SUPPLEMENT
The Glycobiology of Human Milk Oligosaccharides

Sialic Acid Utilization[1–3]

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ABSTRACT

Early postnatal development encounters milk as a key environmental variable and yet the sole nutrient source. One evolutionary conserved constituent of milk is sialic acid, which is generally displayed on glycoconjugates and free glycans. During early postnatal development, high sialic acid need was proposed to be unmet by the endogenous sialic acid synthetic capacity. Hence, milk sialic acid was proposed to serve as a conditional nutrient for the newborn. In the elderly, at the other end of ontogeny, decreased sialylation in the brain, saliva, and immune system is observed. Analogous to the neonatal situation, the endogenous synthetic capacity may be unable to keep up with the need in this age group. The data discussed here propose a functional dietary role of sialic acid as a building block for sialylation and beyond. *Adv. Nutr. 3: 392S–397S, 2012.*

Introduction

Postnatal mammalian development is special in that mothers provide their infant with the sole nutrient source and an important part of their environmental stimuli in the form of milk. Generally speaking, milk is one of the most complex biological fluids providing nutrients, protective compounds, and growth and development factors (1,2). Depending on the species, the duration of lactation and the quantity of milk and its constituents vary. Also among different mammalian species, some constituents are more and others less evolutionarily conserved (3–6). For the conserved constituents, this suggests that there are universal roles in newborn development, despite the varied life histories and wide diversity of habitats that mammals occupy. That there are differences in milk composition also suggests that each mammal has adapted its milk composition to cope with the specific needs of their neonates.

Based on anatomic and metabolic comparisons of primitive egg-laying and more modern mammals, several theories have been put forward regarding the origin of mammary glands and their secretions. One such theory is that the provision of innate protective factors was at the base of lactation and that the provision of nutrients was secondary (4,7).

Milk oligosaccharides are an important compound group in most animal milk, with some oligosaccharides showing high and others low evolutionary conservation (3). In general, free milk oligosaccharides are extensions of the disaccharide lactose with galactose, *N*-acetylglucosamine (GlcNAc),[4] *N*-acetylgalactosamine, fucose, and sialic acid [*N*-acetylneuraminic acid (Neu5Ac), and/or *N*-glycolylneuraminic acid (Neu5Gc)]. Although milk from farm animals is relatively poor in oligosaccharide amount and structural diversity, human milk is particularly rich in both (8). Sialylated oligosaccharides, especially the structurally simpler sialyllactoses, are widespread and can be found in the milk from mice to humans. In humans, some of the variation in milk oligosaccharides can be attributed to differences in genetic background, which also manifest as differences in blood group determinants. Other mammals such as rats and mice have only few milk sialylated oligosaccharides, which are in the form of sialyllactoses and

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[4] Abbreviations used: GlcNAc, *N*-acetylglucosamine; GNE, glucosamine (UDP-*N*-acetyl)-2-epimerase/*N*-acetylmannosamine kinase; ManNAc, *N*-acetylmannosamine; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid.

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are present in relatively high amounts. This suggests an ecological niche and species-dependent selective pressure for milk oligosaccharide types, diversity, and amounts (9). Likely sources of selective pressure come from both commensal and pathogenic microbes, rendering milk glycans important modulators of the microbiota (10,11).

The role and potential of milk sialic acid in human nutrition and especially as food for the brain were recently reviewed (12,13). Here, we discuss sialic acid as a glyconutrient for the newborn and elderly with an emphasis on possible metabolic fates.

Current status of knowledge

What is sialic acid and why control over sialic acid levels is central

Sialic acid was initially described in saliva (Greek: *sialon*) and brain glycolipids and thus received its name. Sialic acids are in fact a family of monosaccharides with a 9-carbon backbone incorporating a keto acid functional group from which the negative charge and acidity of these compounds derive. Sialic acids are found in the deuterostome lineage of the animal kingdom and in some bacteria. Plants are generally considered not to produce sialic acids (14), although there is evidence in another recent report (15). The most common sialic acids in mammals are Neu5Ac and Neu5Gc. However, humans have a deletion mutation in the gene *CMAH* (cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase) that normally encodes the enzyme to convert Neu5Ac into Neu5Gc. Therefore, contrary to most mammals, humans can only synthesize Neu5Ac (16). However, specific human cells, such as endo- and epithelial cells, display Neu5Gc incorporated from dietary sources rich in Neu5Gc (17). As most humans have anti-Neu5Gc antibodies, its possible impact on human physiology is the subject of ongoing research in both humans and using a Neu5Gc-deficient mouse model (18–21). On endothelia, interaction of displayed Neu5Gc with circulating anti-Neu5Gc antibodies could lead to and exacerbate vascular inflammatory damage and pathology.

