the clinically approved glucocorticoid dexamethasone, it has been
reported that its primary effect is to induce the synthesis of receptor protein through an increase in the level of mannose receptor mRNA (Shepherd et al., 1985; Cowan et al., 1992). Here, we demonstrated that treatment in vitro with dexamethasone resulted in an increase in mannose receptors specifically on a macrophage cell line but not liver sinusoidal endothelial or hepatocyte cell lines as evidenced by a greater uptake of Cerezyme. The enhanced uptake was consistent with being strictly mannose receptor dependent as it could be completely inhibited by yeast mannan. This differential effect of dexamethasone on uptake by the different cell types would appear to be recapitulated in the spleens and livers of animals treated with Cerezyme. Immunofluorescence studies using a human glucocerebrosidase-specific antibody indicated that a greater amount of the infused Cerezyme was internalized by the Kupffer cells in dexamethasone-pretreated animals. A similar increase in Cerezyme uptake by splenic macrophages was also detected with dexamethasone pretreatment. Dexamethasone-treated rats had greater amounts of enzyme delivered to their splenic macrophages than did non-treated animals despite the use a 4-fold higher dose of enzyme in the latter group. When adjusted for dose, this represented an approximately 3-fold increase in Cerezyme uptake by the dexamethasone-treated splenic macrophages. The extent of the increase in uptake is consistent with that reported for the synthesis of mannose receptor following dexamethasone treatment (Shepherd et al., 1985; Cowan et al., 1992). This effect of dexamethasone was also realized in lipid-laden macrophages as maybe encountered in Gaucher-affected tissues. Sphingomyelin-filled alveolar macrophages isolated from a mouse model of Niemann-Pick A disease that had been pretreated with dexamethasone also exhibited a higher propensity to internalize Cerezyme compared to untreated controls. This indicates that the mannose receptors on alveolar macrophages were also upregulated. Hence, it is likely that this strategy should also allow for improved delivery of Cerezyme to Gaucher-affected cells. By extension, it should also apply to the improved delivery of acid sphingomyelinase to Niemann-Pick A-affected macrophages.
1,25-Dihydroxyvitamin D3 has also been reported to upregulate the mannose receptor on macrophages in vitro (Clohisy et al., 1987). However, attempts to use this agent in combination with Cerezyme in the clinic revealed no apparent increase in efficacy (Grabowski et al., 1998). Since no details of this trial has been reported thus far, the reason for the discrepancy in our animal studies with the human study using this strategy is difficult to reconcile. However, in light of this observation, we should be cautious about extrapolating the possible application of this strategy to humans.

**Potential benefits of pretreatment with dexamethasone on enzyme replacement therapy for Gaucher disease**

Although enzyme replacement therapy for Gaucher Type I disease has been shown to be effective, there remains debate as to the optimal dose and frequency of administration of the enzyme (Altarescu et al., 2000; Beutler, 2000). Studies to evaluate various treatment regimens, including the use of a high dose with a low frequency of re-infusion or a low dose with a high frequency of re-infusion have been equivocal due to the broad spectrum of disease severity among patients (Beutler, 2000). A regimen that is commonly used to attain a clinical response in patients involves the use of a high dose (60 U/kg) and low frequency of re-infusion (biweekly). Since infusion time (for 60 U/kg) can be approximately 2 hours, interventions that support a reduction in dose or infusion interval are desirable. The demonstration here that pretreatment with dexamethasone could effect a greater uptake of Cerezyme by tissue macrophages might allow for a reduction in dose and therefore the time for infusion of the enzyme.

Another aspect of enzyme replacement therapy for Gaucher disease is the relatively long duration of treatment (several months) necessary to completely reverse the disease manifestations (Brady and Barton, 1994; Weinreb et al., 2002). A possible explanation may be that the amount of enzyme delivered to the target macrophages, despite the use of 60 U/kg, is suboptimal, and that complete elimination of the
accumulated substrate can occur only after several repeated infusions. Although increasing the dose may aid in rectifying this problem, higher doses of Cerezyme may also saturate the mannose receptors and thereby limit the benefit that could be provided to the affected macrophages. In contrast, the strategy to increase the surface density of the mannose receptor specifically on the affected macrophages using dexamethasone should promote a greater uptake of the infused enzyme and thereby enhance the efficacy of enzyme replacement therapy. This increase in effectiveness could translate to a more rapid response time and to a reduction in dose and perhaps even decrease the frequency of infusions.

