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JB Reflections and Perspectives

Akira Kobata: a man who established the structural basis for glycobiology of N-linked sugar chains

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Akira Kobata is a pioneer of the glycobiology of N-linked sugar chains. He established the basis of glycobiology by developing a series of reliable methods to analyse the structures of N-linked sugar chains. The sensitive methods established by him greatly contributed to our understanding of the structural characteristics of the sugar chains and the biosynthetic mechanisms responsible for the production of such characteristics. He also provided new aspects that the sugar chains of glycoproteins play an important role in cell-to-cell recognition, and that the structures of sugar chains are altered under physiological and pathological conditions, including many tumours and diseases on a structural basis. In this article, the author would like to sketch out Kobata's main contributions to glycobiology for the sake of young scientists, who are planning to enter this scientific field in the future.

Keywords: γ-Glutamyltranspeptidase/glycoproteins/human chorionic gonadotropin/immunoglobulin G/milk oligosaccharides/N-linked sugar chains.

Abbreviations: GnT, *N*-acetylglucosaminyltransferase; γ-GTP, γ-glutamyltranspeptidase; hCG, human chorionic gonadotropin; IFN, interferon; IgG, immunoglobulin G.

Akira Kobata was born in 1933, in Nemuro, Hokkaido (Fig. 1). He graduated from the University of Tokyo in 1956 with a BA and received his MS in pharmaceutical sciences from the University in 1958. He then joined the Central Research Institute of Takeda Chemical Co., Ltd, where he started investigating the topics on nutrition. He found that human milk contains a large amount of various nucleotides, which are not found in cow's milk (*1*). Among the nucleotides, he found two novel nucleotides named UDP-X_1 and UDP-X_3. Structural studies of these nucleotides revealed that they are UDP-sugars, the sugar portions of which are not monosaccharides but di- and tri-saccharide: Galβ1-4GlcNAc and Fucα1-2Galβ1-4GlcNAc (*2*, *3*). He was awarded a PhD from the University of Tokyo in 1962 for his dissertation titled 'Comparative study of nucleotides in human milk and cow's milk'.

Journey from milk oligosaccharides to N-linked sugar chains

Longing to elucidate the physiological roles of the two novel nucleotides in human milk, he joined to the laboratory of Victor Ginsburg in National Institutes of Health (NIH) as a visiting scientist in 1967 (Fig. 2). In spite of his exertions, the functional roles of these nucleotides have not yet been elucidated until today. During his stay in the laboratory of Ginsburg, he devised an analytical method to obtain the oligosaccharide profiles of human milk by using small amount of samples (*4*). By analysing many milk samples, he found that human milk can be classified into three groups by their oligosaccharides profiles (Table 1). Approximately 80% of milk samples contains all nine neutral oligosaccharides known at that time. In contrast, 15% of samples lack four oligosaccharides containing the Fucα1-2Gal group, and 5% lack three oligosaccharides containing the Fucα1-4GlcNAc group. This finding greatly contributed to the successful elucidation of the whole biosynthetic scheme of ABO- and Lewis-type blood group determinants (*5*).

In 1971, Kobata returned Japan as the professor of the first Department of Biochemistry, Kobe University School of Medicine. Upon starting his own laboratory, Kobata considered thoroughly to what field he should develop his future research. It was the time when cell biologists started to realize important roles of the sugar chains of glycoconjugates on the cell surface. As the techniques used for the structural analysis of the sugar moiety of a glycoprotein were still immature, it was not so easy to study the molecular basis of the structure and the function of a glycoprotein produced by cells. Based on his experience during the successful study of human milk oligosaccharides for the elucidation of the biosynthesis of blood group determinants, Kobata stumbled upon a strategy that the characteristic features of the N-linked sugar chains of a glycoprotein might be accurately elucidated, if an appropriate method is established to quantitatively release the sugar chains as oligosaccharides. In order to shape up this strategy, he concentrated his research on the development of enzymatic and chemical means to quantitatively release the N-linked sugar chains of glycoproteins as oligosaccharides.

