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No overall hyposialylation in hereditary inclusion body myopathy myoblasts carrying the homozygous M712T *GNE* mutation

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Abstract

Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult-onset, slowly progressive distal and proximal muscle weakness, which is caused by mutations in UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE), the key enzyme in the biosynthetic pathway of sialic acid. In order to investigate the consequences of the mutated GNE enzyme in muscle cells, we have established cell cultures from muscle biopsies carrying either kinase or epimerase mutations. While all myoblasts carrying a mutated *GNE* gene show a reduction in their epimerase activity, only the cells derived from the patient carrying a homozygous epimerase mutation present also a significant reduction in the overall membrane bound sialic acid. These results indicate that although mutations in each of the two GNE domains result in an impaired enzymatic activity and the same HIBM phenotype, they do not equally affect the overall sialylation of muscle cells. This lack of correlation suggests that the pathological mechanism of the disease may not be linked solely to the well-characterized sialic acid pathway.

Keywords: Hereditary inclusion body myopathy; Muscle cells; GNE; UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; Sialic acid; Sialylation

Hereditary inclusion body myopathy (HIBM) is a neuromuscular disorder characterized by adult onset, slowly progressive distal and proximal muscle weakness, and a typical muscle pathology consisting of cytoplasmic rimmed vacuoles and filamentous inclusions in the cytoplasm or the nucleus [1]. Mutations in *GNE*, the gene encoding the enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (UDP-GlcNAc 2-epimerase/ManNAc kinase), have been identified as the molecular basis of the disease [2,3]. GNE is the key enzyme in the biosynthetic pathway of sialic acids [4], which are the most abundant terminal monosaccharides on glycoconjugates in eukaryotic cells. The first two steps of sialic acid biosynthesis are catalyzed by each one of the two distinct functional domains of GNE. First, the UDP-GlcNAc 2-epimerase domain forms ManNAc from UDP-GlcNAc with simultaneous

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release of UDP. ManNAc is then phosphorylated at C6 by a specific kinase, and subsequently sialic acid is formed by condensation of *N*-acetylmannosamine-6-phosphate and phosphoenolpyruvate, and activated by CTP to form CMP-sialic acid.

Sialic acids are known to affect many cellular functions and adhesion processes, such as cell migration, transformation of tissues and metastasis, inflammation, and wound healing. HIBM was first characterized as an ethnic disorder occurring at a high prevalence (1:1500) in the Persian Jewish community, however, with the identification of the gene, the same hereditary recessive disease was recognized not only in additional Middle Eastern populations but worldwide [3,5]. Most GNE-linked HIBM patients present an unusual feature, the sparing of the quadriceps. A single homozygous missense mutation in the kinase domain of *GNE*, M712T, was identified in all HIBM patients from Jewish Middle Eastern descent, while HIBM patients worldwide display mutations in both the epimerase and the kinase domains of the enzyme, either in a homozygous form, or, more commonly, as compound heterozygotes. Although the role of GNE has been thoroughly characterized as the key enzyme in the biosynthetic pathway of sialic acid [6,7], the process by which these mutations lead to the pathophysiology of HIBM is not yet understood. Our previous studies have shown that although the enzyme activity of GNE has been reduced in lymphoblastoid cell lines from HIBM patients carrying the homozygous M712T mutation in the kinase domain of GNE, the overall sialylation remained unchanged in those cells [8]. To investigate the possible role of sialylation in the pathophysiology of the myopathy in HIBM, we have analyzed the GNE enzymatic activity and its effect on the overall sialylation on muscle cell cultures from patients carrying the M712T mutation and from patients carrying 3 additional mutations affecting either the kinase or the epimerase domains.

