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Establishment of an Analytical Method for Sialyl Glycoprotein Extraction from the Experimental Hormone-Induced Estrous Cycle of the Mouse Ovary

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Abstract

Ovaries are often found in pairs as part of the vertebrate female reproductive system that maintains immature egg cells until they become mature with ovulation. The ovary is also an internal secretion organ for both estrogen and progesterone. Estrogen is responsible for the appearance of secondary sex characteristics of females at puberty and for the maturation and maintenance of the reproductive organs. Progesterone functions mainly to regulate the condition of the endometrium, preparing it to accept a fertilized egg. On the other hand, glycans are a main component of the animal cell surface and show structural changes during early development and cell differentiation. In the present study, to clarify the relation between the changes in glycan structures and ovarian functions such as ovulation, we used a system in which ovulation was induced by sex hormone treatment. As a result, it was found that glycoproteins including terminal sialic acids, especially N-glycolyl neuraminic acid-type glycans, increased during development of the ovary. Furthermore, to find specific glycoproteins with the N-glycolyl neuraminic acid-type glycan that were induced by hormone treatment, we established a method for the purification of glycoproteins, using sialic acid followed by detection using mass spectrometry. Applying the modified protocol, transferrin could be detected as the protein that changed the amount of glycans in the developmental cycle of the ovary. Thus, this protocol will be a useful tool for the detection of glycoproteins with sialic acid residues. It may also be a valuable method for use with other organs.

Keywords: Ovary; Sialyltransferase; Transferrin; MALDI-TOF-MS; LC-MS

Abbreviations: ACN: Acetonitrile; Ac_2O: Acetic Anhydride; AcOH: Acetic Acid; AcONa: Sodium Acetate; aoWR: ((aminooxy) acetyl)Tryptophanylarginine Methyl Ester; CBB: Coomassie Brilliant Blue; DHB: 2,5-Dihydroxybenzoic Acid; DPD: Dimethyl Pimelimidate Dihydrochloride; DTT: Dithiothreitol; FSH: Follicle-Stimulating Hormone; Fuc: L-Fucose; Gal: D-Galactose; Glc: Glucose; GlcNAc: N-acetyl-D-glucosamine; GnRH: Gonadotropin Releasing Hormone; hCG: Human Chorionic Gonadotrophin; HCl: Hydrochloric Acid; Hex: Hexose; HILIC: Hydrophilic Interaction Chromatography; HRP: Horseradish Peroxidase; IAA: Iodoacetamide; LH: Luteinizing Hormone; LC-MS: Liquid Chromatography Mass Spectrometry; MALDI-TOF-MS: Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry; Man: D-Mannose; MeOH: Methanol; MTT: 3-Methyl-1-P-Tolyltriazene; NaCl: Sodium Chloride; $NaIO_4$: Sodium Periodate; NaN_3: Sodium Azide; NaOH: Sodium Hydroxide; $Na_2S_2O_5$: Sodium Disulfite; NeuAc: N-Acetylneuraminic Acid; NeuGc: N-Glycolylneuraminic Acid; PBS: Phosphate Buffered Saline; PBST: Phosphate Buffered Saline with Tween 20; Purpald®: 4-Amino-3-Hydrazino-5-Mercapto-1,2,4-Triazole; PMSG: Pregnant Mare Serum Gonadotropin; PNGaseF: Peptide-N-Glycosidase F; PVDF: Polyvinylidene Difluoride; SSA: Sambucus Sieboldiana Agglutinin; TBS: Tris-Buffered Saline; TBST: Tris-Buffered Saline with Tween 20; TCEP: Tris(2-Carboxyethyl)Phosphine; TFA: Trifluoro Acetic Acid

Introduction

In recent years, glycans linked to proteins have attracted attention as the third chain of biological molecules following nucleic acids and proteins [1]. The glycans may play important roles in cell differentiation, proliferation, cancer metastasis, immunoreaction, intracellular transport and adhesive interaction between cells [2-4]. Nucleic acids and proteins have many features that are common beyond species. On the other hand, the glycans show many differences between species, organs, tissues and cells. Typically, glycans are linked to serine or threonine residues (O-linked glycosylation) or to asparagine residues (N-linked glycosylation) [5]. N-linked glycosylation sites generally fall into the N-X-S/T sequence motif in which X denotes any amino acid except proline [6]. Changes in the extent of glycosylation and the carbohydrate structures of proteins on the cell surface have been shown to correlate with cancer and other disease states such as metastasis and malignancy [7-9]. Therefore it is thought that the study of glycans helps to elucidate biodiversity and complicated life phenomena [10,11]. The female-specific phenomenon called the estrous cycle is controlled closely by sex hormones (estrogen, progesterone, follicle-stimulating hormone and luteotrophic hormone). The estrous cycle comprises the recurring physiologic changes that are induced by reproductive hormones in most mammalian placental females [12,13]. Estrous cycles start after puberty in sexually mature females and are interrupted by pregnancies temporarily. The follicle is a functional unit of ovulation. The follicles exist in the ovary as lumps of spherical cells. The estrous cycle is roughly distributed between three phases: a follicular period, an ovulation period and a luteinization period. There are many variations among animals in terms of their estrous cycles. Some animal species such as deer and foxes may undergo cycles only once a year during

