The 6th Symposium
of Japan Consortium for Glycobiology and Glycotechnology

Integration of Glycoscience and Beyond
-Researches based on free ideas and innovative approaches-

December 3 - 4, 2008 at Tokyo Conference Center (Shinagawa)

Program December 3 (Wednesday), 2007

Opening Address
Yoshitaka Nagai (President, JCGG)

Invited Lectures
Chair : Naoyuki Taniguchi (RIKEN)

Nuclear protein transport and its significance to cell function
Yoshihiro Yoneda (Osaka University)

In eukaryotic cells, cell functions are maintained through the continuous traffic of various proteins between the cell nucleus and the cytoplasm. The nuclear import of proteins is mediated by specific amino acid sequences, nuclear localization signals (NLSs). The scheme of the molecular mechanism of nuclear protein import has been clearly elucidated. Typically, the nuclear import of the classical basic-type NLS-containing cargoes is initiated by the formation of a cargo-containing complex, which has targeting activity to the nuclear pore complex (NPC) components and is composed of two essential factors, importin α and importin β. Other key molecule is a Ras-related small GTPase Ran. Nuclear RanGTP dissociates the complex, thus releasing the cargo from the carrier molecules. On the other hand, it has recently been shown that these nuclear transport factors are also involved in diverse nuclear processes. We found that cellular stresses induce the nuclear accumulation of importin α. Our findings suggest that the nuclear accumulation of importin α can be involved in a common nuclear response to various stresses. Moreover, we found that the expression of importin α subtype is switched during neural differentiation and demonstrated that the subtype switching has a strong impact on cell fate determination.

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How Sweet it is: applying new glycomics technologies to identify stem cell and cancer markers

Michael Pierce (UGA Cancer Cent)

Glycosylation is a dynamic post-translational modification that displays precise, spatio-temporal changes during cell differentiation, as well as during the onco-genesis and progression of malignancies. Identification of these specific glycan changes on the surfaces of cells, therefore, is a logical target for the development of cell surface biomarkers. In order to identify these glycan changes and understand their regulation, our NCRR Center for Biomedical Glycomics has developed a toolbox of technologies that are being applied to the analysis of glycoproteins during embryonic stem cell differentiation, and during the formation and progression of invasive carcinomas. Several of these tools involve relative quantification of glycans released from glycans and include analyses by TIM (Total Ion Mapping), isotopic permethylation (QUIBL), and metabolic isotopic labeling (IDAWG).

These tools are coupled with a protocol to prepare total glycoproteins from cell/tissue sonicates prior to trypsinization and glycopeptide production, glycan release with concomitant labeling of the peptide sites of glycosylation, parent protein identification, and glycan relative quantitation. In addition, a comprehensive library of transcript amplimers (~800 each for human and mouse) allows the quantitation and comparison of cell/tissue glycotranscriptomes — those gene transcripts that are involved in the regulation of glycan expression and recognition[1]. Bioinformatic tools for the analysis and synthesis of all of these data have been developed. Results have identified well over 150 glycans in mouse embryonic stem cells, embryoid bodies, and retinoic acid-differentiated ES. Integration of glycan structural data and transcript expression data highlight likely regulation points of N-linked glycan biosynthesis, and several O-linked glycans change dramatically during differentiation. Significant changes in glycosaminoglycan expression have

also been noted, and stage-specific differentiation glyco-markers have been identified[2]. We have, in addition, applied some of the glycoproteomic tools to discovery of potential cancer biomarkers. The expression of a specific branched N-glycan, the β(1, 6) branch, is known to be turned-on in late breast adenoma and is continued to be expressed in carcinoma, due to expression of the glycosyl-transferase GnT-Va. A lectin, L-PHA, binds specifically this branch, and was used to develop a protocol to isolate L-PHA-binding glycoproteins from invasive breast carcinoma tissue. Matched, non-diseased breast tissue served as controls for each of 4 breast carcinomas. Nanospray ionization mass spectrometry (NSI-MS/ MS) was used to analyze tryptic digests of L-PHA bound glycopeptides to identify parent glycoproteins. Glycoproteins found to be common to non-diseased and carcinoma tissues were excluded. Comparative analysis of the glycopeptide data identified 34 proteins that were enriched by L-PHA fractionation in tumor tissue, relative to normal for at least 2 cases of ductal invasive breast carcinoma. Of these 34 L- PHA tumor enriched proteins, 12 were common to all 4 matched cases analyzed in detail. An antibody to one of these potential markers was obtained and used to determine that this glycoprotein with L-PHA-binding glycans was significant non-diseased tissues, thereby corroborating the results obtained by MS analysis[3]. These results indicate that lectin enrichment strategies targeting a particular glycan change can be an effective method of identifying potential biomarkers for breast carcinoma. These types of targeted glycoproteomics strategies are being applied to other stages and types of malignancies, including ovarian endometrioid carcinoma[4].

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Role of Glycans in Intracellular Events

Session 1 Quality Control and Metabolism of Glycocojugates
Chairs : Yasuyuki Igarashi (Hokkaido University)
Kenji Yamamoto (Kyoto University)

The biological function of glycosphingolipids: Lessons learnt from the

knockout mice of the glycosphingolipid metabolism related genes.

