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Reduced sialylation status in UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE)-deficient mice

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Abstract Sialic acids are widely expressed as terminal carbohydrates on glycoconjugates of eukaryotic cells. They are involved in a variety of cellular functions, such as cell adhesion or signal recognition. The key enzyme of sialic acid biosynthesis is the bifunctional UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), which catalyzes the first two steps of sialic acid biosynthesis in the cytosol. Previously, we have shown that inactivation of the GNE by gene targeting causes early embryonic lethality in mice, whereas heterozygous GNE-deficient mice are vital. In this study we compared the amount of membrane-bound sialic acids of wildtype mice with those of heterozygous GNE-deficient mice. For that we quantified membrane-bound sialic acid concentration in various organs of wildtype- and heterozygous GNE-deficient mice. We found an organ-specific reduction of membrane-bound sialic acids in heterozygous GNE-deficient mice. The overall reduction was 25%. Additionally, we analyzed transferrin and polysialylated neural cell adhesion molecule (NCAM) by one- or two-dimensional gel electrophoresis. Transferrin-expression was unchanged in heterozygous GNE-deficient mice; however the isoelectric point of transferrin was shifted towards basic pH, indicating a reduced sialylation. Furthermore, the expression of polysialic acids on NCAM was reduced in GNE-deficient mice.

Daniel Gagiannis and André Orthmann have contributed equally to this work.

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Introduction

Sialic acid is the most abundant terminal monosaccharide of glycoconjugates of the eukaryotic cell surface [1, 2]. It is involved in a variety of cellular functions, such as cell–cell interaction including metastasis formation and progression of a variety of tumors, virus infection, and the biological stability of glycoproteins [3, 4]. Of particular interest is the unique polysialylation of the neural cell adhesion molecule (NCAM) [5].

The expression of the polysialylated form of NCAM is high during embryogenesis, peaks in the perinatal phase [6], and decreases rapidly in the adult, where only plastic regions in the brain express polysialic acid [7]. In addition, polysialylation of NCAM often correlates with tumor progression and prognosis [8, 9].

Sialic acids are synthesized in the cytosol from UDP-*N*-acetylglucosamine by four reactions. The first two reactions (Fig. 1) are catalyzed by a bifunctional enzyme, the UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE). GNE catalyzes both reactions as a multimer in the cytosol [10, 11]. A very recent study suggests expression of GNE not only in the cytosol but also in the nucleus [12]. GNE is ubiquitiously expressed, but Northern-blot analysis and *in situ* hybridization revealed the highest expression in liver [10]. During mouse development GNE is expressed at all stages investigated so far [13]. GNE is associated with the protein kinase C and the phosphorylation of GNE is regulating its enzymatic activity [14].

The clinical relevance of the GNE was demonstrated by the detection of a binding defect of the feedback inhibitor