Characterization of three endo-β-*N*-acetylglucosaminidases, endo D, endo H and endo C_{II}, which hydrolyze specifically the *N,N′*-diacetylchitobiose moiety of the trimannosyl core of N-linked sugar chains, had opened a way for the investigation of the high mannose-type and hybrid-type sugar chains of N-linked sugar chains (*6*). These enzymes have different substrate specificities. By using the enzymes in a proper way, Kobata along with Tai and Yamashita could determine the structures of all major N-linked

Fig. 1 Akira Kobata.

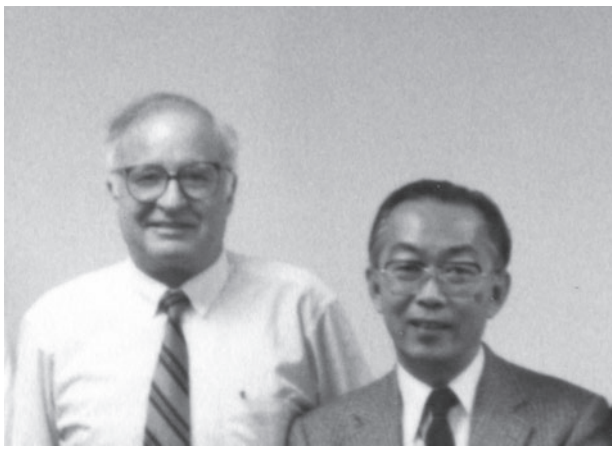

Fig. 2 V. Ginsburg and A. Kobata at NIH in 1987.

sugar chains of ovalbumin, indicating the effectiveness of his strategy (*7*–*9*). Because of the strict specificities, none of these enzymes could release the complex-type sugar chains, which are the largest population of N-linked sugar chains. Thus, Kobata along with Takasaki and Mizuochi (deceased in 2002) searched for several chemical methods suitable for releasing the complex-type sugar chains, and finally reached to hydrazinolysis (*10*). The reducing termini of oligosaccharides, thus released from glycoproteins, were quantitatively labelled by NaB^3H_4 reduction (*11*), and subjected to structural studies. In addition to the sequential exoglycosidase digestion, which had been developed for the study of milk oligosaccharides, many novel sensitive methods to investigate the structures of tritium-labelled oligosaccharides were developed. Gel-permeation chromatography, using a column containing ultra-fine Bio-Gel P-4, effectively fractionated oligosaccharides by their sizes (*12*, *13*).

Muramatsu and Kobata found that oligosaccharides and glycopeptides could be fractionated into three groups by concanavalin A (Con A)-Sepharose column chromatography according to their binding affinities: first group included those containing one Manα1- or -2Manα1- residue; the second included those containing two Manα1- or -2Manα1- residues; and the third related to those containing more than three Manα1- or -2Manα1- residues (*14*). This finding was successfully extended to the establishment of a method for separating mono-antennary complex-type, bi-antennary complex-type and high mannose-type sugar chains, from higher antennary complex-type sugar chains, and effectively applied to the elucidation of the molecular basis of Warren–Glick phenomenon (*15*), which is now considered as one of the most important tumour-related alterations of N-linked sugar chains (*16*). The usefulness of the Con A-Sepharose column accelerated the analysis of behaviour of oligosaccharides on many other immobilized lectin columns, and lectin columns were found to be useful for fractionating the sugar samples. Based on these studies, serial lectin column chromatography has been established as a useful fractionation method of oligosaccharides (*17*). As many lectins recognize quite a large portion of an oligosaccharide, the method has also become a useful technique to determine the structures of oligosaccharides as well. Purification and characterization of many exo-glycosidases, especially those with unique aglycon specificities, improved the effectiveness of sequential exo-glycosidase digestion (*18*). Methylation analysis devised by Hakomori has been useful, because complete methylation of oligosaccharides can be performed by a single procedure (*19*). The difficulties of N-methylation of aminosugar residues and poor recovery of *O*-methylated 2-*N*-methylaminosugars by gas chromatography were overcome by the successful synthesis of all partially *O*-methylated 2-*N*-methylglucosamines as standards, and by introduction of a column packed with Gas-chrom Q coated with OV-17 (*20*).

