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EXOGENOUS MANNOSE DOES NOT RAISE STEADY STATE MANNOSE-6-PHOSPHATE POOLS OF NORMAL OR N- GLYCOSYLATION-DEFICIENT HUMAN FIBROBLASTS

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Abstract

Increasing intracellular mannose-6-phosphate (Man-6-P) was previously reported to reduce the amount of the major lipid linked oligosaccharide (LLO) precursor of N-glycans; a loss that might decrease cellular N-glycosylation. If so, providing dietary mannose supplements to glycosylation-deficient patients might further impair their glycosylation. To address this question, we studied the effects of exogenous mannose on intracellular levels of Man-6-P, LLO, and N-glycosylation in human and mouse fibroblasts. Mannose (500μM) did not increase Man-6-P pools in human fibroblasts from controls or from patients with Congenital Disorders of Glycosylation (CDG), who have 90–95% deficiencies in either phosphomannomutase (CDG-Ia) or phosphomannose isomerase (MPI) (CDG-Ib), enzymes that both use Man-6-P as a substrate. In the extreme case of fibroblasts derived from *Mpi* null mice (<0.001% MPI activity), intracellular Man-6-P levels greatly increased in response to exogenous mannose, and this produced a dose-dependent decrease in the steady state level of the major LLO precursor. However, LLO loss did not decrease total protein N-glycosylation or that of a hypoglycosylation indicator protein, DNaseI. These results make it very unlikely that exogenous mannose could impair N-glycosylation in glycosylation-deficient CDG patients.

INTRODUCTION

Mannose-6-P (Man-6-P) is a precursor for N-glycan synthesis. Man-6-P is converted to Man-1-P, then to GDP-Man and dolichol-P-Man for synthesis of the universal lipid linked oligosaccharide (LLO) donors of N-glycans. (See Scheme 1) (Eklund, et al. 2007). Two pathways can generate Man-6-P: phosphorylation of mannose via hexokinase or conversion of fructose-6-P via phosphomannose isomerase (MPI) (Eklund, et al. 2007). Both pathways contribute mannose to N-glycosylation. Patients with congenital disorder of glycosylation (CDG) Type Ib have an 85–95% deficiency in MPI activity (Eklund and Freeze 2006, Freeze 2006), resulting in unoccupied N-glycosylation sites in many proteins. Dietary supplements of mannose correct the patients' abnormal glycosylation and pathological features (Eklund and Freeze 2006, Freeze 2006). In mice, ablation of *Mpi* is lethal by embryonic day 10.5 due to intracellular accumulation of toxic levels of Man-6-P that can reach 25mM (DeRossi, et al. 2006).

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LLO and protein synthesis are coordinated, but LLO is likely in excess since intact LLO glycan is also hydrolyzed from the lipid carrier (Lehrman 2006, Moore and Spiro 1994, Spiro and Spiro 1991). Recently, an in vitro study using streptolysin-O-permeabilized cells showed that modest concentrations of Man-6-P (10–100 μ M) specifically decreased the predominant LLO species, Glc₃Man₉GlcNAc₂-PP-Dol (Gao, et al. 2005), generating the free intact glycan. Decreasing LLO might also decrease protein N-glycosylation. Since previous studies suggested that the intracellular concentration of Man-6-P in rat liver was ~100 μ M (Asikin and Koeppe 1979), even slight increases might decrease LLO and limit N-glycosylation (Gao, et al. 2005).

Under this scenario, providing mannose to phosphomannomutase- (PMM2, Man- 6P→Man-1-P) deficient CDG-Ia patients, who already have reduced N-glycosylation, might further compromise their glycosylation by increasing intracellular Man-6-P. Although mannose therapy in CDG-Ia patients showed no benefit, it did not appear to further decrease glycosylation of plasma glycoproteins (Kjaergaard, et al. 1998, Mayatepek and Kohlmüller 1998, Mayatepek, et al. 1997).