Junko Matsuda (Tokai University)

Nervous system is rich in glycosphingolipids (GSLs), of which expressions are specifically regulated by the synthesizing and degrading enzymes in tissues, cells, and during development. Sphingolipid activator proteins (saposins A, B, C, D) are small homologous glycoproteins which are required for *in vivo* degradation of some sphingolipids and also lipid antigen presentation. To understand the physiological and pathophysiological roles of GSLs in the nervous system, we have generated specific saposin A, D, and C knockout mice. Saposin A knockout mice showed the clinical, biochemical and pathological phenotype of late-onset, slowly progressive form of globoid cell leukodystrophy (Krabbe disease). Saposin D knockout mice showed progressive polyuria and impaired motor coordination tasks with the accumulation of α-hydroxyl fatty acid-ceramide. Neuropathologically, patterned cerebellar Purkinje cell degeneration was the most conspicuous finding. Saposin C knockout mice developed tremor and ataxia at around 5 months of age. Neuropathologically, cerebellar Purkinje cell degeneration, aberrant lamination of hippocampal mossy fibers, and the increase of ubiquitin-immunoreactive cells were observed. The phenotypes observed in these mouse models provide us insights into the regional role of GSLs in the nervous system. In addition, these mouse models would be a useful to develop new therapeutic strategies for the neurodegenerative diseases.

Protective effect of GnT-III on β-amyloid production in Alzheimer's disease

Hiroshi Manya (Tokyo Metropolitan Institute of Gerontology)

Alteration of glycoprotein glycans often changes various properties of the glycoprotein. We examined whether modifying the N-glycans on amyloid precursor protein (APP) affected β-amyloid (Aβ) production. To understand the significance of the N-glycans in the pathogenesis of early-onset familial Alzheimer's disease (AD), we produced normal and mutated APP (both the Swedish type and the London type) in transfected neuroblastoma cells and determined the N-glycan structures of these three recombinant APPs. In comparison with normal APP, both mutant APPs showed higher contents of bisecting N-acetylglucosamine and core-fucose residues. We then investigated the expression levels of N-acetylglucosaminyltransferase III (GnT-III) mRNAs in the brains of AD patients, because GnT-III is the glycosyltransferase responsible for the synthesis of bisecting N-acetylglucosamine residues; the mRNA level of GnT-III was increased in AD brain. In addition, the secretion of both Aβ40 and Aβ42 was significantly decreased when neuroblastoma cells were transfected with GnT-III. Taken together, these results suggest that bisecting N-acetylglucosamine residues on APP may protect against Aβ accumulation in the brains of AD patients.

Endoplasmic reticulum (ER) quality control of glycoproteins by ER lectins

Nobuko Hosokawa (Kyoto University)

Glycoproteins synthesized in the endoplasmic reticulum (ER) are regulated by the ER quality control mechanism, and terminally misfolded proteins are degraded by a mechanism known as ER-associated degradation (ERAD). Yos9p is a yeast lectin with a mannose 6-phospate receptor homology (MRH) domain, and enhances glycoprotein ERAD by making a complex with ER membrane-embedded ubiquitin ligase complex Hrd1p-Hrd3p and an ER chaperone Kar2p. Herein, we analyzed how mammalian MRH domain-containing lectins OS-9 and XTP3-B contribute to ER quality control of glycoproteins. We have elucidated that both human OS-9 and XTP3-B are ER resident proteins, and that these lectins make a large complex containing HRDl-SEL1L ubiquitin ligase complex and an ER chaperone BiP. Human XTP3-B has two transcriptional splice variants, and the long isoform is incorporated in this large quality control complex, while short transcriptional variant is not. This ER quality control scaffold regulates the ERAD of both glycoproteins and non-glycosylated proteins. We are now examining the lectin activity of human OS-9 and XTP3-B both *in vivo* and *in vitro*. We are also analyzing the functional difference between these MRH-domain containing lectins and EDEMs, which enhance glycoprotein ERAD.

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Catabolic pathway for free N-glycans

Tadashi Suzuki (RIKEN)

There is growing evidence that N-linked glycans play pivotal roles in various cellular events. It has long been shown that during the N-glycosylation of proteins, significant amounts of free oligosaccharides (free OSs) are generated in the lumen of the endoplasmic reticulum (ER) by an unclarified mechanism. More recently, free OSs were also found to be generated in the cytosol by deglycosylation of misfolded glycoproteins, which are subjected to destruction by a cellular system called "ER-associated degradation" (ERAD). We have been studying enzymes involved in the formation and degradation of free OSs generated in the cytosol, i.e. peptide:N-glycanase (PNGase), endo-β-N-acetylglucosaminidase (ENGase), and α-mannosidase (Man2C1)[1]. However, there are more players, that remain to be identified, involved in this process (Figure). Moreover, recent finding of large accumulation of free sialooligosaccharides in MKN7/MKN4S cells[2] indicated that there may also be a catabolic processing path for more processed, complex-type glycans which

contain various sugar components such as galactose or sialic acids. Recently, we have established an HPLC method to identify the structures of high mannose-type free glycans in mammalian cells[3], and we are currently analyzing the change of free OS structures in cells under various conditions

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JCGG Luncheon Seminar 1 (sponsored by Shimadz Co.)

New chemical methods for selective protein labeling

Tadashi Hamachi (Kyoto University)

Selective protein labeling is one of the essential methods for facilitating many aspects of chemical biology researches. My group has recently demonstrated that the ligand-directed affinity labeling coupled with the subsequent chemical reactions can provide powerful and unique methods for targeting and subsequently functionalizing a specific protein (lectins, enzyme, GPCR and others). Here, I would like to briefly introduce new strategies for chemical labeling of proteins in a target-selective manner based on the combination of molecular recognition and protein surface modification. These include (i) acyl transfer reaction directed by the ligand-tethered DMAP (4-dimethylaminopyridine), (ii) a nucleophilic substitution (SN2) reaction using the affinity-labeling strategy, (iii) a new peptide/probe pair displaying controlled reactivity (a reactive tag). By using these methods, we successfully introduced a variety of functional units such as fluorescent tag, biotin, F-probe, photo-crosslinker into target proteins such as lectin with site-selective manner, which are useful for bio-imaging, biochemical assay and protein engineering.

