Glycoconjugate Journal (1996) **13:** 123–126

MINI REVIEW

A reflection on the early history of glycosphingolipids*

TAMIO YAMAKAWA

The Tokyo Metropolitan Institute of Medical Science, 18-22, Honkomagome 3-chome, Bunkyo-ku, Tokyo 113, Japan

Received 12 September 1995

The ground-breaking work of Thudichum

In the textbooks of biochemistry printed before 1950, we can find brief descriptions for phrenosine, kerasine, nervone, and hydroxynervone, but nothing more for glycosphingolipid related substances. These names are not used now but these four names indicate galactosylceramides containing C24 normal fatty acid, C24 α-hydroxy fatty acid, C24 monounsaturated fatty acid, and C24 monounsaturated α-hydroxy fatty acid, respectively. Phrenosine and kerasine were isolated from human brain and named by J.L.W. Thudichum in 1884 [1].

Thudichum was a German clinician with training in chemistry. He also isolated and named several lipids such as cerebroside, sphingosine, ceramide, sphigomyelin, and cephalin from human brain. Thus, Thudichum can be considered the father of sphingolipid research. Glycosphingolipids and sphingolipids were originally derived from the brain, thus, these molecules were postulated to be important for neural functions. Thudichum seems to have been quite a determined person. He was not accepted by German academic society and moved to London where he started his own laboratory in his house and did experiments by himself. He published his life's work in a book entitled *A Treatise on the Chemical Constitution of the Brain* in 1884 [1].

In May, 1965, the 16th Colloquium of The German Society for Physiological Chemistry was held in Mosbach, a small city by the Neckar river, and I was invited to this meeting by E. Klenk. After the meeting, a ceremony took place in Büdingen, near Frankfurt, to dedicate a memorial plate indicating the house where Thudichum was born on the 27th of August, 1829, and I had the pleasure of attending the ceremony. His research was centred on the chemical analysis of the brain, to search for changes of brain components in diseases, such as cholera and typhoid fever, and to understand the pathogenesis of these diseases.

Forty years after the isolation of phrenosine and kerasine by Thudichum, Klenk isolated and named nervone and hydroxynervone, also from the brain, in 1924 [2].

Sulfatide, from discovery to structural determination

Another glycosphingolipid, which has a long history, is sulfatide. In 1933, G. Blix in Sweden isolated sulfatide (sulfated galactosylceramide) from human brain [3]. Sulfatide is a major component in myelin, and sulfatase deficiency causes sulfatide accumulation in metachromatic leukodystrophy. Jatzkewitz [4] and Austin [5] almost simultaneously discovered this. Establishment of the complete structure of sulfatide took 29 years. In 1962, we applied methylation and gas chromatographic analysis to determine the structure of sulfatide and found that the sulfate group was attached to the C3 of galactose [6].

The colourful history of Forssman antigen

Forssman antigen was described by J. Forssman in 1911 [7]. Forssman injected guinea pig kidney homogenates into rabbits and found that the rabbit antiserum haemolyzed sheep erythrocytes. Then, Forssman haptene or heterophile antigen was found to occur in horse kidney, and goat and cat erythrocytes, but not in the tissues of bovine, pig, rabbit, and human. To understand the nature of Forssman haptene, Landsteiner and Levene of the Rockefeller Institute of Medical Research tried to isolate Forssman

*This paper is dedicated to Dr Sen-itiroh Hakomori's 65th birthday.

haptene from horse kidney in 1925 to 1927 [8–11]. They isolated a 'lipoid' fraction which gave a purple colour reaction when heated with orcinol, hydrochloric acid and copper sulfate, the so-called orcinol reagent. Unknown to them at that time, this description indicates the presence of sialic acid, which has nothing to do with Forssman antigen. This may be the first suggestion for the presence of glycolipid containing sialic acid. In 1926, E. Walz in the laboratory of H. Thierfelder in Tübingen isolated a similar kind of glycolipid fraction, showing a purple colour reaction with the orcinol reagent, from bovine spleen, and published this result with the discussion that Thierfelder, his mentor, had observed the occurrence of a similar substance in bovine brain as unpublished data [12]. Klenk in Cologne, who was also a student of Thierfelder, eventually showed that this colour was associated with neuraminic acid and opened a new research field for ganglioside biochemistry.

The structural determination of Forssman antigen in the 1960s was quite competitive between S. Hakomori's and my [13] groups. Finally, Hakomori published the correct structure in 1971 [14]. The critical point turned on how the molar ratio of *N*-acetylgalactosamine to glucose was determined. Hakomori used alditol acetate derivatives for GLC, a method developed by B. Lindberg in Sweden, and we used trimethylsilyl (TMS) derivatives as developed by C. Sweeley and ourselves. Alditol acetates gave better ratios by GLC analysis for hexosamine to hexose than TMS derivatives. Thus, our conclusion was 1 mol less *N*-acetylgalactosamine than Hakomori's. Sometimes, the limitation of the methods used is critical for accurate interpretation of the results.

