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RECENT PROGRESS IN THE ANALYSIS OF GANGLIOSIDES BIOSYNTHESIS

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ABSTRACT

Recently, a number of glycosyltransferase genes have been isolated. The majority of glycosyltransferase genes responsible for the synthesis of acidic glycosphingolipids (gangliosides) have been cloned in Japan. Therefore, the total make-up of the regulatory mechanisms for ganglioside synthesis can now be addressed, even though there are still issues remaining as to the detailed specificities of many enzymes. The availability of these glycosyltransferase genes enables us to directly analyze the biological functions of gangliosides by remodeling carbohydrate profiles in cultured cells and experimental animals.

Key Words: ganglioside, glycosyltransferase, carbohydrate, glycosphingolipid, expression cloning

INTRODUCTION

Gangliosides, acidic glycosphingolipids have been thought to be important molecules in the cell to cell recognition process and in the modulation of cellular signals on the cell surface membrane.[1,2] Particularly, they have been considered to be essential in the morphogenesis and function of the central nervous system of vertebrates, since they are highly conserved among almost all vertebrates, and are prerequisites for life, proven by the fact that no living vertebrates have been seen with defects in the ganglioside synthesis pathway.[3]

Recently, a number of glycosyltransferase genes responsible for the synthesis of gangliosides have been cloned,[4-6] and our understanding of their biosynthesis and expression has been markedly enhanced (Table 1). Molecular cloning of these glycosyltransferases has been approached in three ways: (1) using synthetic probes based on the partial amino acid sequences obtained from purified enzymes,[7] (2) performing expression cloning with specific ligands such as monoclonal antibodies to detect newly synthesized carbohydrate structures[8] and (3) using PCR in the case of sialyltransferase genes since known sialyltransferases contain very similar sequences named "sialyl motifs",[9] by which researchers can prepare degenerate primers. In particular, approaches (2) and (3) have been very useful, because purification of glycosyltransferases is notoriously difficult.

Successfull cloning of ganglioside synthase genes should enable us to further and precisely analyze the biological functions of gangliosides, and it now is an appropriate time to summarize the findings from cloned genes relevant to the biosynthesis of gangliosides.

Table 1 Glycosyltransferases involved in the biosynthesis of gangliosides

Enzyme	Other name	Specificity (Products)	Ref.
Glc-Cer synthase	β1,1Glc-T	Glc-Cer	10
Lac-Cer synthase	β1,4Gal-T (Gal-T2)	Lac-Cer	11
GM3 synthase	α2,3S-T (SAT-I)	GM3	13
GD3 synthase	α2,8S-T (SAT-II)	GD3, GT3, GD1a	23
		GQ1b, GD1c	24,25
GM2/GD2 synthase	β1,4GalNAc-T (GalNAc-TI)	GM2, GD2, GA2	15
GT3 synthase	α2,8S-T (SAT-III)	same as SAT-II?	30
GM1/GD1b/GA1 synthase	β1,3Gal-T (Gal-T3)	GM1, GD1b, GA1	31
GT1b/GD1a/GM1b synthase	α2,3S-T (SAT-IV)	GT1b, GD1a, GM1b	39
GQ1b/GT1a/GD1c synthase	α2,8S-T (SAT-V)	GQ1b, GT1a, GD1c	40

CLONING OF GLYCOSYL TRANSFERASE GENES

1) Glucosylceramide synthase gene: this gene product catalyzes the first step in the assembly of the sugar chains of gangliosides (Fig. 1). A cDNA for this enzyme was isolated by expression cloning using a mouse B16 melanoma mutant cell as the recipient cell of transfection and anti-GM3 monoclonal antibody.[10] The deduced amino acid sequence predicted a transmembrane protein with a type III orientation, which was different from the majority of already cloned glycosyltransferases. Moreover, this enzyme seemed to have the catalytic domain in the cytoplasm, which was also different from other transferases. mRNA for this gene could be found in all the tissues examined.