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mating season, whereas other animal species such as horses and sheep may undergo a succession of cycles if they do not become pregnant. Mice, cows and swine may undergo cycles all year round [14]. Mice typically have rapid cycle times of 4 to 5 days. Although they ovulate spontaneously, they do not develop a fully functioning corpus luteum unless they receive coital stimulation [15]. There are many studies about proteins controlling the estrous cycle [15,16]. However, there are few studies on the estrous cycle and glycans. In addition, little is known about the glycans that show changes specific for the estrous cycle, or the proteins that carry them. Therefore, the functional changes of glycans that are specific to the estrous cycle and the proteins are not yet known. In this study, we demonstrated that structural changes of glycans occur during the estrous cycle in the whole ovary. We found that the amount of high mannose-type glycans increased from the early follicular phase. They maintained the amount until the luteinization phase. The expression of some N-acetyl neuraminic acid (NeuAc)-type glycans increased slowly from the early follicular phase and reached the maximum in the luteinization phase. The amount of N-glycolyl neuraminic acid (NeuGc)-type glycans, sialic acid containing biantenna carbohydrate chains, increased in the ovulation phase. Some of them decreased in amount in the luteinization phase. Both NeuAc-type glycans and NeuGc-type glycans are called sialic acid. To discover the glycoproteins that show the changes of the amount of sialic acid residue(s) during the estrous cycle, a new analytical method was established. This novel, modified protocol using internal organ samples consisted of the selective oxidation of the sialic acid residues, selective chemical biotin modification, capture of the proteins that used affinity to the biotin-avidin and mass spectrometry for detection of proteins. Using by this method, we detected transferrin, on which the amounts of sialic acid residues such as NeuGc changed during the estrous cycle.

Materials and Methods

Animals

All mice (ICR) were purchased from Charles Liver Laboratories Japan, Inc. (Yokohama, Japan) and maintained in light-controlled (0800 to 2000), air-conditioned rooms (22°C, humidity 50-60%). They were fed a standard mouse diet and water ad libitum. All animal procedures conformed to the Guidelines of Hokkaido University for the Care and Use of Laboratory Animals published by the Animal Research Committee, Hokkaido University.

Preparation of ovarian tissues

All ovaries were obtained from three immature mice at 3 weeks of age in one experiment. The ovaries from each animal were collected at 0 (nontreated as control), 24 or 48 h after an intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG) (ASKA Pharmaceutical Co., Ltd. Tokyo, Japan). In addition, ovaries were collected from animals with combined treatment at 48 h after an intraperitoneal injection of 5 IU of PMSG followed by 5 IU of human chorionic gonadtropin (hCG) (ASKA Pharmaceutical Co., Ltd. Tokyo, Japan) 10 or 20h later.

Analysis of N-glycan in mouse ovary by glycoblotting and MALDI-TOF MS

Six mouse ovaries were homogenized in 500 µl of TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) at 4°C. The lysates were centrifuged at 20,400 ×g for 30 min at 4°C and the supernatant was collected. The extracted protein (100 µg) quantified by Bradford assay (BIO-RAD) was reduced with Tris (2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich Co. MO, USA) to a final concentration of 5 mM at 60°C for 30 min, followed by alkylation with iodoacetamide (Wako, Osaka, Japan) to a final concentration of 10 mM at room temperature for 1 h in the dark. The mixture was digested by 400 U of trypsin (Wako, Osaka, Japan) at 37°C for 2 h in 20 µl of 20 mM ammonium bicarbonate buffer (pH 7.8). After heat-inactivation of trypsin at 90°C for 10 min, the sample was lyophilized. Then N-glycan was released by 2 U of recombinant PNGase F (Roche Diagnostics, Penzberg, Germany) at 37°C for 16 h in 20 µl of 100 mM ammonium bicarbonate buffer. The released N-glycan was analyzed according to the previously reported glycoblotting technique [17-19] and MALDI-TOF MS. Briefly, N-glycan released from the mouse ovary was spiked with A2 amide glycan (an internal standard, 16.8 pmol) and enriched using BlotGlyco H beads (Sumitomo Bakelite Co., Tokyo, Japan). The blotted glycan was recovered upon adding the aminooxy-functionalized labeling reagent, aoWR. The excess reagent (aoWR) was removed with a HILIC microelution plate (Waters, Milford, MA, USA). The recovered aoWR derivatized glycan (20 µl) was concentrated using a centrifugal concentrator, then the sample was dissolved in 1.0 µl of 2,5-dihydroxybenzoic acid (DHB; 10 mg/ml in 30% acetonitrile; Bruker Daltonics, Bremen, Germany) and subjected to MALDI-TOF MS analysis using an ultraflex II time-of-flight mass spectrometer (Bruker Daltonics Inc., MA, USA) controlled by the FlexControl 3.0 software package. MS spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV and a pulsed ion extraction of duration of 160 ns in the positive ion mode. The spectra were the results of signal averaging of 1000 laser shots. All peaks were picked using FlexAnalysis 3.0 with the SNAP algorithm that fits isotopic patterns to the matching experimental data.