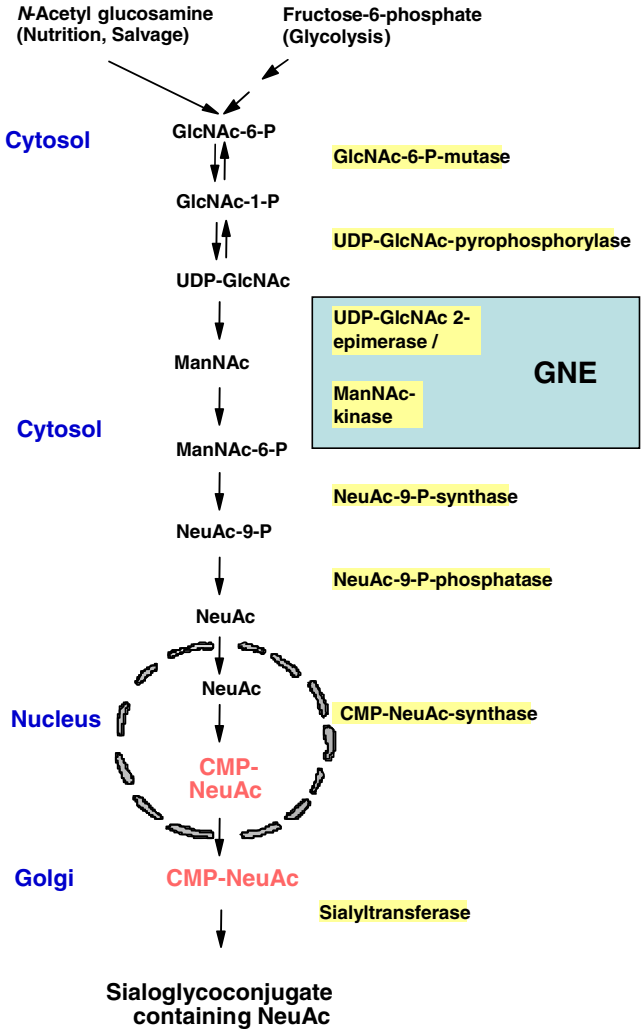

Fig. 1 Scheme representing the biosynthesis of sialic acid

CMP-sialic acid leading to Sialuria [15]. In this sialic acid storage disease, free sialic acid accumulates in the cytoplasm resulting in mental retardation of the patients. The significance of the enzyme is further illustrated by the observation that in a variant of HL60 cells the low expression of sialic acids is correlated with a dramatically reduced enzyme activity [16]. Recently, it has been proposed that mutations in the human GNE-gene are responsible for hereditary inclusion body myopathy, a unique group of neuromuscular disorders characterized by adult onset, slowly progressive weakness and a typical muscle pathology [17]. Sialylation is essential for early development in mice. The inactivation of the GNE by gene targeting causes early embryonic lethality, thereby emphasizing the fundamental role of this bifunctional enzyme and sialylation during development [18].

In this study we analyzed the sialylation of heterozygous GNE-deficient mice in more detail. Quantification of the membrane-bound sialic acid concentration in heterozygous GNE-deficient mice revealed a decrease of membrane-bound sialic acid in most organs compared with wildtype mice. Separation of transferrin by electrophoresis can be used to monitor the status of cellular protein glycosylation [19]. Therefore, we analyzed transferrin of wildtype and heterozygous GNE-deficient mice by Western blot analysis and found no change of expression, but a shift of the isoelectric point (pI) towards basic pH in heterozygous GNE-deficient mice, indicating less sialylated transferrin. In addition, we analyzed brain solubilisates from wildtype- and heterozygous GNE-deficient mice for expression of polysialylated NCAM. In agreement with our previous results, we found less polysialylated NCAM in GNE-deficient mice compared with wildtype mice.

Results and discussion

Homozygous GNE-deficiency is lethal at embryonic day 8.5 [18]. However, heterozygous GNE-deficient animals are vital and have no obvious defects. Since GNE is the key enzyme of sialic acid biosynthesis *in vitro* and *in vivo* we tested the hypothesis that heterozygous GNE-deficient animals have reduced sialic acid compared to wildtype animals. This is of special interest because until now there is no information available how much sialic acid is necessary for life (e.g. in mice). Therefore we analyzed the expression of membrane-bound sialic acid in wildtype and heterozygous GNE-deficient mice. For that we isolated brain, spinal cord, heart, lung, liver, kidney, spleen, blood cells, abdominal muscle or submandibulary gland and prepared membrane fractions from these organs.

Sialylation of mouse organs

In wildtype animals we found two groups of organs: organs with high levels of membrane-bound sialic acid (40–60 nmol sialic acid per mg membrane) and organs with low levels of membrane-bound sialic acid (10–25 nmol sialic acid per mg membrane). The first group (high sialic acid concentration) includes brain, spinal cord, lung, spleen, blood cells and submandibulary gland. The second group (low sialic acid) is represented by heart, liver, kidney, abdominal muscle. All data are summarized in Fig. 2 and Table 1.

