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Comparison of lectin-binding patterns between young adult and older rat glycoproteins in the brain

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Glycoproteins in the soluble fraction and in the membrane fraction of various portions of brains and spinal cords, obtained from 9-week-old rats and 29-month-old rats, were comparatively analyzed by SDS-polyacrylamide gel electrophoresis and lectin staining. The glycoprotein patterns of each brain part showed marked differences by the age of donors. The most prominent evidence in the soluble fractions of white matter, basal ganglia, and spinal cord detected by WGA is that the glycoproteins with an apparent molecular weight of 123K and 115K have increased in the aged rats. In addition, the reactivity of 115K with Con A and PNA has also increased in the aged rats. On the other hand, reactivity of an apparent molecular weight of 115K with WGA has increased in the membrane fractions of white matter, basal ganglia, hippocampus, cerebellum, and spinal cord from the aged rats. In contrast, by MAM, which is specific for Siaα2$\rightarrow$3Gal linkage, an apparent molecular weight of 115K has been detected only in the membrane fraction of cerebellum and it has decreased in the aged rats. Reactivity of an apparent molecular weight of 133K and 125K in the membrane fractions of white matter and basal ganglia with LCA has decreased in the aged rats. In contrast, reactivity of the front band with LCA and AAL has increased and that of 130K with AAL has decreased in spinal cord from the aged rats, respectively. These results indicate that the glycosylation state of the protein in the brain changes during aging.

Introduction

Glycosylation is one of the common posttranslational modifications of a protein that can affect its function in many ways. The functional importance of glycosylation in cell-cell and cell-extracellular matrix, as well as intracellular events, has been recognized by the explosion of the science of glycobiology. In the development of brain nervous system, glycans have been implicated as important mediators of adhesive interactions among neural cells [1,2]. Although several studies testify to the importance of the structural changes of glycans during development, limited data are available concerning the alteration of glycans during aging. We and others showed previously that the galactose content of human serum IgG decreases with age [3,4]. Since degalactosylated human IgG binds less effectively to C1q and Fc-receptors [5], this alteration will partly explain the phenomenon of immunodeficiency observed in the aged people. Because the biosynthesis of glycans is not controlled by the interaction of a template and depends on the concerted action of glycosyltransferases and glycosidases in endoplasmic reticulum and Golgi apparatus, the structures of glycans are much less rigid than those of proteins and nucleic acids. Therefore, the structures of glycans can be easily altered by physiological conditions of the cells. Accordingly, age-related alterations of the glycans of various glycoproteins are relevant to the understanding of pathological conditions found in aged individuals.

As the first step toward such study, the change of glycosylation of brain proteins during aging was investigated. To identify the nature of the glycans, we chose lectins that specifically bind to different sugar moieties. In the present study, we found that the glycosylation pattern of the brain glycoproteins changes during aging.

Materials and methods

Chemicals and lectins

Biotin-labeled *Lens culinaris* agglutinin (LCA), *Aleuria aurantia* lectin (AAL), *Sambucus sieboldiana* agglutinin (SSA), *Maackia amurensis* agglutinin (MAM), concanavalin A (Con A), peanut agglutinin (PNA), *Datura stramonium* agglutinin (DSA), wheat germ agglutinin (WGA), *Ricinus communis* agglutinin-I (RCA-I) and *Phaseolus vulgaris* agglutinin E_4 (E-PHA) were purchased from Seikagaku Corporation (Tokyo, Japan). *Biotinylated Ly-*

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copersicon esculentum lectin (tomato lectin, TL) was from Sigma Chemical Co., (St Louis, MO). Vectastain ABC kit was purchased from Vector laboratories (Burlingame, CA). *Arthrobacter ureafaciens* sialidase was purchased from Nacalai Tesque Inc. (Kyoto). All other reagents were of the highest quality available.

Preparation of soluble fractions and membrane fractions from rat brain and rat spinal cord

The cerebrum was separated into four parts, white matter, gray matter, basal ganglia, and hippocampus. In addition, cerebellum and spinal cord were also isolated. Each sample, thus obtained from 9-week and 29-month old female Fisher rats, was homogenized with 10 ml/g wet tissue of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 250 mM sucrose buffer (SET buffer) in a Potter homogenizer. After the homogenates were centrifuged at 100,000 × g for 1 h at 4°C, the supernatants (soluble fractions) and the pellets (membrane fractions) were obtained. The pellets were then suspended in SET buffer. Protein was determined by the method of Lowry *et al.* [6]. In order to examine the individual difference, same analyses were made on the samples from additional five young adult and aged rats, respectively.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

SDS-PAGE was performed on a 7.5% slab gel as described by Laemmli [7]. Samples of 15 μg proteins were applied on each lane. After electrophoresis, the proteins were transferred on a polyvinylidene difluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA). Western blot analysis using lectins was performed as follows: After blocking with 3% bovine serum albumin (BSA) in 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (TBS) for 30 min at room temperature, the membranes were incubated with various biotin-conjugated lectins each in TBS containing 0.05% Tween 20 and 1% BSA for 1 h at room temperature. The PVDF membranes were then incubated with avidin-conjugated horseradish peroxidase for 1 h at room temperature, and finally with the peroxidase substrate, 3,3′-diaminobenzamide tetrahydrochloride, to detect lectin-reactive glycoproteins. In some experiments, the PVDF membranes were treated with 0.5 units of *A. ureafaciens* sialidase in 1 ml of 0.5 M acetate buffer, pH 5.0, at 37°C for 20 hr prior to incubation with PNA and RCA-I.

