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CELL SURFACE GLYCOPROTEINS: BIOCHEMICAL, IMMUNOLOGICAL AND MOLECULAR BIOLOGICAL STUDIES

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ABSTRACT

This article briefly summarizes the author's contribution to the study of cell surface glycoproteins. At first, much effort was devoted to developing analytical methods, especially exoglycosidases and endoglycosidases. An endo-β-*N*-acetylglucosaminidase found in *Streptococcus pneumoniae* was the first example of an endoglycosidase acting on glycoproteins. A combination of radioactive labeling, glycosidase digestion and lectin affinity chromatography enabled the characterization of carbohydrate moieties of cell surface glycoproteins derived from cultured cells. Application of the method to teratocarcinoma stem cells and preimplantation mouse embryos led to the discovery of marked changes in the carbohydrate moieties of cell surface glycoproteins during embryogenesis. Combining biochemical and immunohistochemical methods, an overall picture was obtained for carbohydrate changes in early embryogenesis of the mouse, and molecular biological approaches have been adopted to determine their biological significance. Furthermore, some core proteins carrying developmentally regulated carbohydrate markers were found and characterized by molecular cloning. One example of such a protein is embigin, which enhances integrin-mediated cell-substratum adhesion and established the existence of a new group in the immunoglobulin superfamily. Carbohydrate immunochemical markers useful in the analysis of mouse embryogenesis were also found to be effective in the classification of human carcinomas with respect to metastatic potential.

INTRODUCTION

Molecular interactions at the cell surface play important roles in the regulation of cellular activities such as growth, differentiation, migration and adhesion.[1] The author has been engaged in studies on cell surface molecules, namely cell surface glycoproteins and a novel heparin binding growth factor, midkine,[2–25] the latter of which has recently been reviewed.[2,3] Taking this opportunity, I wish to summarize our contribution to a research area, namely cell surface glycoproteins, with some personal reflections.

EXOGLYCOSIDASES

As a graduate student, I joined the laboratory of Prof. F. Egami, Faculty of Science, Tokyo University. Prof. Egami identified ribonuclease T_1, which is useful in structural studies of ribonucleic acid. He proposed that I study carbohydrases of a marine gastropod, *Charonia lampas* (Japanese name: *bōshubora* or *horagai*), considering that studies of complex carbohydrates would become important and that the identification and characterization of enzymes capable of their degradation would be highly desirable.

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Among the wide variety of complex carbohydrates, I chose to study glycoproteins, since little was known about these molecules and enzymes acting on the carbohydrate moieties. The apparent specificity of the glycosylation products promised both interesting and important results. I prepared extracts from the hepatopancreas of *C. lampas*, and examined enzymes acting on glycopeptides isolated from both ovalbumin and ovomucoid. Enzymes releasing mannose, N-acetylglucosamine and galactose were found, but no oligosaccharides were detected even for short reaction times, suggesting that the sugars were released by the step-wise activity of exoglycosidases.[26] The enzyme releasing mannose was found to be novel, and was considered to be an α- mannosidase. Thus, attempts were made to purify it using p-nitrophenyl α-mannoside as a substrate. The purified enzyme indeed released mannose from glycopeptides.[27] However, an α-mannosidase was also purified from jack bean meal, and was shown to act on glycoprotein carbohydrates. The characterization of the jack bean enzyme was published slightly earlier than that of the *C. lampas* enzyme.

The importance of enzymes acting on glycoprotein carbohydrate moieties became apparent to several investigators at the time. I, together with co-workers, systematically purified various exoglycosidases from *C. lampas*, and also from another marine gastropod, *Turbo cornutus* (Japanese name: *sazae*) (Table 1).[26–33] Among these, β-mannosidase and β-xylosidase were the first to be purified. These enzymes act on glycoprotein carbohydrates, and have since proved useful in structural and biological studies of glycoproteins as well as bacterial lipopolysacaharides. (Table 1). They became commercially available from Seikagaku Kogyo Co. During my time in Prof. Egami's laboratory, I also studied both glycoproteins from the jelly coat of starfish eggs[34] and sulfated glycopeptides from bovine brain,[35] the former of which were later shown to participate in fertilization by Prof. M. Hoshi, Tokyo Institute of Technology.

