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ABSENCE OF METABOLIC CROSS-CORRECTION IN TAY-SACHS CELLS: IMPLICATIONS FOR GENE THERAPY

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We have investigated the ability of a receptor-mediated gene transfer strategy (cross-correction) to restore ganglioside metabolism in fibroblasts from Tay-Sachs (TS) patients *in vitro*. TS disease is a GM2 gangliosidosis due to the deficiency of the lysosomal enzyme β-hexosaminidase A (Hex A, E.C. 3.2.1.52). The hypothesis is that transduced cells over-expressing and secreting large amounts of the enzyme would lead to a measurable activity in defective cells via a secretion-recapture mechanism. We transduced NIH3T3 murine fibroblasts with the LαHexTN retroviral vector carrying the cDNA encoding for the human Hex α-subunit. The Hex activity in the medium from transduced cells was about 10 fold higher (up to 75 mU) than observed in untransduced cells. TS cells were cultured for 72 hrs in the presence of the cell medium derived from the transduced NIH3T3 cells and were analyzed for the presence and for the catalytic activity of the enzyme.. Although TS cells were able to efficiently uptake large amount of the soluble enzyme, it failed to reach the lysosomes in a sufficient quantity to hydrolyze the GM2 ganglioside to GM3 ganglioside. Thus, our results showed that delivery of the therapeutic Hex A was not sufficient to correct the phenotype of TS cells.

RUNNING TITLE: Restoration of a functional hexosaminidase A in Tay-Sachs cells

INTRODUCTION

Tay-Sachs (TS) disease is a GM2 gangliosidosis due to the deficiency of the lysosomal enzyme β-hexosaminidase A (Hex A, E.C. 3.2.1.52). Hex A is a heterodimer of an α- and β-subunit, encoded by two different genes (HEXA and HEXB) located on chromosome 15 and chromosome 5 respectively. Inherited defects in the α-subunit gene lead to the absence of the Hex A and a massive accumulation of the GM2 ganglioside and related lipids, primarily in neuronal lysosomes. Consequences are a severe cellular dysfunction and a rapid progressive neurodegeneration (1-3).

In human and other mammalian tissues, β-hexosaminidase exists in two major forms: Hex A (αβ structure) and Hex B (ββ structure) (1). Minor forms of Hex have also been described and characterized (4-6). The homodimer αα, Hex S, represents the residual Hex activity in Sandhoff disease patients, a type 0 GM2 gangliosidosis due to inherited defects in the HEXB gene and predominates in the presence of an altered balance between the α- and β-subunit (i.e. in leukaemic cells) (7,8-10). Recently Sandhoff and collaborators (11) demonstrated that this form has catalytic activity, such as Hex A, towards anionic glycolipidis, anionic glycans and neutral N-glycans.

The formation of the Hex A is controlled by a complex mechanism which ultimately results in association of α- and β-subunit (1-2,12-13). This event requires the transport of both subunits to the Golgi Apparatus compartment and regulation of the ratio between them. In the Golgi Apparatus a second important event occurs: the generation of the mannose-6-phosphate recognition marker. As result of this modification, the mannose-6-phosphate receptor recognizes and targets the enzyme to the lysosomes. The enzyme is then subjected to a final proteolytic processing, and in the presence of the GM2-activator protein, hydrolyzes the β-GalNAc-(1-4)-β-Gal glycosidic linkage (1,8,11,14-15).

No effective treatment is currently available for TS disease. Gene therapy holds the greatest promise for genetic diseases in which single gene mutations are responsible for the metabolic alterations. Vector–mediated gene transfer (direct correction) or receptor-mediated gene transfer (cross-correction) represent two potential approaches for lysosomal storage disorders (16-18). The first is based on the introduction of the missing gene to the deficient cells. In this case, the deficient cells produce the lacking enzyme. The second is based on the rationale that secreted lysosomal enzymes can be uptaken by the neighboring cells through the binding with plasma membrane mannose-6-posphate receptor.

In this paper we have focused on the understanding of the mechanisms leading the effectiveness of the cross-correction strategy using human TS cells as model. Moreover, because of the combination of the two approaches is requested for the diffusion of the therapeutic enzyme and for the success of the gene transfer treatment in the patients (i.e. bone marrow gene transfer, local gene transfer delivery) (19-22), we have compared the efficacy of both approaches to restore the Hex A activity in TS cells.

We first transduced human fibroblasts from TS patients with a retroviral vector carrying the cDNA encoding for the human Hex α-subunit, and defined the ability of the recombinant Hex A to

restore the GM2 ganglioside metabolism; second, we have cross-corrected human TS fibroblasts by using the Hex A secreted either by transduced murine fibroblasts or by human transduced TS cells and investigated the efficiency of this mechanism.

All our results demonstrate that although both strategies were able to give rise to adequate levels of the missing enzyme in the deficient cells, the GM2 metabolism was only restored in transduced TS cells.

MATERIALS AND METHODS

Materials. The GM2-activator protein was kindly provided by Professor Yu-Teh Li, Tulane University, New Orleans, LU, USA. The fluorogenic substrates 4-methylumbelliferyl-derivative of ß-N-acetylglucosaminide (MUG), of ß-N-acetylglucosamine-6-sulphate (MUGS) of ß-D-galactoside (ß-Gal), of α-D-mannopiranoside (α-Man), 4-methylumbelliferone, Nonidet NP40, agarose gel for electrophoresis, mannose-6-phosphate were from Sigma Chemical Co. Bovine serum albumin and Bio-Rad protein assay reagent were from Bio-Rad Laboratories, DE-52 DEAE-Cellulose was from Whatmann Biochemicals. The medium for tissue culture was from Euro-Clone, Celbio Lab., the fetal calf serum and Colorado serum were from Mascia Brunelli, penicillin/streptomycin was from Gibco BRL, Gentamicin (G418), Polybrene and restriction enzymes were from Boeringer. Centriprep 30 was from Amicon. All other reagents were of analytical grade.

Cell cultures. Human fibroblasts, established from three patients with the late infantile form of TS disease were kindly provided by the "Laboratorio di Diagnosi Pre/Post-natale Malattie Metaboliche, Istituto G. Gaslini Hospital, Genova, Italy. Normal human fibroblasts are routinely used in our laboratory (23). Fibroblasts were cultured in EMEM medium containing 10 % (v/v) heat-inactivated fetal calf serum in 25 cm^2 culture flasks in a humidified atmosphere containing 5% CO2, at 37°C. NIH3T3 mouse fibroblasts were cultured in DMEM medium containing 10 % (v/ v) heat-inactivated Colorado serum in 25 cm^2 culture flasks in the same conditions as above. Cell growth was determined by counting cells in a haemocytometer. The viability of the cells was estimated by examining their ability to exclude Trypan Blue (0.1 % in 0.9 % NaCl).

At confluence, cells were trypsinized and harvested by centrifugation (1000 rev/min, rav 0.5 cm, for 10 min in an MSE bench top centrifuge). After two washings with 0.9% NaCl, the pellet was resuspended in 10 mM-sodium phosphate buffer, pH 6.0, containing 0.1 % (v/v) Nonidet NP40 detergent and sonicated. The lysates were centrifuged at 12,000 rpm in a Eppendorf microfuge for 20 min and supernatants used as cell extracts for enzyme analysis. All procedures were carried out at 4°C.