Gangliosides and sialic acids

In 1934, Klenk isolated phospholipids from the spleen of a patient with Niemann-Pick's disease and identified the accumulated phospholipid to be sphingomyelin [15]. In the next year, he isolated a lipid fraction from the brain of the same patient. The fraction was composed of a white phosphorus-free substance which gave a purple colour reaction with the orcinol reagent. He named this 'Substance X' [16]. He subsequently obtained Substance X in much better yield from the brains of patients with Tay-Sachs' disease [17]. The nature of Substance X became of much interest. In 1941, Klenk named Substance X ganglioside [18]. The reason why he applied this name was that Substance X was most abundant in grey matter. He thought that ganglioside was one molecule, not a mixture, which contained 1 mol each of stearic acid, sphingosine, and neuraminic acid and 3 mol of galactose.

In 1941, Klenk treated Substance X with methanolic hydrochloric acid and isolated a component responsible for the colour reaction as a crystallized substance. He named this 'Neuraminsäure, neuraminic acid' [19]. At almost the same time, in 1936, Blix isolated a substance from bovine submaxillary mucin by hot water extraction. Blix purified a polyhydroxyamino acid like substance. He claimed that this substance is the component responsible for the purple colour reaction with *p*-dimethylaminobenzaldehyde, Ehrlich reagent [20]. In 1938, he confirmed that a Substance X like material isolated from bovine brain gave the purple colour reaction. Blix seems to have believed that the polyhydroxyamino acid was a component of Substance X. Later, in 1952, his group named this substance sialic acid [21].

Determination of the structure for sialic acid or neuraminic acid was an exciting topic and many groups including mine were involved. Finally, A. Gottschalk in Australia succeeded in proposing the correct structure [22]. Later on, S. Roseman discovered that pyruvate and *N*-acetylmannosamine are the precursors of sialic acid [23]. R.K. Yu and R. Ledeen found that the linkage of sialic acid in gangliosides is α but not β [24]. Blix, Gottschalk, and Klenk agreed to the proposal that the name sialic acid be used as the acyl derivative of neuraminic acid [25], and we now use the term sialic acid to describe a group of molecules containing neuraminic acid as the core structure. This agreement was based on the history of the discovery and structural determination of sialic acid and neuraminic acid.

In 1950, I tried to separate lipids from horse erythrocytes [26]. I was in the Institute for Infectious Diseases, University of Tokyo, and the Institute was responsible for supply of anti-sera and vaccines. Horses were immunized with diphtheria toxin, tetanus toxin, and so on for production of large amounts of anti-sera. Serum was valuable but nobody paid attention to clotted erythrocytes. I prepared the so called stroma (erythrocyte membranes) from the clotted erythrocytes and extracted lipids. Based on the solubility differences of extracted lipids in several organic solvents, I was able to separate several lipid fractions and one fraction gave the purple colour reaction with the orcinol reagent. I learned about gangliosides from the article by S.J. Thannhauser in a copy of *Annual Review of Biochemistry* published in 1943 [27], and I thought my fraction from horse erythrocytes might be ganglioside. Thus, I prepared Klenk's ganglioside from bovine brain and compared the two fractions. I named the horse erythrocyte glycolipid haematoside to differentiate this glycolipid from Klenk's ganglioside. The name haematoside derived from the fact that it was isolated from haematic cells. In L. Svennerholm's nomenclature, haematoside is called GM3. Methanolysis of haematoside according to Klenk's procedure gave a crystallized compound and I named this substance haemataminic acid. Analytical results indicated that haematoside was composed of 1 mol each of fatty acid, sphingosine, and haemataminic acid, and 2 mol of galactose.

I submitted a paper describing these results to the *Journal of Biochemistry* published by The Japanese Biochemical Society and at the same time sent a letter to Klenk for his opinion on my results. Six months later, I received a letter from him indicating that he had read my paper with much interest. He also mentioned that he had studied a glycolipid isolated from human erythrocytes, and this glycolipid did not contain neuraminic acid but galactosamine. All ganglioside preparations he had obtained so far contained galactosamine, therefore, I should check for the presence of galactosamine in haematoside. Furthermore, I needed to determine the optical rotation of haemataminic acid before I reached the conclusion that neuraminic acid and haemataminic acid were the same molecule. Subsequently, I confirmed that haematoside did not contain hexosamines, therefore, haematoside is different from brain ganglioside, and that optical rotation values are the same for haemataminic acid and neuraminic acid, thus both should be the same molecule. I also decided to confirm Klenk's results for human erythrocyte glycolipid [28].