Table 1. Exoglycosidase Purified from Marine Gastropods

Enzyme	Substrates including applied subjects
α-Mannosidase	Ovalbumin glycopeptide, Stem bromelain glycopeptide, Japanese encephalitis virus receptor, Bacterial lipopolysaccharide
β-Mannosidase	Bacterial lipopolysaccharide
α-L-Fucosidase	Blood group substances, Milk oligosaccharides
β-Xylosidase	Stem bromelain glycopeptide, Xylosyl serine linkage in proteoglycan
β-*N*-Acetylhexosaminidase	Ovomucoid
α-*N*-Acetylgalactosaminidase	—

MAJOR HISTOCOMPATIBILITY ANTIGEN

After receiving my Ph. D., I wished to study the biological roles of cell surface glycoproteins, and joined the laboratory of Dr. Stanley G. Nathenson, Albert Einstein College of Medicine, as a postdoctoral fellow. He published a paper on the purification of H-2 antigen (major histocompatibility antigen of the mouse), and demonstrated that it is a glycoprotein. I felt that the H-2 antigenic determinant may be carbohydrate as is the case for the ABH blood group antigen.

The H-2 antigen could be obtained in only very small amounts, making biochemical studies difficult. However, Einstein College at that time was a major world center of cell biology, and new techniques including SDS polyacrylamide gel electrophoresis were developed there. In

Nathenson's laboratory, radioactively labeled H-2 antigen was prepared by culturing H-2 bearing tumor cells in the presence of radioactive amino acids. Following this method, I isolated carbohydrate-labeled H-2 antigen, which could be used to characterize the carbohydrate portion.[36–39] A central question was whether carbohydrate is the antigenic determinant. While an article in *Nature* reported that this was indeed the case, all my data strongly suggested otherwise, and the *Nature* paper was subsequently shown to be mistaken. Although results obtained were not dramatic, I learned how powerful the application of radiolabeling methods to glycoprotein research could be. Using the technique, we characterized the thymus leukemia (TL) antigen.[40]

ENDOGLYCOSIDASES AND FIBROBLAST OLIGOSACCHARIDES

Until 1970, all enzymes acting on glycoprotein carbohydrates were thought to be exoglycosidases. When I was analyzing radioactively labeled H-2 glycopeptides using an enzyme preparation from *Streptococcus pneumoniae*, I found an unusual product, best explained by cleavage by an endoglycosidase. Changing the substrate to carbohydrate-labeled IgG glycopeptide, I indeed showed the presence of an endoglycosidase acting on the glycoprotein.[41] The enzyme appeared to be an endo-β-*N*-acetylglucosaminidase.

After my appointment at the laboratory of Prof. A. Kobata, Kobe University School of Medicine, I began systematic studies of the endoglycosidase. The ovalbumin glycopeptide $(Man)_5(GlcNAc)_2$ An was [^{14}C]-*N*-acetylated and used to assay the enzyme; Asn [^{14}C]-acetyl GlcNAc released was quantified. The enzyme was purified and its specificity determined.[42–46] It was shown to cleave di-*N*-acetylchitobiose structure, and have strict specificity for the oligomannosyl cores of glycopeptides. This endoglycosidase, named endo-β-*N*-acetylglucosaminidase D (Endo D), acts on complex-type carbohydrate chains after releasing external side chain sugars. After the discovery of Endo D, another endo-β-*N*-acetylglucosaminidase was found in culture filtrates of *Streptomyces* by A. Tarentino. We found Endo D and the *Streptomyces* enzyme (endo-β-*N*-acetylglucosaminidase H: Endo H) to have complementary specificities (Fig. 1).[46] The exact specificity of Endo D was elucidated by structural studies of the substrates.[45,47–49] We also studied endo-β-*N*-acetylglucosaminidases from rat liver,[50] *Clostridium perfringens*[51] and fig.[52] Endo-β-*N*-acetylglucosaminidases have recently been reviewed.[53]