Our understanding of the mechanisms and regulation of dietary sialic acid (Neu5Ac and Neu5Gc) uptake is rather limited. However, recent in vitro studies show that Neu5Ac can outcompete Neu5Gc uptake, suggesting that the same pathway via pinocytosis and the lysosomal sialic acid transporter SLC17A5 are used (22). The implications of this observation for the uptake and utilization of Neu5Ac and Neu5Gc from dietary sources with a predominance of one or the other form of sialic acid deserves further investigation.

On mammalian cells, sialic acids are displayed terminally either as a single sialic acid moiety or as polysialic acid chains on *N*- and *O*-linked glycans of glycoproteins and glycolipids. Similar to other glycans, the addition and removal of sialic acids are a means to modulate structural stability, cell recognition, and communication processes (23–25). This implies that the regulation of sialylation and the availability of sialic acid are of paramount importance.

In mice, a loss of function mutation in *Gne* [glucosamine (UDP-*N*-acetyl)-2-epimerase/*N*-acetylmannosamine kinase], the key enzyme for sialic acid synthesis, resulted in an embryo-lethal phenotype (26). Similarly, mice with a human M712T *Gne* knockin mutation showed greatly reduced GNE activity and died at postnatal day 3, although the same mutation in humans is not lethal (27). These results suggest that sialic acid provided from the mother to the fetus can maintain only to a limited extent mouse embryo development and that ultimately endogenous sialic acid synthesis is essential. In humans, several mutations in the *GNE* gene leading to decreased enzyme activity are described (including M217T) (28,29). Briefly, this group of *GNE* mutations results in hereditary inclusion body myopathy characterized by adult onset of muscle weakness and pathology (30). Recent preclinical data suggest that patients might at some point benefit from Neu5Ac or its precursors downstream of *GNE* as a cure (31). Another mutation in *GNE* leads to loss of feedback inhibition by the end product of the sialic acid biosynthetic pathway, CMP-Neu5Ac (32). This mutation leads to accumulation of sialic acid in the cytoplasm and sialuria and excretion of large amounts of sialic acid in urine. Sialuria primarily affects infants, who generally show multiple developmental problems such as neonatal jaundice, an enlarged liver and spleen, small red blood cells, weak muscle tone, frequent upper respiratory infections, and episodes of dehydration and stomach upset (gastroenteritis). Older children may have seizures and learning difficulties, although this is not universal. Rather little is known about the long-term effects of this sialic acid overproduction disease, but many of the associated problems appear to improve with age.

Another group of mutations related to control of sialic acid levels affects the sialic acid transporter SLC17A5 (sialin) and leads to sialic acid storage disease. The resulting accumulation of sialic acid in lysosomes negatively affects nervous system development and function. SLC17A5 not only transports sialic acid, but also glutamate and aspartate into synaptic vesicles. Therefore, some of the symptoms of this disease could be caused by deficits in amino acids for neurotransmission (33). Likewise, mutations in the neuraminidase *NEU1* gene lead to sialidosis characterized by the accumulation of sialylated glycans in tissues and numerous developmental problems (34).

Together, control over sialic acid levels and distribution is crucial. Compared with humans, mice appear to be more affected by low or insufficient endogenous sialic acid synthesis.

Milk as nature's model for the supply of glyconutrients such as sialic acid

When milk is the exclusive source of nutrients, are dietary sialic acid and the sialic acid salvage pathway (i.e., biochemical recycling of sialic acid into complex biomolecules) used in preference to endogenously de novo synthesized sialic acid? In other words, are there times of high metabolic demand for sialic acid with insufficient endogenous synthetic capacity?

Human milk is a rather rich source of sialic acid, ranging from ~1.5 g/L in colostrum to 1 g/L in transition milk and 0.3 g/L in mature milk at 3 mo postpartum (35). In general, infant formulas contain considerably less sialic acid than

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human breast milk, and the ratio of Neu5Ac to Neu5Gc in formula may vary depending on the animal milk used. Cow's milk–derived formulas generally provide >95% of sialic acid as Neu5Ac, whereas goat's milk–derived formulas could provide relatively much higher Neu5Gc (17). Interestingly, most sialic acid in human milk is oligosaccharide bound, and this fraction is much smaller in cow's milk– based infant formula. However, the level of protein-bound sialic acid in human milk at ~3 mo postpartum is similar to the protein-bound sialic acid level in whey-predominant infant formula (35,36)