Klenk seemed to think that ganglioside was a single molecule, but this was not the case. Svennerholm in Sweden [29] separated Klenk's ganglioside into two fractions by cellulose column chromatography, and R. Kuhn in Germany separated it into four components. GI to IV. It took another 5 years to determine the structures of the gangliosides. Many scientists were involved in the determination and finally Kuhn and H. Wiegandt completed the work [30]. For doing this, we needed a new method, silica gel thin layer chromatography, which has proven to be an excellent method for the separation of glycolipids.

Erythrocyte glycolipids and ABO blood group antigens

I prepared human erythrocyte membrane to confirm Klenk's results [31] and obtained a glycolipid. Human erythrocyte glycolipid contained galactosamine but not neuraminic acid and these results were consistent with those of Klenk. I named this glycolipid globoside [32]. The term of Globo is now used as the prefix to mean glycolipids containing Galα1-4Galβ1-structure in the core. However, Klenk is the first to describe human erythrocyte glycolipid, globotetraosylceramide.

In 1953, we found that glycolipid fractions of human erythrocytes inhibited haemagglutination of erythrocytes by ABO blood group antiserum [33]. In the 1960s, W.T.J. Morgan and W. Watkins, and independently, E. Kabat and his group isolated and determined the structures of ABO blood group antigens from various body fluids and the mucin of ovarian cysts. These antigens were glycoproteins in nature. However, I believed that major ABO antigens on the surface of erythrocytes were glycolipids and not glycoproteins. Hakomori completed the structural determination of ABO blood group active glycolipids and his group recently gave clear answers to the molecular mechanism responsible for the production of ABO antigens by the determination of the glycosyltransferase gene structures [34].

I was very much interested in erythrocyte glycolipids, because they were so different among species. I analysed erythrocyte glycolipids of various species of mammals [35]. I believed that erythrocyte glycolipids reflect certain properties of mammalian species such as the shape of animals. And I thought that glycolipid differences might be the basis of immunological differences between mammalian erythrocytes [36].

Individual differences in dog erythrocyte glycosphingolipids

In 1964, we reported on dog erythrocyte glycolipids. The major glycolipid was GM3 containing 73% *N*-acetylneuraminic acid and 27% *N*-glycolylneuraminic acid [37]. However, Klenk had reported that dog erythrocyte GM3 contained 100% *N*-acetylneuraminic acid [38]. We examined erythrocyte glycolipids of various breeds of dogs with the help of veterinarians. The difference turned out to be due to the geographical origin of the dogs. Dogs originating from Europe, Russia and America exclusively have *N*-acetylneuraminic acid containing GM3. However, several breeds of Japanese, Korean, and Chinese dogs have *N*-glycolylneuraminic acid containing GM3 [39]. Klenk used European dog erythrocytes in his experiment and we used pooled Japanese dog erythrocytes in our 1964 experiment. Shiba dogs are a breed of traditional Japanese dogs and we found that they have *N*-glycolylneuraminic acid containing GM3. We did pedigree analysis for one family of Shiba dogs, and the result indicated that the expression of *N*-glycolylneuraminic acid GM3 was dominantly inherited.

N-Glycolylneuraminic acids

R. Schauer and his group have extensively analysed sialic acid structures, distributions, degradation, and biosynthesis [40]. They proposed that the expression of *N*-glycolylneuraminic acid is regulated by CMP-*N*-acetylneuraminic acid hydroxylase [41]. Quite recently, A. Suzuki, Y. Kozutsumi, and T. Kawano, my co-workers, succeeded in the purification and cDNA cloning of CMP-NeuAc hydroxylase [42]. They discovered that the hydroxylation is due to a series of reactions including the electron transport system composed of NADH, cytochrome b5, and cytochrome b5 reductase, and the terminal enzyme of hydroxylase. I hope that this evidence will shed new light on the molecular mechanism responsible for the polymorphic differences of *N*-glycolylneuraminic acid in dog erythrocytes.

Sphingosines

In 1867 to 1880, Thudichum gave the name sphingosine to a substance obtained by acid hydrolysis of glycolipids or sphingomyelin. In 1947, H.E. Carter, one of the great pioneers of sphingolipid research, proposed the correct structure which has the amino group at the β position [43]. Carter performed extensive chemical analysis of sphingosine and plant derived glycolipids and many excellent researchers from his laboratory have contributed greatly to the development of the glycolipid research field.

The present

Glycobiology and sphingolipid biology have gained the attention of investigators in different fields. Cell and molecular biological approaches will assist us in understanding the functions of glycosphingolipids and sphingolipids. However, we need also to pay more attention to glycosphingolipids and sphingolipids themselves as chemical compounds, since we still do not completely understand their physical and chemical properties. In my lifetime, great advances have been made in understanding the important roles glycosphingolipids play in nature, and I hope to see more exciting developments in the future.

