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# Diversity at the 5-Carbon Position of Sialic Acids

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This chapter discusses our recent work on two modifications at the 5-position of sialic acids. Earlier studies suggested that humans may not express *N*-glycolyl-neuraminic acid (Neu5Gc), a hydroxylated form of *N*-acetyl-neuraminic acid (Neu5Ac). We found that while traces of Neu5Gc are detectable in human body fluids and tissues, it is a major component in all the great apes which are our closest evolutionary relatives. This correlates with a human deficiency of the hydroxylase that converts CMP-Neu5Ac to CMP-Neu5Gc. While the chimpanzee hydroxylase gene is similar to the murine homologue, the human gene contains a 92 bp deletion, resulting in a frame-shift mutation. Thus, the lineage leading to modern humans suffered this mutation sometime after our common ancestor with the chimpanzee and bonobo, potentially affecting recognition of cell surface sialic acids by many endogenous and exogenous sialic acid binding lectins. The expression of Neu5Gc previously reported in human fetuses and tumors, as well as the traces detected in normal humans must be mediated by an alternate pathway. In unrelated work, we found that monoclonal antibody SGR37 detects de-*N*-acetylation of the 5-*N*-acetyl group of the disialoanglioside $G_{D3}$(giving DeNAc-$G_{D3}$). While this antigen is expressed at low levels in a few normal human cells, it accumulates at high levels in many melanomas and in some lymphomas, but not in carcinomas. Isotype-matched antibodies against $G_{D3}$, 9-OAc-$G_{D3}$ and DeNAc-$G_{D3}$ showed that while the first two are predominantly on the cell surface and partly in lysosomal compartments, DeNAc-$G_{D3}$ has a diffuse intracellular location, which appears to result via endocytosis from the cell surface. Rounding up of melanoma cells during growth is associated with relocation of the internal pool to the cell surface. Thus, a minor modification of a tumor-associated ganglioside can markedly affect subcellular localization and trafficking. There is also potential for use of the antibody in diagnostic or therapeutic approaches to these tumors.

## Structural Diversity at the 5-Carbon Position of Sialic Acids

The sialic acids are a family of 9-carbon monosaccharides that occur in animals of the Deuterostome lineage (vertebrates and "higher" invertebrates) (1). They are typically located at the outermost ends of *N*-glycans, *O*-glycans and Glycosphingolipids (they can also occasionally cap the side chain of GPI-anchors). Sialic acids are subject to a remarkable variety of modifications (1–3) which are the subjects of several presentations in this volume. This chapter focuses exclusively on structural variations that can occur at the 5-carbon position of these molecules. Most commonly, there is an *N*-acetyl group at this position (giving *N*-acetyl-neuraminic acid or Neu5Ac). Less commonly there is hydroxyl group (giving keto-deoxy-nonulosonic acid, KDN) (4). The 5-*N*-acetyl group can also be hydroxyl the addition of this single oxygen atom gives *N*-neuraminic acid (Neu5Gc) (1–3). The hydroxyl this glycolyl group can even be further substitute acetyl group (3). Rarely, the 5-*N*-acetyl group is de lated, giving neuraminic acid (Neu)(5–9). This fr group at the 5-position can in turn, react covalently carboxylate group at the 1-carbon position, giving lactam (10) . Unsaturated and dehydro-forms of these sialic acids are also known to exist (1). Thi focuses on our recent work on the natural occurr biology of two of these sialic acids: *N*-glycolyl-ne acid (Neu5Gc) (11,12) and neuraminic acid (Neu

### Human deficiency of *N*-glycolyl-neuramir

Neu5Gc differs from Neu5Ac by a single oxygen,

added by the action of a specific hydroxylase that can convert the nucleotide sugar donor CMP-Neu5Ac to CMP-Neu5Gc (13–23). The expression of Neu5Gc is known to be widespread in mammalian cells - in fact, it seems to be the major sialic acid in some species. It also shows tissue-specific and developmentally-regulated expression in a variety of systems (1,2,24–26). This modification of the parent molecule Neu5Ac can affect interactions involving many known endogenous and exogenous receptors for sialic acids such as the siglecs and virus hemagglutinins (27–30).