2) Lactosylceramide synthase gene: many attempts have been made to purify this enzyme or to isolate its cDNA resulting in failure. Nomura et al. succeeded in purifying this enzyme from rat brain tissues.[11] The purified enzyme showed 61 kD in SDS-PAGE, and 51 kD after N-glycanase digestion, suggesting its nature as a glycoprotein. Its optimal pH was 7.2, and it required Mn^{2+}. This enzyme was also located on the cytoplasmic side of the Golgi apparatus.[12]

3) GM3 synthase gene: recently, a cDNA for this gene was isolated by expression cloning from a cDNA library prepared from HL60 treated with TPA.[13] The deduced amino acid sequence predicted a type II membrane protein containing sialyl motifs. Although purified GM3 synthase showed multiple specificity,[14] this gene product was specific for lactosylceramide as a substrate.

4) GM2/GD2 synthase gene: this gene was the first glycolipid-specific glycosyltransferase to be isolated.[15] The strategy was to screen transfectants of a mouse B16 derivative with an anti-GM2 monoclonal antibody. Analysis of the substrate specificity *in vitro* and of the transfectant cells indicated that this enzyme could utilize GM3, GD3 and lactosylceramide as an acceptor, as had been suggested by Pohlents et al.[16] However, the efficiency of the GalNAc transfer onto lactosylceramide was much lower than that onto GM3 and GD3[17] indicating the priority of GM3 as a substrate of this enzyme. The cDNA had homology with another β1,4GalNAc-transferase which catalyzes the synthesis of the Sd^a blood group structure, probably on glycoproteins.[18] The corresponding mouse cDNA has also been isolated.[19,20] The expression of this