Accumulation of the structural information of the N-linked sugar chains of various glycoproteins, by using a series of methods so far introduced, afforded important bases, which have become essential for the later development of glycobiology and glycotechnology. The studies of the sugar chains of ovalbumin, unit A of bovine thyroglobulin (*21*), and ovomucoid (*22*) confirmed that the N-linked sugar chains of glycoproteins can be classified into three sub-groups: complex-type, high mannose-type and hybrid-type sugar chains (Fig. 3). This finding led Kornfeld (*23*) and Robbins (*24*) to elucidate the unique pathway of the biosynthesis of N-linked sugar chains. The finding that mono-, bi-, tri-, tetra- and penta-antennary sugar chains exist in complex-type sugar chains, and

Table 1. Three groups of human milk classified by the presence or absence of neutral oligosaccharides.

Names	Structures	Le^{a+b+}	Le^{a+b-}	Le^{a-b-}
2′-Fucosyllactose	Fucα1-2Galβ1-4Glc	+	–	+
3-Fucosyllactose	Galβ1-4Glc (3: Fucα1)	+	+	+
Lactodifucotetraose	Fucα1-2Galβ1-4Glc (3: Fucα1)	+	–	+
Lacto-*N*-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	+	+	+
Lacto-*N*-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	+	+	+
Lacto-*N*-fucopentaose I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	+	–	+
Lacto-*N*-fucopentaose II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc (4: Fucα1)	+	+	–
Lacto-*N*-difucohexaose I	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc (4: Fucα1)	+	–	–
Lacto-*N*-difucohexaose II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc (4: Fucα1; 3: Fucα1)	+	+	–

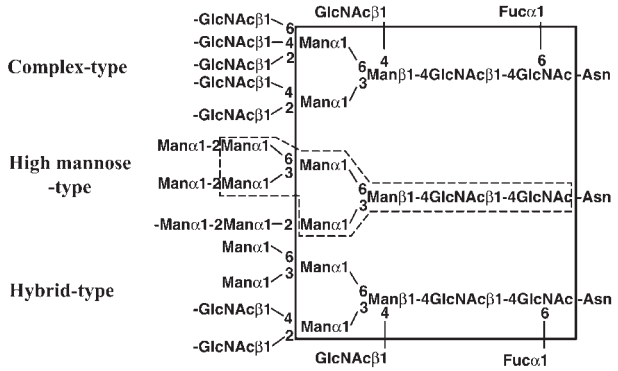

Fig. 3 Three subgroups of N-linked sugar chains. Structures within the solid line are the trimannosyl core commonly included in all N-linked sugar chains. The structure enclosed by dashed line is the common heptasaccharide core of high mannose-type sugar chains. Structures, outside the solid line and dashed line, can vary in the subgroups. The dashes on the monosaccharides at the non-reducing termini indicate that the residues can further be elongated by adding monosaccharides. Taken from Kobata, A. (2000) A journey to the world of glycobiology. *Glycoconj. J.* **17**, 443–464 (Fig. 3 on page 449 with slight modification, original copyright 2001 Kluwer Academic Publishers) with kind permission of Springer Science and Business Media.

Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc is the largest high mannose-type sugar chain were also confirmed by Kobata's studies (Fig. 3). The occurrence of various outer chain structures in complex-type and hybrid-type sugar chains was found by the studies of N-linked sugar chains of many glycoproteins, and additional modifications, the presence or absence of the α-fucosyl residue linked to the C-6 position of the proximal *N*-acetylglucosamine residue and the bisecting GlcNAc, which contributes to the structural variation of complex-type and hybrid-type sugar chains, were also confirmed. The bisecting GlcNAc is never elongated by the action of glycosyltransferases. In most glycoproteins, the fucosyl residue linked to the proximal *N*-acetylglucosamine is not elongated further, but the fucosyl residue of the hybrid-type sugar chains of octopus rhodopsin is substituted by a β-galactosyl residue at its C-4 position (*25*).

Many structural characteristics of the N-linked sugar chains of various glycoproteins were elucidated by Kobata's groups in Kobe University School of Medicine, and in the Institute of Medical Science, the University of Tokyo, where Kobata became the professor and chairman of the newly founded Department of Biochemistry in 1982. Tomoya Ogawa of RIKEN, a leader of chemical synthesis of oligosaccharides, told us "Thanks to the work of Kobata's group, we now have many reliable targets for our synthetic research."