Materials and methods

Establishment of muscle cell cultures. These studies were approved by the Institutional Review Board of Hadassah Hospital, Jerusalem, and Wolfson Hospital, Holon, Israel. Fresh biopsies were processed as described [9]. In brief, once fat and connective tissue were removed, the muscle was cut into small pieces in 0.05% trypsin/0.02% EDTA solution. Tissue pieces were stirred vigorously for 15 min at 37 °C, debris were allowed to settle for 1 min, and the solution with the dissociated cells was transferred to 50 ml tubes containing 15 ml DMEM/10% FCS and 2.5 μg/ml gentamicin. The procedure was repeated 2 more times and finally cells were collected by spinning and resuspended in Ham's F10 medium supplemented with 15% FCS, 2.5 μg/ml gentamicin, 3 mg/ml glutamine, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, 0.18 mg/ml insulin, 0.39 μg/ml dexamethasone, 10 ng/ml EGF, and 0.05 mg/ml uridine. Cells were plated and grown in a humidified CO_2 incubator at 37 °C. In order to isolate myoblasts from the culture, cells were sorted by a magnetic cell sorting kit (MACS, Miltenyi Biotech, USA) with anti-N-CAM mAb as a primary antibody. The sorted cells were grown and analyzed for N-CAM by immunofluorescence. Prior to membrane analyses, medium was changed to serum-free medium (no sialic acid) for at least 48 h, and cells were harvested with 2 mM EDTA to ensure the intactness of the membranes.

Epimerase assay. UDP-GlcNAc 2-epimerase activity in muscle cells was determined as previously described [8]. Briefly, cytosolic supernatants were prepared by resuspending 10^7 cells in 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and lysed by 20 strokes through a syringe with a 26 gauge needle. The lysate was centrifuged at 20,000*g* for 20 min. The protein concentration in the supernatant was determined by the Bradford method and assayed for epimerase activity. Assays were performed in a final volume of 200 μl, containing 35 mM sodium phosphate, pH 7.5, 4 mM $MgCl_2$, 0.5 mM UDP-GlcNAc, 50 nCi [^{14}C]UDP-GlcNAc, and 100 μg of cytosolic protein. Incubations were carried out at 37 °C for 4–6 h and stopped by addition of 300 μl ethanol. Radiolabelled substrates were separated by descending paper chromatography and quantified by liquid scintillation analysis. All specific activities were given as U/mg of protein. One unit is defined as the formation of 1 μmol product per minute.

Sialic acid quantification. Membrane-bound sialic acid was quantified by the periodate/resorcinol method [10]. In brief, lysates from 10^7 cells obtained as described above were oxidized in 250 μl with 5 μl of 0.4 M periodic acid at 37 °C for 90 min, followed by 15 min boiling in 500 μl of 6% resorcinol/2.5 mM CuSO4/44% HCl. After cooling for a few minutes, 500μl *tert*-butyl alcohol was added, the samples were vortexed and centrifuged for 5 min to precipitate cell debris. Immediately after spinning, the supernatants were poured into OD cuvettes and read at 630 nm. Sialic acid concentrations were calculated by comparison with a standard curve (usually in a range of 0–250μM sialic acid) and expressed in nanomoles per mg cytosolic protein.

Flow cytometry analysis. Cells (10^6) were incubated for 45 min directly with 5 μg of either FITC-*Maackia amurensis* agglutinin (MAA) (EY Laboratories, CA, USA), FITC-Sambucus nigra agglutinin (SNA) (EY Laboratories), or with 1 μg biotin-TML (Calbiochem, CA, USA) followed by an additional 60 min incubation with 5 μg FITC–streptavidin (Sigma). Two washes with at least 1 ml PBS each were performed between all steps. Cell fluorescence was analyzed in a Becton–Dickinson FACScan apparatus. Anti-PSA antibody M735 was kindly provided by Dr. Rita Gerardy-Schahn.

Western blot analysis. For Western blot analysis, usually 30 μg of cytoplasmic or membrane protein was loaded in a 7.5% SDS–PAGE gel and blotted according to standard procedures. TML, MAA, and SNA were from Calbiochem (USA) and Vector (USA). Anti-PSA antibody M735 was used at a 1/5000 dilution, as described [11].