We asked whether exogenous mannose increases Man-6-P in fibroblasts from control subjects or from CDG-Ia or CDG-Ib patients. Our results showed no detectable increase, making it unlikely that mannose would decrease glycosylation in patients. To test the extreme case, we also asked whether exogenous mannose would decrease LLO levels in mouse Mpi^{-/-} fibroblasts that accumulate extremely high levels of Man-6-P in response to exogenous mannose. We found that Mpi^{-/-} cells accumulated Man-6-P and specifically lost a portion of their Glc₃Man₉GlcNAc₂-PP-Dol, but this loss does not affect overall N- glycosylation or that of a sensitive hypoglycosylation marker protein, DNaseI (Eklund, et al. 2005, Fujita, et al. 2008).

MATERIALS AND METHODS

Human and Mouse Fibroblasts

Human control fibroblasts were obtained from Advanced Tissue Science or Coriell Cell Repository. Fibroblasts from patients were obtained from physicians as part of their CDG diagnostic workup following informed consent approval from parents or guardians.

Mannose-6-P analysis

Cells were incubated in DMEM medium with 5.5mM glucose and 20% fetal bovine serum with the indicated amount of mannose for 8 hrs before analysis. In some cases, serum was dialyzed to remove endogenous mannose prior to use. The extraction, HPLC analysis, and quantitation of Man-6-P was done as previously described (DeRossi, et al. 2006).

DNaseI and Protein Glycosylation Analysis

DNaseI construct, infection protocols and analysis of its glycosylation status were described previously (Eklund, et al. 2005, Fujita, et al. 2008). To determine protein glycosylation, mouse fibroblasts in the presence or absence of mannose were labeled with ³⁵S-amino acids for 4hr. Labeled proteins in the medium were bound to ConcanavalinA and eluted with 100 mM α -methyl mannoside. The resulting material was analyzed by reducing SDS-PAGE and visualized by flouorography as described for analysis of DNaseI.

Analysis of LLOs

Cells were grown in DMEM medium with 20% FBS and the indicated amount of mannose. Approximately 1.0×10^7 cells were suspended in methanol and dried under a stream of dry N₂. LLOs were extracted as previously described (Gao and Lehrman, 2002) using chloroform–

methanol–water (CMW; 10:10:3). Intact glycan chains were liberated from LLO by mild acid hydrolysis and then loaded onto C18 SepPak column directly connected to 3 ml ENVI-CARB solid phase extraction tube to remove residual salt and lipids (Gruberman et al, 2004). After loading, columns were washed with 9 ml of 2% acetonitrile/0.1 M ammonium acetate in H₂O and the glycans eluted from the ENVI-CARB with 6 ml of H₂O-acetonitrile (3:1, v/v), dried and labeled with 2-aminopyridine for HPLC analysis.

RESULTS

Effects of exogenous mannose on intracellular Man-6-P in human and mouse fibroblasts

We analyzed fibroblasts from normal controls and from three different types of CDG patients, who exhibit protein hypoglycosylation. Cells were grown in medium containing physiological glucose (5.5mM) and 0 or 500 μ M mannose for 24 hours and intracellular amount of Man-6-P was determined. PMM2-deficient CDG-Ia, cells and MPI-deficient, CDG-Ib cells have defects which could increase the intracellular level of Man-6-P. ALG6-deficient cells from CDG-Ic patients have a defect in LLO biosynthesis and would not be expected to show any change in Man-6-P levels. (Freeze 2006). There was no detectable increase in Man-6-P in any of the human cell lines in response to mannose. Representative data are shown in Figure 1.

We next studied fibroblasts derived from wild-type $Mpi^{+/+}$ mice. They also did not have detectable intracellular Man-6-P (<0.5 nmole/100 μ g protein), but addition of 500 μ M mannose to the medium increases the level to 1.3 nmole/100 μ g protein. However, fibroblasts derived from $Mpi^{-/-}$ cells ($<0.001\%$ normal MPI activity) and grown in 10% serum with 5 μ M mannose accumulated detectable Man-6-P (1.2nmole/100 μ g protein); addition of 500 μ M mannose to the medium further increased that level ~ 10 -fold (10nmole/100 μ g protein), similar to that seen in previous studies of $Mpi^{-/-}$ mouse fibroblasts (DeRossi, et al. 2006).