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Session 2 Protein Transport and Glycosylation
Chairs : Yoshifumi Jigami (AIST)
Taro Kinoshita (Osaka University)

Specificity of lectins involved in quality control of glycoproteins
Kazuo Yamamoto (The University of Tokyo)

Glycosylation is one of the most sophisticated posttranslational modifications of proteins. The vital biological functions of protein glycosylation are classified into two kinds. One is the role of glycosylation that functions outside of the cells, where a variety of sugar chains are participated in secretion and stability, cell-cell adhesion and signaling, innate immunity, embryogenesis, morphogenesis and underlying diseases. Another role is quality control of glycoproteins in the endoplasmic reticulum (ER), which is a current topic in glycobiology. Quality control of glycoproteins is categorized into three kinds of reactions; one is folding of newly synthesized glycoproteins, the second is ER-associated glycoprotein degradation of terminally misfolded or unassembled glycoproteins, and the last is sorting and transport of glycoproteins among several organelle. In every three processes, high mannose-type glycans on glycoproteins are used as tags to enter such reactions. These intracellular functions of glycosylation are conserved in yeast, plants, invertebrates and vertebrates. We focused on the intracellular lectins participated in these processes and discuss on the role of glycosylation based on the structural difference of N-glycans.

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Role of glycosylation in the production of recombinant glycoproteins through the secretory pathway of yeast cells
Kaoru Takegawa (Kyushu University)

The fission yeast *Schizosaccharomyces pombe* is the most intensely studied and well-characterized yeast species in terms of molecular genetics and cell biology,

along with budding yeast, *Saccharomyces cerevisiae*. *S. pombe* has not been used actively to make products such as wine, beer and bread industrially, unlike other yeast species. Although *S. pombe* belongs to the yeast group and is subject to manipulation similar to other yeasts, it has many features different from the budding yeast *S. cerevisiae*. For example, it is one of the best experimental models for the study of cell-cycle control, chromosomal structure, and signal transduction for sexual differentiation.

In recent years, *S. pombe* has attracted interest as a promising unicellular eukaryote model for studies on the biosynthetic pathway and oligosaccharide structures of glycoproteins. There are also prospective biotechnological applications for this microorganism, such as in heterologous eucaryotic gene expression, and *S. pombe* is considered to be an attractive host for secretory proteins. The glycoproteins of *S. pombe* contain a large amount of galactose in addition to mannose, indicating that *S. pombe* is equipped with mechanisms for galactosylation of glycoprotein, like mammalian cells. We will focus on recent works on the biosynthetic pathway and physiological role of oligosaccharide moiety in fission yeast

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Acidification of Golgi apparatus by GPHR, a novel anion channel, is critical for glycosylation

Yusuke Maeda (Osaka University)

Golgi apparatus is an indispensable place for glycosylation of proteins and lipids. Proteins involved in glycosylation, such as glycosyltransferases, are aligned at the appropriate cisternae of the Golgi, which may be preferable to add sugars in order. The luminal pH of the Golgi is regulated to be acidic, which is considered to be important for Golgi functions, although the issues as to how the Golgi luminal acidification is regulated are largely unknown. Here we report a mutant cell line, which exhibits abnormally high Golgi pH and accompanying three defects, namely, impaired glycosylations, the delayed transport of proteins and glycolipids, and disorganized structure of the Golgi. By means of expression cloning we identified the gene responsible, termed GPHR (Golgi pH Regulator), which encoded a Golgi-resident membrane protein. After reconstitution in planar lipid bilayers, GPHR functioned as a novel class of anion channel, indicating that GPHR may function in counter ion conductance to regulate Golgi acidification. We will discuss the relationship between glycosylation and acidification of Golgi.

Regulation mechanisms of glycosylation

Satoshi Goto (Mitsubishi KagakuInstitute of Life Science)

To seek novel mechanisms of regulation of glycosylation and directed secretion, we performed genome-wide screen using a Drosophila RNAi library. For this screen, we selected Chaoptin (Chp) as a model glycoprotein because Chp has multiple glycosylation sites with various types of N-glycans (Kanie Y et al.: submitted). This enables us to identify genes responsible for various glycosylation processes. The nature and extent of glycosylation were assayed by biochemical methods. We screened about 7000 genes in the library and identified 99 genes responsible for glycosylation (Y-H M et al.: in preparation).

We classified the identified genes by functional domain analysis of their products. There are five groups of genes that could be involved in transcription, mRNA regulation, membrane trafficking, signal transduction, or cytoskeletal regulation and some genes were classified as being of unknown function. Based on this information, we are now analyzing how glycosylation processes are influenced by the genes that we had identified as regulators. From these analyses, we will reveal a novel mechanism to regulate glycosylation *in vivo*.

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New Waves in Glycoscience I

Session 3　Functions of Unusual forms of Glycosylation

Chairs : Reiji Kannagi (Aichi Cancer Center)
Katsuko Yamashita (Tokyo Institute of Technology)

Roles of the monosaccharide O-fucose modification of Notch in Notch signaling

Kenji Matsuno (Tokyo University of Science)

Notch (N) is a transmembrane receptor with homology to epidermal growth factor (EGF)-like repeats and mediates cell-cell interactions necessary for many cell-fate decisions. These EGF-like repeats are O-fucosylated by the protein O-fucosyl-transferase 1 (O-fut1), which is essential for N signaling. However, roles of mono-saccharide O-fucose modification in Notch signaling became elusive, because it was

proposed that O-fucsyltransferase activity independent Notch-specific chaperon activity of O-fuc1, is per se essential for Notch signaling in Drosophila. In this study, we show that monomeric O-fucose modification of Notch is essential for Dl-Notch but not Ser-Notch signaling activity in the signal receiving cells of imaginal organs. In addition, defects of Notch signaling associated with the lack of O-fucose modification could be partly rescued by over expression of enzymatic activity-depleted O-fut1. These results suggest that monomeric O-fucose modification of Notch has a novel role for the signaling activity of Notch, which collaborates with enzymatic activity-independent functions of O-fut1.