Because of such differences, breastfed and formula-fed infants were studied to compare whether a high or low sialic acid intake in early life influences sialic acid display in the neonate. Saliva contains relatively high amounts of sialic acid, mainly bound to glycoproteins such as mucin and secretory IgA, and can easily be collected noninvasively. In infants born at term, at a mean age of 5 mo, higher free and total sialic acid levels were reported in saliva when infants were exclusively breastfed compared with infants exclusively formula fed (37). Bound sialic acid in saliva showed only a trend toward higher levels in the breastfed infants. In a second study from the same research team, saliva sialic acid levels were compared over time from 2 wk to 4–5 mo postpartum in formula-fed and breastfed preterm infants (38). Again, total saliva sialic acid levels were higher in the human milk–fed group, particularly in the first 3 mo. However, a potential problem in these studies is that part of the measured difference could be due to constituents of the suckled milk that remained in the sampled saliva. In a third correlation study, higher sialic acid levels were measured in the frontal cortex of the brain from a cohort of predominantly breastfed compared with predominantly formula-fed sudden infant death syndrome infants (39). Differences were observed both in protein-bound and ganglioside-bound sialic acid.

This series of observations prompted the proposal that milk sialic acid serves as a glyconutrient in the newborn via the salvage pathway, thus leading to increased sialic acid levels in tissues and saliva of breastfed infants (37–39). Whether the increases in saliva and brain sialic acid levels are derived from dietary sialic acid per se and whether in humans this influences health, physiology, and nervous system function remain to be shown.

Animal models to study milk sialic acid utilization

Rats and mice are widely used as research models, and this is especially true for the study of milk sialic acid. Their milk contains 3′- and 6′-sialyllactoses (Neu5Ac-α2,3-lactose and Neu5Ac-α2,6-lactose) as the only milk oligosaccharides, which are present in rather high amounts (~1–6 g/100 g milk dry matter). Currently, no Neu5Gc has been reported in the oligosaccharides of rat and mouse milk (10,40). More than 80% of total milk sialic acid is actually presented in the form of sialyllactose (40,41). In both mouse and rat milk, 3′-sialyllactose is highest 5–7 d postpartum and decreases thereafter. In rats, 6′-sialyllactose increases in parallel with 3′-sialyllactose, but only to ~10% of 3′-sialyllactose levels, and remains constant thereafter (40). In slight contrast, in early mouse milk, 6′-sialyllactose levels are approximately half those of 3′-sialyllactose and remain constant throughout lactation (10).

The changing expression profile of 3′-sialyllactose in rat and mouse milk during lactation has been used as the basis for correlation studies between milk and the suckling pups. For example, Dickson and Messer (42) showed that in mice and rats, small intestinal neuraminidase enzyme activity correlated with milk sialic acid content, and this especially in the middle and distal small intestine. In accordance with this, rat neuraminidase *Neu1* gene expression was observed to decrease from postnatal days 17 to 21 in the jejunum and ileum (43). In the rat colon, *Neu1* transcripts were detectable at birth and decreased toward weaning at postnatal day 21 (40). Neuraminidase 1 is thought to have a preference for α2,3- and α2,6-linked sialic acid from a β-galactoside such as is present on 3′- and 6′-sialyllactose (44). Together, these findings suggest that milk sialyllactoses serve as sialic acid carriers delivering sialic acid to the suckling pups.

Once cleaved from lactose, sialic acid can be imported into the cells through the SLC17A5 transporter (22,45). Expression levels of the *Slc17a5* transcript also correlated with milk 3′-sialyllactose levels (40), which suggests that at least part of neuraminidase-liberated sialic acid from sialyllactose is taken up by small intestinal and colonic SLC17A5 into cells. Once internalized, sialic acid can then have one or several fates, including being a) further transported systemically intact, b) activated with CMP and used for subsequent glycosylation, or c) catabolized to pyruvate and *N*-acetylglucosamine.

The precise fate of internalized sialic acid is not known, but significant insight has been obtained from a series of pioneering studies with ^{3}H and ^{14}C single- and double-radiolabeled Neu5Ac and derivatives in suckling and weaning rats and mice (46–48).