Lesson from immunoglobulin G

Together with ovalbumin, immunoglobulin G (IgG) has been used in Kobata's laboratory as a standard glycoprotein for the investigation of the substrate specificities of endo- and exo-glycosidases, because they are inexpensive and commercially available. Accordingly, Kobata's group thoroughly investigated the structures of the sugar chains of IgG. Mizuochi and Kobata found that the sugar patterns of IgGs obtained from sera of healthy individuals exhibited extremely high micro-heterogeneity and 75% of them occurred as non-sialylated form in contrast to other serum glycoproteins. This micro-heterogeneity is produced by the presence or absence of two galactoses,

the bisecting GlcNAc, and the fucose residue of the usual bi-antennary complex-type sugar chain: Galβ1-4GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)-GlcNAc. Interesting evidence was that the molar ratio of each sugar chain of the serum IgG samples is quite constant in healthy individuals. By adding the information of the characteristic feature of the sugar patterns of myeloma IgG samples and glycosylated Bence Jones proteins, Kobata proposed that B-cells are a mixture of clones with different sets and ratios of glycosyltransferases, and speculated that each glycoform of IgG might have a different function. This hypothesis was recently confirmed by the comparative studies of the function of IgGs before and after removal of galactose residues, fucose residue or sialic acid residues. In collaboration with Dwek's group of Oxford University and Miyamoto's group in the University of Tokyo, Mizuochi and Kobata also found that an extensive deletion of galactose residues occurs in the N-linked sugar chains of IgGs obtained from the sera of rheumatoid arthritis patients. These results were reviewed several times by Kobata, and the most recent invited review was written in 2008 (*26*).

Lesson from γ-glutamyltranspeptidase

γ-Glutamyltranspeptidase (γ-GTP) is a glycoprotein bound to the apical membrane of the epithelial cells in various organs of mammals. It is composed of a heavy and a light subunit of glycoprotein nature, and is embedded in the plasma membrane of cells by the N-terminal portion of the heavy subunit (*27*). In collaboration with Katsunuma's group of Tokushima University, and Sakamoto's group of Osaka University, Yamashita and Kobata revealed many important lines of evidence in terms of the N-linked sugar chains of a membrane bound glycoprotein.

Investigation of rat kidney γ-GTP revealed the presence of a series of high mannose-type sugar chains and from bi- to tetra-antennary complex-type sugar chains with bisecting GlcNAc and core fucose (*28*). Sialylated and non-sialylated Galβ1-4GlcNAcβ1, and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1 were found in the outer chain moieties of the complex-type sugar chains. When Yamashita and Kobata examined comparatively the sugar patterns of the two subunits of rat kidney γ-GTP, they found that non-sialylated and non-fucosylated bi-antennary sugar chains are included only in the light subunit, and high mannose-type sugar chains are included only in the heavy subunit, indicating the presence of site-specific glycosylation (*29*). Presence of site-specific glycosylation was later detected in many other glycoproteins.

The presence of isozymes with different isoelectric points was already known in rat kidney γ-GTP at that time (*30*). By comparative study of the sugar patterns of four isozymes purified by DE-52 column chromatography, Yamashita and Kobata confirmed that the total numbers of sialic acid residues showed a reciprocal relationship to the isoelectric point of each isozyme (*29*). Further, they continued their works. When they compared the sugar chains of γ-GTPs purified from the kidney and the liver of various mammals (*31–36*), they found presence of both organ-specific and species-specific glycosylation of γ-GTP (Fig. 4). They also found that the bisecting GlcNAc is detected in the sugar chains of all kidney γ-GTPs, but not in those of liver enzymes. This evidence, together with the fact that none of the glycoproteins produced by the liver, so far studied, contains bisected sugar chains, indicated that expression of *N*-acetylglucosaminyltransferase III (GnT-III), which catalyzes the addition of the bisecting GlcNAc residue to N-linked sugar chains, is suppressed in the adult liver or by differentiation to hepatocytes. Later it was confirmed that the GnT-III activity was high in rat fetal liver and new born liver as compared to in adult liver (*37*). In contrast, the enzyme should be strongly expressed in the kidney of all mammals.