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New functions of polysialic acid

Chihiro Sato (Nagoya University)

Polysialic acid (polySia) is a polymerized structure of sialic acid with a degree of polymerization (DP) ranging from 8 to 200. There are six proteins so far identified as polysialylated glycoproteins in vertebrates. Among these, NCAM modified with a polySia chain is well studied in the nervous system. PolySia is a negative regulator of neuronal cell - cell and cell-extracellular matrix interactions through its steric and repulsive hindrance due to its bulky polyanionic structure. On the other hand, whether polySia functions as a positive regulator through binding to specific ligand molecules have remained unknown. In the present study, we demonstrate that a brain-derived neuro trophic factor, BDN F, is a ligand of polySia and that its dimer directly binds to polySia to form a large complex under physiological conditions. While a little affected by the linkage and type of sialic acid components in the polySia the complex formation is highly dependent on the chain length. This is the first study clearly demonstrating the biological significance of the DP of polySia in eukayotes. The fine-tuning of DP or amounts of polySia might be involved in the unknown phenomena in some neural diseases through retaining BDNF by polySia.

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Biological functions of C-mannosylation in cell signaling

Yoshito Ihara (Wakayama Medical University)

C-Mannosylation is unique in that an alpha-mannose is directly bound to the indole C2 carbon atom of a Trp residue through a C-C bond to produce C-mannosyl Trp (C-Man-Trp), and it occurs at the first Trp in the Trp-X-X-Trp motif, which is found in the thrombospondin type 1 repeat (TSR) of proteins. There are a number of examples of C-mannosylated proteins, such as thrombospondin, complements, Interleukin-12, and the erythropoietin receptor. However, the biological function of C-mannosylation is not fully understood.

We previously reported that C-mannosylation is increased in specific tissues or cell types under hyperglycemic conditions, suggesting a pathological role for the increased C-mannosylation in the development of diabetic complications. We also found that synthesized C-mannosylated TSR-derived peptides specifically enhanced LPS-induced signaling in macrophage-like RAW264.7 cells. In addition, we searched specific binding partners for C-mannosylated peptides in the cells, and identified heat shock cognate protein 70 (Hsc70) as a binding protein for the C-mannosylated peptides, and the binding was further characterized. In this symposium, we would like to demonstrate our recent results concerning the effect of C-mannosylated peptides on Hsc70-induced signaling in the cells, and discuss about the possible functions of C-mannosylation in the cell signaling.

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Molecular mechanism of photoreceptor synapse formation by a novel extra cellular matrix protein pikachurin

Takahisa Furukawa (Osaka Bioscience Institute)

The retinal photoreceptor cells play a primal and central role in the phototransduction system. The understanding of the molecular mechanism of photoreceptor develop-ment is an interesting and important issue. We have been demonstrated that transcription factors, *Otx2* and *Crx* play critical roles in retinal photoreceptor development. We previously reported that the cell fate of both rod and cone photoreceptors is converted to that of amacrine-like cells in the *Otx2* conditional knockout (CKO) mouse line in developing photoreceptors. In order to identify genes which regulate photoreceptor development, we performed microarray analysis comparing the retinal gene expression profile between wild-type and *Otx2* CKO mouse retinas.

In this screen, we identified various Otx2 downstream factors possibly involved in photoreceptor development. Among these genes, we identify pikachurin, a novel extra-cellular matrix-like retinal protein. Pikachurin null mutant mice display improper apposition of the bipolar terminus to the ribbon synapse, resulting in alterations in synaptic signal transmission and visual function as detected by electroretinogram. We observed colocalization of pikachurin with dystrophin-glycoprotein complex (DGC) molecules and direct binding of pikachurin with α-dystroglycan, an extra-cellular component of DGC. Together, our results identify a novel dystroglycan ligand, pikachurin, and demonstrate its essential role in the precise interactions between the photoreceptor ribbon synapse and the bipolar terminus.

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Program December 4 (Thursday), 2008

New Waves in Glycoscience II

Session 5 Interface of Biology and Chemistry

Chairs : Makoto Kiso (Gifu University)
Ken Kitajima (Nagoya University)

Synthesis and biofunction of innate immunostimulating glycoconjugates from bacteria

Yukari Fujimoto (Osaka University)

Bacterial cell wall peptidoglycan (PGN) is a potent immunopotentiater and an adjuvant for antibody production. We have recently revealed that intracellular proteins, Nod1 and Nod2, are innate immune receptors of PGN, which recognize diaminopimelic acid (DAP) containing peptide components, and muramyldipeptide (MDP), respectively. Genetic studies have shown that polymorphisms in human NOD1 gene significantly associated with susceptibility to several diseases including allergic diseases such as asthma. However, the function and the immunostimulating mechanism of *Nod1* have not yet been well understood. We thus synthesized DAP-type PGN fragments including tracheal cytotoxin (TCT), based on a newly developed synthetic method of DAP. We hence achieved the first chemical synthesis of TCT and a repeating unit of DAP-type PGN fragment. We also prepared a library of N-acyl-γ-D-glutamyl-DAP (N-acyl-iE-DAP) to enhance the activity with increasing the membrane permeability, and succeeded to find that N-myristoyl-iE-DAP has several hundred times stronger activity than iE-DAP. The compound enabled the investigation of Nod1 function *in vivo*. We now analyze the function of the bacterial glycoconjugates including PGN for understanding the activation mechanism and also applications to regulate the immune system.