Ingested Neu5Ac, both as free sialic acid or sialyllactose, was readily taken up from the intestine and passed into the blood and tissues within 6 h (46,48). However, only a relatively small fraction was retained in the body; the majority was excreted into urine both as sialyllactose and liberated sialic acid (40,46). The absolute amounts of sialic acid absorbed and retained in the body likely depend on many variables including the form of sialic acid ingested (i.e., free or bound), the age, and the nutritional and health status of the animal. After oral administration of ^{14}C-Neu5Ac (given as sialyllactose or free sialic acid) 3-d-old suckling rats retained ~30% of ^{14}C in the body after 6 h (48). In food-deprived mice of weaning age (20 d), only ~1.5% of ^{14}C from administered ^{14}C-sialyllactose was retained in tissues after 6 h (46). When free double-labeled Neu5Ac [*N*-acetyl-(2-^{14}C,9-^{3}H)neuraminic acid] was administered to food-deprived mice, ~4% of intubated ^{3}H radiolabel was retained in the organs after 6 h, whereas <1% of ^{14}C was retained (47). Sialic acid can be metabolized by *N*-acetylneuraminate lyase (Npl) cleavage into the products *N*-acetylmannosamine (ManNAc) and pyruvate. This double-labeling study indicates that a greater fraction of ^{3}H-ManNAc (or ^{3}H-GlcNAc or other metabolite) is retained by the body compared with that of ^{14}C-pyruvate, which is further metabolized and exhaled as CO_2.

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Interestingly, well-fed mice exhaled more radiolabeled CO_2 derived from ingested sialic acid compared with food-deprived animals (47), indicating different metabolic use depending on metabolic state.

Intestinal gene expression profiles correlating with milk 3′-sialyllactose and *Slc17a5* gene expression also suggest that ingested sialic acid can be metabolized by Npl to ManNAc and pyruvate and that ManNAc can in turn be epimerized to GlcNAc by renin-binding protein (*N*-acetyl-D-glucosamine-2-epimerase) (40). The overall contribution and importance of the dietary sialic acid to the cellular GlcNAc pool, hexosamine flux, and *N*-linked glycosylation and consequent physiological effects are not known. Our recent gene expression study suggests that when the milk sialic acid supply is high, endogenous colonic sialic acid and GlcNAc biosynthesis may be low, as based on *Gne* and *Gfat* (glucosamine-fructose-6-phosphate aminotransferase) expression, respectively (40).

The use of dietary sialic acid for glycosylation or further metabolism may be a feature of intestinal epithelial cells. However, that ingested sialyllactose can be found intact in plasma (40,46) and that radiolabel from ingested Neu5Ac was detected in distal tissues such as the brain suggest that other tissues may make use of dietary sialic acid. As mentioned previously, in humans, epithelial and endothelial cells lining the blood vessels can incorporate the nonhuman dietary-derived sialic acid Neu5Gc (17). Consequently, the reported increases in Neu5Ac in tissues after Neu5Ac administration might to a large extent localize to the endothelial cells of the tissue blood vessels.

The brain is the major site of Neu5Ac display and incorporation during development and in suckling rats was found to retain ~4% of ingested ^{14}C from Neu5Ac after 6 h (48). When correlating milk sialyllactose uptake during the suckling period with global gene expression profiles in the brain, the slight changes that we observed in the expression of genes involved in endogenous synthesis, uptake (*Neu1*, *Slc17a5*), catabolism (*Npl*, *Renbp*), or uptake into the Golgi apparatus for glycosylation (*Slc35a1*) did not point to a dominant pathway activated at the transcriptional level during early postnatal brain development (40). However, feeding or intraperitoneal injection of Neu5Ac in suckling rats from postnatal days 14 to 20 increased cerebral and cerebellar protein and ganglioside-bound Neu5Ac (49,50). On injection, rats also showed behavioral changes that remained beyond the treatment period (50). Likewise, studies in piglets also showed better cognitive performance in animals fed a sialic acid–enriched diet (51). The mechanisms leading to altered behavior and learning are not well understood. One possibility is the provision of sialic acid as a building block for sialylation. However, other mechanisms are possible and include a direct signaling role of sialic acid and an increased flux of the sialic acid catabolic product GlcNAc via the hexosamine pathway that leads to increased *N*-linked glycosylation and improved receptor half-life with consequent decreased signaling threshold (52).

Interestingly, in the liver of suckling rats, GNE enzyme activity increased in parallel to ingested 3′-sialyllactose and remained high after postnatal day 7 (40). How and why this occurs are unknown, but a metabolic signal might link dietary sialic acid intake with GNE enzyme activity in the liver, which is one of the major sites of endogenous sialic acid synthesis. Our results are in line with those of sialic acid feeding studies in young piglets and rats that showed increased liver and colon *Gne* mRNA levels on receiving a sialic acid–rich diet (40,51,63).

Specific absorption studies of dietary sialic acid are scarce, but several lines of evidence suggest that dietary sialic acid is taken up and used during the suckling period. At least partly, dietary sialic acid can be catabolized to ManNAc and GlcNAc for further use. The exact metabolic use and fate are not fully understood and warrant further investigation, at both the cellular and tissue levels. Knowledge of the use of dietary sialic acid in newborns could improve our understanding of the evolutionary advantages and roles of milk sialic acid compared with GlcNAc or ManNAc and lead to hypotheses for dietary sialic acid use beyond infancy.