Analytical procedure and SDS-PAGE analysis

Six mouse ovaries were homogenized in 500 µl of 100 mM sodium acetate buffer (pH 4.5) with detergent and a protease inhibitors (20 mM EDTA, 1% Triton X-100, 10 µg/ml approtinin, 10 µg/ml leupeptin, 1mM PMSF) at 4°C. The lysates were centrifuged and quantified as in the N-glycan analysis method. The extracted protein (100 µg) was oxidized using sodium periodate (Wako, Osaka, Japan) to a final concentration of 5 mM at 4°C for 5 min, followed by reduction with sodium disulfite (Wako, Osaka, Japan) to a final concentration of 6 mM at 4°C for 10 min. Following this, the lysates were biotinylated with 150 nmol of aminooxybiotin (Biotium Inc, CA, USA) at 4°C 16 h. Then the mixture was ultrafiltered using YM-10 (Nihon Millipore K.K., Tokyo, Japan) and additional aminooxybiotin was removed. The concentrated liquid (50 µl) was added to 100 µg of Dynabeads® MyOneTM streptavidin C1 (Life Technologies Japan Ltd., Tokyo, Japan) and the biotinylated sialoglycoproteins were incubated with streptavidin beads at 4°C under rotary agitation for 30 min. Nonspecific glycoproteins were washed out with TBST and the biotinylated sialoglycoprotein was released by treatment with TBST and loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 8% 2-mercaptoethanol) at 90°C for 5 min. Equivalent amounts of protein in each fraction were separated by 12% SDS-PAGE gel and the gel was stained according to the Silver Stain MS kit procedure (Wako Pure Chemical Industries, Osaka, Japan). A schematic showing the modified protocol for the analytical procedure is shown in Supplementary Figure 1.

Protein identification by LC/ESI-MS

The protein fraction purified by the modified protocol was separated on 12% SDS-PAGE gel and stained using a Silver Stain MS kit (Wako Pure Chemical Industries, Osaka, Japan). The gel pieces were excised and digested with trypsin as described elsewhere (http://donatello.ucsf.edu/ingel.html). The extracted digest was concentrated to 10 µl.

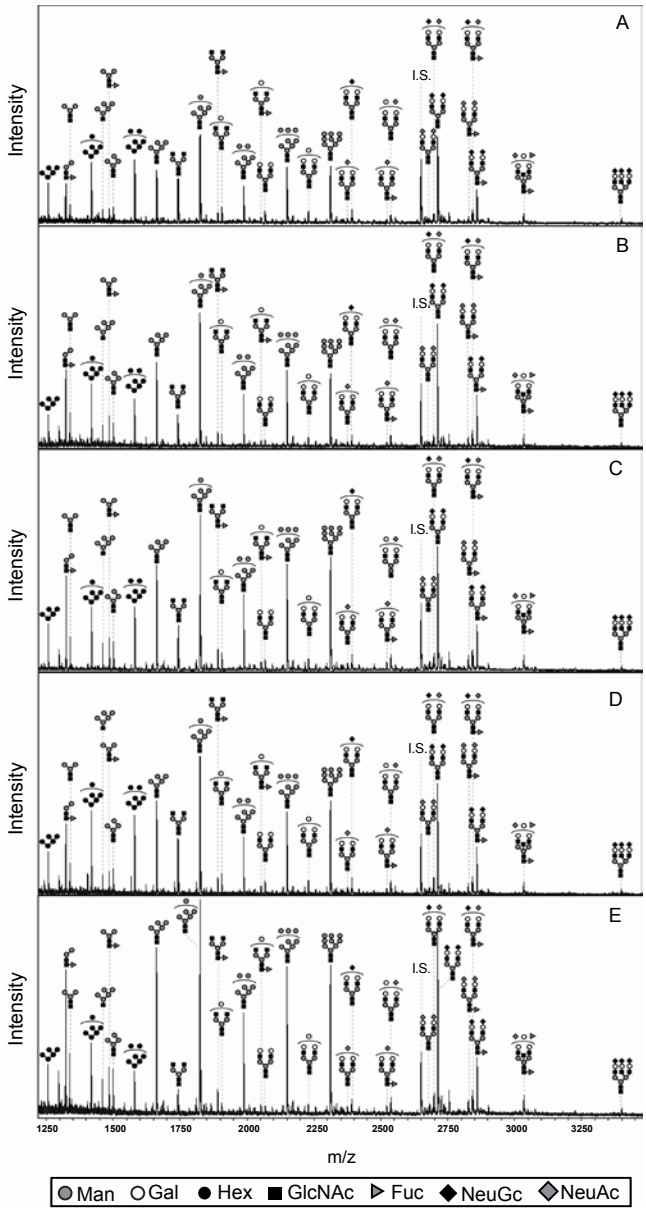

Figure 1: Structural characterization of N-glycans during the mouse estrous cycle, immature (A), early follicular phase (B), middle follicular phase (C), ovulation phase (D), and luteinization phase (E). The intensities of the N-glycan ms spectra were compared with A2 amide as an artificial glycan (as internal standard; I.S.).