Results

Establishment of cell cultures

Cell cultures from muscle biopsies of 11 clinically and genetically confirmed HIBM patients with mutations in the *GNE* gene, and of 9 normal controls, were established as described. Nine of the patients were of Jewish Persian descent, carrying the founder homozygous mutation M712T; one Iranian non Jewish patient carried a homozygous epimerase mutation, V367I [12], and another patient from German origin was a compound heterozygote, with one mutation at the epimerase domain (R11W) and the second at the kinase domain (F537I) (H. Lochmuller, unpublished data).

Enzymatic activity of GNE in patient-derived muscle cultures

GNE possesses UDP-GlcNAc 2-epimerase activity as well as ManNAc kinase activity. In this study, we only determined the epimerase activity of the cells for two reasons. First, the epimerase activity is rate limiting for the overall reaction of GNE and is, in contrast to the kinase activity, regulated by several mechanisms [6]. Second, detection of specific ManNAc kinase activity in lysates of cells with low GNE expression, as muscle cells, is almost impossible due to the high background activities of other sugar kinases.

We first detected UDP-GlcNAc 2-epimerase activity in cells expressing the M712T mutant and in control cells (Fig. 1). The epimerase activity has a variable range between the cells deriving from different individuals of the same group of more than twofold, and overlapping values were detected between the two groups. However, the mean values for each group (60 ± 13 μU/mg versus 41 ± 11 μU/mg, p value $= 0.002$) point to a significant decrease of about 32% in the epimerase activity of the HIBM cells (Table 1). This decrease is consistent with the 35% decrease value we found in the lymphocytes of HIBM patients [8]. Interestingly, the patient cell line with the highest activity, which was in the range of control cells, derives from the unaffected quadriceps muscle. Next we analyzed the UDP-GlcNAc 2-epimerase activity of two additional cell cultures derived from patients with the V367I and the R11W/F537I mutations (Fig. 1). The activities were reduced for 60% and 57%, respectively, compared to the control cells. They are therefore lower than the activities of cells with the M712T mutant, indicating that the degree of epimerase activity may be dependent on the localization of the mutation.

Table 1
UDP-GlcNAc 2-epimerase activity (μU/mg protein)

Muscle cells	Epimerase activity (μU/mg protein)	p value
Normals	60 ± 13 (9)	
All affected	41 ± 11 (11)	0.002
M712T	$45 + 9$ (9)	0.007
Epimerase mutations	$25 + 2$ (2)	0.004

Epimerase activity values are the average of 4 independent experiments. Values are means + SD of the data of the indicated number of muscle cell cultures.

Characterization of sialylation in muscle cultures

In order to investigate the effect of the decrease in the epimerase activity of GNE on the sialylation pattern of the cultured HIBM muscle cells, membrane-bound sialic acid was evaluated by various means. Membrane-bound sialic acids (including plasma membrane, membranes of the Golgi apparatus, and lysosomal membranes) were quantified by the colorimetric periodate/resorcinol assay (Fig. 2). Here again a broad range of membrane-bound sialic acids were observed between the cell cultures of the various individuals, with overlapping values between affected and controls. When comparing the cell lines carrying the M712T mutation with the control cells a slight, but not statistically significant, decrease in sialylation was observed (Table 2). In contrast, the membrane-bound sialic acid content of the homozygous V367I cells was reduced by 35% (Fig. 2), in agreement with the strong reduction of UDP-GlcNAc 2-epimerase activity in these cells.