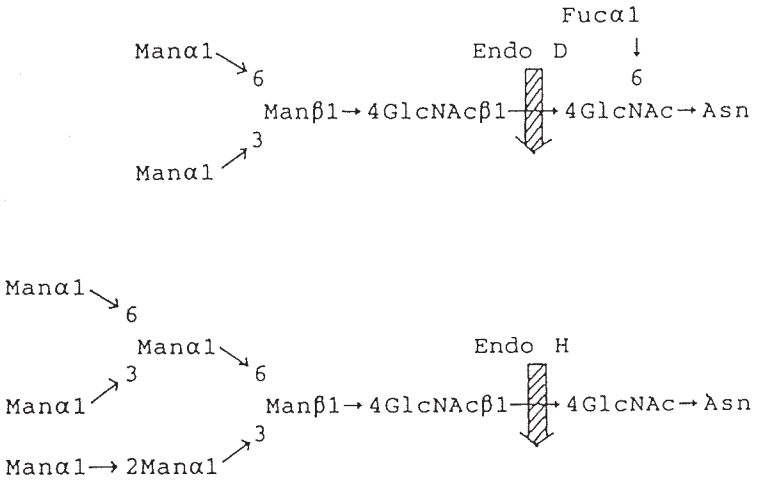

Fig. 1. Different specificities of Endo D and Endo H. The upper substrate was hydrolyzed by Endo D, but not by Endo H; the lower substrate was hydrolyzed by Endo H, but not by Endo D.

The endoglycosidases have been used in structural studies of ovalbumin glycopeptides[47] and IgG glycopeptides.[49] Furthermore, combination of exoglycosidase and Endo D proved useful in the removal of carbohydrates from IgG.[54] This enzymatic digestion procedure demonstrated the importance of carbohydrates in Fc region functions such as binding to Fc receptors. The restricted and complementary specificities of Endo D and Endo H and related enzymes were used by three groups in the USA and, consequently, they identified and characterized a processing pathway for asparagine-linked oligosacchrarides.[53]

Radiolabeled glycopeptides were also used to study the specificity of concanavalin A columns. We found that two α-mannosyl residues, which may be substituted only at C-2, are required for binding to the column.[55] We also extended studies on exoglycosidases using radiolabeled substrates.[56–58]

Soon after the discovery of Endo D, I felt that it would be useful for the characterization of radioactively labeled glycopeptides from the cell surface of cultured fibroblast cells.[59] Using the complementary specificities of Endo D and Endo H, we found that the level of high mannose oligosaccharides is elevated in growing cells as compared with nongrowing cells.[60,61] Endo D and Endo H digestion was useful in determining the overall structure of asparagine-linked cell surface oligosaccharides.[61–64] Furthermore, in addition to the enzymatic method, concanavalin A affinity chromatography was employed. Consequently, we identified increased branching in cell surface oligosaccharides from virally-transformed cells.[65]

TERATOCARCINOMAS AND EARLY MOUSE EMBRYOS

After studying endo-β-*N*-acetylglucosaminidases and fibroblast glycopeptides, I wished to study more directly the function of cell surface carbohydrates, and selected early mouse embryogenesis as the experimental system. As a Franco-Japanese exchange scholar, I joined the laboratory of Prof. F. Jacob, Pasteur Institute. At that time, Prof. Jacob concentrated on the mouse teratocarcinoma system to study the molecular mechanisms of early embryogenesis. Since nothing was known about carbohydrates of cell surface glycoproteins in early embryonic cells, I analyzed [^{3}H]-fucose-labeled glycopeptides from plasma membranes and whole cells of teratocarcinoma stem cells (EC cells), which resemble the multipotent cells of early embryos.[66,67] Unexpectedly, high levels of the label were eluted in the excluded volume of Sephadex G-50 columns. This radioactivity progressively decreased when EC cells differentiated *in vitro*. Preimplantation and early postimplantation mouse embryos also synthesized large fucosyl glycopeptides, while during the midgestational period, the synthetic activity became scarcely detectable.[66,68] Various biochemical experiments excluded the possibility that the large glycopeptides were mucopolysaccharides, mucin-type glycopeptides or glycolipids.[67] Thus, we concluded that there exists a class of unusual glycoproteins in early embryonic cells, and that the carbohydrate profile changes markedly during embryogenesis.[66–72] The large carbohydrates were found to carry certain cell surface markers of EC cells, such as *Lotus tetragonolobus* agglutin binding sites, and glycosidase digestion showed them to be poly-*N*-acetyllactosamines.[69]