Yamashita and Kobata collaborated with Taniguchi of Hokkaido University in comparatively studying the sugar chains of γ-GTPs purified from rat AH-66 hepatoma cells and rat liver, and revealed that the two enzymes contain very different sets of sugar chains (*34*). The sugar chains of the liver enzyme were all acidic, while the hepatoma enzyme had the neutral oligosaccharides composed of both high mannose-type and complex-type sugar chains in addition to the acidic sugar chains. Three prominent structural differences were found in the acidic sugar chains of the two enzymes. (i) The hepatoma enzyme has incomplete outer chains lacking the galactose residues; (ii) Galβ1-4GlcNAc tandem repeats were found in the liver enzyme, but not in the hepatoma enzyme; and (iii) more than 40% of the sugar chains of the hepatoma enzyme contain bisecting GlcNAc, which is not found in those of the liver enzyme. As the expression of GnT-III should be suppressed in the normal livers of all mammals as described already, occurrence of the bisecting GlcNAc residue was considered the most interesting. Therefore, this phenomenon seems to indicate that GnT-III is ectopically expressed in hepatoma. However, since bisected sugar chains were not prominently detected in the human hepatoma γ-GTP (*36*), this interesting finding could not be applied to the diagnosis of the human hepatoma. These results indicated that the structural change of the sugar chains of tumour glycoproteins could be species specific. Alterations of the sugar chain structures of glycoproteins have later been found to occur in various other tumours by Kobata's group. It was confirmed that three β-*N*-acetylglucosaminyltransferases, GnT-III, -IV and -V, play major roles in the structural alterations of the complex-type sugar chains produced in various tumours. The importance of these phenomena for the diagnosis and immunotherapy of tumours was discussed in the review written by Kobata and Amano (*38*) in 2005.

Lesson from human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is a heterodimer composed of α- and β-subunits. Both subunits contain two N-linked sugar chains (*39*,*40*). The whole structures of N-linked sugar chains of urinary