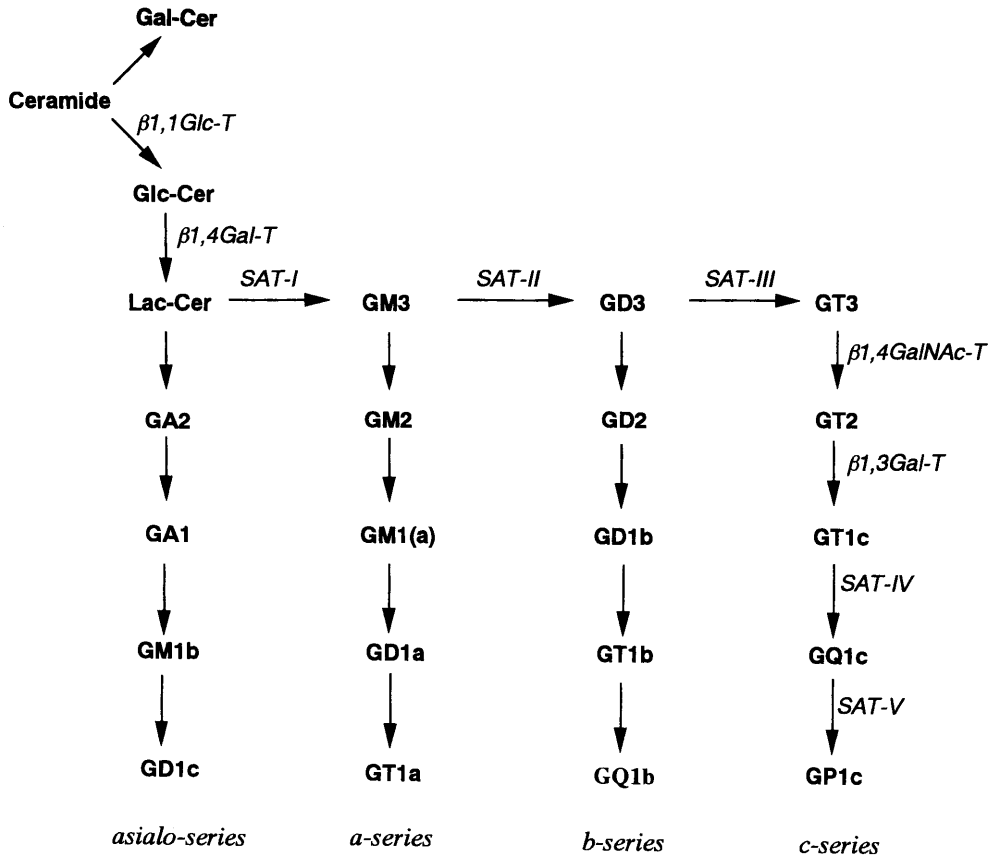

Fig. 1. Biosynthetic pathways of gangliosides: SAT-I, α2,3-sialyltransferase; SAT-II, α2,8-sialyltransferase; SAT-III, α2,8-sialyltransferase. This enzyme might be identical with SAT-II as described in the text. SAT-IV, α2,3-sialyltransferase; and SAT-V, α2,8-sialyltransferase.

β1,4GalNAc-transferase gene in human cells has been determined showing a characteristic pattern in neuroectoderm-derived tumors and HTLV-1 infected cell lines.[21,22]

5) GD3 synthase gene: this cDNA (α2,8 sialyltransferase) product was one of the key enzymes in ganglioside biosynthesis, regulating the entry into the b-series (and also c-series) (Fig. 1). cDNAs coding for this enzyme were isolated almost simultaneously by three independent research groups (including us) in 1994.[23-25] All these groups conducted expression cloning using anti-GD3[24,25] or anti-GD2[23] monoclonal antibodies. The deduced amino acid sequences predicted a type II membrane protein and the presence of sialyl motifs. The L-sialyl motif contained one more amino acid than other sialyltransferases cloned so far. GD3 synthase has also been isolated from rat brain.[26,27] As for the substrate specificity of this enzyme, there is some controversy, though all studies agree that GM3 is the best acceptor for this enzyme.[28] The expression of the GD3 synthase gene was analyzed in human cancer cells and lymphocytes. Melanoma cells among cell lines examined specifically expressed GD3 synthase mRNA.[29] Acute T-cell lymphoblastic leukemia cells from patients also expressed this gene, whereas the majority

of T-ALL cell lines did not.

6) GT3 synthase gene: expression cloning using COS-1 cells with an anti-GT3 monoclonal antibody unexpectedly resulted in the identification of a cDNA identical to GD3 synthase.[30] These results suggested that the same enzyme can synthesize both disialyl and trisialyl sequences. However, it is not certain at this moment that GD3 synthase physiologically generates GT3 in the tissues, since melanoma cells expressing high levels of GD3 do not necessarily express GT3. We can not neglect the possibility that there is another GT3 synthase.

7) GM1/GD1b/GA1 synthase gene: GM1 is one of the most extensively studied gangliosides, mainly because it is one of the major gangliosides in the vertebrate brain. A cDNA coding for this GM1 synthase has recently been isolated by our group[31] from the rat brain cDNA library. The deduced amino acid sequences predicted a type II membrane protein with 371 amino acids. This gene showed no significant homology with other galactosyltransferase genes such as blood group B synthase,[32] Gal-Cer synthase,[33,34] β1,4Gal-transferase[35] or α1,3Gal-transferase.[36,37] Analysis of the enzyme substrate specificity revealed that it was capable of synthesizing GM1, GD1b and GA1, confirming earlier enzymological data.[38] In adult rat tissues, this gene was highly expressed in the thymus, spleen, kidney and testes. The adult brain showed a low level of mRNA expression, although embryonal rat brain showed much stronger intensity of bands in the Northern blot, indicating that GM1 (and GD1b/GA1) play important roles in the development of the rat brain.