Construction of a suitable retroviral vector for Hex α-subunit cDNA transfer and transduction. We cloned the cDNA encoding for α-subunit of human Hex A (was from ATCC) into the Moloney Leukemia retroviral vector backbone (MoLV, LXTN). In this vector the cDNA is under the LTR promoter. This vector contained also the neomycin resistance gene. The tk-neo fusion gene is under the control of the tk promoter (24). The procedure for the production of the retrovirus was as follows. The E86 ecotropic packaging cell line was transfected with the vector carrying the α-subunit cDNA by standard calcium phosphate co-precipitation. The E86 supernatants were collected, filtered (∅ 0.45 μm) and used to infect the amphotropic packaging cell lines AM12 in the presence of polybrene. After selection with neomycin, (G418), the best AM12 transduced clone was selected by titration and used as viral producer packaging cells. Viral supernatants were collected, filtered through 0.45 μm pore size filters and used to infect the designed cells. One day before the infection, TS cells were plated at 75 % of confluence. The following day cells were incubated with viral supernatant, in the presence of

polybrene, for 16 hr at 37°C. After G418 selection, transduced cells were analyzed for the α-subunit gene expression and for the Hex A properties. The same procedure was used to transduce murine fibroblasts NIH3T3.

Administration of the Hex A to TS cells. Human TS cells were cultured in the medium (conditioned medium) containing Hex A secreted by transduced NIH3T3 cells for 72 hr in 25 cm^2 culture flasks at 37°C. The total enzyme activity in the conditioned medium was up to 75 mU. The medium was changed every 24 hours for three days. Untreated and treated TS cells were harvested and evaluated for the Hex A properties. As experimental control, TS cells were cultured in conditioned medium containing different amount of Hex A secreted either by transduced TS cells or normal fibroblasts.

In some experiments, mannose-6-phosphate was added to the conditioned medium to have a 5 mM final concentration. These cells were then analyzed for hexosaminidase, galactosidase and mannosidase activities.

b-Hexosaminidase activity assay. Enzyme activity was determined by using the two fluorogenic substrates 3 mM MUG or MUGS in 0.1 M-citrate/0.2 M-disodium phosphate buffer pH 4.5 (25). Fluorescence of the liberated 4-methylumbelliferone was measured on a Perkin Elmer LS3 fluorimeter (excitation 360 nm, emission 446 nm).

The optimum pH for the Hex A activity was determined by testing the enzyme activity in citrate/sodium phosphate buffers, in the pH range 3.5 to 7.5 (5). To define the thermal stability, 50 µl of each sample were incubated at 52° C for different time, then cooled on ice for 1 hour and assayed at 37 °C for the Hex activity. Results represented the average of at least three independent experiments and are expressed as a percentage of the activity found in the controls, kept on ice (5). The inhibitory effect of glucosamine towards the Hex A was by incubating Hex isoenzymes at 37 °C in the presence of 100 mM glucosamine. Results are the average of at least three independent experiments and are expressed as a percentage of the activity of treated samples with respect to untreated control. (25)

ß-Hexosaminidase isoenzymes analysis. Cell lysates were analyzed by the ion-exchange chromatography on DEAE-cellulose (25). The chromatography was performed by using 1 ml column equilibrated with 10 mM-Na phosphate buffer, pH 6.0 (buffer A). The flow rate was 0.5 ml/min. Enzyme activity retained by the column was eluted by a linear gradient of NaCl (0.0-0.5 M in 40 ml of buffer A). Finally, the column was eluted with 1.0 M-NaCl in the same buffer. Fractions (1ml) were collected and assayed for the Hex activity with the two substrates MUG and MUGS.

Sub-cellular fractionation. Cells at confluence were harvested and resuspended in 0.25M-sucrose, then homogenized in a Potter Elveheim type homogenizer until more than 90% of the cells were disrupted. Differential centrifugation of the homogenate was performed at 800g for 10 min at 4^oC in the AJ20 rotor of a Beckman J2-21 centrifuge to sediment the nuclear fraction. The supernatant was

then centrifuged at 13.000g for 15 min to sediment the lysosomal fraction (L) and the supernatant (post-lysosomal fraction, PL) from this step was decanted (25).

Preparation of radioactive gangliosides GM1 and GM2. Gangliosides GM1 and GM2 were extracted from calf brain (26) and purified to 99% by silica gel- ion exchange, dialysis and precipitation from acetone. Their structural and homogeneity characterization was performed as described elsewhere (27,28). [^{3}H]GM1 and [^{3}H]GM2 containing *erythro* C18-sphingosine, isotopically tritium labeled at position 3, were prepared, from GM1 and GM2, by the dichloro-dicyano-benzoquinone/sodium boro[^{3}H]hydride method followed by reversed phase HPLC purification (26,29) (homogeneity over 99%; specific radioactivity of 1.2 and 1.3 Ci/mmol, respectively). Radioactive standard gangliosides and sfingolipids are available in the laboratory (30)

Feeding experiments. [^{3}H]GM1 or [^{3}H]GM2, dissolved in propan-1-ol/water, 7:3 by vol, were pipetted into a sterile tube and dried under a nitrogen stream. The residue was solubilised in an appropriate volume of pre-warmed (37°C) EMEM, to obtain a ganglioside concentration of $5x10^{-7}$ M. After removal of the original medium and rapid washing of cells with EMEM, 2 ml of the medium containing the radioactive lipid were added to each 60 mm dish and the cells (TS fibroblasts, normal fibroblasts, transduced TS fibroblasts, cross-correct TS fibroblasts) were incubated for 3 hr at 37°C. After incubation, the radioactive medium was removed and the dishes were washed, first with EMEM solution (for 5 min), then with 10% FCS-EMEM (for 30 min). After a chase of 15 hr, cells were washed twice with PBS, scraped off with a rubber policeman, centrifuged at 1000g for 10 min. The pelletted cells were subjected to lipid extraction (27) resulting in a delipidized pellet and a total lipid mixture. Radioactivity imaging of TLC plates of lipid extracts was acquired with a β-imager 2000 instrument (Biospace, Paris). The radioactivity associated with individual lipids was determined with the specific β-vision software provided by the manufacturer (Biospace) and the radioactivity associated with total lipid extracts was determined by liquid scintillation counting (30).

Degradation of GM2 by secreted Hex A. Transduced confluent NIH3T3 cells were maintained in culture for 72h in the presence of the 10mM NH4Cl which specifically increases all secreted lysosomal enzyme activities (31). The medium was collected and partially purified by ionic exchange chromatography on DEAE-cellulose as described above. The assay was performed using 50 mU of secreted Hex A (toward MUG) in 170 µl of 10 mM, pH 4.5 citrate buffer containing 10 µg GM2-activator protein, 0.1% bovine albumin, 52 µg of sodium taurodeoxycholate and 20.000 dpm of GM2 (3 µg). The enzyme reaction mixture was maintained at 36°C under continuous vortexing for 12 hr. The reaction mixture was mixed with 3 volumes of tetrahydrofurane, the mixture centrifuged, the clear solution dried and the residue resuspended into a few µl of chloroform/methanol 2:1. The solutions were analyzed by HPTLC followed by radioimaging and quantitative detection of the separated GM2 and GM3.