The same groups of organs were also identified in heterozygous GNE-deficient animals. However, the levels of membrane-bound sialic acids were significantly lower. The high sialic acid concentration group represented by brain, spinal cord, lung, spleen, blood cells and submandibulary gland contained 30–40 nmol sialic acid per mg membrane; the only exception was spleen with more than 55 nmol sialic acid per mg membrane. The low sialic acid group is represented by heart, liver, kidney, abdominal muscle and

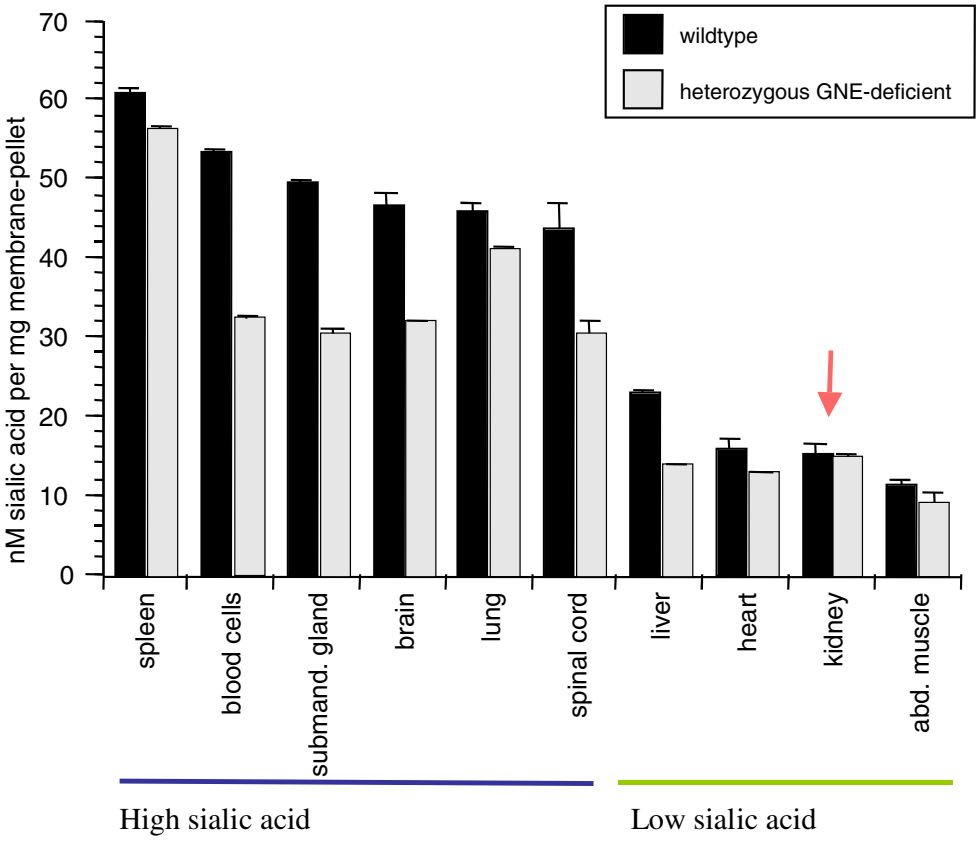

Fig. 2 Quantification of sialic acid in several organs. Organs were homogenized and membrane fractions were prepared. Sialic acid concentration was measured using the periodate/resorcinol method. *Each bar* represents mean values +/− SD of at least three animals. All experiments were carried out in duplicates. *Red arrow* indicates kidney, the only organ with unchanged membrane-bound sialic acids

contained only between 9 and 15 nmol sialic acid per mg membrane. All data are summarized in Fig. 2 and Table 1. The overall reduction of membrane-bound sialic acid in organs of heterozygous GNE-deficient animals is 25%. Each bar in Fig. 2 represents at least three animals. To our surprise we found in our analysis very little individual variation of the sialic acid concentration from animal to animal (see the SD in Table 1).