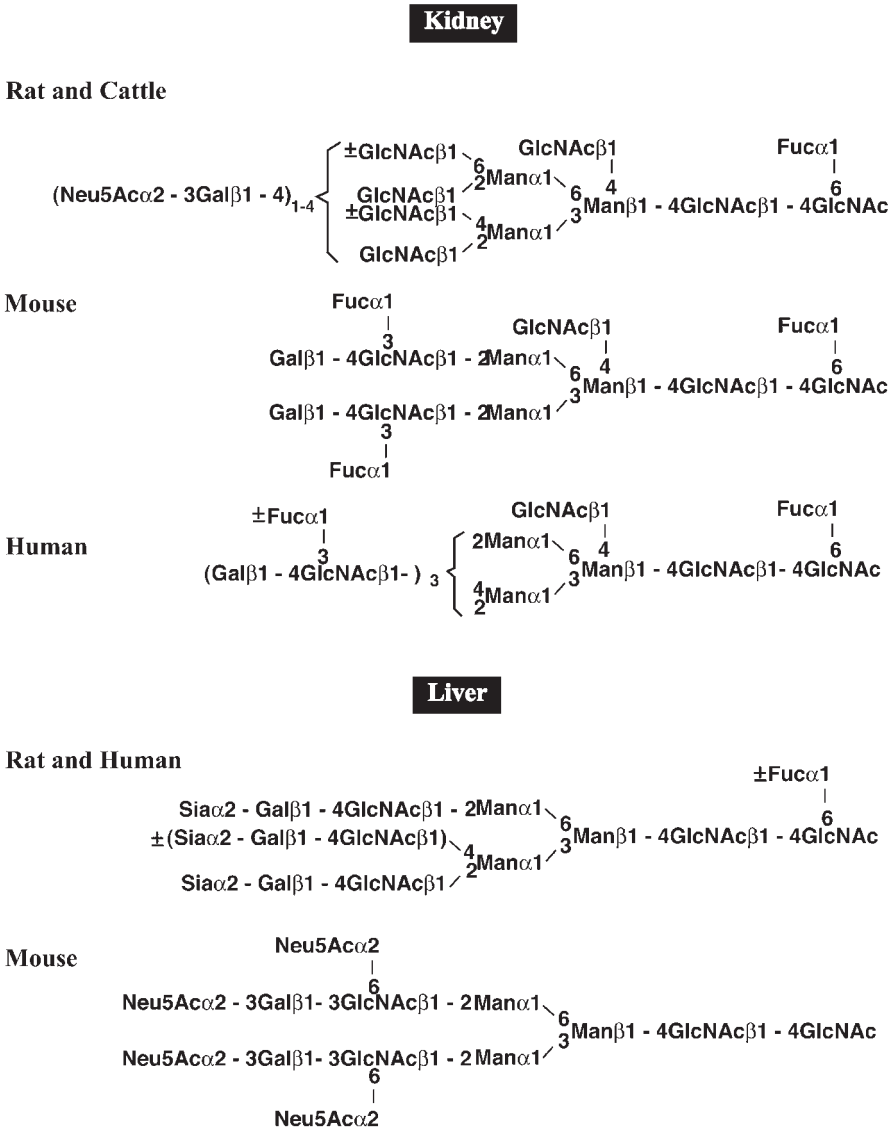

Fig. 4 Major N-linked sugar chains of γ-GTPs purified from the kidney and the liver of various mammals. Taken from Kobata, A. (2000) A journey to the world of glycobiology. *Glycoconj. J.* **17**, 443–464 (Fig. 8 on page 455, original copyright 2001 Kluwer Academic Publishers) with kind permission of Springer Science and Business Media.

hCG were elucidated by Kobata's group in collaboration with Tojo's group of Kobe University in 1979 (*41*). The hormone contains mono- and di-sialylated E and F and mono-sialylated H in Fig. 5. HCG can be dissociated into α- and β-subunits (named hCGα and hCGβ, respectively) by 8 M urea. By comparative study of the sugar chains of hCGα and hCGβ, Mizuochi and Kobata found that the five sialylated complex-type sugar chains of hCG are not evenly distributed at the four asparagine loci of hCG molecule, indicating the presence of site-specificity (*42*). The two N-linked sugar chains of α-subunit are never fucosylated, and one of them remains at mono-antennary stage (sialylated oligosaccharide H).

By collaboration with Ryuichiro Nishimura of Hyogo Cancer Center, Kobata, Mizuochi and the author performed the following series of studies on the sugar chains of hCG. A small amount of α-subunit occurs in free form in the urine of pregnant women. Interestingly, this free α-subunit cannot bind to the hCGβ in contrast to hCGα. Structural studies of the sugar chains of free α-subunit revealed that it contains only one N-linked sugar chain, the 9% of which were sialylated oligosaccharide E and remainder were sialylated oligosaccharide F (*43*). Based on this interesting structural difference found in the sugar chains of free α-subunit and hCGα, Kobata speculated that the steric hindrance of bulky sialylated oligosaccharides E and F on the free α-subunit might inhibit its association with hCGβ. Therefore, uneven distribution of the N-linked sugar chains at the four N-glycosylation sites of hCG might be produced only when the two subunits are associated before the N-linked sugar chains start maturation at the Golgi apparatus.

Studies of the N-linked sugar chains of hCGs, purified from urine of various trophoblastic diseases, afforded another interesting information, which are useful for improving the diagnostic value of hCG for