Other analytical methods. Proteins were measured by the method of Bradford (32) using the serum bovine albumin as standard. Lipids were analyzed by chromatography on silica gel on HPTLC plates (Merck), using the solvent system chloroform/methanol/0.2% aqueous CaCl2, 50:42:11 by vol., in comparison with standard compounds, followed by radioactivity imaging (28).

RESULTS

Construction of a retroviral vector for Hex α-subunit cDNA. The recombinant retrovirus based on a Moloney leukemia retroviral (MoLV) backbone LXTN vector containing the wild-type Hex α-subunit cDNA was produced (Fig.1). In this vector, the α-subunit cDNA is expressed under the control of the promoter/enhancer sequence in the viral LTR; the tk-neo fusion gene is under the control of the tk promoter. The HSV-tk gene is contained in the vector as a safety mechanism (24). The LαHexTN is used to produce the LαHexTN recombinant retrovirus and to transduce human fibroblasts from TS patients and NIH3T3 cells, as described in the methods section. The presence of Hex α-subunit cDNA in the retroviral transduced cells was evaluated by southern blot (data not shown).

Vector-mediated gene transfer strategy for TS cells. After selection with G418, transduced TS cells display an Hex specific activity of 4.2±0.8 mU/mg, as evaluated with the fluorogenic substrate MUGS, which is hydrolyzed only by the α-subunit (33). This value is comparable to that measured in control fibroblasts (Fig. 2A). The secreted Hex A activity in the culture medium of transduced TS cells was comparable to that of normal fibroblasts and much higher with respect to TS cells (Fig.2 B).

The isoenzyme pattern of Hex in transduced TS cells has been determined by ionic exchange chromatography on DEAE-cellulose (25). Under our experimental condition, Hex B was unretained by the column and eluted with void volume, while Hex A or other minor forms (Hex S or Hex intermediate forms), were eluted by a linear gradient of NaCl (0.0-0,5M in buffer A). Besides, the combination of the chromatographic analysis and the specific enzymatic assay with the two substrates, MUG, which is hydrolyzed by both α- and β-subunit, and MUGS, which is hydrolyzed only by α-subunit, gives information about the subunits composition of the Hex isoenzymes. In LαHexTN transduced human TS cells, the Hex activity was composed of Hex A and Hex B with a Hex isoenzymes pattern similar to that of control cells (Fig.3b, 3c). No presence of Hex S was detected. On the contrary, TS cells were characterized by the complete absence of Hex A, whereas they have Hex B, and a second form eluted by the gradient at the NaCl concentration required to elute the intermediate forms of Hex (Hex I) (5). The ββ structure of this form was demonstrated by its inability to hydrolyze the MUGS substrate (Fig.3a) .

Recombinant Hex A from transduced TS cells and Hex A from control untrasduced cells were separated from the other isoenzymes by preparative DEAE-cellulose chromatography and analyzed for their biochemical properties **(**5, 25,34**)**.. They showed the same optimum pH of 4.5 towards both MUG and MUGS substrates (data not shown), and displayed comparable curves of heat inactivation when incubated at 52°C for different intervals of time, with their almost complete inactivation after 15 min (FIG. 4A). In the presence of 100 mM glucosamine (the specific inhibitor of the β-subunit) Hex A (αβ-dimer), from transduced and control cells, loose about 50 % of its original activity (FIG. 4B). As internal control, the effect of glucosamine was also tested on Hex B (ββ dimer), isolated either from control cells or from transduced TS cells, and on Hex S (αα dimer, obtained from HL 60 cell line – see Ref. 9).

Hex B loose about 80 % of its original activity, in agreement with previous data **(**25), whereas Hex S is activated.

GM2 ganglioside hydrolysis in transduced human TS cells Figure 5 shows the radioactive lipid pattern of transduced cells fed with [^{3}H]GM1. Radioactive ganglioside was taken up by the cells and catabolyzed to GM2->GM3->neutral sphingolipids with a hydrolysis rate comparable to that of control cells demonstrating the restoration of the metabolic defect in transduced TS cells.

Production of a suitable enzyme producer cell lines. After transduction with the retroviral vector LαHexTN, Hex A was over-expressed in NIH3T3 cells. An increase of about 10 folds of the cell enzyme activity towards the substrate MUGS was observed (Fig 2A). Furthermore, transduced NIH3T3 cells released high levels of Hex activity in the medium of culture (Fig.2B). No atypical intracellular Hex isoenzymes, such as Hex S, were detected by DEAE-cellulose chromatography in transduced NIH3T3 cells, but an increase of the Hex B activity was observed (data not shown). These data were confirmed by ionic exchange chromatography analysis of secreted Hex activity of both transduced and untransduced cells. Fig.3d and Fig.3e showed a successful production of high levels of the secreted Hex A which was eluted about 10 ml before of the intracellular Hex A activity. The amount of the enzyme secreted into the medium of culture was in the proportion to the cell number.

As control, NIH3T3 cells were also transduced with a similar retroviral vector carrying as report gene the cDNA encoding for the enzyme β-galactosidase. There were no changes in the intracellular or in the secreted Hex activity in transduced cells; no increase of Hex B activity was revealed by DEAE-cellulose chromatography (data not shown).

Hex isoenzymes secreted by transduced NIH3T3 cells were partially purified by preparative DEAE-chromatography and were analyzed for their biochemical properties as above described. The curves of heat inactivation, the assay in the presence of 100 mM glucosamine, the chromatographic pattern itself, confirmed that the released Hex activity corresponds to Hex B and Hex A with the total absence of Hex S (data not show).

The ability of the Hex A secreted by transduced NIH3T3 cells to hydrolyse the natural ganglioside GM2 was determined in an *in vitro* assay in the presence of GM2-activator protein. The retroviral transduced NIH3T3 were maintained in culture for 72h then the secreted Hex A was separated from the other protein of the culture medium by DEAE-chromatography. The enzyme activity on the natural substrate GM2 ganglioside was tested (31). Although the enzyme activity was low (Fig.6), the measured hydrolysis rate was comparable to that determined for the enzyme secreted from normal human fibroblasts.

Receptor-mediated gene transfer strategy for TS cells. TS cells from three patients were incubated for 72 hr with the medium from LαHexTN transduced NHI3T3. The conditioned medium was changed every 24hr. After 72 hr, cells were harvested and the Hex A activity was evaluated. The cross-correction procedure was able to restore the Hex activity towards MUGS substrate in all TS

fibroblasts from patients considered in this study (Fig.7A). The internalization of the Hex A was time dependent, the maximum uptake of the enzyme being after 72 hr of incubation. Fig. 7B shows the Hex isoenzyme pattern in TS cross-corrected cells and demonstrates the presence of up-taken Hex A. This enzyme displayed optimum pH of 4.5, versus both MUG and MUGS substrates, and a curve of heat inactivation when incubated at 52°C for different intervals of time comparable to that of the control enzyme (data not shown)