There is only one organ, which had the same membrane-bound sialic acid in wildtype- and heterozygous GNE-deficient animals. This is kidney (an organ with low concentration of membrane-bound sialic acid). There is no obvious explanation why the sialylation in kidney is the same in both animals. To our knowledge there is no salvage pathway in kidney, although no detailed organ-specific analysis of sialic acid biosynthesis can be found in the literature. As mentioned, GNE-deficient mice are vital and appear normal, although they have an overall reduction of membrane bound sialic acid of 25%. As mentioned in the introduction, all patients suffering from the hereditary inclusion body myopathy have mutations in the GNE-gene. Therefore, the heterozygous GNE-deficient mice could be a model system for hereditary inclusion body myopathy. Unfortunately, the GNE-deficient mice cannot be used as an animal model for hereditary inclusion body myopathy, since none of our animals developed a myopathy, not even after 2 years, the maximal live span of a mouse.

Table 1 Summarizes all data of the quantification of membrane-bound sialic acid in nmol per mg membrane.

Organ	wildtype	heterozygous GNE-deficient
Spleen	60.8+/−0.51	56.2+/−0.5
Blood cells	53.5+/−0.22	32.3+/−0.14
Submandibulary gland	49.3+/−0.38	30.3+/−0.77
Brain	46.6+/−1.4	31.8+/−0.1
Lung	45+/−1.7	42.2+/−0.7
Spinal cord	43.7+/−3.1	30.3+/−1.7
Liver	22.8+/−0.5	13.8+/−0.14
Heart	15.9+/−1.2	12.7+/−0.28
Kidney	15+/−1.4	14.8+/−0.21
Abdominal muscle	11.2+/−0.7	8.9+/−1.2

Sialylation of transferrin

Serum transferrin is a generally accepted glycoprotein to study glycosylation patterns [19]. We therefore analyzed transferrin from wildtype and heterozygous GNE-deficient mice by SDS-page and Western blotting. The serum concentration of transferrin is similar in wild type or heterozygous GNE-deficient mice (Fig. 3a). However, when we

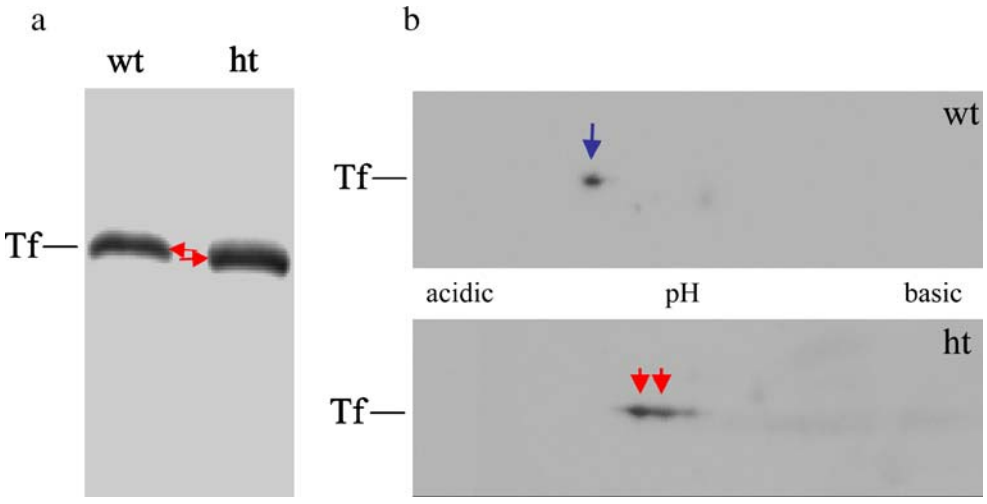

Fig. 3 Analysis of transferrin. Serum samples of wildtype (wt)- or heterozygous (ht) GNE-deficient mice were separated on 7.5% SDS-PAGE (**a**) or 2D gels (**b**) and analyzed by Western blot using polyclonal anti-transferrin antibodies. Note that the *red arrows* in A indicate a slightly different molecular weight

