N-glycolylneuraminic acid deficiency in humans

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Abstract — Classic studies suggested that the common mammalian sialic acid N-glycolyneuraminic acid (Neu5Gc) is an oncofetal antigen in humans, being immunogenic in adult humans and yet apparently expressed in human fetuses and tumors. We and others have recently found that the human deficiency of Neu5Gc can be explained by an inactivating mutation in the gene encoding CMP-N-acetylneuraminic acid hydroxylase. Thus, Neu5Gc is not an oncofetal antigen in the classical sense, and other explanations must be found for the observed expression pattern. This review provides an update on this matter, and considers a variety of other old and new questions that arise from it. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

1. N-glycolyl-neuraminic acid (Neu5Gc) is a common form of mammalian sialic acid

The sialic acids are a large family of acidic 9-carbon sugars found primarily in animals of the deuterostome lineage, that all appear to be biosynthetic derivatives of N-acetylneuraminic acid (Neu5Ac) or ketodeoxynonulosonic acid (KDN) [1–7]. One of the commonest biosynthetic transformations of N-acetylneuraminic acid in mammals occurs at the sugar nucleotide level, wherein a cytochrome b5/b5 reductase dependent CMP-Neu5Ac-specific hydroxylase generates CMP-N-glycolyneuraminic acid (CMP-Neu5Gc) [8–21].

These two nucleotide sugar donors appear to be used interchangeably by the Golgi CMP-sialic acid transporter [22] by most mammalian sialyltransferases that add terminal sialic acid residues to cell surface and secreted sialoglycoconjugates [23], and by the lysosomal sialic acid transporter that allows sialic acids to be recycled back to the cytosol [24]. Perhaps because there is no known pathway for converting Neu5Gc back to Neu5Ac, the latter tends to recycle and accumulate to high levels in cells that express the hydroxylase [18].

2. Evidence for a human-specific loss of Neu5Gc expression

The original studies of sialic acid distribution in nature noted that Neu5Gc was not easily detected in human tissues (reviewed in [1–3]). This was in contrast to most other mammalian tissues, wherein Neu5Gc was often found to be the major sialic acid. In separate studies it was found the serum-sickness antibody reaction to infusion of horse serum into humans (so-called Hanganutziu-Deicher or HD antibodies) was partly directed against Neu5Gc [25, 26]. This strengthened the notion that adult humans may not express Neu5Gc. However, several studies provided direct and indirect evidence that Neu5Gc was expressed in some human fetal tissues and cancers [27–32]. Thus, it was assumed that Neu5Gc was a classic ‘onco-fetal antigen’ which was expressed in fetal tissues prior to post-natal immune tolerization, suppressed during adult life, and then re-expressed during malignant transformation.

3. Genetic basis for the human-specific loss of Neu5Gc expression

The cloning of the mouse [18] and pig [19] CMP-sialic acid hydroxylase gene allowed a search for the human counterpart. The existence of homologous sequences in human EST (expressed sequence tag) databases suggested that messenger RNA for the human enzyme could be found in adult human tissues. Indeed, two groups independently cloned hydroxylase human cDNAs and noted a 92-bp deletion in the coding region sequence, in comparison to the corresponding regions of mouse and pig cDNAs [33, 34]. This deletion includes sequences coding for the putative Rieske-iron-sulfur binding motif of the murine hydroxylase protein that is thought to be critical for enzyme activity [18]. Irie et al. reported a cDNA sequence which did not include the initial 5’ translation start site [33]. This cDNA gave them a truncated inactive protein product in transfected cells comprising ~80% of the car

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boxyl end of the hydroxylase, leading to the assumption that an in-frame methionine codon downstream of the 92 bp deletion was the natural translation initiation site. However, Chou et al. cloned a more complete cDNA which included a primary initiator methionine codon corresponding to that of the mouse and pig protein, thus indicating production of a much shorter truncated polypeptide involving the amino terminus of the protein, with a frame-shift mutation resulting from the loss of the 92-base pair exon \[^{34}\]. It remains to be shown if the larger protein product studied in vitro by Irie et al. actually exists in vivo. Regardless, both groups came to the conclusion that the loss of Neu5Gc expression in humans was due to the lack of a functional CMP-Neu5Ac hydroxylase. Irie et al. also sequenced the entire corresponding region of the human genome, showing that the 92-bp deletion represented loss of a single human exon corresponding to exon 6 of the mouse hydroxylase gene \[^{33}\].