Effects of exogenous mannose on LLO levels in $Mpi^{-/-}$ cells

$Mpi^{-/-}$ fibroblasts require mannose for survival and must either import exogenous mannose and/or salvage it from degraded glycans (DeRossi, et al. 2006). $Mpi^{-/-}$ cells can grow normally in 100% serum, showing that they salvage sufficient mannose from serum glycoproteins. If the salvage pathway is blocked, adding 10–15 μ M free exogenous mannose restores normal glycosylation (Fujita, et al. 2008). A combination of 100 μ M mannose and 10–20% serum is cytostatic over several days, and 500 μ M mannose becomes cytotoxic beyond 24 hr due to the accumulation of Man-6-P (DeRossi, et al. 2006).

Previous *in vitro* studies using streptolysin-O permeabilized mammalian cells showed that Man-6-P selectively reduced the amount of LLO available for glycosylation (Gao, et al. 2005). By using intact $Mpi^{-/-}$ cells, we could manipulate Man-6-P levels with exogenous mannose and then determine effects on the steady-state amount of Glc₃Man₉GlcNAc₂PP-Dol and other LLO species (DeRossi, et al. 2006). Representative elution profiles (Figure 2) and quantitation from several experiments (Table 1) show the amount of the predominant LLO species. Wild-type mouse cells made nearly the same amount and spectrum of LLO species regardless of the addition of exogenous mannose. (Figure 2, A and B and Table 1, experiments 1 and 2). $Mpi^{-/-}$ cells grown in dialyzed 10% serum with 100 μ M mannose made a smaller amount of Glc₃Man₉GlcNAc₂PP-Dol and higher amount of Man₉GlcNAc₂PP-Dol than controls under similar conditions (Figure 2, C and Table 1, experiment 1). In 500 μ M mannose, $Mpi^{-/-}$ cells maintained about the same amount of Man₉GlcNAc₂PP-Dol, but specifically decreased the amount of Glc₃Man₉GlcNAc₂PP-Dol (Figure 2, D). Without addition of mannose to $Mpi^{-/-}$ cells, 20% serum was insufficient to generate a normal amount of LLO seen in control cells (Experiments 3 and 4, Table 1); however, the size distribution was similar to that seen in control cells (Figure 2, E). Adding mannose at 20 μ M nearly normalized the

amount of LLO (Table 1, experiments 2, 3 and 4), but providing 100 μ M mannose reduced the steady state amount Glc₃Man₉GlcNAc₂-PP-Dol, and consistently increased Man₉GlcNAc₂-PP-Dol (Table 1). Adding 500 μ M mannose reduced the total amount of LLO in Mpi^{-/-} cells, and especially decreased Glc₃Man₉GlcNAc₂-PP-Dol. Taken together, results using intact Mpi^{-/-} cells confirm previous in vitro studies showing Man-6-P- dependent depletion of steady state Glc₃Man₉GlcNAc₂-PP-Dol (Gao, et al. 2005).

Increased Man-6-P and decreased Glc₃Man₉GlcNAc₂PP-Dol does not reduce N-glycosylation

We wanted to know if the reduced amount of Glc₃Man₉GlcNAc₂-PP-Dol also reduced N-glycosylation in the Mpi^{-/-} cells. An engineered form of DNaseI containing a single N-glycosylation site is a sensitive indicator of insufficient glycosylation because the consensus sequon, -NDS-, is poorly glycosylated (Eklund, et al. 2005, Fujita, et al. 2008). If LLO is limiting or an incomplete LLO glycan species is inefficiently transferred, DNaseI should show hypo-N-glycosylation, i.e., unoccupied N-glycosylation sites, as it does in CDG-Ia, -Ib and -Ic cells (Freeze 2006) and in Mpi^{-/-} fibroblasts without exogenous mannose or mannose salvage pathway (Fujita, et al. 2008). Mouse cells were transfected with an adenovirus construct harboring DNaseI, and then incubated with increasing amounts of mannose to increase intracellular Man-6-P. Cells were labeled with ³⁵S-amino acids, secreted DNaseI isolated from the medium, and glycosylated and non-glycosylated forms resolved by SDS-PAGE. As shown previously, the upper band is N-glycosylated, while the lower band is not, based on digestion with PNGaseF (data not shown). Results in Figure 3a show that even though the steady state level of Glc₃Man₉GlcNAc₂PP-Dol decreased in response to increased Man-6-P, the amount of DNaseI and its degree of N- glycosylation was unaffected, remaining at 75–77%. Likewise, overall protein glycosylation as measured by binding of ³⁵S amino acid-labeled proteins to Concanavalin A, also remained normal (Figure 3b). These results show that even though exogenous mannose greatly increased intracellular Man-6-P and decreased the steady state level of the major LLO, it did not alter the extent of protein N-glycosylation.