From neonates to elderly

As proposed for early postnatal development, old age might be a second condition in which dietary sialic acid could compensate for an apparent deficiency of endogenous sialic acid. In elderly humans, lower sialic acid levels were reported on saliva proteins (53), brain gangliosides (54–56), and specific immune cell components (57). The reasons leading to decreased sialylation are not understood. With old age, numerous organ and body functions are affected. For example, salivation and muscle function are affected in disorders such as xerostomia and dysphagia and gastrointestinal function in gastroesophageal reflux disease and chronic constipation (58–60). Loss of neuronal and receptor function is associated with these problems, and in this respect, the sialic acid present on gangliosides and proteins may play a role because it is important for their function (23,61,62).

In 24-mo-old rats, but not in 3-mo-old rats, we also observed increased sialic acid levels in the ganglioside fraction of the right hemisphere of the brain on sialic acid feeding (63). Total protein-bound sialic acid was not affected in this setting. Dietary sialic acid was shown to increase brain ganglioside-bound sialic acid in malnourished rat weanlings (49,50) and in 8-wk-old rats (64). It is worth noting here that free sialic acid and oligosaccharide-bound sialic acid given in the diet do not necessarily have the same effects (64). The reasons for this are not known.

With the above in mind, we hypothesized that endogenous sialic acid synthesis or availability is limited in old age and that dietary sialic acid compensates for the postulated shortage. Hence, we looked at the RNA expression of *Gne* in the liver and colon, two of the major sites of sialic acid synthesis. The *Gne* transcript level was unchanged in the liver of aged compared with young adult rats, whereas in the colon, it was significantly increased (63). Therefore, although the sialic acid synthetic capacity of the liver may not change with age, that of the colon may increase. On Neu5Ac feeding, colon and liver *Gne* transcript levels increased in young adult

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animals. In aged rats, Neu5Ac feeding did not affect liver *Gne* expression in contrast to the colon, where *Gne* transcripts decreased (63). The differential response in *Gne* expression to Neu5Ac in young versus aged colon remains to be explained but suggests a signaling role for sialic acid. Nevertheless, the findings do not support the notion of a general endogenous sialic acid shortage in aged rats.

Because dietary Neu5Ac supply can affect brain ganglioside sialic acid levels, we wondered whether dietary Neu5Ac could also influence neuronal function. To this end, we measured pilocarpine-, a muscarinic acetylcholine receptor agonist, stimulated salivation in young and aged rats fed or not fed a Neu5Ac-enriched diet. We found reduced salivation in aged rats compared with young rats and that Neu5Ac feeding restored the salivation function in aged animals (63). This suggests that neuronal circuits and receptor function could be altered by Neu5Ac feeding. Mechanistically, this may occur via stabilization of receptor or neuronal membrane function by increased ganglioside-bound sialic acid. An additional and so far unexplored role could be via the free radical scavenger activity reported for sialic acid (65). Thus, supplemental sialic acid may limit neuronal damage and permit recovery of function, or it may exert direct signaling functions as seen in vitro by neuronal differentiation (66,67).

It is currently not known whether dietary Neu5Ac is used as such in aged animals or whether it is catabolized to ManNAc or GlcNAc with subsequent reentry into the sialic acid synthetic pathway or parallel metabolic pathway. With regard to early postnatal development, additional metabolic fate studies, such as those initiated by Nohle and Schauer (47) with double-labeled *N*-acetyl-(2-^{14}C,9-^{3}H) neuraminic acid would greatly advance our current understanding of dietary sialic acid utilization.

Conclusions

Although human correlation and animal sialic acid feeding studies suggest that dietary sialic acid can be utilized, the fundamental question as to whether we need dietary sialic acid remains unanswered? During early postnatal nutrition, nature provides the best model for sialic acid utilization. The fact that sialyllactoses are found in most mammalian milks strongly suggests that they play a conserved role(s) in postnatal development. Whether these roles include protection against infection, establishment of commensal microbes, promotion of nervous system performance, or simply nutrition awaits detailed preclinical studies and clinical trials. The ultimate goal will be to reestablish the natural course of events in the case of deficiency.

With the advent of new technologies and processes, it seems timely to continue the investigations into the use of diet-derived sialic acid from metabolic fate to biological effects and especially in at-risk populations from premature infants to the elderly. However, with presumed nonhuman sialic acid Neu5Gc–associated problems in mind, the sialic acid sourcing needs to be carefully evaluated to select Neu5Ac-rich sources.

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