The obtained tryptic peptides were analyzed using an UltiMate 3000 nanoHPLC system (Dionex Co., CA, USA) connected to a 4000QTRAP hybrid triple quadrupole linear ion trap mass spectrometer equipped with a NanoSpray source (Applied Biosystems/MDS Sciex, Toronto, Canada). The HPLC and MS systems were both controlled using Analyst software version 1.4.1. The samples (1 µl) were directly loaded on a C18 reversed-phase column (MonoCap for fast flow, 0.05 mm I.D. × 250 mm, GL Sciences Inc., Tokyo, Japan) in a column oven at 40°C and eluted with an acetonitrile gradient (0-40% acetonitrile in 40 min, followed by a 5 min wash with 70% acetonitrile) containing 0.1% formic acid at a flow rate of 500 nl/min. Mass spectra were recorded in the information-dependent acquisition (IDA) mode with an enhanced MS scan (EMS), enhanced resolution scan (ER) and enhanced product ion scan (EPI) for the two most intense ions. The total cycle time for this method was 2.4 sec. The ESI settings were: interface heater temperature, 150°C; ion source and curtain gas, 18 and 10, respectively; capillary voltage, +3000 kV; declustering potential, 70. The obtained MS/MS spectra were searched for in the mass spectrometry protein sequence database (MSDB) using the Mascot search engine (Matrix Science, London, UK) to assign peptide sequences.

Coupling anti-mouse transferrin antibody to Protein G Sepharose

Thirty microliters of Protein G Sepharose 4 Fast Flow (GE Healthcare, Tokyo, Japan) was transferred into a spin filter column and washed with 0.1 M borate buffer (pH 9.5) three times and then added to 500 µg of anti-mouse transferrin (Rockland Immunochemicals Inc., PA, USA). The antibody solution was gently rotated at 4°C 16 h. Then beads were washed with 500 µl of 0.1 M borate buffer (pH 9.5) three times. Following this they were crosslinked with dimethyl pimelimidate dihydrochloride (Sigma-Aldrich Co. MO, USA) to a final concentration of 20 mM. Then the antibody solution was gently rotated at room temperature for 1 h. Next, the beads were washed with 500 µl of 200 mM ethanolamine (pH 8.0) three times. One milliliter of 200 mM ethanolamine (pH 8.0) was added to the beads and incubated at room temperature for 2 h. The beads were washed with 500 µl of 0.2 M NaCl/50 mM Tris-HCl, pH 8.0, 0.1 M Gly-HCl, pH 3.0 and 0.2 M NaCl/50 mM Tris-HCl, pH 8.0, three times.

Collection of mouse transferrin by immunoprecipitation

Two hundred microliters of the homogenized ovarian solution (0.25 mg/ml) was added to Protein G Sepharose coupling anti-mouse transferrin antibody. The lysate-bead mixture was incubated at 4°C for 16 h under rotary agitation. After the incubation, the tubes were centrifuged and the supernatant was removed. The beads were washed with 500 µl of TBST buffer three times and eluted using 200 µl of 0.1 M Gly-HCl, pH 3.0, five times. Each transferrin eluate was neutralized with 10 µl of 1 M ammonium bicarbonate.

Western blot and lectin blot analyses

Equivalent amounts of protein (7.5 µg) in each fraction were separated by 12% SDS-PAGE gel and transferred to an immobilon-PVDF membrane (Nihon Millipore K.K., Tokyo, Japan). The blots were probed with an anti-transferrin polyclonal primary antibody (Stressgen, Victoria, BC, Canada) followed by incubation with an anti-rabbit polyclonal secondary antibody conjugated to alkaline phosphatase (Chemicon International Inc., CA, USA) and detected by chemiluminescence (CDP-Star Detection Reagent, Roche Applied Science, Inc., Penzberg, Germany). The mouse transferrin (400 ng) collected by anti-mouse transferrin coupling beads was dotted on an activated immobilon-PVDF membrane with a dot blot apparatus (Bio-Rad Laboratories, Inc., CA, USA). The membrane was then stood at room temperature for 15 min. Each spot was washed using 50 µl of PBS two times and nonspecific binding was blocked by soaking in 10 mM Tris-HCl, pH 7.4/0.5 M NaCl/0.05% Tween 20 at room temperature for 10 min three times. The blots were probed with primary biotin conjugated to Sambucus sieboldiana agglutinin (J-OIL MILLS INC, Tokyo, Japan) and secondary avidin conjugated to horseradish peroxidase (Bio-Rad Laboratories, Inc., CA, USA) and detected by chemiluminescence (ECL Plus, GE Healthcare, Tokyo, Japan).

Statistical Analysis

Results are presented as means ± SD of three independent experiments. Results from experimental groups and controls were compared by Student's t test and were considered significant if two-tailed P values were <0.05.