Early pioneers in the sialic acid field noted that in contrast to many other animals, human tissues did not have easily detectable Neu5Gc (1). In other work, it was shown that the immune response of humans to the infusion of animal serum was at least partly directed against the Neu5Gc epitope present in the infused serum (31,32). Some humans with cancer or certain other diseases can also spontaneously express antibodies directed against Neu5Gc (31,33–35). Human fetal tissues (36) and certain human tumors (35–37) were also reported to contain small amounts of Neu5Gc. However, one study did not support the presence of Neu5Gc in human tumor cells (38). Also, most of the above observations were made using monospecific antibodies and/or TLC analysis. Analysis by TLC can be limited in sensitivity and specificity, and antibodies against carbohydrates are known to cross-react with other related structures. We therefore decided to re-explore the issue of Neu5Gc expression in humans, and in their evolutionary cousins, the great apes.

**Great apes express *N*-glycolyl-neuraminic acid at levels comparable to that of other animals.** All humans living today belong to a single species with limited genetic diversity (39). Darwin and Huxley correctly deduced that humans are most closely related to the African great apes (40,41), and this prediction has been amply confirmed by a variety of methods (42–51). From the strictly genetic point of view humans should be classified simply as specialized members of the great ape family, since we share an almost 99% genomic DNA sequence identity with *Pan troglodytes* (the chimpanzee) and *Pan paniscus* (the bonobo) which, in turn, have a lesser genetic identity with *Gorilla gorilla* (the gorilla) and *Pongo pygmaeus* (the orangutan) (49-51). Thus, the obvious morphological and functional differences between humans and these great apes should be based on relatively few changes in gene expression or function. However, despite a large amount of compar
the years, there have been no clearcut
in the expression of gene products betv
great apes.

We found that while Neu5Gc is un
plasma proteins and erythrocytes, it is
in all four great apes, as well as in m
(11). With our collaborator Elaine M
that this marked difference in Neu5Gc
seen amongst cultured lymphoblastoi
and great apes, as well as in a variety
pared between humans and chimpanze
rebral cortex and the cerebrospinal flu
the above findings, CMP-sialic acid h
was detected in chimpanzee cells, bu
(11). However, we did notice traces of
sponding to Neu5Gc in some normal
firmation of this finding by mass spec
under way). As mentioned above, Neu
also been reported by others in human
mors. Furthermore, the regulation of
tivity is very complex, and involves n
tors (13, 15, 16, 18, 19, 21–23). Thu
appeared that the activity of this hydr
ply suppressed in adult humans, rathe
together.

**A mutation in human CMP-sialic a
curred after our last common ance
apes.** To explore this issue, we identifi
base sequences homologous to the prev
hydroxylase cDNA, and used them to
cDNAs for the chimpanzee and huma
Sequencing indicated a 92 bp deletion
of the human cDNA. In contrast, the c
cDNA sequence is functionally intact a
tical to that of the mouse. The deletion
results in a frame shift, which would ca
cation of the coding sequence. Since t
sequences coding for the putative Reis
ing region of the murine hydroxylase p
cated molecule is highly unlikely to h
activity. This deletion in the cDNA is
reported independently by Irie, *et al.*
prior to our publication. However, th

isolate the complete 5' region of the human cDNA that includes the primary initiator methionine. Thus their 5' truncated cDNA gave a different protein product in an in vitro translation reaction, leading to the assumption that a methionine codon downstream of the 92 bp deletion is the primary initiator for translation of a truncated protein representing >80% of the carboxyl end of the hydroxylase. They went on to show that such a truncated protein had no residual hydroxylase activity when expressed in COS cells. However, we find an intact primary initiator methionine codon corresponding to that of the mouse and chimpanzee protein, and therefore predict a much shorter truncated polypepide involving the amino terminus of the protein. Thus, it remains to be shown that the protein product studied *in vitro* by Irie et al. actually *exists in vivo*. Regardless, both groups agree that humans are genetically deficient in the activity of the hydroxylase.