Two years after returning from France, I was appointed as a professor of biochemistry, Kagoshima University Faculty of Medicine, and opened a new laboratory. We continued studies on the large carbohydrate from EC cells and isolated and biochemically characterized it.[73–81] In particular, we showed it to be highly branched, and to have a molecular weight of around 10,000 or more. On the basis of its complexity and the markers it carried, this large glycan was different from other poly-*N*-acetyllactosamines so far identified, and we named it embryoglycan.[75] The protein-carbohydrate linkage of embryoglycan is of asparagine-linked one.[76,80]

Embryoglycan has Le^X structure, epitope of SSEA-1, which is the most well-known cell surface marker of EC cells.[80] Furthermore, the major carrier of SSEA-1 in EC cells was shown to be high molecular weight glycoproteins with embryoglycan.[82]

In addition to biochemical analysis, we employed immunohistochemical methods to analyze carbohydrate changes during development.[83–95] For example, an *N*-acetylgalactosamine-recognizing lectin, *Dolichos biflorus* agglutinin (DBA), specifically reacted with extraembryonic endoderm in early postimplantation embryos,[86] and in the cells derived from pluripotent EC cells.[91]

Summarizing all these results and citing works performed in other laboratories, I presented an overall picture of carbohydrate changes during early mouse embryogenesis.[96–98]

The epitope structure of the binding site of DBA was determined as shown in Table 2.[99] DBA also specifically reacts with thymocytes in early developmental stages,[83] some leukemia cells[100] and restricted tissues in adult mice.[84] EC cells whose direction of differentiation is restricted to extraembryonic endoderm cells are also reactive to DBA.[101]

Table 2. Carbohydrate Markers Present on Poly-*N*-acetyllactosamine (embryoglycan) and Useful for Analysis of Early Mouse Embryogenesis

Name	Determinant
Antigens	
SSEA-1 (Le^X)	Galβ 1→4(Fuclα 1→3)GlcNAc
I	Galβ1→4GlcNAcβ1 ↘ 6 Galβ1→4GlcNAcβ1→3Gal ↗ 3 Galβ1→4GlcNAcβ1
i	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Gal
Lectin receptors	
LTA (FBP)	Galβ1→4(Fucα1→3)GlcNAc
PNA	Galβ1→3GalNAc
DBA	GalNAcβ1→4(NeuAcα2→3)Galβ1→4GlcNAcβ1→3Gal
GSI-B_4	Galα1→3Gal

The basis of the carbohydrate change is alteration in glycosyltransferase activity. Indeed, we verified that the reduction in SSEA-1 antigenicity after differentiation of EC cells was accompanied by a concomitant reduction in α1-3 fucosyltransferase activity.[102] The key fucosyltransferase was purified and characterized.[103]

Embryoglycan carries numerous markers found in early embryonic cells (Table 2).[82,95-98,104–107] In addition, sera from patients with germ cell tumors frequently have precipitating antibodies which recognize an epitope on embryoglycan.[108–110] Using endo-β-galactosidase C, a novel endo-β-galactosidase identified in our laboratory, which acts on Galα1-3Gal β1-4 GlcNAc sequence releasing Gal α1-3 Gal disaccharide,[111–112] the epitope recognized by the patients' sera was shown to be Gal α1-3Gal.[113] Endo-β-galactosidase C was also useful in correcting the structure of glycopeptides from Ehrlich carcinoma cells.[114]