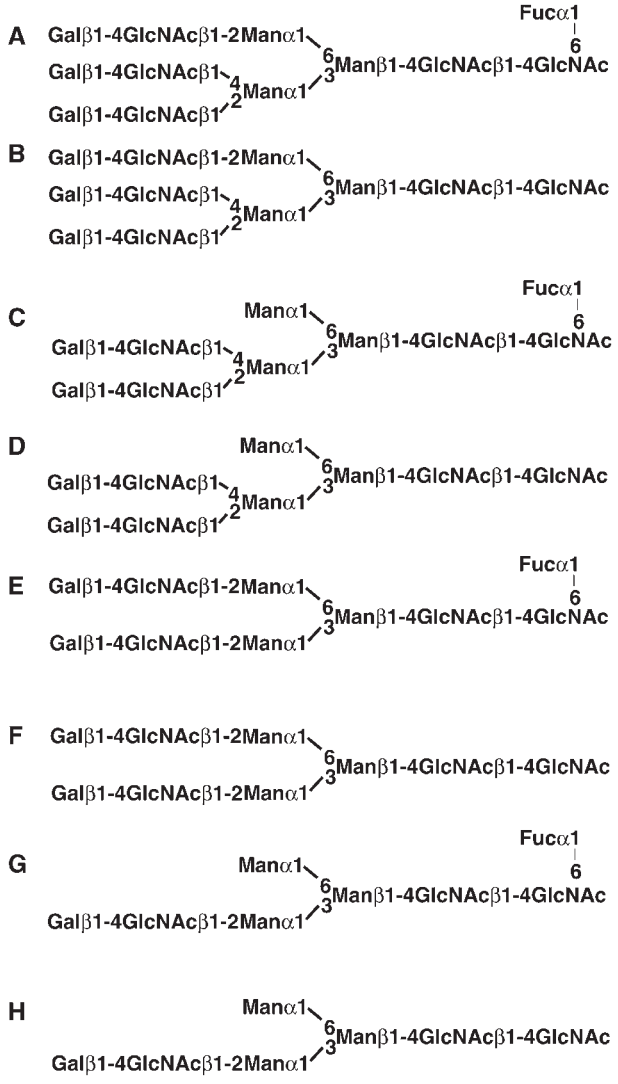

Fig. 5 Structures of N-linked sugar chains found in various hCGs.

these diseases. HCGs purified from hydatidiform mole patients showed the same pattern of N-linked sugar chains as those from pregnant women (*44*). In contrast, hCG samples purified from choriocarcinoma patients contain sialylated forms of all eight oligosaccharides shown in Fig. 5 (*44*, *45*). It was also found that some of the choriocarcinoma hCGs contain non-sialylated forms of the eight oligosaccharides. Another interesting evidence was that hCGs obtained from invasive-mole patients contained six oligosaccharides except for C and D in Fig. 5 (*46*). Therefore, a part, but not all, of the abnormalities found in the neutral portion of the N-linked sugar chains of choriocarcinoma hCG is induced in the invasive-mole hCGs. Detection of oligosaccharides A and B in invasive-mole hCGs indicated that ectopic expression of GnT-IV occurs in this lesion. However, absence of oligosaccharides C and D indicated that the newly expressed GnT-IV transfers an *N*-acetylglucosamine residue to bi-antennary complex-type sugar chains but not to mono-antennary sugar chains. This substrate specificity is the attribute of GnT-IV in normal tissues, because oligosaccharides C and D are not detected in the glycoproteins produced by normal cells. Therefore, in terms of GnT-IV, transformational change induced in choriocarcinoma is considered to take place in two steps. The first is the ectopic expression of the regular GnT-IV, and the second is the modification of the substrate specificity of the enzyme. Elucidation of the enzymatic basis of this interesting phenomenon is a target for future study. In a review article, Kobata thoroughly discussed the structures and functional roles of N-linked sugar chains of glycoprotein hormone (*47*).

Lesson from recombinant glycoproteins

Many recombinant glycoproteins have been produced by using various animal cell lines as hosts. Occurrence of both organ- and species-specific differences in the N-linked sugar chains of glycoproteins, as already discussed, suggested the importance of comparative studies of biological activities of these recombinant glycoproteins. In addition, altered glycosylation phenomena found in various malignant cells could be reflected in the recombinant glycoproteins, because many of the cell lines used as hosts are somewhat malignant cells. A comparative study by Kobata's group of the human interferon-β1 (IFN-β1) and three recombinant IFN-β1s produced by different cell lines revealed that their sugar patterns were different in spite of containing the same number of complex-type sugar chains (*48*). The differences occur both in the antennary structures and in the structures of outer chain moieties. Therefore, recombinant glycoproteins could become useful clues to investigate the biological function of the sugar chains of glycoproteins. By comparative studies of the sugar patterns and *in vivo* activities of several preparations of recombinant human erythropoietin, Takeuchi and Kobata confirmed that the activity is proportional to the ratio of tetra-antennary to bi-antennary oligosaccharides (*49*). Unfortunately, Takeuchi could not continue his research as he died in 2001.