Genomic PCR analysis indicated that the corresponding 92 bp region is present in total genomic DNA of all the African great apes, indicating that this region of the human genome is either deleted or markedly altered in sequence (12). We also found that human genomic BAC clones spanning the hydroxylase gene failed to hybridize with a chimpanzee cDNA probe corresponding to the 92 bp human cDNA deletion (12). Irie, *et al.* (53) went further and actually sequenced the entire human genomic region in question, proving that the 92-bp loss represents an exon deletion corresponding to exon 6 of the mouse hydroxylase gene. Chromosomal localization studies by our collaborator David Nelson showed that the hydroxylase gene is located at 6p22-p23 (12). This is not close to the site of any of the major chromosomal rearrangements known to have occurred in hominoid evolution (54).

**The 92 bp deletion is found in diverse human populations.** Our studies of Neu5Gc deficiency in humans included a survey of more than 70 individuals from many ethnic backgrounds (11). To confirm that the hydroxylase mutation is not simply a human polymorphism, our collaborator S. Warren carried out a similar PCR analyses on DNA samples from 18 "Caucasians", 4 African-Americans, 4 !Kung bushmen, 4 Khwe pigmies and 6 Japanese. All these samples failed to give the 92 bp PCR product, while all control samples from chimpanzees and bonobos showed the product (12). Thus, the human CMP-Neu5Ac hydroxylase gene seems to have undergone this inactivatin after the time of the last common ancestor with and chimpanzee, but before the common origin ern humans.

**What are the consequences for human ev** change from Neu5Ac to Neu5Gc could theoreti several of the known endogenous and exogenou for sialic acids (27, 28, 30). The interactions o pathogens such as Influenza A and B viruses (: E. coli K99 (58–61) are known to be affected extents. There are many other microbes that u acids as specific binding sites on mammalian ce ing major pathogens such as *Helicobacter pylori modium falciparum* (62–65). In most such case sequences of having Neu5Gc or Neu5Ac have n studied. Sialic acids are also ligands for several e vertebrate lectins (27, 28, 30, 63, 66–68). Fo Siglecs such sialoadhesin on macrophages, and sociated glycoprotein on neuronal axon myelin sh a lack of binding to Neu5Gc (27). Thus, the de mans causing loss of Neu5Gc expression could influence a variety of normal and abnormal pro terestingly, even in animals that express large a Neu5Gc in other tissues, the level in the brain extremely low (69–71). The reason for this is un is tempting to speculate that humans have gained tage in brain development and/or function by e the last traces of Neu5Gc in the brain, while "sac throughout the rest of the body.

Finally, we have also isolated alternative hu scripts with various deletions and insertions. Alt did not conduct an extensive search in a chimpanz we encountered only one such anomaly among panzee clones that we obtained. Thus, we spec the many abnormalities and variations of the 3' the human hydroxylase are indicative of a gene th non-functional, and is undergoing a random deg as a pseudogene. Regardless, if it were possibl mine when exactly the loss of Neu5Gc expressior in relation to hominid evolution, this could help controversial question of the origin of modern hur as well as make predictions regarding the functio quences of Neu5Gc loss. Unfortunately, autosom extremely difficult to recover from fossils >30,

old (73). We are currently extracting and studying the sialic acids themselves from fossil samples.

**What about the Traces of Neu5Gc Found in Human Tissues, Fetuses and Tumors?**

Despite the finding of an inactivating mutation in the human hydroxylase gene, human fetal tissues (36) and certain human tumors (36, 37,74) have been reported to contain small amounts of Neu5Gc . In keeping with this, some humans with malignancies or with certain inflammatory or infectious diseases are known to spontaneously develop antibodies against Neu5Gc (31, 33, 34). As previously mentioned, we have also found small traces of a HPLC peak corresponding to Neu5Gc in some human tissues (11). Taken together, these data suggest that the ability to express Neu5Gc may not be completely lost in humans. We need to explore if this represents the upregulation of another minor pathway for the synthesis of Neu5Gc, such as the use of the donor glycolyl-CoA (75,76), or the incorporation of Neu5Gc derived from dietary sources (77).