Results

Structural analysis of hormone-induced ovary N-glycans

ICR female mice at 3 weeks of age were used for ovary maturation by two kinds of gonadtropins, pregnant mare serum gonadtropin (PMSG) and human chorionic gonadtropin (hCG). Hormone induction at 3 weeks of age can induce the first estrous cycle. After the first estrous cycle, the ovary maintains the cycle and there are follicles of the different periods in the same ovary. Generally, to induce ovulation, 5 IU of two gonadtropins is administrated at 48 hr intervals by intraperitoneal injection. The early follicular phase is induced 24 hrs later by PMSG injection. In a similar manner, the middle follicular phase is induced 48 hrs later by PMSG injection. The ovulation phase is induced 8 hrs later by hCG injection and the luteinization phase 24 hrs thereafter by hCG injection. Enriched ovarian carbohydrate chains were analyzed by MALDI-TOF MS and identified (Figure 1). There was no marked difference in the identified glycans in the various estrous phases. On the other hand, as a result of relative quantitative analysis of each glycan compared with A2 amide, which is not observed in the living body, a difference was observed (Figure 2 A-D). Glycans containing 6 hexoses tended to increase in the middle follicular phase (Figure 2A) and the amount of high-mannose-type glycans started to increase from the early follicular phase. Maximum intensity was observed in the lutenization phase (Figure 2B). We observed 4 kinds of structures containing NeuAc-type glycans, whose expression increased slowly from the early follicular phase. Some of them reached their maximum expression levels in the lutenization phase (Figure 2C). For NeuGc-type glycans, structures consisting of biantenna-type glycan increased significantly in the ovulation phase. Two structures, one with fucose and the other with tri-antenna-type glycan, had low level expression (Figure 2D). In addition, a specific protein carrying biantenna-type glycan with NeuGc-type glycans was observed in this phase.

Detection of glycoproteins of ovarian origin by a modified method

To detect specific glycoproteins containing NeuGc-type glycan in the ovulation phase, a modified protocol was established. This was similar to the method using serum and body fluid such as urine [20]. In this study, we optimized it for organs and detected the increased NeuGc-type glycan glycoproteins in the ovulation phase. This was established as a protocol for the detection of glycoproteins without enzymatic digestion into peptides. The method consisted of the following three steps (Supplementary Figure 1): (1) selective oxidation of sialoglycoproteins, (2) modification of sialoglycoproteins by aminooxybiotin and (3) reaction with magnetic beads carrying avidin. Prior to using hormone-induced samples from the ovary, the protocol was tested using authentic samples that had and did not have any sialic acid in their structure: fetuin (sialic acid +) and RNaseB (sialic acid -), respectively. In a previous study, both sialic acid and mannose were oxidized under neutral conditions [21]. However, with the modified protocol, sialic acid was oxidized selectively under acidic conditions. As a result of these tests, we decided that hydrogen ion activity in a solution at pH 4.5, with an $NaIO_4$ concentration of 5 mM and a reaction time of 5 minutes was optimal for selective oxidation of sialic acids (Supplementary Figure 2A and 2B). Following this, we decided that a 5