4. When did loss of Neu5Gc occur in human evolution?

The closest evolutionary cousins of humans are the African great apes, with the chimpanzee/bonobo clade considered by most to have shared a last common ancestor with us (likely about 5–7 million years ago) \[^{35, 41}\]. Blood samples from all the great apes showed high levels of Neu5Gc \[^{42}\] and genomic PCR studies indicated that the 92-bp exon was intact in these species \[^{34}\]. Cloning and expression of the chimpanzee hydroxylase cDNA confirmed that the great apes indeed have an intact functional hydroxylase gene \[^{34}\]. Thus, the 92 bp exon deletion must have occurred after the last common ancestor of humans with the great apes. On the other hand, the mutation was found in all human populations studied \[^{34}\], indicating that it occurred before the emergence and world-wide diaspora of modern humans (likely about 100–200 thousand years ago) \[^{43, 44}\].

5. Why did the human lineage lose expression of Neu5Gc?

There is no way to be certain why this human-specific mutation became universal in our species. Given the prominence of sialic acids cell surface receptors for a variety of microbes \[^{7, 45–47}\] a likely explanation is negative selection by a lethal pathogen that selectively recognized Neu5Gc \[^{48}\]. The fortuitous occurrence of the hydroxylase inactivation could thus have provided a survival advantage to humans who became homozygous for this mutated allele. Another possibility is that the deletion allowed positive selection, due to some favorable biological consequence of losing Neu5Gc expression. A final possibility is that the loss of the hydroxylase had neither positive nor negative selective value, but simply happened to occur in a human population that underwent a sudden constriction due to some unrelated factors, and later expanded to represent modern-day humans. Such a population bottleneck could have facilitated the drift of this mutation to fixation in the subsequent absence of positive or negative selection. Regardless of what the original reason was, this global change affecting almost all cell surfaces and sialylated glycoconjugates could potentially explain some of the obvious morphological and functional differences between humans and great apes.

6. Consequences of Neu5Gc loss for the functions of sialic acids in humans

The functions of sialic acids \[^{7}\] can be divided into two major categories: those involving the physical properties of the molecule (such as charge and hydrophilicity) and those in which sialic acids are specifically recognized by receptor proteins of endogenous or exogenous origin. With regard to the former category, the loss of Neu5Gc expression in humans is accompanied by a corresponding increase in the less hydrophilic sialic acid Neu5Ac. It remains to be explored if this change in the properties of cell surfaces has any biological consequences, particularly with regard to unusual molecules like polysialic acid, which is enriched in the brain. With regard to the category of sialic acid recognition there are several interesting effects of Neu5Gc loss to consider. For example, the presence or absence of Neu5Gc could affect the interactions of microbial pathogens such as influenza viruses \[^{49–53}\] E. coli K99 \[^{54}\] and rotavirus \[^{58}\]. There are many other microbes that also use sialic acids as specific binding sites on mammalian cells, including major pathogens such as Helicobacter pylori and Plasmodium falciparum \[^{45–58}\]. However, in most such instances, the consequences of having less Neu5Gc and more Neu5Ac on human cells has yet to be studied. Sialic acids also serve as ligands for a variety of endogenous vertebrate lectins \[^{47, 62–72}\]. With regard to the selectins a careful study has not been done, but available data would suggest that the type of N-acyl group of sialic acids should not affect recognition \[^{62, 63}\]. An as yet uncloned uterine agelutin reported in rats showed a preference for Neu5Gc \[^{73}\] and it remains to be seen if a human homologue exists. Recent evidence indicates that the recognition of alpha-dystroglycan by the G-domain of laminins involves recognition of sialic acid \[^{74}\]. However, the effects of changing an N-acyl group to an N-glycolyl group has not been examined.