Cell surface expression of sialic acids was further analyzed in HIBM and control cells using various sialic

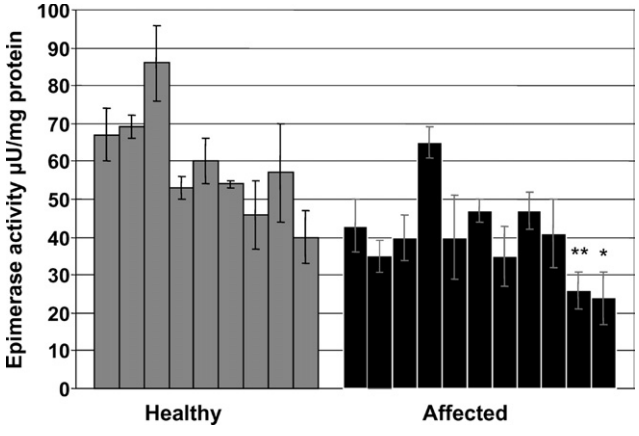

Fig. 1. UDP-GlcNAc 2-epimerase activity in cultured muscle cells' cytoplasm. Each column value represents the average of 4 independent experiments. All muscle cultures carried the homozygous mutation in the kinase domain (M712T), except ** that carried a mutation at the epimerase domain (R11W) and a mutation at the kinase domain (F537I), and * that carried a homozygous mutation at the epimerase domain (R367I).

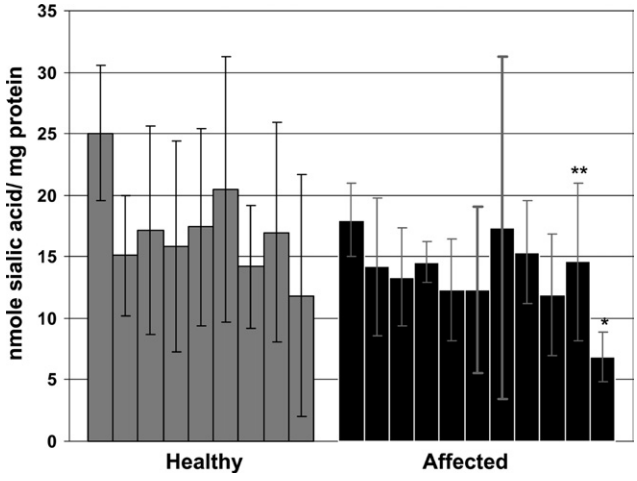

Fig. 2. Membrane-bound sialic in cultured muscle cells. Each column value represents the average of 5 independent experiments. All muscle cultures carried the homozygous mutation in the kinase domain (M712T), except ** that carried a mutation at the epimerase domain (R11W) and a mutation at the kinase domain (F537I), and * that carried a homozygous mutation at the epimerase domain (R367I).

Table 2
Overall sialic acid in muscle cultures by the periodate/resorcinol assay

Muscle cells	Bound sialic acid (nmol/mg protein)	*p* value
Normals	17 ± 3.8 (9)	
All affected	13.7 ± 3 (11)	0.038
M712T	14.3 + 2.2 (9)	0.079
Epimerase mutations	10.7 + 5.4 (2)	0.073

Values are means + SD of the data of the indicated number of muscle cell cultures.

acid specific lectins, such as *Tritrichomonas mobilensis* (TML) which binds to sialic acid in α2,3-, α2,6-, and α2,8-linkages. No significant difference in TML binding was found between HIBM and control cells by FACS analysis (Fig. 3). Analysis with two linkage-specific lectins, MAA for α2,3-linked sialic acids, and SNA for α2,6-linked sialic acids to trace more subtle changes of sialylation did not indicate any difference either (Fig. 3). Finally, Western blots were performed with the same lectins to examine more specifically the sialic acid pattern on membrane glycoproteins. No changes could be observed in the individual cells from either patients or control individuals, instead a broad range of variability among the different individuals from either group was observed in these analyses (data not shown).