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Synthetic N-glycans for quantitative analysis of calreticulin

Ichiro Matsuo (Gumma University)

Among various functions of glycoprotein glycan chains, the roles of asparagine (N-) linked oligosaccharides in glycoprotein quality control system are attracting particular attention[1]. In order to gain precise understanding of the system, homogeneous and structurally defined oligosaccharides are highly desired. Recently, we established a highly stereoselective synthetic route for the series of endoplasmic reticulum compartment (ER) related N-linked oligosaccharides[2]. These oligosaccharides will be useful as molecular probes to clarify various issues related to glycoprotein quality control system, including protein-oligosaccharide interactions and substrate specificity of processing enzymes.

We report herein the following two topics; 1) the systematic syntheses of ER related high mannose type glycans ($Glc_{0-3}Man_{7-9}GlcNAc_2$) using convergent manner and 2) interaction analysis of synthetic oligosaccharides with calreticulin (CRT) using isothermal titration calorimetry. CRT is molecular chaperone that recognizes $Glc_1Man_9GlcNAc_2$ on glycoprotein. The interaction studies revealed that CRT binds mono-glucosylated oligosaccharides. However, the binding constants varied strongly depending on the sugar chain structure of non-reducing end (B or C arm). Based on these results, the structure requirement for the recognition of CRT will be discussed.

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Structural glycobiology for understanding of glycan functions

Yoshiki Yamaguchi (RIKEN)

It is becoming more and more important to elucidate the role of glycans from the structural aspects. In this situation, we have been developing a methodology for analyzing the structure of glycoconjugates by NMR spectroscopy. In particular, we have established a systematic method for stable isotope labeling of glycoproteins for NMR study. Based on these techniques, we are analyzing in detail the conformation of oligosaccharides and oligosaccharide-protein interactions.

Hydrogen bonds play key roles in stabilizing oligosaccharide conformations and mediating oligosaccharide-protein interactions. Hence, an identification method of hydrogen bonds is highly desirable for the conformational analyses of oligosaccharides. We report a novel NMR method to gain information on exchange ratios of the hydroxyl protons of oligosaccharides. We have successfully estimated the exchange rates of individual hydroxyl protons on the oligosaccharides.

We have also constructed a glycans conformation database using the structural data in the Protein Data Bank. Our analysis revealed some relationships between glycosidic torsion angles and inter-residue hydrogen bonds. The newly constructed database on glycan conformation becomes a powerful tool for analyzing the 3D structures of glycans and glycans-binding proteins.

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Analysis of N-Glycan Structure from a pmol Level of Glycoprotein

Kazuhiro Ikenaka (National Institute for Physiological Sciences)

Sugar chains play important roles in maintaining protein conformation and in cell to cell interaction. Thus determination of sugar chain structure attached to a protein is very important. In spite of recent progress in sugar chain structure determination by mass spectrometry, it still requires a large amount of sample to determine the structure of sialylated sugar chains or to determine isomeric structure. Structure determination of sugar chains using HPLC after pyridylamination has been used frequently by others and us, however, this method also requires pmol amount of samples. Recent improvement in the fluorescent detection system allows us to detect purified pyridylaminated sugar chains as little as 10 fmol, thus by increasing the recovery yield and effectively eliminating impurities it should be possible to determine the structure of N-linked sugar chains by the pyridylamination method from very small amount of samples. We have achieved this goal by utilizing two chromatography steps. In my talk I will present data showing that sugar chain structure of glycoproteins separated on two dimensional gel electrophoresis or from human biopsy samples can be determined effectively by our method.

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Introduction to data mining techniques applied to glycan structure analysis

Kiyoko Aoki-Kinoshita (Soka University)

Data mining techniques have recently applied to the analysis of glycan structures due to the increase of data in publicly available data resources. Such techniques are especially useful due to the noise that is often found in the data because of difficulties in fully characterizing glycan structures. Fortunately, data mining methods can overlook such noise to extract important information. One of these methods is based on probabilistic modeling, where profiles can be learned from the data. This method can extract complex patterns from glycan structures that may not necessarily be easily found with the naked eye. Another is the kernel method, which can classify data in multiple dimensions. This method takes as input a vector of

"features" which can be sub-structures or pathway information or localization information, for example. It then uses a "kernel trick" to compare feature vectors for different glycans. Thus glycans from different classes can then be trained to learn the features that most distinguish between the classes. Feature extraction methods based on trained kernels can then be used to predict possible glycan biomarkers.

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Role of Glycans in Extracellular Events
Session 6 Roles of Glycans on Extracellular Matrices
Chairs : Tasturo Irimura (University of Tokyo)
Koji Kimata (Aichi Medical University)

Mechanism of the neuritogenic activity of chondroitin sulfate chains
Hiroyuki Kitagawa (Kobe Pharmaceutical University)

Chondroitin sulfates are universally ubiquitous molecules distributed on cell surfaces and in extracellular matrices. Chondroitin sulfate is a linear, sulfated polysaccharide composed of repeating disaccharide units consisting of alternating glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) residues, and synthesized as a proteoglycan bound to specific Ser residues in the core protein. Compelling evidence has shown that chondroitin sulfate proteoglycans regulate diverse physiological phenomena such as cell adhesion, morphogenesis, cell division, and infections with viruses and bacteria. In particular, chondroitin sulfate plays critical roles in central nervous system development and regeneration, and individual modifications of chondrotin sulfate form a "sulfation code" that regulates growth factor signaling or neuronal growth. Although chondroitin sulfate-E polysaccharide (rich in the E disaccharide unit [GlcA-GalNAc(4,6-O-disulfate)], but not chondroitin sulfate-A (rich in the A disaccharide unit [GlcA-GalNAc(4-O-sulfate)] or -C (rich in the C disaccharide unit [GlcA-GalNAc(6-0-sulfate)] polysaccharide, is reported to have an inherent ability to promote neurite outgrowth toward

primary neurons, its molecular mechanism remains elusive. Here, I show the identification of a functional receptor for neuroregulatory chondroitin sulfate-E. Our data provide the first evidence that biological functions of chondroitin sulfate are exerted through the chondroitin sulfate receptor-mediated signaling pathway(s).