When cross-correction experiments were performed in the presence of 5 mM mannose-6-phosphate, no Hex A activity could be recorded in TS cells (Fig. 7A). This suggests that the uptake process of Hex A by TS cells occurs through the mannose-6-phosphate surface receptor mechanism. Cross-corrected TS cells exhibiting a MUGS specific activity of 4.5-4.8 mU/mg cell protein, were fed with radioactive gangliosides GM2 or GM1. Figure 8 shows that in cross-corrected TS cells the hydrolysis of GM2 to GM3 does not occur. GM2 ganglioside was taken up by the cells but was not catabolized (Fig. 8 lines e-i) whereas GM1 ganglioside was taken up by the cells and hydrolyzed to GM2 that was not further processed but was stored (Fig.8, lines e-i). Identical results were obtained when TS cells were cross-corrected with Hex A secreted either by LαHexTN transduced human TS cells or by control human fibroblasts. In these experimental conditions, in TS cells the internalized MUGS activity was 22.7% and 14.5 %, respectively, as respect to the normal fibroblasts (Fig. 9). In both cases the uptaken MUGS activity was lower than that observed in cross-corrected TS cells with Hex A secreted by transduced NIH3T3, but this value was in the range of the activity required to correct the phenotype (1). The lower enzyme activity is not surprising considering that the enzyme activity in the cell medium collected from normal cells and transduced TS cells was about 20 times lower than that present in the medium collected from NHI3T3 transduced cells (Fig. 2B). Therefore, these cross-corrected TS cells were fed with radioactive GM1 ganglioside as previously described, but again the uptaken Hex A was not able to hydrolyze the GM2 ganglioside to GM3 ganglioside (Fig. 8, lane l). The same results was also obtained by using a 10X conditioned medium containing Hex A secreted either by LαHexTN transduced human TS cells or by control human fibroblasts (Fig.9), where the percent of internalized activity was significantly increased, so the absence of hydrolysis was not related to the amount of up-taken Hex A activity (data not showed) .

These findings suggest that the up-taken enzyme could not be transported to the lysosomes where the hydrolysis of natural substrate process occurs. This aspect was further investigated. The nuclear, lysosomal and post-lysosomal fractions were obtained by the differential centrifugation. The lysosomal fraction of cross-corrected cells had MUGS activity (Fig. 10) which was about one tenth of that associated to the normal fibroblast lysosomal fraction. These data suggest that the amount of the lysosomal Hex A requested to prevent the metabolic phenotype (1) may be inadequate to hydrolyze the GM2 when affected lysosomes are considered.

DISCUSSION

The restoration of the metabolic defect in human Tay-Sachs cells has been evaluated by the receptor-mediated gene transfer strategy (cross-correction). This strategy is based on the rationale that part of each lysosomal enzyme is secreted and can be up-taken by the neighboring cells via the mannose-6-phosphate receptor (35-37). The hypothesis is that transduced cells over-expressing and secreting large amount of these enzymes would lead to a measurable activity in defective cells via secretion-recapture mechanism.

We explored cross-correction by using an *in vitro* model. Human TS fibroblasts were incubated in Hex A-containing medium, and were evaluated for Hex A properties, localization and activity on radioactive natural compounds previously fed to the cells. To mimic the gene transfer procedures in the clinic, we have used, as enzyme producer, cells transduced with a MoLV retroviral vector carrying the cDNA encoding for the human α-subunit of the Hex A (LαHexTN, Fig.1). The efficacy of the vector to restore the enzyme activity within the TS cell on natural compounds was demonstrated by transduction of human TS fibroblasts (Fig. 5). In this context, it is known that a correct α- and β- subunit association is required (2,11). A combination of the DEAE-cellulose chromatography and the enzyme assay with the two fluorogenic artificial substrates, MUGS (which is hydrolyzed only by Hex isoenzymes that contain α-subunit) and MUG (which is hydrolyzed by Hex isoenzymes that contain α- and/or β-subunit) was used to verify the correct Hex A formation (Fig. 3a, 3b, 3c). LαHexTN transduced human TS fibroblasts had Hex A activity and properties (optimum pH, thermal stability behavior and similar sensibility to the glucosamine) similar to that of control cells (Fig. 4).

The Hex A activity on natural compounds in the transduced TS cells was evaluated by cell feeding experiments with radioactive gangliosides. In both TS (Fig.5, lane c) and transduced TS cells (Fig. 5, lane d) the exogenous [H^3]GM1 reached the lysosomes where it was correctly converted, by the galactosidase, to GM2 ganglioside. Only in transduced TS cells the GM2 ganglioside was hydrolyzed to GM3 ganglioside and neutral sphingolipids. Since the GM2 ganglioside hydrolysis requires a concerted action between both α- / β-subunit of Hex A, and the small GM2 activator protein (1), this hydrolysis confirms the correct structure of the recombinant enzyme. Thus detection of the GM3 ganglioside in transduced TS cells demonstrates the restoration of the metabolic defect.

We have used the murine cell lines NIH3T3 transduced with the retroviral vector Lα-HexTN as enzyme producer cells in order to over-express Hex A. Many authors have used these cells in similar experiments (38). Transduced NIH3T3 released high levels of Hex A activity into the culture medium. Unexpectedly we also observed an increase in Hex B levels in transduced cells in comparison to the untransduced cell lines. Since NIH3T3 cells constitutively express the α-subunit, its over-expression could negatively influence the stoichiometric process of α– and β–subunit association and lead in the formation of Hex S (αα–dimer) (9,25,39). However, in our experimental conditions the α– and β-subunit balance was naturally resolved, in fact no Hex S was detected.. Moreover, we are investigating whether the increase in β-subunit level is related to the α-subunit over-expression. Lacorazza and

collaborators showed similar findings in C17 neuroblastoma clone transduced with a different retroviral vector expressing the α-subunit gene (40).

To perform the cross-correction experiments, the medium containing Hex A secreted by transduced NIH3T3 was used to culture TS cells. Culture media with different amount of Hex activity were used. TS cells uptake Hex A from the medium of culture (Fig. 7) by a mannose-6-phosphate receptor dependent mechanism (Fig 7A). The level of Hex A activity restored in TS cells was comparable to that measured in control human fibroblasts (Fig. 2A, Fig. 7A). Nevertheless, the up-taken Hex A could not catabolyze GM2 ganglioside (Fig. 8). Identical finding were obtained by culturing TS cells in medium containing different amount of enzyme activity (1X vs. 10X) secreted either by human transduced TS cells or human normal fibroblasts. In this context we observed that the MUGS activity internalized in TS cells was lower if compared with that measured in cross- corrected TS cells with the medium containing Hex A secreted by transduced NIH3T3, but it was sufficient to give rise to the normal phenotype (1). We may address the observed discrepancy on the amount of the internalized MUGS activity, to the higher level of the Hex A released by transduced NIH3T3 in the medium of colture with respect to that released by normal fibroblasts. However, all these data indicate that the chimerical structure of the internalized enzyme (human - the exogenous α-subunit - and mouse - the endogenous β-subunit) is not responsible for the missing GM2 ganglioside hydrolysis. Therefore it is well known that the murine and human α-subunit cDNAs have very high homology, more than 80% (41-42)

Moreover, the Hex A secreted by transduced NIH3T3 hydrolyzes, as does the enzyme secreted by normal human fibroblasts, GM2 ganglioside to GM3 ganglioside. These data, all together, suggest that the structure of the recombinat enzyme is not responsible for the missing GM2 ganglioside hydrolysis and that, most likely, the enzyme does not localize into the lysosomes.