The most extensive family of sialic acid-binding lectins are the Siglec, which are sialic acid-recognizing immunoglobulin superfamily lectins that share close homologies in the amino terminal V-set carbohydrate recognition domain \[^{65, 66, 70, 72, 75–77}\]. The effect of Neu5Gc on binding has only so far been reported for Siglec 1–6 \[^{63, 68, 71, 78, 79}\]. A clear-cut example is Siglec-1 (siaload
With regard to Siglec-2 (CD22), there are species-specific effects. While mouse CD22 shows a strong preference for Neu5Ac over Neu5Gc [81], it remains to be seen what if any consequence this has for the biology of sialoadhesin in humans versus great apes. It shows a pattern more similar to that of the rat [81]. It remains to be seen what if any consequence this has for the biology of sialoadhesin in humans versus great apes. With regard to Siglec-2 (CD22), there are species-specific effects. While mouse CD22 shows a strong preference for Neu5Gc over Neu5Ac [63, 79], human (and great ape) spleen of humans was found to be markedly altered in frequency of sialoadhesin positive macrophages in the situation amongst higher primates, there has not been a systemic evaluation of the distribution of Neu5Gc in multiple tissues from any of these species. Thus, some of these may well represent instances of post-natal suppression of normal hydroxylase expression, as originally postulated for humans. Caution should also be exercised in making broad statements about the distribution of Neu5Gc expression in various animal taxa on the basis of sampling just a few tissues in one or two representative species. For example, recent studies show that ducks express Neu5Gc [102], indicating that Neu5Gc deficiency is not a general feature of birds, as was previously suggested based on the immune reactions of chickens. Studies of the hydroxylase gene from some of these species are warranted. Regardless, the human-specific mutation in the hydroxylase gene is obviously an event unique to the higher primate lineage.

8. Neu5Gc expression in human tumors and fetuses

Reports of Neu5Gc expression in cultured human cell lines [10, 92, 103–105] may be explained by the incorporation of Neu5Gc from fetal bovine serum used in the culture medium [32, 106, 107]. A similar consideration applies to human tumors grown in immunodeficient but Neu5Gc-rich mice [105, 108]. However, there have been several reports of detection of Neu5Gc in fresh samples of humans tumors and fetuses [29, 30, 32, 95]. While most of these studies utilized unpurified polyclonal antibodies raised in chickens or HD antibodies from human patients [96, 109–112], and a few instances in which the presence of Neu5Gc was confirmed by mass spectrometry [30–32]. The exon deletion in the human hydroxylase gene likely involves the active site of the enzyme and it is hard to imagine how this could be repaired. Even if this were possible by some unusual mechanism operating in the genomic disarray of human tumors, one cannot explain reports of Neu5Gc detection in normal human fetal tissues. On the other hand, there are no homologous genes found in any genome, and no clear-cut alternative pathway for the synthesis of Neu5Gc has been demonstrated — although reports of De-N-acetylation of sialic acids [113, 115] and of a pathway for glycolyl-CoA production [116, 117] raise the possibility of a de-acylation/re-acylation mechanism. A simpler and more attractive possibility is that the Neu5Gc in tumors and fetuses originates from dietary sources (see below).