analyzed transferrin by two dimensional gel electrophoresis we found different spot patterns in both mouse-lines, indicating differentially modified transferrin. In wild type mice, we found only one specific spot (Fig. 3b, wt, blue arrow). In contrast, in heterozygous GNE-deficient mice (Fig. 3b, ht), we found several spots (red arrows) with higher pI, indicating loss of (negative) charged sialic acids, which is a common posttranslational modification of transferrin. A slight reduction in the molecular weight of transferrin of heterozygous GNE-deficient mice is already visible in Fig. 3a, which could be explained by the loss of sialic acid (compare red arrows in Fig. 3a).

Polysialylation of NCAM

Since polysialylation is a unique posttranslational modification of NCAM and polysialylated NCAM is easily detectable by Western blot analysis, we compared the expression of polysialylated NCAM in total mouse brain solubilisates of wildtype with heterozygous GNE-deficient mice (Fig. 4). We found high expression in both animals when loading more than 20 μg sample per lane. After detailed analysis of 1–4 μg sample per lane, we found dramatic differences between both mouse lines (as shown in Fig. 4 for 3 μg). Heterozygous GNE-deficient mice express significant less polysialic acids on NCAM. Quantification revealed a decrease of polysialylation by 54% in heterozygous GNE-deficient mice compared with wildtype animals.

From our previous studies we know that heterozygous GNE-deficient animals are vital without any further histological findings. Therefore, we concluded in our earlier manuscript [18] that one allele of the GNE is enough to express sufficient levels of GNE to ensure physiological sialic acid concentration. To our surprise we found in heterozygous GNE-deficient animals 25% reduced membrane-bound sialic acid. Interestingly this reduction is organ-specific. In liver, blood cells and submandibulary gland the reduction is 40%, whereas in kidney there is no reduction at all. This would implicate a fundamental role of sialylated membrane proteins in the kidney. The heterozygous GNE-deficient animals could display an animal model for minimal concentration of (membrane-bound) sialic acid, without having obvious defects in the architecture or function of organs. However, the fact that heterozygous GNE-deficient mice express less polysialic acids might have consequences on their behavior, since polysialyltransferases knockout mouse have been reported to have some deficits in learning and memory [20, 21]. Further experiments using a conditional GNE-deficient mouse system will help to answer, which concentration of membrane-bound sialic acid might be necessary for a functional organ and to ensure learning and memory.

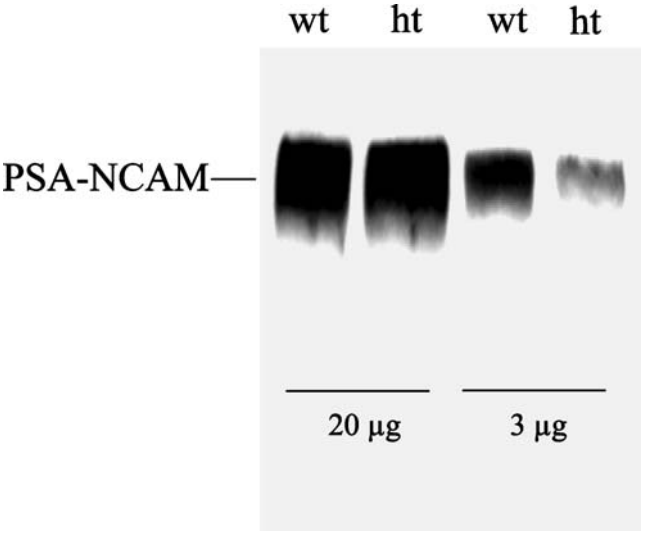

Fig. 4 Analysis of polysialylated NCAM. Brain samples (20 or 3 μg per lane) of wildtype (wt)- or heterozygous (ht) GNE-deficient mice were separated on 7.5% SDS-PAGE and analyzed by Western blot using polysialic acid-specific antibodies

Experimental

Animals and genotyping

DNA of individual female C57 BL/6 mice was digested with BamH1, subjected to Southern blotting, then analyzed by hybridization (labeled to 10^8 cpm/μg by random priming) as described [18].