**De-*N*-Acetyl-Gangliosides in Humans as Novel Tumor Antigens**

Gangliosides are sialic acid-containing glycosphingolipids that are typically concentrated on the outer leaflet of the plasma membrane of vertebrate cells (78,79). They contribute to the physical properties of the membrane (80), can act as receptors for bacterial and viral adhesins (62), and can interact with and modulate the functions of growth factors and extracellular matrix receptors (81,82). Gangliosides are also thought to be potential ligands for endogenous animal lectins such as selectins (83, 84) and the siglecs (29, 30). The common monosialoganglioside $G_{M3}$ (Siaα2-3LacCer) can be modified by the ST8Sia I sialyltransferase(85,86) to give $G_{D3}$ (Siaα2-8Siaα2-3LacCer), which is a known marker for cell activation and transformation (87–90). Such ganglioside antigens are also major targets for the immunotherapy of cancer (91–98).

The monoclonal antibodies (Mabs) SGR37 and SMR36 were raised by our collaborator Tadashi Tai, using chemically synthesized de-*N*-acetyl-$G_{D3}$ as an immunogen. We had previously reported reactivity of these antibodies with human melanoma cell lines such as Melur and M21 (8). More recently, we compared the distribution of the SGR37 antigen (de-*N*-acetyl-$G_{D3}$) with that of $G_{D3}$ in normal human tissues and in hu SGR37 is an $IgG_3$ antibody that was highly specific manner with de-*N*-ace recognition a non-acetylated amino g acid residue, as well as the intact sic sialic acid unit. As expected for glycc section reactivity was markedly dimi organic solvents (*e.g.* methanol) or by affin embedding process(99). Even r caused some loss of the SGR37 antig colipid antigens, the way to circumve to avoid using organic solvents, and ir tions with aldehydes. While this appr mAb R24 (anti-$G_{D3}$), SGR37 reactiv creasing paraformaldehyde fixation (r diminished at concentrations of parafo This is probably because the free amin cal determinant of the SGR37 antige modified by paraformaldehyde. In k tion, similar problems were noted with therefore turned to the use of fresh u sections (100), which, while more diffi sistent reactivity with the SGR37 anti

**The SGR37 antigen is an uncomme curring structure in normal human**

tion of both R24 and SGR37 antigens normal human tissues using unfixed R24 antigen positivity was seen in ma skin melanocytes, blood vessels, panc medullary cells, marginal zone lymph interfollicular zone lymphocytes in th of the testis, and smooth muscle cells. sonably well with prior reports (90,9· trast, reactivity to SGR37 was found and in blood vessels of some tissues, and in the pia mater and white matter o cells that were clearly negative for the l tive for the SGR37 antigen were a fe nuclear cells seen in the skin and colo

Can we be certain that the antige tissue sections are indeed gangliosides? are known to be highly specific for de side targets. The extractability of the

solvents or paraffin embedding (see above) suggests that the natural epitope is on a lipid, and the sensitivity of the SGR37 antigen to aldehydes (see above) fits with the requirement of this antibody for a free amino group. We also found that strong periodate oxidation (46 mM, neutral pH) abolished both SGR37 and R24 reactivity, indicating that the epitopes in the tissues for both antibodies are carbohydrate-dependent . Overall, the sensitivity to organic solvents and aldehydes as well as the abolition of reactivity by strong periodate reassures us that the molecule being detected by SGR37 in the tissues is either de-*N*-acetyl $G_{D3}$, or some closely related structural analogue. Thus, we can conclude that MAb SGR37, which was originally raised against a synthetic ganglioside recognizes a naturally occurring ganglioside antigen in a few normal human cell types . Since many R24 positive areas did not also stain for SGR37, it appears that GD3 expression is not always accompanied by de-*N*-acetylation.