To clarify these findings we evaluated the localization of Hex A in cross-corrected TS cells. Sub-cellular fractionation of cross-corrected TS cells showed an atypical pattern of enzyme distribution, with unexpectedly high levels of MUGS activity in all sub-cellular fractions. Thus, only a minor part of the cell restored activity was associated to the lysosomes. Although this activity, if all belonging to the inner volume of lysosomes, should be sufficient to restore the GM2 ganglioside metabolism (1), probably it may be inadequate to hydrolyze the GM2 when affected lysosomes are considered. Moreover, we cannot exclude that the enzyme associates to the lysosome membranes but does not belong to the lysosomes.

In addition storage of lipids in the lysosomes may change membrane trafficking along the lysosomal pathway (43). In this regard, Kobayashi and colleagues (44), have shown that lysobisphosphatidic acid is localized to the late endosomes and may have an important role in the protein-sorting functions of endosomes. In particular they showed that the lysobisphosphatidic acid is the main component of the endosome membrane domains and that these specialized domains are involved in sorting the multifactor receptor for insulin-like growth factor 2 and ligands bearing mannose-6-phosphate, in particular lysosomal enzymes (45). Thus, the mannose-6-phoshate mechanism may be not sufficient to target the enzyme to the lysosomes.

Alternatively the membrane fluidity of the endosomes may be altered in the mutant cells, resulting in an abnormal lipid/protein trafficking (38). An abnormal transport along the lysosomal pathway has been described in the mucolipidosis type IV disease by Cheng et al. (46). Altered endocytic environment on the biogenesis of the lysosomes was also demonstrated in the fibroblasts of patient suffering from sialic acid storage disease (47).

The overall results reported in this study, demonstrate that the gene therapy of TS disease could be achieved by a direct gene transfer strategy. Nevertheless, the therapy of the lysosomal storage disorder is based on the combination of direct and cross-correction gene transfer strategies. Indeed, the endpoint of both approaches is the production of a functional Hex A in the lysosomes. While direct transduction was demonstrated to be able to restore the phenotype in several lysosomal disorders (16,18-19,48-50), the efficacy of the cross-correction has been shown for very few lysosomal diseases (51-52). The clarification of the cross-correction mechanism is necessary for the therapeutic administration of the missing enzyme to patients and for the distribution of the secreted enzyme by implanted engineered cells to neighboring deficient cells and across same non permeable membranes (e.g. the blood-brain barrier, ependyma).

Here, we provide data exploring this process and showing that the internalized enzyme probably undergoes a more complex turnover via a mechanism that is not yet known. Moreover, from our data emerges the necessity to follow the enzyme topology in the cross-corrected cells, by feeding natural compounds to cells, before considering the enzyme replacement therapy as potential cure for TS disease and similar genetic disorders.

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LEGEND TO THE FIGURES

Fig.1. *Recombinant retrovirus containing α-Hex cDNA*. The recombinant retrovirus was produced as described in Material and Method. α-Hex cDNA is expressed under the promoter/enhancer sequence in the viral LTR; the tk-neo is under the control of the tk promoter.

Fig.2. *Hex A activity in cells transduced with the vector expressing α-Hex cDNA*. The intracellular (Panel A) and the secreted (Panel B) Hex activity was assayed towards the synthetic substrate MUGS. One unit (U) is the amount of enzyme that hydrolyses 1 μmol/min of substrate at 37°C. TS, Tay-Sachs cells; NF, normal fibroblasts; t-TS, transduced TS; t-NIH3T3, transduced NIH3T3; NIH3T3, untransduced NIH3T3.

Fig. 3. *DEAE-cellulose chromatography analysis of Hex A in transduced cells*. **a**: TS, Tay-Sachs cells; **b**: t-TS, transduced TS cells; **c**: NF, normal fibroblasts; **d**: untrasduced NIH3T3, secreted activity; **e:** t-NIH3T3, transduced NIH3T3, secreted activity.

Similar amount of protein for each sample was loaded into a 1 ml column equilibrated with 10 mM-Na phosphate buffer, pH 6.0. After loading, the column was eluted for 15 ml with 10 mM-Na phosphate buffer, pH 6.0. Hex A activity, retained by the column, was eluted with 40 ml of a linear gradient of NaCl (0-0.5 M in the above buffer). Fractions (1ml) were collected and assayed for Hex activity towards the two substrates MUG (•) and MUGS (o).

Fig. 4. *Hex A biochemical properties.* Hex A from transduced human TS cells (•) and from control cells (o) displayed a comparable behavior of heat inactivation when incubated at 52°C for different intervals of time, with their almost complete inactivation after 15 min (Panel A). In the presence of 100 mM glucosamine Hex A from transduced and control cells loose about 50 % of its activity (Panel B). Results are the mean of three different experiments.

Fig. 5. *Radioactive lipid pattern from transduced cells fed with [^{3}H]GM1*.. The total cell lipid extract was separated by HPTLC using the solvent system chloroform/methanol/0.2% aqueous CaCl2, 50:42:11 by vol. Radioactive lipids were detected by digital autoradiography; 200-400 dpm were applied on a 4 mm line; time of acquisition: 24 hr. Lane **a**, standard GM1; lane **b**, normal fibroblasts; lane **c**, TS fibroblasts; lane **d**, transduced TS fibroblasts; lane **e** standard GM2; lane **f**, standard GM3.

Fig.6. *Degradation of GM2 ganglioside by Hex A secreted in to the culture medium by retroviral transduced NIH3T3 cells*. The assay was performed in the presence of the GM2-activator protein.

Fig. 7. *Hex A activity in cross-corrected TS fibroblasts.* Enzyme activity was evaluated using the substrate MUGS. Panel A; TS cells were incubated in conditioned medium containing 75 mU of Hex A secreted by transduced NIH3T3 for 72 hr; the conditioned medium was changed every 24 hr in order

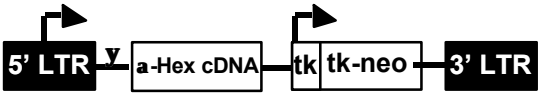
to maintain constant concentration of fresh Hex A; in some experiments confluent TS cells were grown for 4 days in conditioned medium containing 5 mM mannose-6-phosphate (M6P); TS, Tay-Sachs cells; cc-TS, cross-corrected TS; cc-TS+M6P, TS cells cross-corrected in the presence of M6P. Panel B; ionic exchange chromatography pattern of Hex A from cross-corrected TS cells (cc-TS).

Fig. 8. *Radioactive lipid pattern of cross-corrected cells fed with [^{3}H]GM2 and [^{3}H]GM1.* The total cell lipid extract was separated by HPTLC using the solvent system chloroform/methanol/0.2% aqueous CaCl2, 50:42:11 by vol. Radioactive lipids were detected by digital autoradiography; 200-400 dpm were applied on a 4 mm line; time of acquisition: 24 hr. Lane a, standard lactosylceramide; lane b, standard GM1; lanes c, standard GM2; lanes d, standard GM3; lanes e, normal fibroblasts; lanes f and h, TS cells (patients 1 and 2); lanes g and i, TS cells cross-corrected with the conditioning medium from transfected NIH3T3 cells (patients 1 and 2); lane l, TS cells cross-corrected with the conditioning medium from transduced TS cells; lane m, mixture of radioactive standard gangliosides.

Fig.9. *% of MUGS activity in cc-TS***.** TS cells were incubated in conditioned medium containing Hex A secreted by transduced TS (t-TS) cell or secreted by normal fibroblasts (NF), in the same condition described in the legend of the Fig. 6. In the same experiment TS cells were incubated in 10 X concentrated conditioned medium containing Hex A secreted by transduced TS (t-TS) cell or secreted by normal fibroblasts (NF). Enzyme activity was evaluated using the substrate MUGS.