Results and discussion

Brains of a young adult (9-week-old) and an aged (29-month-old) rats, were separated into white matter, gray matter, basal ganglia, hippocampus, and cerebellum, respectively. The soluble fractions and membrane fractions of these five brain portions and spinal cords were prepared as described in "Materials and methods". After SDS-PAGE, proteins of the twelve preparations were transferred on PVDF membranes and stained with wheat germ agglutinin (WGA). It is well known that the lectin binds oligosaccharides terminating with sialic acid and *N*-acetylglucosamine [8]. As shown in Fig. 1A, each soluble portion from the young adult and the aged rats showed a few number of positive bands. Among them, triplet bands around 123K molecular-marker were observed in the soluble fractions of white matter, basal ganglia, and spinal cord from both rats: apparent molecular weights were 130K, 123K, and 115K, respectively. Although reactivity of the lectin with 130K did

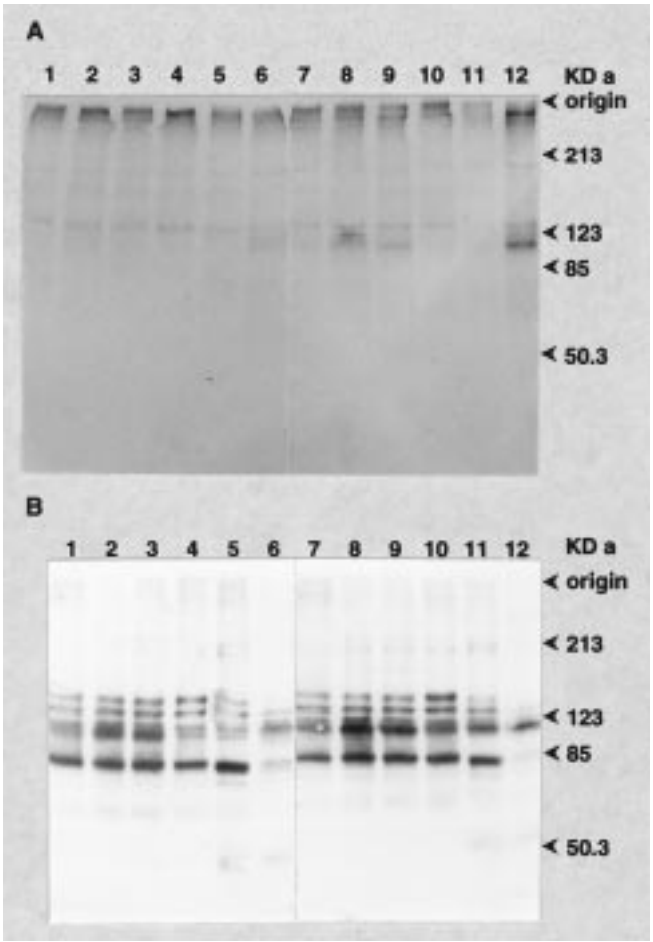


Figure 1. Western blot analysis of the glycoproteins of various portions of rat brain and rat spinal cord using WGA. The samples of rat gray matter (lanes 1 and 7), white matter (lanes 2 and 8), basal ganglia (lanes 3 and 9), hippocampus (lanes 4 and 10), cerebellum (lanes 5 and 11), and spinal cord (lanes 6 and 12) were analyzed by SDS-PAGE using 7.5% acrylamide gel as described in "Materials and methods". Samples in lanes 1–6 and lanes 7–12 were obtained from 9-week-old and 29-month-old rats, respectively. (A), The soluble fractions; (B), the membrane fractions. After electrophoresis, the proteins were transferred on a PVDF membrane, and then stained with WGA as described in "Materials and methods".

not change, that with 123K and 115K bands increased in the fractions of white matter, basal ganglia, and spinal cord in the aged rats (Fig. 1A, lanes 2 and 8, lanes 3 and 9, lanes 6 and 12, respectively). The appearance of WGA-positive glycoproteins may be due to the presence of *O*-linked *N*-acetylglucosamine residue because it is known that many cytosolic proteins were modified by this monosaccharide [9]. Therefore, *N*-acetylglucosaminylation of proteins may have been changed during aging, although the possibility of the presence of *N*-glycans terminating with sialic acid and *N*-acetylglucosamine will not be denied. In contrast, as shown in Fig. 1B, several heavily stained bands were observed in the membrane fractions of both samples. Reactivity of the lectin with 115K band increased in white matter, basal ganglia, hippocampus, cerebellum and spinal cord of the aged rat.