Biological roles of highly sulfated chondroitin sulfate in cancer
Shuhei Yamada (Hokkaido University)

Altered expression of cell surface chondroitin sulfate (CS) and dermatan sulfate (DS) in cancer cells has been demonstrated to play a key role in malignant transformation and tumor metastasis. In the present study, structural analysis of CS/DS from mouse Lewis lung carcinoma-derived two different metastatic clones revealed the upregulation of GlcA-GalNAc(4,6-O-disulfate) (E unit) in highly metastatic cells than in low metastatic cells. This key finding prompted us to study the role of CS-E-like structures in experimental lung metastasis. The metastasis of the highly metastatic cells to mouse lungs was effectively inhibited by enzymatic removal of cell surface CS/DS or by pre-administration of CS-E (rich in E-unit), in a dose-dependent manner. In addition, immunocytochemical analysis showed that highly metastatic cells rather than low metastatic cells expressed more strongly the CS-E epitope, which was specifically recognized by the phage display antibody GD3G7. More importantly, this antibody and a CS-E decasaccharide fraction, the minimal structure recognized by GD3G7, strongly inhibited the metastasis of the highly metastatic cells at least by interfering with the proliferation and invasion during the metastatic cascade. These results suggest that the E-unit-containing epitopes on cancer cells may be promising therapeutic targets.

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Function of proteoglycans in the extracellular matrix
Hideto Watanabe (Aichi Medical University)

Versican/PG-M is a large chondroitin sulfate proteoglycan (CSPG), whose core protein consists of two globular domains, G1 and G3, and two CS-attachment domains, CSα and Csβ. The N-terminal G1 domain comprises three subdomains, A, B and B'. The B-B' segment binds hyaluronan (HA), which is enhanced by the A subdomain. The C-terminal G3 domain interacts with other ECM molecules, including tenascins, fibulin-1, -2, and fibrillin-1. Versican is characterized by distinct expression patterns. Where constitutively expressed, it serves as a structural

macromolecule of the extracellular matrix (ECM). In embryonic tissues, it is transiently expressed and regulates cell behavior. We generated and analyzed knock-in mice *Cspg2*$^{\Delta3/\Delta3}$ whose versican lacks the A subdomain of G1. They survived up to a perinatal period exhibiting cardiac abnormalities including ventricular dilatation associated with decreased deposition of the mutant versican. We have found that the A subdomain is necessary for versican deposition in the ECM; 2) its decreased deposition impairs BMP-signaling during heart development; 3) decreased versican deposition impairs TGFβ-mediated smooth muscle cell differentiation in developing aorta; and 4) it plays an important role in collagen fiber formation. These observations indicate that versican in the ECM plays the central role in matrix assembly.

JCGG Luncheon Seminar 2 (Sponsored by Seikagaku Biobusiness Co.)

Roles of tumor cell surface heparan sulfate proteoglycans in metastasis
Kayoko Oguri (Nagoya Medical Center)

We have demonstrated that cell surface heparan sulfate proteoglycans (HSPG) of tumor cells show redundant and multiple receptor functions at various stages during metastasis, through cell proliferation, cell adhesion, cell migration or regulation of extracellular molecules. For example, in Lewis lung carcinoma cells, expression level of syndecan-2, transmembrane HSPG, showed inverse correlation to metastatic potential with causal relationship. This was rationally explained by our finding that syndecan-2 suppressed activation of MMP-2 which is essential to metastasis. In addition, we found that CPI-anchored HSPG, glypican, tethered MMP-9 to leading edges of highly invasive tumor cells, and we obtained the preliminary data that the positions and numbers of sulfate residues of heparan sulfate of tumor cell surface were involved in an initial arrest of the cells in blood vessels at the target organ. Based on these results, I'd like to discuss possible functions of HSPG of tumor cell surface to play in metastasis.

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Heparanase regulates immune cell function
Nobuaki Higashi (The University of Tokyo)

Heparanase was originally identified as an endo-β-glucuronidase which cleaves heparan sulfate proteoglycan, and the enzyme's pathological roles were extensively investigated in the field of cancer biology. In the present study, we examined

functional relevance of heparanase in the immune system, focusing on its intracellular distributions. Heparanase is involved in degradation of the basement membranes by monocytes. Capping of the molecule on the cell surface and subsequent redistribution at the leading edge are responsible for the degradation. A series of anti-mouse heparanase monoclonal antibodies were established and used to examine the intracellular distribution of the enzyme. Heparanase accumulation in the granules was detected in skin mast cells and in neutrophils in the inflamed microvasculature. Involvement of heparanase in heparin processing and modulation of granular function was examined. When heparanase gene or recombinant proteins were incorporated into MST mastcytoma cells, active heparanase was localized to the granules. In these modified MST cells, granular heparin was cleaved into a smaller molecular size and activity of granular enzymes seemed to be modulated by the low-molecular-weight heparin. Heparanase mediated cleavage of heparan sulfate and heparin is likely to regulate processes essential to the functions of cells in the immune system.