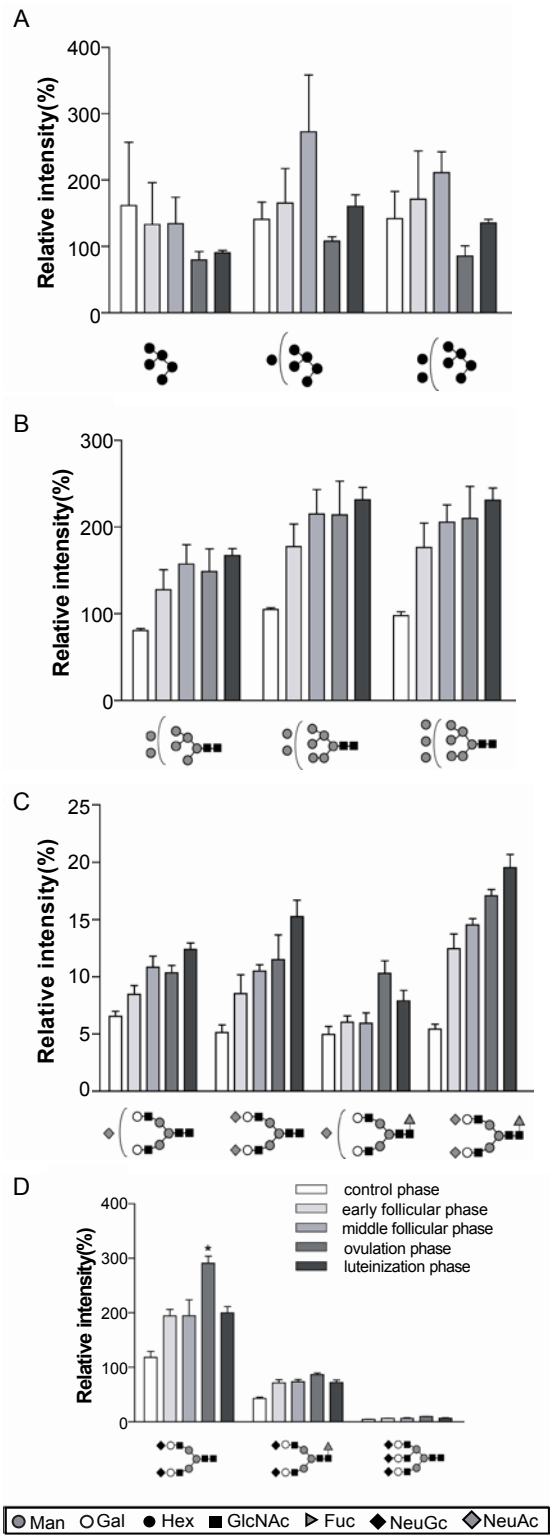

Figure 2: Relative quantitative analysis of each glycan. Hexose-type glycan (A), High-mannose-type glycan (B), NeuAc-type glycan (C), NeuAc-type glycan (D). High-mannose-type glycan and NeuAc-type glycan displayed progressive enhancement through the estrous cycle. However, expression of sialylated NeuGc-type glycan was remarkably promoted only during the ovulation phase. Error bars represent SD. Significance relative to ovulation phase (vs. early follicular phase, middle follicular phase, and luteinization follicular phase; *P< 0.05, vs. control phase; *P<0.01).

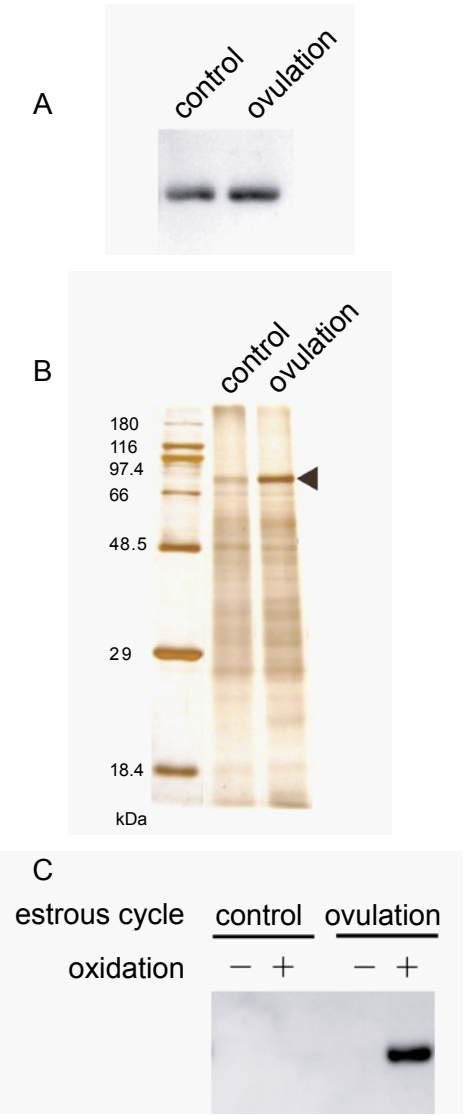

Figure 3: Detection of transferrin in control and ovulation phase. (A) Each lane was loaded with the equivalent of 50 μg of homogenates. The same amount of transferrin in crude extracts was detected by western blot analysis using an anti-transferrin antibody. (B) After performing the modified protocol, collected sialoglycoproteins were separated by SDS-PAGE and the band (80KDa) was visualized by silver staining. (C) With the modified protocol, the band was confirmed to be transferrin by western blot analysis using the anti-transferrin antibody under oxidation conditions (+/-).

nmol concentration of biotin and 12 h reaction time were optimal for effective biotinylation (Supplementary Figure 3). With these optimal conditions, we detected fetuin as a glycoprotein including terminal sialic acids. Supplementary Figure 4 shows that one band was detected in the lane of elution on SDS-page stained with Coomassie brilliant blue. The band of low molecular weight (14.3kDa) was avidin. This demonstrated that RNaseB, which did not have any sialic acid residue as glycan, was washed out and fetuin, which had a sialic acid residue as glycan, was detected in the elute solution. It was confirmed that it was fetuin by MS/MS and Mascot search analyses (Supplementary Figure 4). Next, the ovary subjected to hormone induction was used for the detection of proteins containing glycans in their structures. Figure 3A shows that the same amount of transferrin was detected in crude extracts (50 μg of protein) derived from the control and ovulation phase ovaries before applying the modified protocol. After applying the modified protocol, it was detected as band of 80 KDa in the ovulation phase (Figure 3B) and confirmed to be tranferrin under the oxidation conditions (+/-) in the modified protocol by western blotting (Figure 3C). Furthermore, the band was taken out as pieces of the gel and digested by trypsin. Analysis of the band was performed by LC/ESI-MS and it was confirmed to be transferrin. These results suggested that the state of N-glycoryl sialylation of transferrin changed during the estrous cycle. To confirm the sialylation of transferrin in detail, transferrin was recovered from ovary samples of the ovulation phase by the immunoprecipitation method. Figure 4A shows that transferrin adhered to immunoprecipitation beads and was collected. Lectin blot analysis was performed on ovaries to check the total sialic acid quantity. In comparison with the resting stage of ovary activity, an increase of the quantity of sialic acid of transferrin was observed in the ovulation phase (Figure 4B). Although the same amount of transferrin