**The SGR37 antigen accumulates in most melanomas and some lymphomas, but not in carcinomas.** Many clinical samples of human melanomas (which are known to be $G_{D3}$ positive) were found to also immunostain strongly for the SGR37 antigen (9). Indeed, in some samples, reactivity with the R24 antibody was found to be more focal, compared to that with SGR37. This implies that naturally occurring tumors *in situ* may have higher levels of de-*N*-acetylation than that of the corresponding cultured cells. Immunoreactivity with SGR37 was also seen in some malignant-appearing cells in lymphoma samples (particularly of the T cell type). Unlike the case with melanomas and lymphomas, there was no SGR37 immunoreactivity in a number of carcinomas studied (R24 immunostained stromal elements in these tumors) (9).

## De-*N*-Acetyl Gangliosides Have an Uunusual Subcellular Localization.

We used the IgG3 antibody SGR37, and isotype-matched antibodies against $G_{D3}$ (R24) and 9-*O*-acetyl-$G_{D3}$ (27A) to study the distribution of these antigens in cultured Melur and M21 human melanoma cells,(9) Flow cytometric analyses indicated that while $G_{D3}$ and 9-*O*-acetyl-$G_{D3}$, are easily detected on the cell surface, SGR37 reactivity is very low. Glycolipid epitopes could be cryptic because of shielding by cell surface glycoproteins (105) or possibly because of interactions with other lipids (106). To evaluate Melur cells were treated with either trypsin or sap used a concentration of trypsin known to cleave face glycoproteins, exposing underlying glycc probes (105). This treatment did not increase SGI tivity on the cell surface. However, saponin, a reag cally used to permeabilize intact cells, clearly incr activity to SGR37. In contrast, R24 reactivity o melanoma cells was somewhat increased after tryp somewhat decreased after the saponin treatment (9

Some gangliosides are thought to be selectively in an intracellular compartment (107–111). Indirec nofluorescence studies of the saponin-permeabiliz showed that while the melanoma cells displaye amounts of cell surface $G_{D3}$ and 9-*O*-acetyl-$G_{D3}$ ar reactivity in some internal vesicle-like elements. I ing contrast, the SGR37 antigen was found to be di cytoplasmic, and concentrated in the perinuclear some cells, with very low levels seen on the cell s Saponin is known to pemeabilize cells specifically b calating with cholesterol in cell membranes. Thus, v cluded that SGR37 antigen is either completely intr lar and/or is partly cryptic on the cell surface, in a c terol-rich domain which can be disrupted by sap Permeabilization of cells with streptolysin O (whic makes holes only in the plasma membrane, without a ing cholesterol content) revealed only a small amo the SGR37 antigen. Thus, the intracellular pool can accessed easily without a membrane-perturbing reagen saponin. As an independent check, we also used the MAbs SMR36 (anti de-*N*-acetyl-$G_{M3D3}$), GMR14 (anti- and Jones (anti- 9-*O*-acetyl-$G_{D3}$). Again, we observe same differential distribution of these gangliosides. S both saponin and streptolysin O perturb the plasma m brane in different ways, we also carried out immuno rescence studies on semi-thin sections of the cultured that were harvested without permeabilization( sectionin the cells was directly done without disrupting memb structures). This approach strongly corroborated the al results, *i.e.*$G_{D3}$ and 9-*O*-acetyl-$G_{D3}$ are predominantly on cell surface, whereas de-*N*-acetyl-$G_{D3}$ is detected in a fuse cytoplasmic internal distribution. The overall con sion is that the intracellular distribution of the SGR37 a gen is markedly different from those of the other two clo

related structures(9).