7. Neu5Gc deficiency in other animals

Since Neu5Gc has been reported in deuterostome lineage animals ranging from sea urchins to fish to primates, the hydroxylase gene must be at least 500 million years old, evidently having evolved at the same time as the sialic acids themselves [1–3, 42–83]. However, similar to humans, a serum-sickness like reaction against Neu5Gc has been reported in chickens, where the Neu5Gc can be of exogenous (horse serum infusion) or endogenous (viral-induced lymphoma expressing Neu5Gc) origin [87–89]. Indeed, both polyclonal and monoclonal antibodies against Neu5Gc have been raised in chickens [87, 90–96] and used to detect this sialic acid in tissue sections and lipid extracts from human tumors and fetuses (see below). Also, a cat blood group system with naturally occurring antibodies against Neu5Gc has been reported [97]. In addition, marked variations in Neu5Gc expression have been reported in tissues from various species, including inbred strains of mice [98–101]. However, unlike the situation amongst higher primates, there has not been a systemic evaluation of the distribution of Neu5Gc in multiple tissues from any of these species. Thus, some of these may well represent instances of post-natal suppression of normal hydroxylase expression, as originally postulated for humans. Caution should also be exercised in making broad statements about the distribution of Neu5Gc expression in various animal taxa on the basis of sampling just a few tissues in one or two representative species. For example, recent studies show that ducks express Neu5Gc [102], indicating that Neu5Gc deficiency is not a general feature of birds, as was previously suggested based on the immune reactions of chickens. Studies of the hydroxylase gene from some of these species are warranted. Regardless, the human-specific mutation in the hydroxylase gene is obviously an event unique to the higher primate lineage.
9. Traces of Neu5Gc may be present in normal human tissues

It used to be thought that normal human tissues did not contain Neu5Gc. A 1983 symposium abstract mentioned possible traces of Neu5Gc in human liver (Schroder C., Nohle U., Shukla A.K., Schauer, R.). Improved methods for the isolation and structural analysis of trace amounts of new sialic acids - identification of N-glycolyneuraminic acid in man. Abstract #162 at the 7th. International Symposium on Glycoconjugates, 1983). Using more recently developed methods, we found that a small HPLC peak corresponding to Neu5Gc is present in purified sialic acid preparations from several normal human tissues [12]. Confirmation of this finding by mass spectrometry is under way.

10. Do humans incorporate Neu5Gc from animal foods?

Based on all the above facts, the possibility arises that the Neu5Gc found in human tumors, fetuses and normal tissues is derived from dietary sources. In keeping with this notion, human cells grown in fetal calf serum (a source of Neu5Gc-containing glycoconjugates) express easily detectable levels of Neu5Gc [10, 106, 118], which disappear when the cells are cultured in serum-free media for a period of time [42]. Furthermore, orally administered sialic acids in rats can be absorbed and incorporated into tissues at a very low rate [119–121] possibly via formation of an acylmannosamine intermediate that is presumably reconverted to sialic acid after cellular uptake. An additional possibility is that sialic acids that reach the lysosomal compartment via fluid-phase pinocytosis can be exported into the cytosol by the lysosomal membrane transporter [24], and thus be available for activation to CMP-Neu5Gc. It remains to be shown in humans or any other primates if ingested Neu5Gc can indeed be directly absorbed and/or incorporated via acylmannosamine intermediates. If so, the enhanced levels of Neu5Gc detected in tumors and fetuses might be explained by their higher degree of vascularity and cell growth rates.

11. Spontaneous development of antibodies to Neu5Gc in humans

Screening of hospitalized patients showed that several human diseases such as cancer, leprosy, rheumatoid arthritis and infectious mononucleosis are associated with the spontaneous appearance of HD antibodies directed against Neu5Gc [107, 122–125]. In some instances, there was even evidence for circulating HD antigens [122]. The mechanisms and consequences responsible for these observations remain obscure. One possibility is that the same pathways responsible for Neu5Gc accumulation in tumors and fetuses are operative in these other situations as well, resulting in an immune reaction.