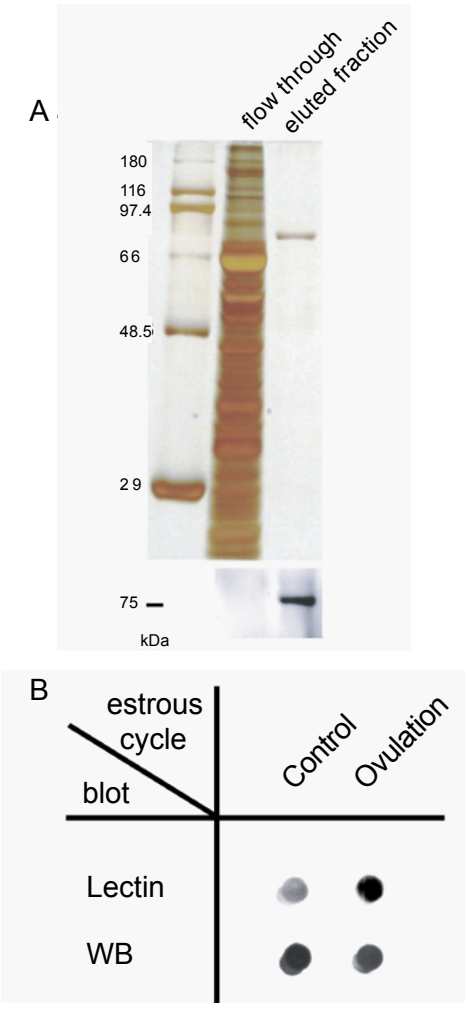

Figure 4: Comparison of sialylation of transferrin. (A) After applying the modified protocol, transferrin was recovered from the eluted fraction by the immunoprecipitation method. Upper is silver staining. Lower is western blot analysis using an anti-transferrin antibody. (B) Lectin blot analysis using Sambucus sieboldiana agglutinin was performed on ovaries to check the total sialic acid quantity. Western blot analysis was performed using the anti-transferrin antibody.

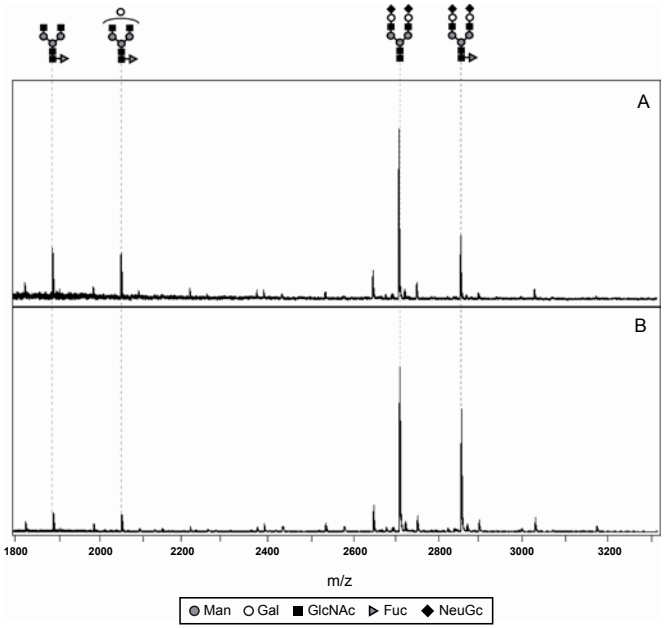

Figure 5: Changes in the carbohydrate chain structure of transferrin during the ovulation phase. (A) Glycans of transferrin in the control. (B) Glycans of transferrin in the ovulation phase. Although sialic acid residues were not detected in major ion peaks observed at m/z 1892 and 2054, sialic acid residues were detected in ion peaks observed at m/z 2712 and 2858.

was detected in both the control and ovulation phase by western blot analysis, more sialic acid residues were detected in the ovulation phase than in the control. This suggested that sialic acid residues were added to transferrin in the ovulation phase.

Changes of the carbohydrate chain structure of transferrin

Glycans of the transferrin that markedly changed in the ovulation phase are indicated by relative comparison with the internal standard A2 amide in Figure 5. Although the amounts of the glycans without neuraminic acid residues of transferrin were markedly reduced in the ovulation phase, the glycans containing NeuGc of transferrin showed a marked increase in the ovulation phase. These findings indicated that transferrin, which is closely related to the estrous cycle, underwent a change in its glycan structure during the cycle. Furthermore, NeuGc-type glycans remarkably increased. This is the first report demonstrating that glycans of the glycoprotein transferrin, NeuGc-type glycans in particular, change with the estrous cycle.