Characterization of PSA in muscle cultures

Although polysialic acid (PSA) is not naturally present in adult muscle tissue [13], it can be detected in cultured dividing cells. Furthermore, expression of PSA is the most sensitive marker for alterations in UDP-GlcNAc 2-epimerase activity [14]. We have therefore analyzed the PSA pattern of the different muscle cultures. Western blots were performed with proteins extracted from cell cultures of both HIBM and control individuals. As shown in Fig. 4, protein extracts derived from M712T HIBM patients as well as the healthy controls display PSA at their surface (lanes 3–9), and no significant difference either in the PSA quantity or in the molecular weight of the polysialylated glycoproteins could be observed. However, in the ms86 culture (lane 10) there is a strong decrease in PSA and in the ms312 cell culture (lane 11) almost no PSA could be detected. As expected, PSA could not be detected in protein samples from HIBM and control muscle biopsies (lanes 1 and 2).

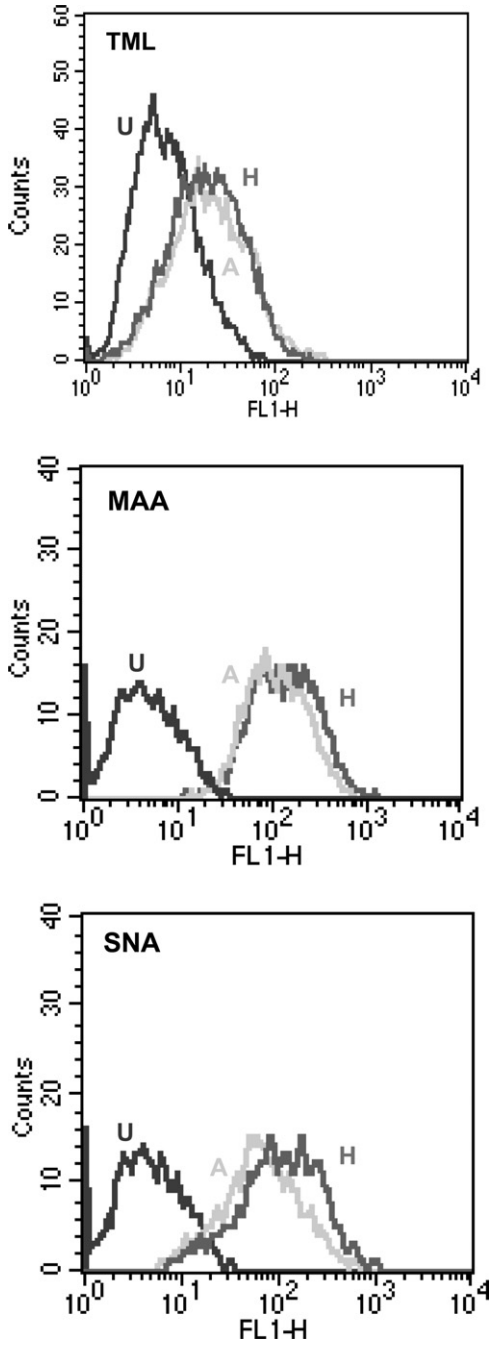

Fig. 3. Cell surface sialylation of muscle cell cultures. Sialylation was FACS analyzed by the sialic acid-specific lectins TML, MAA, and SNA. U, untreated cells; H, muscle cultured cell from a healthy individual; A, muscle cultured cells from an HIBM affected patient.

Discussion

HIBM patients worldwide, presenting the same unique phenotype, carry different mutations. The homozygous M712T mutation, occurring in all our Persian Jewish patients (more than 120), leads to the same phenotypic disorder as in all the other identified compound heterozygous mutations in both GNE domains. All the to date recognized mutations in HIBM result in a decrease of the epimerase activity of GNE in recombinant protein experiments ([8,15], and unpublished data). We have shown here that cultured muscle cells from M712T carrying patients reduce epimerase activity by