Acknowledgements

I would like to thank Professor Yu-Teh Li, Tulane University, for valuable advice and manuscript editing, and Dr Akemi Suzuki, Tokyo Metropolitan Institute of Medical Science, for his help in manuscript preparation.

References

1. Thudichum JLW (1884) *A Treatise on the Chemical Constitution of the Brain*. London: Bailliere, Tindall and Cox.
2. Thierfelder H, Klenk E (1930) *Die Chemie der Cerebroside und Phosphatide*. Berlin: Verlag von Julius Springer.
3. Blix G (1993) *Z Phys Chem* **219**, 82–98.
4. Jatzkewitz H (1958) *Z Phys Chem* **311**, 279–82.
5. Austin JH (1959) *Proc Soc Exp Biol Med* **100**, 361–64.
6. Yamakawa T, Kiso N, Handa S, Makita A, Yokoyama S (1962) *J Biochem* **52**, 226–27.
7. Forssman J (1911) *Biochem Z* **37**, 78–115.
8. Landsteiner K, Levene PA (1925) *J Immunol* **10**, 731–33.
9. Landsteiner K, Levene PA (1925) *Proc Soc Exp Biol Med* **23**, 343–44.
10. Landsteiner K, Levene PA (1927) *Proc Soc Exp Biol Med* **24**, 693–95.
11. Landsteiner K, Levene PA (1927) *J Immunol* **14**, 81–83.
12. Walz E (1927) *Z Phys Chem* **166**, 210–22.
13. Ando S, Yamakawa T (1970) *Chem Phys Lipids* **5**, 91–95.
14. Siddiqui B, Hakomori S (1971) *J Biol Chem* **246**, 5766–69.
15. Klenk E (1934) *Z Phys Chem* **229**, 151–56.
16. Klenk E (1935) *Z Phys Chem* **235**, 24–36.
17. Klenk E (1939) *Z Phys Chem* **262**, 128–43.
18. Klenk E (1942) *Z Phys Chem* **273**, 76–86.
19. Klenk E (1941) *Z Phys Chem* **268**, 50–58.
20. Blix G (1936) *Z Phys Chem* **240**, 43–54.
21. Blix G, Svennerholm L, Werner I (1952) *Acta Chem Scand* **6**, 358–62.
22. Gottschalk A (1955) *Nature* **176**, 881–82.
23. Comb DG, Roseman S (1960) *J Biol Chem* **235**, 2529–37.
24. Yu RK, Ledeen RK (1969) *J Biol Chem* **244**, 1306–13.
25. Blix FG, Gottschalk A, Klenk E (1957) *Nature* **179**, 1088.
26. Yamakawa T, Suzuki S (1951) *J Biochem* **38**, 199–212.
27. Thannhauser SJ, Schmidt G (1943) *Annu Rev Biochem* **12**, 233–50.
28. Yamakawa T (1988) *Trend Biochem Sci* **13**, 452–54.
29. Svennerholm L (1956) *Nature* **177**, 524–25.
30. Kuhn R, Wiegandt H (1963) *Chem Ber* **96**, 866–80.
31. Klenk E, Lauenstein K (1951) *Z Phys Chem* **288**, 220–28.
32. Yamakawa T, Suzuki S (1952) *J Biochem* **39**, 393–402.
33. Yamakawa T, Iida T (1953) *Jpn J Exp Med* **23**, 327–31.
34. Yamamoto F, Clausen H, White T, Marken J, Hakomori S (1990) *Nature* **345**, 229–33.
35. Yamakawa T, Suzuki S (1953) *J Biochem* **40**, 7–10.
36. Yamakawa T (1983) *Glycopipids of the red blood cells in Red Blood Cells of Domestic Mammals* (Agar NS, Board PG, eds), pp. 37–55. Amsterdam: Elsevier Science Publishers.
37. Handa S, Yamakawa T (1964) *Jpn J Exp Med* **34**, 293–304.
38. Klenk E, Heuer K (1960) *Deutsch Z Verdau Stoffwechsel* **20**, 331–41.
39. Yasue (Hamanaka) S, Handa S, Miyagawa S, Inoue J, Hasegawa A, Yamakawa T (1978) *J Biochem* **83**, 1101–7.
40. Schauer R (1982) *Sialic Acids, Chemistry, Metabolism and Function*. Wien, New York: Springer-Verlag.
41. Shaw L, Schauer R (1988) *Biol Chem Hoppe-Seyler* **369**, 477–86.
42. Kawano T, Koyama S, Takematsu H, Kozutsumi Y, Kawasaki H, Kawashima S, Kawasaki T, Suzuki A (1995) *J Biol Chem* **270**, 16458–63.
43. Carter HE, Glick FJ, Norris WP, Phillips GE (1947) *J Biol Chem* **170**, 285–99.