Discussion

Glycosylation is the enzymatic process that links saccharides to produce glycans, attached to proteins, lipids and other organic molecules. This enzymatic process produces one of the fundamental biopolymers found in cells along with DNA, RNA and proteins. Glycosylation is a form of cotranslational and post-transcriptional modification. Glycans serve a variety of structural and functional roles in cell membranes and secreted proteins [5]. The majority of proteins synthesized in the rough ER undergo glycosylation [22-25]. Understanding the state of glycans as a post-transcriptional event is important for basic reproductive biology and for practical applications in animal and human reproductive science. The complicated glycan biology process during the estrous cycle is a critical event following gene expression and protein transcription. To date, to identify ovarian genes and proteins regulated by PMSG and hCG injections, a large number of transcriptional analyses using DNA microarrays and proteomic analyses using MALDI-TOF MS have been performed [26-28]. Furthermore, a genome-wide rat ovarian gene expression database has been constructed [29]. Generally, these studies provide valuable evidence for global gene and protein expression changes in the ovary. However, the glycomic approach to post-transcriptional events does not reflect the translational rates of particular genes and proteins. Thus far, carbohydrate chain analysis using MALDI-TOF MS and the detection of the protein has been carried using serum and body fluid [20]. In the mouse ovary controlled by sex hormone treatment, a previous study using comparative proteomic analysis clearly showed that protein profiles during the mouse ovary cycle changed [15]. A total of 1028 common protein spots were found in representative gels. Among these, 253 protein spots were differentially expressed during the mouse ovarian cycle. On the other hand, few studies have examined the relations of the mouse estrous cycle and glycans of the glycoproteins. In the present study, we found the differential expression of glycans according to the period in the estrous cycle. As a result of detailed glycan analysis, the sialic acid NeuGc-type glycan was found to change in the ovulation phase. To find the carrier protein for NeuGc-type glycan, we developed a modified protocol for specific enrichment and purification of sialylated glycoconjugates from crude live samples. Using the modified protocol, we have shown that glycans of transferrin, sialic acid(s) in particular, change with the estrous cycle. As a conventional method for collection of sialylated glycoproteins, Yodoshi et al. reported the serotonin method using serotonin-bound silica [30]. Serotonin is a small molecule consisting of an indol ring and aminoethyl group. The indol ring seems necessary for binding of sialic acid residues [31]. This protocol is based on the fact that serotonin has a characteristic feature of binding with NeuAc-containing glycoconjugates such as glycolipids and glycoproteins with high affinity. On the other hand, our modified protocol is novel for detection of sialylated glycoproteins using selective oxidation of sialic acid(s), chemical modification of biotin and avidin-biotin binding affinity as the third step. With the modified protocol established, we aimed to detect sialylated glycoproteins whose expression was elevated during the ovulation cycle. It was demonstrated that transferrin had a different state of sialylation during the ovulation cycle. It has been reported that, during the estrous cycle, the amount of transferrin in follicles increases due to the ovary maturation [32]. This seems to be sufficient to suggest that transferrin participates in the estrous cycle. Briggs et al. demonstrated that transferrin could be detected in granulose cells in the human ovary by immunostaining [33]. This suggests that tranferrin synthesis occurs in granulose cells locally and that there is an as yet unknown mechanism for ovarian maturation. In conclusion, we have described a method for enriching and detecting glycoproteins containing sialic acid residue(s) in the hormone-induced estrous cycle of the ovary. This protocol appears to be well suited to enrichment and detection of sialyl glycoproteins from complex crude mixtures of organs. It should be emphasized that the modified protocol greatly facilitates glycoproteomic analysis of a wide range of normal animal organs and various stages of diseases. Here, we demonstrated for the first time that the glycoform-based proteomic approach using the modified protocol is feasible for discovery research into proteins that change the state of sialic acid residues during the estrous cycle in the ovary.

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Supplementary Information

Supplementary Figure 1: General process for the modified protocol. To identify the sialoglycoproteins whose expression level increased during the ovulation phase, we established a modified protocol. It consists of the three following steps: (I) The nonreducing end of a sialoglycan is selectively oxidized. (II) The oxidized sialoglycoproteins are biotinylated by aminooxybiotin. (III) Biotinylated sialoglycoproteins are collected by avidin magnetic beads. Following these three steps, specific proteins including sialic acid residues in the structure can be detected by LC-MS analysis of organ samples.

Supplementary Figure 2: Optimal conditions for selective oxidation of sialic acids. Fetuin (100 mM, sialic acid +) and ribonuclease B (100 mM, sialic acid -) were used to determine the optimal conditions. The optimal conditions for selective oxidation of sialic acids were determined to be pH 4.5 for hydrogen ion activity in solution, a 5 mM $NaIO_4$ concentration (A), and a 5 minute reaction time (B).

Supplementary Figure 3: Optimal conditions for effective biotinylation. To determine the optimal conditions, fetuin (100 mM) was used as a glycoprotein including terminal sialic acids. Five nmol of biotin and a 12h reaction time were determined to be optimal for effective biotinylation.

Supplementary Figure 4: A model experiment with the modified protocol using fetuin and ribonuclease B. Fetuin with the glycans including sailic acid residues in the structure was detected in the eluted fraction. Ribonuclease B without the glycans including sialic acid residues in the structure was not detected.