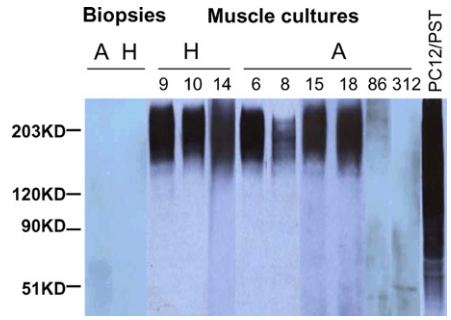

Fig. 4. Western blot for the analysis of PSA in HIBM muscle cultures. Anti-PSAmAb was probed on protein extracts from muscle biopsies (A, HIBM affected; H, healthy) and from cell cultures derived from healthy individuals (9, 10, and 14) and from HIBM patients (6, 8, 15, and 18 carry the M712T mutation in the kinase domain; 86 is a compound heterozygote with mutations at the epimerase domain, R11W, and at the kinase domain F537I; 312 carries a homozygous mutation at the epimerase domain, R367I).

about 35%. This reduced activity, however, does not affect the overall sialylation of the M712T affected muscle cells. These data are in good agreement with results from lymphocytes of the same patients [8]. In contrast, muscle cells carrying an homozygous mutation in the epimerase domain, which markedly reduced the epimerase activity in this cells for about 60%, indeed result in a significant decrease of the sialic acid content. Thus, even though all the different mutations in GNE detected in HIBM patients may affect the epimerase activity of the enzyme in cells, only a basic low level of epimerase activity seems to directly affect the extent of sialylation on the cells. These results are in agreement with the 25% sialylation reduction observed in 5 Japanese patients carrying at least one of the two compound heterozygote mutations in the epimerase domain [15,16]. Polysialic acid measurements in dividing muscle cells are consistent with this observation. Although PSA is not expressed in adult tissue, and in particular not in muscle tissue (and therefore cannot be directly or indirectly involved in the pathogenesis of HIBM), we have taken advantage of the muscle cultures to use it as a possible marker of sialylation. Cell cultures with a slight decrease in epimerase activity (those carrying the M712T mutation) display PSA at their surface; also the compound heterozygote cells, with one mutation in the epimerase domain and a second one in the kinase domain, do carry some PSA at the surface membrane. In contrast in the cells carrying the homozygous epimerase mutation which results in a very strong reduction of the epimerase activity, no PSA could be detected. Although we cannot exclude that this phenomenon is due to the relatively high passage number of this particular culture, this observation indicates that a serious impairment in the biosynthesis of sialic acid occurs only when the epimerase activity of GNE is dramatically reduced. It could be assumed that the normal metabolic range of epimerase activity is quite broad, and that the pathological threshold is most likely below the 35% reduction, since this decrease in the epimerase activity, as seen in the M712T patient muscle cells, does not affect the overall sialylation. This suggestion is in line with the fact that sialic acid biosynthesis is modulated by a feedback inhibition mechanism [17] and therefore, slight changes in the enzymatic activity are expected to be overcome in this manner.

Our studies are the first to analyze a relatively high number of HIBM patients, thereby overcoming the natural individual variability in epimerase activity and sialic acid content observed in several studies. This series of nine patients carrying the M712T homozygous kinase mutation displays a broad and variable range of epimerase activity in muscle cells, which all resulted in a normal sialylation pattern, as previously observed in lymphoblastoid cells. Only muscle cells with a strong reduction in epimerase activity also show a decrease in sialylation. These results suggest that the hyposialylation mechanism, although it may occur in some patients with specific mutations in the epimerase domain, may not necessarily be part of the unique pathophysiology of muscle tissue in HIBM patients, and therefore, other still unknown pathways involving GNE could be responsible for the disease phenotype. Alternatively, it cannot be excluded that a very subtle change in sialylation, that cannot be detected by the methods used in these studies, for example at one single specific glycoconjugate, could be enough to switch on the cascade of events resulting in the HIBM phenotype. Some speculations have been made about the role of α-dystroglycan in HIBM. However, conflicting data have been reported as to its sialylation status in this disease [15,16,18]. Broader studies characterizing the nature of the glycoconjugate sialylation link and identifying single sialylated proteins, as well as further analysis of differentially expressed genes in HIBM tissue, are necessary to gain more insights into the defective muscle cell function leading to the disease.

Acknowledgments

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