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Carbohydrate Ligands for Selectins and Cell Extravasation

Kenji Uchimura (National Center for Geriatrics and Geronrology)

Recirculation of lymphocytes and recruitment of neutrophils and macrophages to inflammatory lesions require adhesion and transmigration through the wall of vessels. A cascade of leukocyte diapedesis includes steps of rolling, activation, firm adhesion and transendothelial cell migration. Selectins are lectin-like adhesive receptors and are known to mediate the rolling by interacting their carbohydrate ligands. The interaction of L-selectin on lymphocytes with sialyl 6-sulfo Lewis X on high endothelial venules (HEVs) is crucial for lymphocyte homing to lymph nodes. We have previously shown elimination of sialyl 6-sulfo Lewis X in HEVs and considerably reduced lymphocyte homing and firm adhesion in mice lacking two N-acetylglucosamine-6-sulfotransferases (GlcNAc6ST-1 and GlcNAc6ST-2) (Uchimura et al.: Nat Immunol 2005). These results established an essential function for the sulfotransferases in lymphocyte extravasation. As a collaborative work, we have

also shown that P-selectin glycoprotein ligand 1 (PSG L-1), a ligand for P-, E-, L-selectins, interacted with chemokines CCL21 and CCL19 and facilitated efficient homing of T lymphocytes to lymph nodes (Veerman et al.: Nat Immunol 2007), In this presentation, recent advances in lymphocyte extravasation will also be discussed .

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Session 7 Role of Glycans on Cell Surface Glycoconjugates

Chairs : Koichi Furukawa (Nagoya University)
Shoko Nishihara (Soka University)

New dimensions of Siglec research

Takashi Angata (AIST)

Studies of glycan-recognition proteins complement those of glycan biosynthesis and structures, and will lead to better appreciation and application of glycan functions.

Siglecs are glycan-recognition proteins of vertebrates, and recognize sialylated glycans. As of 2003, Siglecs-1 through -12 have been found in humans, while our analysis of human genome sequence suggested more Siglecs were to be found. Thus we cloned two of these new Siglecs, Siglecs-14 and -15. We found both recognize sialylated glycans and associate with a signal adapter molecule DAP12. Over-expression of Siglec-14 in a monocytic cell line enhanced TNF-α production, and this effect was dependent on DAP12. Most known Siglecs associate with a protein tyrosine phosphatase SHP-1, and down-regulate cellular activity. Discovery of DAP12-associating Siglecs sheds light on a new aspect of Siglec biology.

N-terminal parts of Siglecs-14 and -5 show extensive sequence identity to each other, and most antibodies against Siglec-5 also recognize Siglec-14. We developed specific antibodies against each of them, and analyzed their expression profiles. Incidentally, we frequently found Siglec-14-deficient people, who are most likely homozygotes of a fusion gene SIGLEC14/5. Genetic polymorphisms of Siglecs and phenotypic variations thereof will be another subject of future studies.

Role of sulfatide in replication of influenza A virus

Takashi Suzuki (Shizuoka University)

Sulfatide is abundantly expressed in various mammalian organs, including the

intestine and trachea, in which influenza A viruses (IAVs) replicate. However, the function of sulfatide in IAV infection remains unknown. Sulfatide is synthesized by two transferases, ceramide galactosyltransferase (CGT) and cerebroside sulfotransferase (CST), and is degraded by arylsulfatase A (ASA). In this study, we demonstrated that sulfatide enhanced IAV replication through efficient translocation of the newly synthesized IAV nucleoprotein (NP) from the nucleus to the cytoplasm, by using genetically produced cells in which sulfatide expression was down-regulated by RNA interference against CSTmRNA or overexpression of ASA gene and in which sulfatide express was up-regulated by overexpression of both CST and CGT genes. Treatment of IAV-infected cells with an anti-sulfatide monoclonal antibody (MAb) or an anti-hemagglutinin (HA) MAb, which blocks the binding of IAV and sulfatide, resulted in significant reduction in IAV replication and accumulation of the viral NP in the nucleus. Furthermore, anti-sulfatide MAb protected mice against lethal challenge with pathogenic influenza A/WSN/33 (H1N1) virus. These results indicate that association of sulfatide with HA delivered to the cell surface induces translocation of the newly synthesized IAV ribonucleoprotein complexes from the nucleus to cytoplasm. Our findings provide new insights into IAV replication and suggest new therapeutic strategies.

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Crucial role of O-glycosylation on herpes simplex virus infection

Hisashi Arase (Osaka University)

Paired immunoglobulin-like type 2 receptor (PILR)-α is one of immune inhibitory receptors expressed on various cell populations. PILRα recognizes CD99 expressed on activated T cells. Interestingly, specific O-glycans on CD99 are required for the recognition by PILRα. On the other hand, recently, we found that glycoprotein B (gB) of herpes simplex virus (HSV)-I associates with PILRα. Furthermore, association of gB with PILRα plays an important role in HSV-1 infection by mediating membrane fusion. Here, we found that O-glycosylation at two threonine residues on gB is essential to associate with PILRα. gB lacking O-glycosylation did not mediate membrane fusion and viral entry against PILRα expressing cells. Furthermore, O-

glycosylation of gB was required for HSV-1 infection not only of PILRα expressing cells but also of PILRα negative HSV-1 susceptible cell lines such as Vero cells. Our results demonstrate that O-glycans on gB plays an important role in HSV-1 infection .