12. Implications of human Neu5Gc deficiency for biotechnology and xenotransplantation

Since normal humans display a delayed serum-sickness like reaction to the Neu5Gc in infused animal serum, it is reasonable to express concern about the presence of Neu5Gc in biotechnology products prepared in animal cells and in the animal organs (e.g., pig livers) being considered for transplantation into humans. With regard to biotechnology products, it is fortuitous that the most commonly used Chinese hamster ovary (CHO) cells tend to express very low levels of Neu5Gc [118, 126]. One of the first licenced products (erythropoietin) was made in CHO cells and later noted to contain very low levels of Neu5Gc [127]. However, analysis of sera from patients who had previously received many doses of erythropoietin failed to show detectable levels of anti-Neu5Gc antibodies [127]. One possible explanation is that glycoprotein-bound Neu5Gc is not as immunogenic as the Neu5Gc-containing glycolipids in horse serum. Another possibility is that the amounts of erythropoietin administered to patients were so low that they failed to evoke an immune response against Neu5Gc and indeed, may even have tolerized the immune systems of the patients. It remains to be seen what will happen when large amounts of other recombinant products (especially those derived from transgenic goat and sheep milk, which have a high Neu5Gc content) are given to patients. Even if there is no immediate immune reaction against such glycoprotein-bound Neu5Gc, the clearance and reprocessing of such glycoproteins by organs such as the liver could eventually result in re-expression of the Neu5Gc on endogenous cell surface glycolipids. Carefully performed clinical studies and/or studies in knockout mice are needed.

13. Why is Neu5Gc so rare in normal mammalian brains?

While Neu5Gc is easily detectable in most animal tissues, it is hard to detect in the brains of the same species [1–3]. In keeping with this, hydroxylase expression is selectively down-regulated in the brain [128]. However, some studies showed that traces of Neu5Gc were indeed present in animal brains [42, 83–85] presumably because complete elimination of hydroxylase expression in the brain is difficult to achieve. This still begs the question of why this systematic down-regulation occurs in the first place.

14. Future directions

The discovery of the genetic mechanism of Neu5Gc deficiency in humans has raised some new questions and revived interest in many old ones. For example, when did
loss of Neu5Gc occur in human evolution? Can such a timing be correlated with some of the major events in the evolution of human uniqueness? Does the global physico-chemical change of the surfaces of human cells resulting from the loss of Neu5Gc and an excess of Neu5Ac have any physiological or pathological consequences? Are there more examples of resistance and susceptibility to various infectious agents that can be explained by human Neu5Gc deficiency? Does the deficiency alter the functions of any of the endogenous sialic acid binding lectins (particularly the Siglecs) in humans? What is the mechanism for Neu5Gc expression in human tumors and fetuses? Can dietary intake of Neu5Gc account for this, as well as the traces of Neu5Gc apparently present in normal human tissues? Do the spontaneously appearing antibodies against Neu5Gc that appear in various human diseases represent a reaction to accelerated incorporation of Neu5Gc from diet? If so, can this reaction be harnessed for therapeutic purposes in cancer? Can lower levels of such reactions occurring in normal humans be involved in triggering diseases of immune origin? Why is the hydroxylase selectively down-regulated in the normal mammalian brain? Does the complete loss of Neu5Gc in humans confer some kind of advantage to the developing or adult brain? Does the loss of the hydroxylase gene have any consequences beyond the loss of Neu5Gc itself? Are there risks involved with the use of Neu5Gc-rich biotherapeutic agents? Will reactions to Neu5Gc pose a serious barrier to organ xenotransplantation? Many of these questions are approachable by relatively straightforward experimentation in mice and/or humans. Of particular interest are the consequences of genetically induced global or conditional Neu5Gc deficiency in mice, and the overexpression of Neu5Gc in the brains of transgenic mice; evaluation of the dietary sources of Neu5Gc and its handling in intact humans; and a wider screening of animal and microbial sialic acid binding proteins for their ability to recognize Neu5Gc – and the biological consequences thereof.

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