DISCUSSION

Metabolic disorders caused by mutations in linear biosynthetic pathways often accumulate substrate proximal to the lesion and decrease the amount of product distal to it. This approach was used to identify many types of CDG, based on the synthesis of incomplete LLO glycan chains (Freeze 2006). So, it was reasonable to predict that PMM2-deficient CDG-Ia cells (and possibly patients themselves) might accumulate Man-6-P. Addition of exogenous mannose might further increase this amount since these cells would not efficiently convert Man-6-P into Man-1-P (Gao, et al. 2005). Accumulation of Man-6-P might further exacerbate the patients' impaired N-glycosylation by cleaving the predominant LLO species (Gao, et al. 2005). This perspective would argue that giving these patients mannose might actually be harmful. However, this is not necessarily true since PMM2-deficient CDG-Ia patients have normal MPI activity, and they could still catabolize Man-6-P. Similarly, providing hyper-physiological levels of mannose to MPI-deficient fibroblasts or patients might also cause accumulation of Man-6-P. However, our results show that exogenous mannose did not detectably increase Man-6-P in patient cell lines. The simplest interpretation of these results is that both groups of patients have sufficient MPI activity to catabolize excess Man-6-P and prevent its accumulation. Previous results showing that dietary supplements of mannose neither improve nor decrease N-glycosylation when given to CDG-Ia patients (Kjaergaard, et al. 1998, Mayatepek and Kohlmüller 1998, Mayatepek, et al. 1997), but mannose rescues deficient glycosylation in partially MPI-deficient, CDG-Ib, patients (Niehues, et al. 1998) by overcoming their Man-6-P deficiency. Taken together, these results make it very unlikely that providing mannose supplements to CDG-Ia patients would increase Man-6-P and further inhibit protein glycosylation.

In contrast to patients with hypomorphic alleles, $Mpi^{-/-}$ mouse fibroblasts have no (<0.001%) residual enzymatic activity and, therefore, accumulate Man-6-P (DeRossi, et al. 2006) because its entry into glycolysis is blocked. We confirmed the in vitro finding (Gao, et al. 2005) that a dose-dependent increase of Man-6-P decreases $Glc_3Man_9GlcNAc_2PP-Dol$ levels, but the loss occurs at very high levels of Man-6-P that can only be achieved in cells lacking all Mpi enzymatic activity. Adding 500 μ M mannose to $Mpi^{+/+}$ control mouse fibroblasts slightly increases intracellular Man-6-P. Surprisingly, the decreased steady state amount of $Glc_3Man_9GlcNAc_2PP-Dol$ LLO seen in $Mpi^{-/-}$ cells exposed to 500 μ M mannose did not reduce N-glycosylation of DNaseI or of total proteins as measured by binding of metabolically labeled proteins to Concanavalin A. This observation shows that LLO glycan is transferred to proteins at normal efficiency, and further suggests that the steady state LLO pool is in excess, at least in these cells. The decreased steady state level of $Glc_3Man_9GlcNAc_2PP-Dol$ seen in mannose-supplemented cells may simply reflect the excess remaining after $Glc_3Man_9GlcNAc_2$ was transferred to protein.