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Molecular recognition and signaling mechanisms for bacterial peptidoglycan and fungal beta-1,3-gucan in insects

Lee Bok Luel (Pusan National University)

Innate immunity is a crucial host defense mechanism against microbial infection in all animals. The ability of a host to distinguish between self and non-self remains a central hallmark of innate immunity. Pathogenic microbes possess distinct pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides of Gram-negative bacteria, peptidoglycans (PG) of Gram-positive bacteria, and β-1,3-glucan of fungi[2]. The Drosophila Toll signaling pathway is responsible for defense against Gram-positive bacteria and fungi, while the immune deficiency (Imd) pathway is activated primarily in defense against Gram-negative bacteria. Both of these pathways lead to the expression of anti-microbial peptides (AMPs) via NF-KB-like transcription factors.

Recognition of these PAMPs is achieved by a group of germ-line encoded receptors and soluble proteins. In insects, bacterial lysine (Lys)-type PGs are recognized by the PG recognition protein-SA (PGRP-SA) and Gram-negative binding protein 1 (GNBPI). These proteins are believed to mediate the activation of a serine protease (SP) cascade and ultimately, the cleavage of Spatzle (Spz). Cleaved Spz serves as a ligand for the cell membrane receptor Toll and induces the production of AMPs. Drosophila GNBP3 (GNBP3), however, is known to be required for the detection of fungal cell wall components in the Toll signaling pathway. These recognition signals against bacteria and fungi are amplified in hemolymph (insect blood) by a proteolytic SP cascade similar to the vertebrate complement system. However, details of the activation mechanism of the extra-cellular SP cascade in the Toll pathway have not been clearly resolved. The amplification of these recognition

signals represents an efficient host defense strategy in insects, which are devoid of an acquired immune system

Here, we demonstrated that the biochemical ordered functions of the extracellular SP cascade in the Toll pathway were determined *in vitro*, suggesting that the activation of a three step-proteolytic cascade is necessary and sufficient for PG recognition signaling. The processed Spätzle by this cascade induced antibacterial activity *in vivo*. These results demonstrate that the three-step proteolytic cascade linking the PG recognition complex and Sptäzle processing is essential for the PG-dependent Toll signaling pathway[1,2)].

The activation of the prophenoloxidase (proPO) cascade leading to melanization is a major innate immune reaction in invertebrates triggered by LPS, PG and β-1,3-glucan and is vital for the survival and development of insects. The melanization reaction induced by activated phenoloxidase in arthropods must be tightly controlled because excessive formation of quinones and excessive systemic melanization damage to the hosts. However, the molecular mechanism by which phenoloxidase-induced melanin synthesis is regulated *in vivo* is largely unknown. It is known that the Sptäzle processing enzyme is a key enzyme in the production of cleaved Sptäzle from pro Sptäzle in the *Drosophila* Toll pathway. Here, we provide biochemical evidence that the *Tenebrio molitor* Sptäzle processing enzyme converts both the 79 kDa *Tenebrio* prophenoloxidase and *Tenebrio* clip-domain serine protease homologue 1 zymogen to an active melanization complex. This complex, consisting of 76 kDa *Tenebrio* phenoloxidase and an active form of *Tenebrio* clip-domain serine protease homologue 1, efficiently produces melanin on the surface of bacteria and this activity has a strong bactericidal effect. Interestingly, we found the phenoloxidase-induced melanization reaction to be tightly regulated by *Tenebrio* pro-phenoloxidase, which functions as a competitive inhibitor of melanization complex formation. These results demonstrate that the *Tenebrio* Toll pathway and the melanization reaction share a common serine protease for the regulation of these two major innate immune responses[3)].

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N-glycan-based regulation of integrin- mediated cell adhesion

Gu Jianguo (Tohoku Pharmaceutical University)

Integrin is a major carrier of N-glycans. Among the integrin superfamily, α5β1 is one of the best characterized integrins. It has been reported that the presence of N-glycan on integrin α5β1 is required for αβ heterodimer formation and proper integrin-matrix interactions. An alteration of expression of N-glycan on α5β1 integrin contributes to adhesive properties of tumor cells and tumor formation. The expression of β1,6 branched N-glycans, which are the products of N-acetylglucosaminyltransferase V (GnT-V), is usually correlated with cancer metastasis. In contrast, the overexpression of GnT-III down-regulated integrin-mediated cell spreading and migration, thereby contributing to the suppression of cancer metastasis. Therefore, GnT-III has been proposed as an antagonistic of GnT-V. Because integrin α5β1 contains 26 potential N-linked glycosylation sites, (14 in the α subunit and 12 in the β subunit), the determination of those crucial N-glycosylation sites for its biological functions is quite important for an understanding of the underlying mechanism. Recently, we determined that 6 of these glycosylation sites are essential to dimmer formation and biological function. Based on these observations, we postulate that those the N-glycosylation on integrin may participate in the supramolecular complex formation on the cell surface, which controls intracellular signal transduction.

N-Acetylglucosaminyltransferase-4a sustains glucose sensor function in pancreatic β cells in normal physiological contexts.

Kazuaki Ohtsubo (University of Occupational and Environmental Health)

We have previously reported that the *Mgat4a*-encoded N-acetylglucosaminyltransferase 4a (GnT-4a) is required for the production of an N-glycan structure that acts as a ligand for endogenous lectins that maintain cell surface residency of glucose transporter-2 (GLUT2). GnT-4a deficiency caused by genetic disruption of Mgat4a or high-fat diet administration to mice attenuated GLUT2 glycosylation and its glycan-mediated lectin bindings that diminished cell surface GLUT2. This thereby abolished glucose-stimulated insulin secretion that evokes hyperglycemia, elevation of free fatty acids and liver gluconeogenic enzymes, and hepatic steatosis. These phenotypic features encompass metabolic disorder diagnostic type-2 diabetes. These findings revealed that GnT-4a expression and GLUT2 glycosylation are under dietary and genetic control that maintains insulin secretion in normal physiological contexts.

Closing Remarks