To investigate the function of carbohydrates on EC cells, we prepared anti-carbohydrate monoclonal antibodies by using EC cells as the immunogen. A monoclonal antibody thus

obtained, 4C9, inhibited cell-substratum adhesion of EC cells.[115] The epitope recognized by the 4C9 antibody is Le^X structure. The distribution of 4C9 antigen is similar to SSEA-1, while 4C9 is more specific for cells in lineages starting from totipotent embryonic cells leading to primordial germ cells.[116] After several control experiments, we proposed that Le^X structure enhances cell-substratum adhesion. This proposal has been supported by a recent finding in our laboratory; transfection with α1-3 fucosyltransferase that forms Le^X structure, resulted in increased cell-substratum adhesion of transfected L cells (Sudou, A. et al., unpublished).

We also searched for lectins in teratocarcinoma cells to find endogeneous sugar-recognizing proteins. A lectin was found in teratocarcinoma OTT6050, but identified as laminin.[117] The finding of lectin activity in laminin is, however, important in considering the function of laminin.

A candidate for a carbohydrate-recognizing protein on the surface of EC cells is β-galactosyltransferase. We prepared a monoclonal antibody to the enzyme and verified its cell surface location.[118]

To obtain a more definitive answer concerning the function of carbohydrates, we decided to use transgenic techniques. We produced transgenic mice carrying α 1-3 galactosyltransferase cDNA, and these mice became the first example of transgenic animals expressing a specific carbohydrate structure ectopically.[119] They tended to show some abnormalities, such as increased protein level in the urine. Undoubtedly, gene targeting experiments will be highly informative, and work along this line is currently in progress.

CORE PROTEINS

We were also interested in proteins that carry developmentally regulated carbohydrate chains. To identify these, glycoproteins were isolated from teratocarcinoma cells by affinity chromatography through lectin-agarose columns. Rabbits were immunized against the glycoproteins, and affinity-purified antibodies were used to immunochemically characterize glycoproteins.[120,121] Subsequently, monoclonal antibody technique was also used.[122] Then, we used the affinity purified antibodies to screen a λgt 11 expression library. When DBA lectin was used, a core protein named embigin was identified, the protein moiety of which had a molecular weight of 30 kDa, while that of the whole glycoprotein was 70 to 90 kDa.[123] Embigin has a transmembrane domain, and the extracellular portion contains two immunoglobulin-like domains. During mouse embryogenesis, embigin is preferentially expressed before 10 days of gestation, and *in situ* hybridization analysis has identified the preferential site of this expression.[124] L cells lack embigin, and those transfected with embigin cDNA exhibited increased cell-substratum adhesion activity.[125] This increased adhesion was inhibited by anti-integrin antibody. Thus, embigin was concluded to have a role in enhancing integrin-mediated cell-substratum adhesion.

Using antibodies raised against glycoproteins binding to *Lotus tetragonolobus* agglutinin (LTA),[126] we also cloned a core protein named basigin.[127,128] Basigin shows strong homology to embigin throughout most of the molecule, indicating that embigin is a prototype of a new class of molecules in the Ig superfamily. The molecular weight of the protein portion of basigin is 27 kDa, while that of the glycosylated molecule is 44 to 66 kDa. The immunoglobulin-like domain close to the transmembrane region is unique, since it has homology to both Ig V domain and MHC class IIβ-chain, which has a C domain (Fig. 2). Basigin may be related to the primordial form of the immunoglobulin superfamily. Basigin was also found independently by other groups and given different names such as gp42 and HT7. Although basigin is broadly distributed in various embryonic and adult tissues, it may still be used as a marker of brain blood vessels; basigin was reported to be absent in neurons and systemic blood vessels. Attempts are currently