Recently, many recombinant N-glycosylated glycoproteins are approved for clinical use as biopharmaceuticals around the world. Kobata's works on recombinant glycoproteins called attention that glycosylation in biopharmaceuticals affects their efficacy and safety, and the glycosylation is dependent on the manufacturing process and the expression system. Nowadays, it is very important for the development of biosimilar products to assure the similarity of N-linked sugar chains to the original products.

Perspectives

The importance of correct structural information of the sugar chains of glycoproteins needs to be stressed. Before Kobata developed a series of reliable methods of N-linked sugar chains, proposed incorrect and incomplete structural information sometimes disturbed and misled the progress of glycobiology. After the establishment of sensitive and quantitative methods, the correct structural information of the N-linked sugar chains of various glycoproteins was

accumulated. Kobata's group proposed structural rules of the N-linked sugar chains (Fig. 3), which led to elucidate the biosynthetic pathway of the N-linked sugar chains. Later, molecular glycobiology (*e.g.* purification, characterization and cloning of glycosyltransferases, and establishment of glycosyltransferase genes knockout mice) has been launched on the basis of structural rules (*50*). Due to the limited space, all of them cannot be listed. One example is that pathological alterations of the N-linked sugar chains of tumour glycoproteins has led to elucidate the molecular and biological importance of the modified N-linked sugar chains by actions of GnT-III and GnT-V. These studies were performed by Taniguchi, Pierce, Dennis, Marth and others (*51*).

After having retired from the University of Tokyo in 1993, Kobata was invited to be director of the Tokyo Metropolitan Institute of Gerontology. The main purpose of gerontology is to elucidate the mechanisms of deterioration, which occur in various parts of the human body through ageing, and use this knowledge to improve quality of life among the elderly. Kobata considered that age-related alteration of the sugar chains of various glycoconjugates could be an important element in solving various pathological problems found in elderly individuals, because as already described, the sugar chains can be altered by the physiological conditions of cells. With this idea in mind, he newly established the Department of Glycobiology in the Institute. In addition to glycobiology and glycopathology, he assumes that glycogerontology will be a new field of life science. The author is now the Head of the Department, and we are mainly studying on age-associated changes of glycoproteins (*52*, *53*), and disease-associated changes of protein glycosylation in Alzheimer's disease (*54*, *55*) and muscular dystrophy (*56*–*58*).

By recent development of the analytical methods for the structural studies of sugar chains, the techniques developed by Kobata's group have now become classical ones. However, the strategy of quantitatively releasing N-linked sugar chains as oligosaccharides, and subjecting them for structural study after proper fractionation will remain as the main stream of structural glycobiology. Furthermore, it must be pointed out that the studies by Kobata's group were performed by using NaB^3H_4 of lower specific activity. Since NaB^3H_4 of 100 times higher specific activity is now commercially available, the sensitivity of analysis can be much improved by simply using a series of methods devised by Kobata's group, if this hotter reagent is introduced in the study. Among the recent developments, nuclear magnetic resonance (NMR) and mass spectrometry are making tremendous developments. Particularly, the introduction of tandem mass spectrometry is expected to offer a very sensitive and reliable method not only to identify the glycoprotein but also to determine the location of the glycosylation sites and the structures of N-linked sugar chains at each glycosylation site (*59*). Future development of labelling and fractionation methods of N-linked sugar chains must be directed to afford suitable samples for applying this useful physical method (*60*).

Epilogue

Kobata is a pioneer of the glycobiology of N-linked sugar chains. For his important contributions to glycobiology during his services as professor in Kobe University School of Medicine, and Institute of Medical Science, the University of Tokyo, he received a number of awards including the Claude S. Hudson Award from American Chemical Society, PSJ Award from the Pharmaceutical Society of Japan and Japan Academy Prize. He was a Fogarty Scholar-in Residence from 1985 to 1987, Auckland Foundation visiting professor in 1988 and also served as the director of Institute of Medical Science. His love for glycobiology is common knowledge. His work on glycobiology is motivated by a desire and curiosity to pursue the truth. His enthusiasm and perseverance to glycobiology is worthy of admiration. The author sincerely hopes that this article will help young scientists seek their own originality in their future research.

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Conflict of interest
None declared.

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