Fig.10. *Sub-cellular fractionation of cross-corrected TS cells*. Sub-cellular fractionation of cross-corrected TS cells (cc-TS), TS cells (TS) and normal fibroblasts (NF). The nuclear (N), lysosomal (L) and post-lysosomal (PL) fractions were obtained by differential centrifugation (25). Results are expressed as relative specific activity (RSA). In the figure is reported also the distribution of the activities of other lysosomal enzymes (α-Mannosidase and β-Galactosidase) as experimental control. RSA is the ratio of the % of the activity of each fractions and the % of the proteins in that fraction.

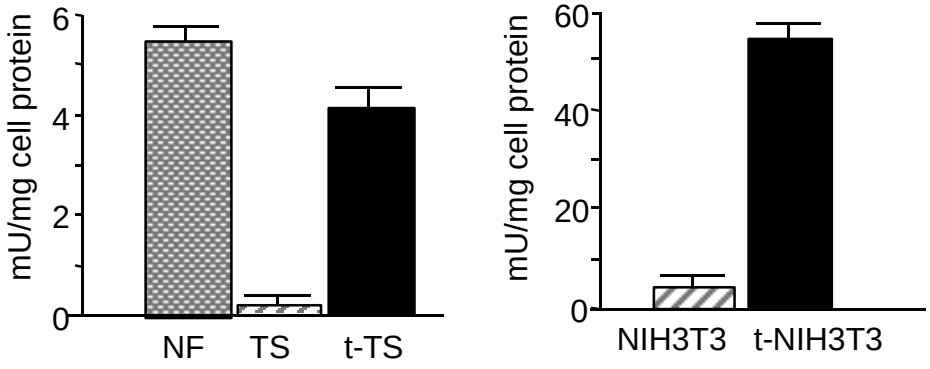
FIG.1



MoMLV retroviral vector LαHexTN

FIG.2

A: intracellular Hex A activity



B: secreted Hex A activity

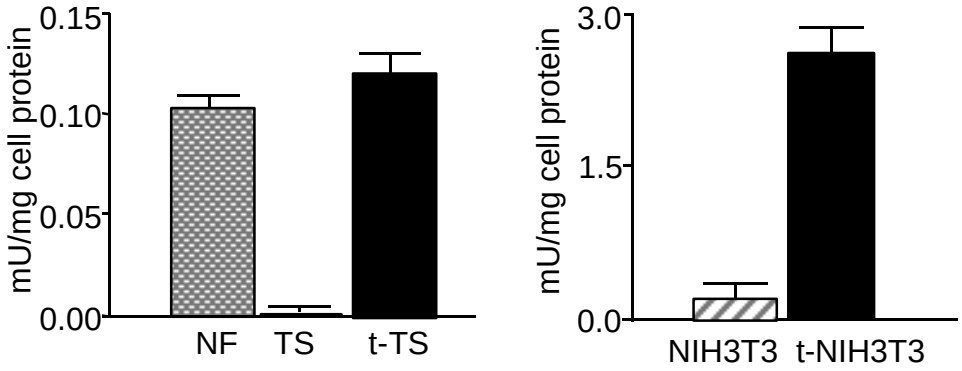


FIG.3

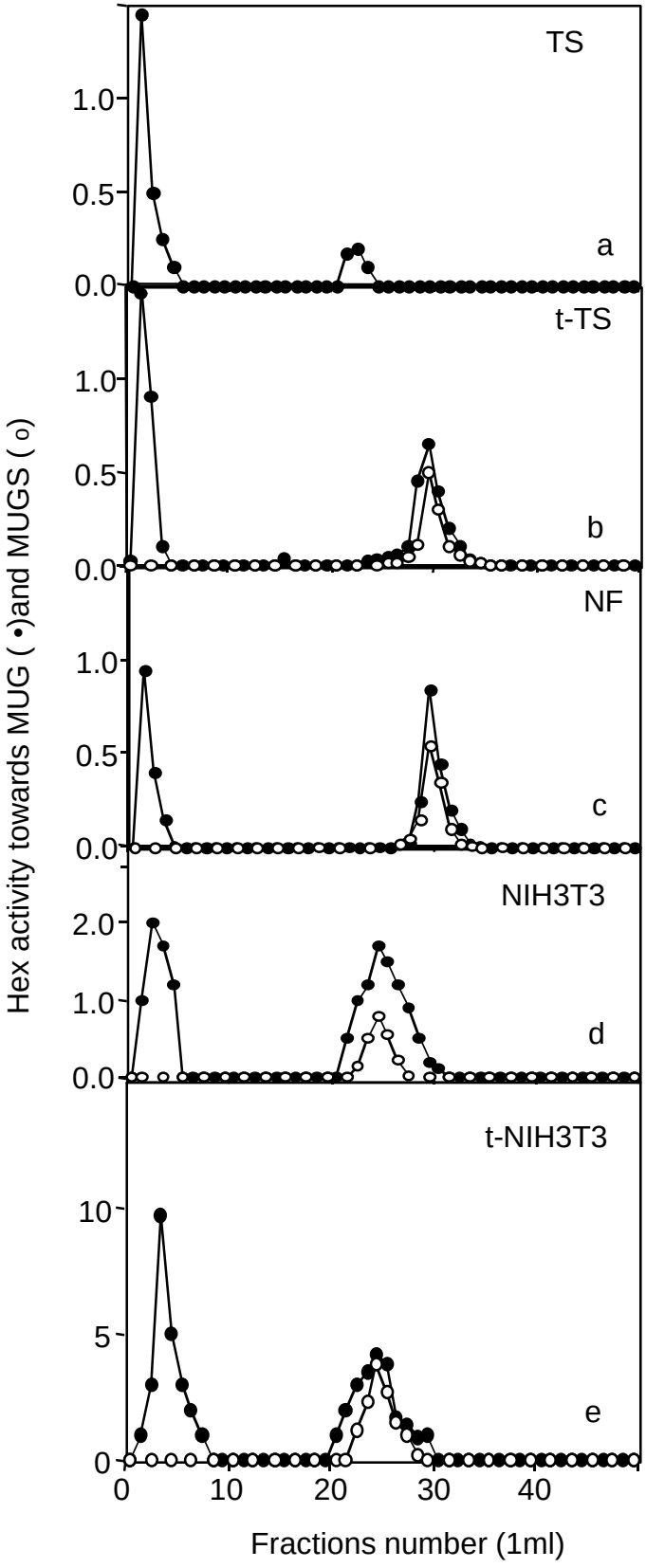


Fig. 4

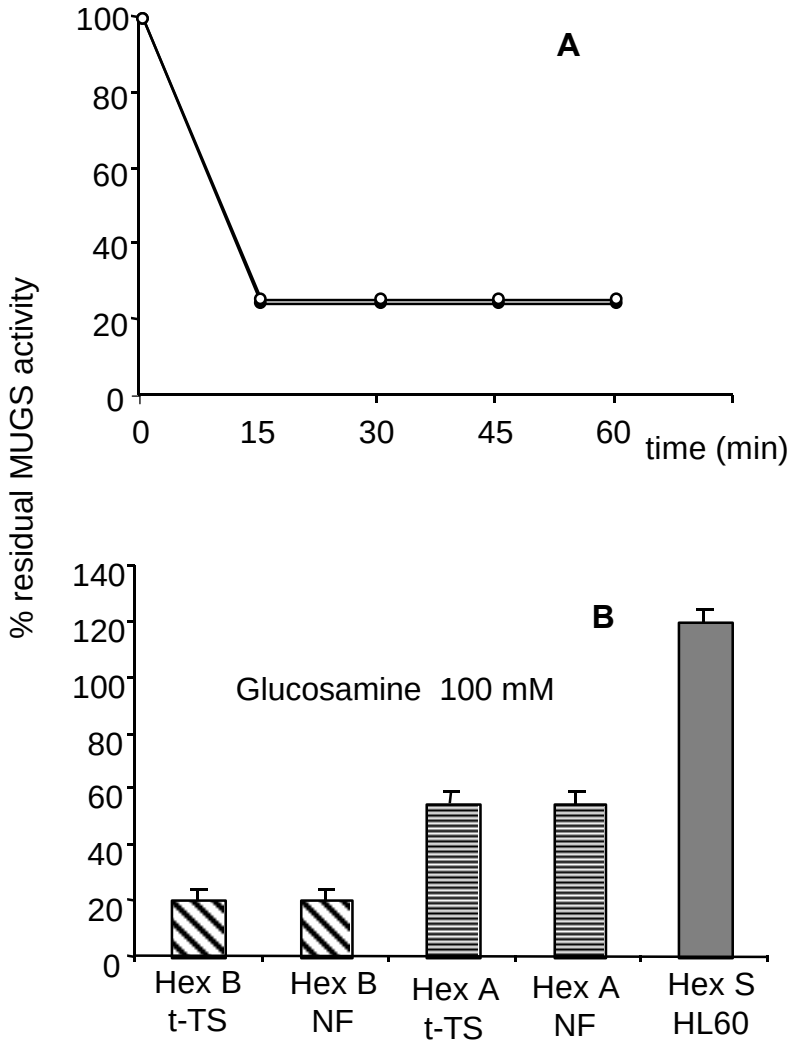