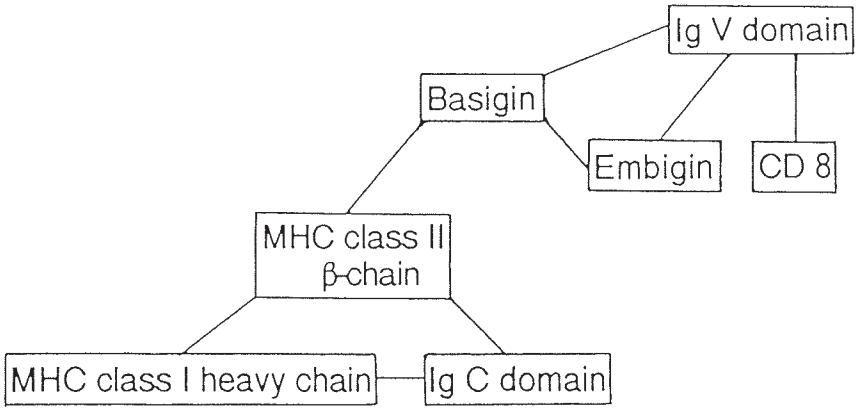

Fig. 2. Relationship between members of the Ig superfamily. Cited from Ref. 128.

underway to determine the function of basigin by gene targeting.

Using antibodies to DBA-binding glycoproteins from teratocarcinomas, we also found two new proteins by cDNA cloning. One is heparin-binding protein-44 (HBP-44),[129–130] which forms a complex with brushin[120,131] (also called GP-330 or Heymann nephritis antigen), a calcium sensor common to the kidney and extraembryonic endoderm. The other is reticulocalbin, a calcium-binding protein which is located in the endoplasmic reticulum and has 4 EF-hand.[132] Antibody screening was also applied to identify a new core protein in peanut agglutinin (PNA)-binding glycoproteins from human gastric carcinomas.[133–135] MGC-24 (*m*ulti-glycosylated *c*ore protein-24) thus found, is a hydrophilic, cysteine-rich protein which carries numerous Galβ1-3 GalNAc oligosaccharides.[135]

CARBOHYDRATES AS METASTATIC AND PROGNOSTIC MARKERS

At Kagoshima University Faculty of Medicine, we embarked upon numerous collaborative studies with clinical departments; the subjects were glycoproteins such as those in photoreceptor cells and human carcinomas.[136–151] The most notable finding produced by these collaborative studies was that the carbohydrate markers we used to analyse mouse embryogenesis were also useful in analyzing the biological properties of human carcinomas. Thus, lung carcinomas with [DBA(+), 4C9(−)] carbohydrate profile are less metastatic, and patient prognoses are better in comparison with carcinomas with different carbohydrate profiles.[146]

In the case of transient cell carcinoma of the urinary bladder, primary tumors that strongly express *Lotus tetragonolobus* agglutinin binding sites (LTA), showed a tendency to be more metastatic than those expressing them only weakly or not at all.[147] Also, expression of LTA-related markers, namely Le^X and sialyl Le^X, correlated with metastatic potential of the tumors.[145,148] The latter is interesting from the point of view that selectins, which are carbohydrate receptors involved in adhesion of leukocytes and which recognize sialyl Le^X, may be involved in metastasis of human carcinomas. These metastasis-associated carbohydrate markers are present on a 60 kDa glycoprotein.[149] The influence of carbohydrate profiles on metastatic potential and prognosis of human carcinomas has been recently reviewed by the author.[152]

CONCLUDING REMARKS

In the three decades from when I began studying glycoproteins, this research area has undergone many major changes. Functions of cell surface carbohydrates have been mentioned in diverse biological systems,[153] carbohydrate structures have been elucidated for many glycoproteins, glycosyltransferases forming the chains have been cloned, and carbohydrate receptors called selections have been identified and cloned. Glycobiology is now the key word for this busy area. Since gene targeting has come to the center stage, the biological functions of the carbohydrate chains will be definitively determined. I wish to contribute to glycobiology also at this important stage, from the Department of Biochemistry, Nagoya University School of Medicine, to which I have recently moved.

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