8) GT1b/GD1a/GM1b synthase gene: a cDNA clone which seemed to be related to this gene, was isolated by PCR cloning using a primer based on sialyl motifs.[39]

9) GT1a/GQ1b/GD1c synthase gene: a cDNA clone, which seemed to correspond to this enzyme was cloned by PCR based on sialyl motifs.[40] However, the product of the GD3 synthase gene could also synthesize GT1a/GQ1b/GD1c as well as GT3.[28] Therefore, it is not clear now which enzyme (or gene) is really responsible for the synthesis of GT1a/GQ1b/GD1c.

10) O-acetyltransferase gene: one of important modifications of gangliosides is the O-acetylation of sialic acid residues on C-9 and/or C-7 observed in some restricted cells and tissues. From its distribution in developing retina[41] and Purkinje cells,[42] it has been suggested that that O-acetylation of gangliosides serves as a cellular recognition signal. O-acetylated gangliosides can also serve as tumor markers, e.g. 9-O-acetyl-GD3 in melanoma.[43] Recently, cDNAs coding for proteins regulating O-acetylation of GD3 have been isolated, whereas the actual nature of their products have not yet been verified.[44,45]

11) N-glycolylneuraminic acid synthase gene: another important modification of the sialic acids in gangliosides is the substitution of N-glycolyl for the more common N-acetyl group on C-5 of this sugar. Since humans and chickens do not synthesize N-glycolylneuraminic acid (NeuGc), this substituent form serves as a "heterophile antigen" known as the Hanganutziu-Deicher (H-D) antigen.[3] This HD antigen has been thought to be a tumor antigen,[46] although this supposition has not been fully confirmed by all investigators. The synthesis of NeuGc has recently been elucidated to occur through hydroxylation of CMP-NeuAc with CMP-NeuAc hydroxylase and NADH-dependent cytochrome b5.[47,48] cDNAs of these two proteins have been cloned from mouse liver.[48] cDNA of the human CMP-NeuAc hydroxylase gene lacked approximately 100 amino acids in the N-terminal region and could not generate a functional

enzyme.[49] This result again raises doubts as to whether human tumors can synthesize and express N-glycolylneuraminic acid-containing gangliosides.

12) Polysialic acid synthase genes: two sialyltransferase genes which code enzymes synthesizing α2,8 NeuAc linkages have been isolated.[50-54] These enzymes (ST8SiaII/STX and STSiaIV/PSA) catalyze the biosynthesis of polysialic acid chains that are characteristic of N-CAM and a few other glycoproteins. These two enzyme products are very similar in terms of substrate specificity, but show distinct expression patterns in tissues and developmental stages. These two enzymes seemed not to be involved in glycolipid synthesis.

REGULATORY MECHANISMS FOR THE EXPRESSION OF GANGLIOSIDE SYNTHASE GENES

Dynamic changes in the expression pattern of gangliosides during tissue development, differentiation or malignant cell transformation have strongly interested us in the regulatory mechanisms for the gene expression of these glycosyltransferases. Although there is some evidence that post-translational factors may influence enzyme activity,[15,19] transcriptional regulation probably plays the major role. Not much information is now available on this point. Our study on the GM2/GD2 synthase gene[55] showed that this gene has three transcription initiation sites and three alternative exons (Fig. 2). Consensus binding sites for EGR-1, HNE-5 and Sp-1 transcription factors were found in the 5′-flanking region. No TATA box was present. The regulatory mechanisms for the gene expression of this GM2/GD2 synthase were more complex than expected, although these different transcription units, including individual promoter/enhancers, may be responsible for cell type-specific expression.