Yet another potential benefit associated with the use of dexamethasone in Gaucher disease is its anti-inflammatory activity. The total weight of accumulated glucosylceramide in the livers of affected Gaucher patients accounts for less than 2% of the observed hepatomegaly (Cox, 2001). It has been proposed that the organomegaly is more likely the result of chronic inflammation induced by the lipid-laden macrophages. This possibility has been substantiated in a murine model of Gaucher disease (Mizukami et al., 2002) which displayed significant systemic inflammation despite exhibiting only a minimal increase in accumulation of glucosylceramide. If this line of reasoning is correct, the anti-inflammatory activity of dexamethasone may provide an additional benefit to the treatment of Gaucher disease beyond improving the delivery of enzyme to the macrophages. The anti-inflammatory activity of dexamethasone may also negate any potential down-regulation of the mannose receptor by interferon-γ (Harris et al., 1992; Shepherd et al., 1994). The dual benefits of dexamethasone at improving the targeting of Cerezyme to macrophages and reducing inflammation may outweigh the potential side effects associated with the use of the glucocorticoid. As such, its inclusion represents a possible strategy to improve the treatment of Gaucher disease. However, safety considerations associated with repeated chronic use of dexamethasone in Gaucher patients will need to be carefully evaluated. Perhaps, other dexamethasone-like
compounds that are capable of facilitating the upregulation of mannose receptors on macrophages but without the associated toxicity may be more appropriate for consideration.
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References


Figure legends

**Figure 1. Differential effects of dexamethasone on uptake of Cerezyme by different cell types.** Cells were pretreated with or without dexamethasone and assayed for Cerezyme uptake as described in materials and methods. (A) NR8383, alveolar macrophage cell line, (B) liver sinusoidal endothelial cell line, and (C), Hep3B, hepatocyte cell line. Values were normalized to endogenous glucocerebrosidase activity, which was set at 100%. Error bars represent standard deviation. The results shown are representative of 5 independent experiments with the macrophages and 3 with both the liver sinusoidal endothelial cells and Hep3B cells. Mannan was included to saturate the mannose receptor. The shaded columns represent cells that were treated with dexamethasone. Error bars represent standard deviation.

**Figure 2. Dexamethasone pretreatment increased the uptake of Cerezyme by liver Kupffer cells in vivo.** Liver thin-sections from control and dexamethasone-treated and Cerezyme injected (18 U/kg) rats were co-immunostained with anti-macrophage (CD11b) and anti-human glucocerebrosidase (1B5) antibodies. The liver Kupffer cells are shown in green and the location of human glucocerebrosidase (Cerezyme) is shown in red. Arrows indicate Kupffer cells that were co-stained with the marker for Cerezyme. Increasing the dose of dexamethasone (from 10 to 100 µg/rat) increased the number of Kupffer cells that stained positive for human glucocerebrosidase. Slides shown are representative of results obtained from two independent experiments with 3 rats used per group in each study.

**Figure 3. Dexamethasone pretreatment increased the uptake of Cerezyme by splenic macrophages in vivo.** Splenic macrophages were isolated from control and
dexamethasone-pretreated rats injected with or without Cerezyme. Purified macrophages (approximately 90% pure) were lysed and assayed for glucocerebrosidase activity. Control rats and rats pretreated with dexamethasone were administered either 18 or 72 U/kg Cerezyme. The data in the 18 U/kg groups were from two studies with a total of 17 rats (p<0.05). The data in the 72 U/kg groups were from two studies with a total of 10 rats (p<0.001). Values were normalized to endogenous glucocerebrosidase activity, which was set at 100%. The shaded columns represent animals that were treated with dexamethasone. Error bars represent standard deviation. The results indicated that dexamethasone pretreatment increased Cerezyme uptake at all the doses tested.

**Figure 4. Dexamethasone pretreatment increased the uptake of unmodified glucocerebrosidase by spleen macrophages in vivo.** Splenic macrophages were isolated from control and dexamethasone-pretreated rats injected with or without unmodified glucocerebrosidase. Purified macrophages (approximately 90% pure) were lysed and assayed for glucocerebrosidase activity. Values were normalized to endogenous glucocerebrosidase activity, which was set at 100%. p<0.001 between the dexamethasone-treated and non-treated groups administered 7 U/kg of Cerezyme (total of 11 rats in two separate experiments). Error bars represent standard deviation.

**Figure 5. Lipid-laiden alveolar macrophages remained responsive to dexamethasone treatment.** Acid sphingomyelinase knockout mice were pretreated with or without dexamethasone for 3 days after which the lung alveolar macrophages were isolated and allowed to attach to a 12-well dish as described in materials and methods. Cerezyme was then added to the cells and the extent of enzyme uptake determined. Values were normalized to endogenous glucocerebrosidase activity, which was set at
100%. Error bars represent standard deviation. In some studies, mannan was included to saturate the mannose receptor. The shaded columns represent animals that were treated with dexamethasone.
Zhu et al., Figure 1
Zhu et al., Figure 3
Zhu et al., Figure 4
Zhu et al., Figure 5