Currently, there is no explanation for how elevating Man-6-P in the cytoplasm specifically cleaves the glycan moiety of $Glc_3Man_9GlcNAc_2PP-Dol$ on the luminal side of the endoplasmic reticulum. Man-6-P is formed in the cytoplasm and is unlikely to enter the ER lumen. If this molecule acts as a metabolic sensor to coordinate glycosylation site occupancy in response to stress, it may be a transmembrane signaling event tied to the rate of protein synthesis (Lehrman 2006, Shang, et al. 2007). Since Man-6-P links glycosylation and glucose metabolism, it is appropriately located to serve as a metabolic sensor. Very high concentrations of Man-6-P inhibit phosphoglucose isomerase and glucose-6-P dehydrogenase (DeRossi, et al. 2006) and so excessive and uncontrolled accumulation of Man-6-P may be undesirable.

Whether modest increases in Man-6-P concentration in the physiological range can control $Glc_3Man_9GlcNAc_2PP-Dol$ level is not proven, but our data show no effect on total protein N-glycosylation or of a sensitive marker for glycosylation site occupancy.

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Figure 1.

Effects of exogenous mannose on intracellular Man-6-P. Human control, CDG-Ia (PMM2 deficient), CDG-Ib (MPI-deficient), CDG-Ic (ALG6-deficient) and mouse fibroblasts from *Mpi*^{+/+} and *Mpi*^{-/-} embryos were incubated with medium containing 10% serum and either 0 or 500µM mannose for 8 hr. Samples were extracted and analyzed by HPLC as previously described to determine Man-6-P (DeRossi, et al. 2006). Controls of known amount of Man-6-P and Glc-6-P were analyzed in parallel and recoveries normalized to an established standard curve to determine nmol Man-6-P/100µg cellular protein.

Figure 2.

LLO profiles in Normal and $Mpi^{-/-}$ fibroblasts in response to mannose. Cells were incubated in the presence of the indicated amount of mannose in medium containing 5.5 mM glucose and 20% FBS for 24 hours and LLO glycans prepared and analyzed in the Experimental Procedures. Identity of the glycans was determined by comparison with authentic standards. Representative experiments are shown Panels: (a) control mouse fibroblasts, 0 μ M mannose; (b), control mouse fibroblasts, 500 μ M mannose; (c), $Mpi^{-/-}$ mouse fibroblasts 0 μ M mannose; (d), $Mpi^{-/-}$ mouse fibroblasts 500 μ M mannose; (e), $Mpi^{-/-}$ mouse fibroblasts 0 μ M mannose; (f), $Mpi^{-/-}$ mouse fibroblasts 20 μ M mannose; (g), $Mpi^{-/-}$ mouse fibroblasts 100 μ M mannose; (h), $Mpi^{-/-}$ mouse fibroblasts 500 μ M mannose.

Figure 3.

Effects of mannose on N-glycosylation of DNaseI and total secreted proteins. *Mpi*^{-/-} fibroblasts were incubated in medium containing the indicated concentration of mannose and for 4 h and labeled with ³⁵S-amino acids. Panel A. DNaseI was precipitated from the medium and glycosylated (upper band) and non-glycosylated (lower band) forms were separated by SDS-PAGE and visualized via fluorography. Numbers indicate the percentage of fully glycosylated molecules. Panel B. Medium from the same experiment using 10 and 500µM mannose was analyzed by binding to Concanavalin A. Figure shows 1× and 5× amounts of total, bound, and unbound proteins from medium containing 10µM and 500µM mannose separated by SDS-PAGE and visualized via fluorography. Both Panels show that increased mannose in *Mpi*^{-/-} cells does not alter the efficiency of N-glycosylation.

SCHEME 1.

Table 1Amount of LLO (pmol/ 10^7 cell)

	Mpi^{+/+}	Mpi^{-/-}	Mpi^{+/+}	Mpi^{-/-}
Exp	M9 (uM Man)	M9 (uM Man)	G3M9 (uM Man)	G3M9 (uM Man)
#1	3.7 (0)	4.5 (100) 3.8 (500)	13.0 (0)	7.7 (100) 2.4 (500)
#2	1.4 (10) 1.4 (500)		9.4 (10) 9.5 (500)	
#3		1.1 (10) 2.5 (20) 6.1 (100) 1.5 (500)		3.8 (10) 7.6 (20) 4.9 (100) 1.8 (500)
#4		0.6 (10) 1.4 (20) 3.1 (100) 3.0 (500)		2.3 (10) 5.8 (20) 5.2 (100) 1.9 (500)