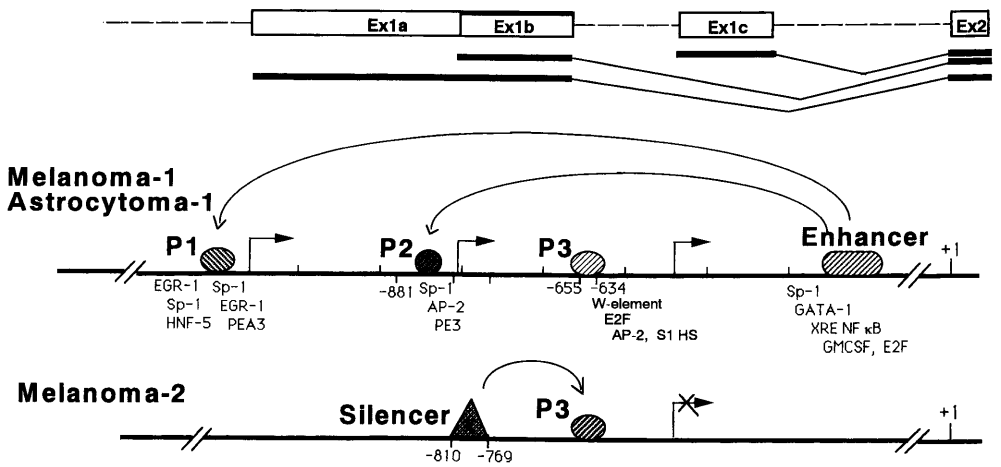

Fig. 2. Summary of the analysis of promoter/enhancer in GM2/GD2 synthase gene. The top panel represents alternative usage of three exons: Ex1a, Ex1b and Ex1c. The bottom panel shows transcription initiation sites in the expressant lines (Melanoma-1 and Astrocytoma-1) and a non-expressant line (Melanoma-2). At least three transcription units were identified. Three promoters (P1–P3) for individual initiation sites were also defined. The activities of the P1 and the P2 promoters were strongly enhanced by an enhancer detected in the original first exon. The P3 promoter was suppresed in "Melanoma 2" cells by a silencer (X).

APPLICATION OF CLONED TRANSFERASE GENES TO THE ANALYSIS OF BIOLOGICAL FUNCTIONS OF GANGLIOSIDES

Using the cloned glycosyltransferase genes, it has become possible to better analyze the biological functions of gangliosides. To elucidate ganglioside functions, four approaches have been performed: (1) observing the effect of the exogenous addition of gangliosides to cultured cells, (2) blocking ganglioside biosynthesis with specific inhibitors, (3) selecting cell variants that lack some or all gangliosides and (4) producing new gangliosides in cells by manipulating cDNAs of glycosyltransferase genes.

The first three methods (1—3) have been used widely and generated various important results for the understanding of ganglioside functions. However, they are all indirect methods and have some limitations when interpreting results obtained by some of these methods. On the other hand, the genetic modification of carbohydrate profiles on some particular cells are much more straightforward and more physiologic. Consequently, results obtained from the experiments with method (4) can be accepted more directly. Tsuji et al. demonstrated the differentiation and arrest in growth of Neuro2a neuroblastoma line after introduction of the GD3 synthase gene.[56] A shift of ganglioside components from the a-series to the b-series seemed to result in the dramatic change in the phenotype.

Ganglioside remodeling in an experimental animal has also been tried, and elucidated new biological functions. Mice with disrupted Gal-Cer synthase gene (knock-out) showed no definite abnormality in myelination, but exhibited serious neurological disorders and died within three weeks of birth.[57] We have also generated mice overexpressing GM2/GD2 synthase gene (transgenic)[58] and knock-out mice of GM2/GD2 synthase gene lacking all complex gangliosides.[59] Particularly, this knock-out mice of GM2/GD2 synthase gene unexpectedly showed no definite abnormality in brain morphogenesis. Furthermore, they exhibited aspermatogenesis which has never been seen before (unpublished data). Thus, genetic modification of gangliosides in animals sometimes results in the discovery of new aspects of ganglioside roles *in vivo*.

In the past 6—7 years, the majority of glycosyltransferase genes responsible for ganglioside syntheses and their modifications have been isolated. Availability of these genes enables us to address the biological functions of glycosphingolipids much more directly than before, and is also dramatically changing the way carbohydrate research is conducted. I believe, therefore, that new findings from these studies on glycosyltransferase genes will promote the remodeling of glycochemistry into glycoscience, and open up new dimensions in this bioscience field.

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