Mice were sacrificed and brain, spinal cord, heart, lung, liver, kidney, spleen, submandibulary gland, abdominal muscle and blood (separated into blood cells and serum) were extracted and frozen for further investigations.

Antibodies

Polyclonal antibodies to transferrin were used as IgGs (gift of Dr. H. Fuchs, Charite, Berlin). Monoclonal anti-PSA antibody 735 was used as IgG (gift of Dr. R. Gerardy-Schahn, MHH, Hannover).

Membrane preparation

Mouse tissue was homogenized in ice-cold buffer containing 1 mM $NaHCO_3$ and 0,2 mM $CaCl_2$ (pH 7,9) using a glass potter. The homogenized material was centrifuged at 4°C for 10 min at 900 g. The supernatant was collected and the pellet was homogenized again in ice-cold buffer containing 1 mM $NaHCO_3$ and 0,2 mM $CaCl_2$ (pH 7,9). After centrifugation (4°C, 10 min, 900 g) both supernatants were combined and further centrifuged at 4°C for 1 h at 30,000 g. The pellet represents the membranes, which was freeze-dried prior to further analysis.

Sialic acid quantification

Free sialic acid was quantified by the periodate/resorcinol method modified from Jourdian et al. [22]. In brief, dried and weighted membrane pellets 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 $CuSO_4$/44% HCl. After cooling for a few minutes, 500 μl tertbutyl 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 0–250 nmol sialic acid) and expressed in nanomoles per mg membrane.

2D-Gelelectrophoresis

2D-gelelectrophoresis was performed using the IEF/SDS-PAGE procedure as described previously [23]. Cell extracts were mixed with 1.2 fold dry strip rehydration buffer in order to reach a final concentration of 2 M thiourea, 7 M Urea, 4% (w/v) Chaps, 0.3% (w/v) DTT and 2% (v/v) IPG buffer pH 4–7. Following a 30 min incubation at 25°C and a subsequent centrifugation for 5 min at 12,000 rpm, pH 4–7 IPG strips (immobilized pH gradient, 18 cm, Amersham Pharmacia, Freiburg, Germany) were rehydrated overnight at room temperature in 360 μl volume of rehydration buffer/cell extract mixture. IEF was carried out for 38,500 Vh at a maximum of 3,500 V using the Multiphor II system (Amersham Pharmacia, Freiburg, Germany). Following end of focusing, IPG strips were treated with equilibration buffer (50 mM Tris, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) supplemented with 0.15% (w/v) DTT, followed by a secondary 15 min treatment with equilibration buffer supplemented with 0.24% (w/v) iodoacetamide. The pretreated IPG strips were then transferred onto SDS-PAGE gels and electrophoresis was carried out at a constant voltage of 100 V at 10°C according to the Amersham Pharmacia instructions.

Immunoblotting

Serum samples of wildtype- or heterozygous GBE-deficient mice were separated on 7.5% SDS-polyacrylamide gels (BioRad) and transferred to nitrocellulose filters. The blots were blocked with 4% fat-free dry milk powder in PBS and incubated with the respective primary antibodies. We used polyclonal anti-transferrin antibodies. After washing, the blots were incubated with peroxidase-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham Buchler) according to the manufacturer's instructions, and visualized by exposing the blots to a Fuji imager system (LAS) for time periods between 10 and 120 sec.

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