Fig. 5

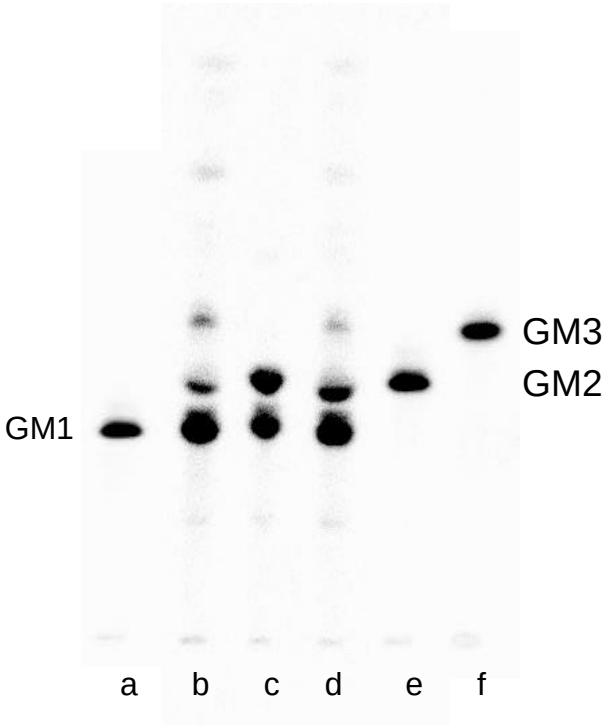


Fig. 6

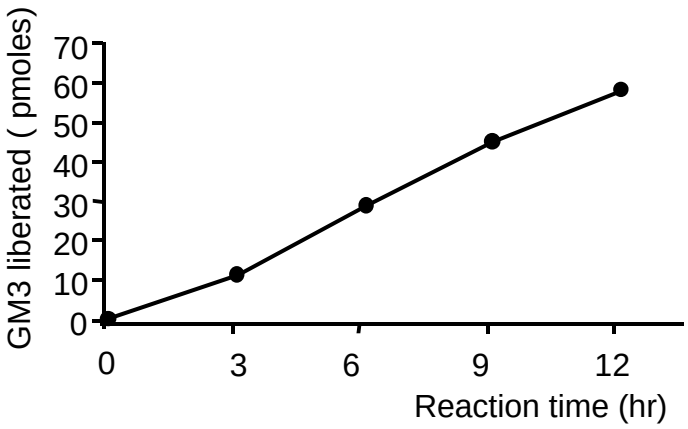


FIG. 7

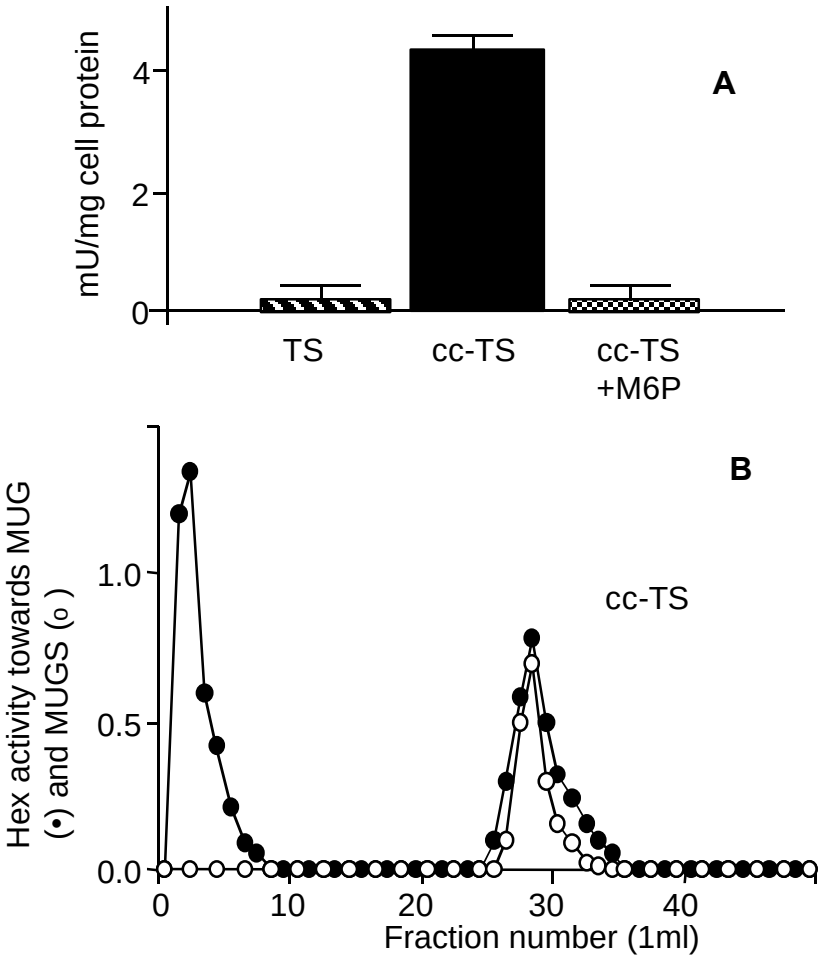


Fig.8

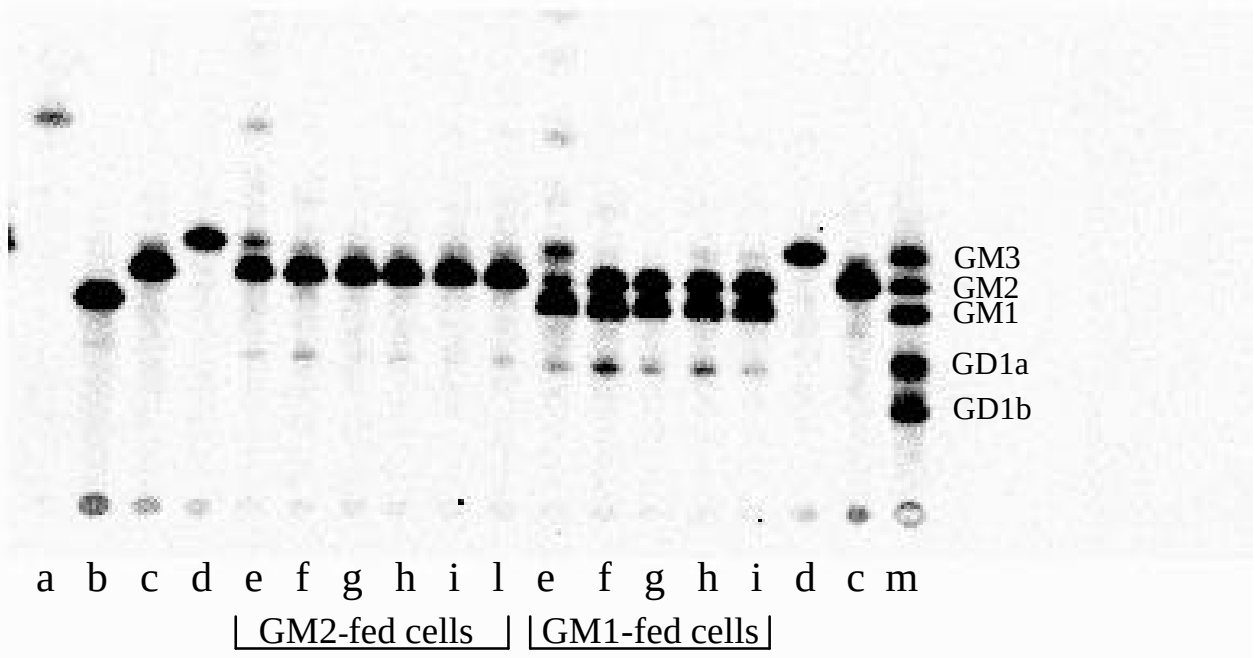


Fig. 9

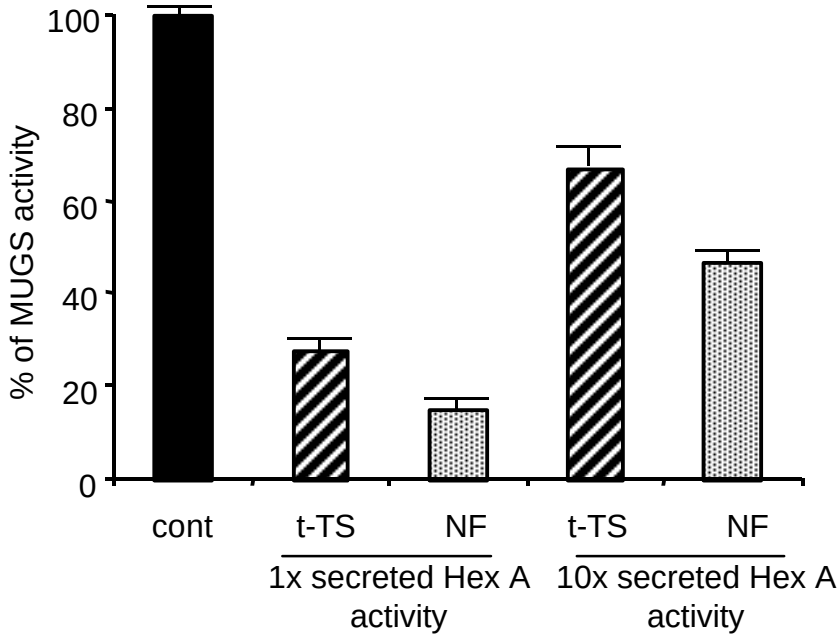
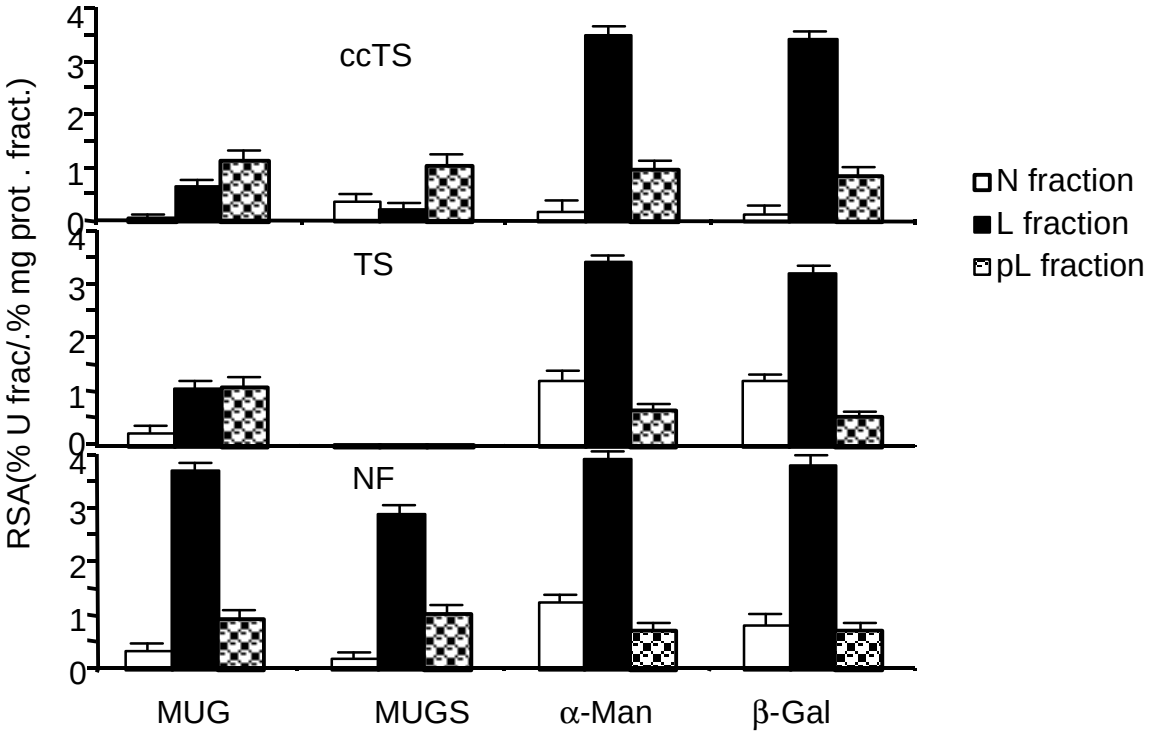


FIG. 10



Absence of metabolic cross-correction in TAY-SACHS cells: Implications for gene therapy

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