**Unlike GD3, de-*N*-acetyl-GD3 antigen is not enriched in plasma membranes or lysosomes.** Double immunofluorescence studies were performed on the semi-thin sections of cultured cells using the same set of antibodies. R24 and 27A stained the cell surface as well as some intracytoplasmic vesicles which were identified as lysosomes (by the lamp-1 lysosomal marker). In contrast, the diffuse cytoplasmic internal distribution of the SGR37 antigen did not overlap with lamp-1, nor with Calnexin (an ER marker). The signal also did not have a perinuclear Golgi-like pattern. Immunoelectron microscopy with gold-bead tagged antibodies confirmed that the SGR37 antigen was simply scattered in the cytoplasm with no labelling of specific membranous structures. All membranous structures that labelled with R24 were completely devoid of label with SGR-37. Taken together, all of the data showed that the R24 and 27A antigens behaved as expected for typical gangliosides, being located mainly on the plasma membrane and in internal organelles. In contrast, the SGR37 antigen appears to be mainly in the cytoplasm, without any clear association with a membrane-bound organelle.

**SGR37 antigen is transiently expressed on the cell surface and then delivered to an internal compartment.** De-NAc-$G_{D3}$ is very likely to be derived from $G_{D3}$ by a de-*N*-acetylation reaction, after the initial synthesis of $G_{D3}$ in the Golgi (6,8). Thus, we can ask if the De-NAc variant is targeted directly to its internal compartment from the Golgi, or whether it first passes through the plasma membrane. To study this, we exploited the fact that when antibodies to potential cell surface antigens are added to the medium of live cells, adsorptive endocytosis is antigen-dependent, and the final intracellular distribution of the antibody probe indicates the distribution of the antigen (112–115). The melanoma cells were incubated with the different monoclonal antibodies under standard culture conditions, and uptake of antibodies monitored. Although it was possible to observe some antibody internalization most of mAb R24 was retained on the cell surface. On the other hand, SGR37 was internalized and concentrated in cytoplasmic vesicular structures. Previous studies have indicated that internalized gangliosides are typically delivered to lysosomes (78,116). In order to test this, cells were simultaneously pulsed with mAb SGR37 along with dextran-FITC, which is known to be concentrated in lysosomes. We found that SGR3 ran-FITC were concentrated into different con Thus, the SGR37 antigen is at least transientl on the cell surface, but then becomes predomin ellular , concentrating initially in an unknown lo than in functional lysosomes.

**SGR37 antigen accumulates on the surface non-adherent cells, but this is not related t status.** In studies of cultured melanoma cells, v that SGR37 reactivity was higher in the small rounded-up cells, as compared to the bulk popul tened cells. Flow cytometry confirmed that the cells had an increased cell surface reactivity to not to R24. This fit with our previous result microtubule-disrupting agent nocodazole increa face density of SGR37 antigen (8). The proport rounded-up cells in different cell cycle phases v termined. No significant variation in either cel global expression of SGR37 antigen was obse ferent phases of the cell cycle (G0/G1 vs. S/G2 the SGR37 antigen accumulates on the surface adherent or rounded-up cells regardless of the phase.

In future, it will be very interesting to explore the markedly differential subcellular localiza NAc-$G_{D3}$, which differs from $G_{D3}$ solely by the a free amino group (the absence of the N-acety this regard, our data suggest that De-N-acety not simply a terminal degradation product of well with our early studies where we had not ence of a re-N-acetylase activity, which could from endogenous De-NAc-$G_{D3}$ (6). The functi quences of De-NAc-$G_{D3}$ expression also need to Others have also shown that exogenously added lated gangliosides can block adhesion of cells lular matrix (117). In other work described e this volume, replacement of the sialic acid *N*-a by *N*-propionyl or other acyl chains achieved cells with mannosamine analogs led to loss of control and loss of contact-inhibition (118). T effects of in vitro addition of $G_{M3}$ (growth supp de-*N*-acetyl-GM3 (growth enhancement) to cu (5,7) also provides a framework for future studi the discovery by Kannagi and colleague

neuraminic acids can be converted into intramolecular lactams (described elsewhere in this volume) adds further complexity to the study of de-*N*-acetyl-gangliosides. Many other questions regarding the biosynthesis, trafficking and function of these novel gangliosides remain to be studied. Meanwhile, the low levels of the SGR37 antigen in normal tissues, the high levels in some tumors, its selective endocytosis from the cell surface, and its high expression on rounded-up/non-adherent cells suggests that it is worthwhile to explore diagnostic or therapeutic approaches using this antibody.

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