Proteins transferred on PVDF membrane were incubated with concanavalin A (Con A), which binds high-mannose type, hybrid type, and biantennary complex-type oligosaccharides [10]. Band around 120K which was heavily stained with the lectin was observed in the soluble fractions of both samples (Fig. 2A). It is interesting that soluble (cytosolic) proteins had Con A-reactive glycans. This result is consistent with a previous report that about 8% of the glycoproteins of rat brain that contain mannose-rich oligosaccharides were recovered in a soluble fraction [11]. It is not clear whether they are originally located in the cytosol or were detached from membrane during homogenization and centrifugation. Reactivity of the lectin with 115K band increased in white matter, basal ganglia and spinal cord of the aged rat (Fig. 2A). On the other hand, many glycoproteins were Con A-reactive in the twelve preparations from the young adult and the aged rats (Fig. 2B). Although showing very similar staining pattern, reactivity of the lectin with 115K band increased in white matter and basal ganglia (Fig. 2B, lanes 2 and 8, lanes 3 and 9, respectively).

We further examined whether any differences could be found between both samples using by two sialic acid specific lectins with showing different linkage specificity: *Maackia amurensis* agglutinin (MAM) (Siaα2→3 linkage) [12] and *Sambucus sieboldiana* agglutinin (SSA) (Siaα2→6 linkage) [13]. Both lectins did not show any distinct positive bands in soluble fractions of either sample (data not shown). On the other hand, a difference was observed in the membrane fractions as shown in Fig. 3. When the PVDF membrane was stained with MAM, a distinct band of molecular weight of 115K has been apparently detected only in the cerebellum but decreased in the aged rats (Fig. 3A, lanes 5 and 11). Staining intensity with SSA has increased in the aged rat samples although the same amounts of proteins were loaded (Fig. 3B), indicating the increase of the Siaα2→6 linkage.

Peanut agglutinin (PNA) is specific for Galβ1→3GalNAcα1→Ser/Thr [14] and does not bind the disaccharide if it carries a substitution either on the Gal or on the GalNAc residue and therefore it will not bind sialylated structures. When the PVDF membranes were treated with *A. ureafaciens* sialidase, which cleaves all sialyl linkages [15], prior to the lectin blotting, reactivity of both fractions increased (Fig. 4 and Fig. 5). Reactivity of the glycoprotein with an apparent molecular weight of 115K increased in the soluble fractions of basal ganglia and spinal cord of the aged rats (Fig. 4B). On the other hand, reactivity of the lectin with 115K band increased in white matter, basal ganglia and hippocampus in the membrane fractions of the aged rat (Fig. 5B, lanes 2 and 8, lanes 3 and 9, lanes 4 and 10, respectively). Additionally, reactivity of PNA with 213K band has increased in the hippocampus and cerebellum of the aged rat (Fig. 5B, lanes 4 and 10, lanes 5 and 11, respectively).

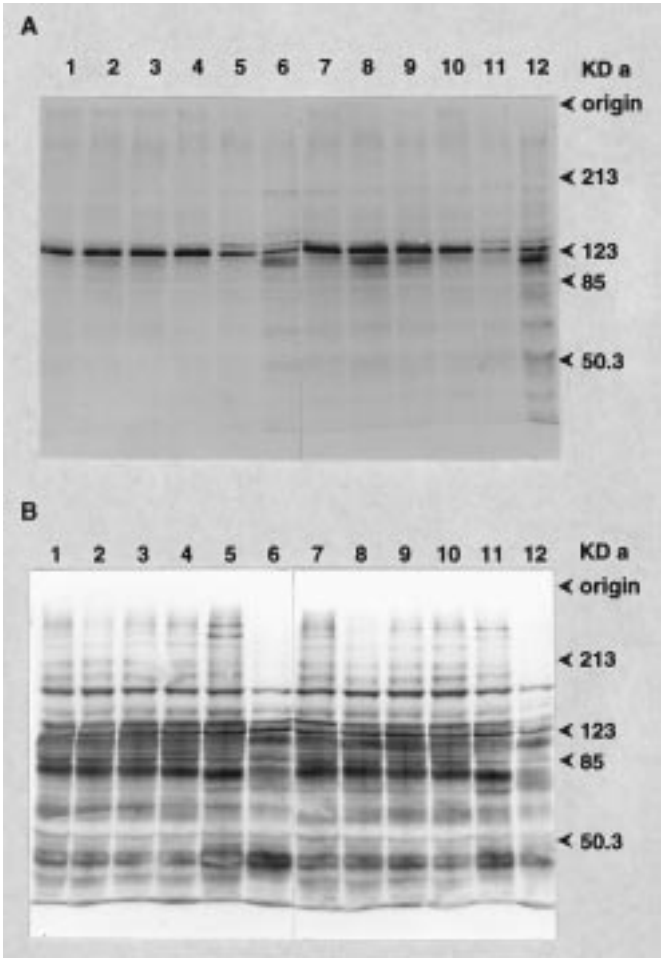


Figure 2. Western blot analysis of the glycoproteins of various portions of rat brain and rat spinal cord using Con A. The samples of rat gray matter (lanes 1 and 7), white matter (lanes 2 and 8), basal ganglia (lanes 3 and 9), hippocampus (lanes 4 and 10), cerebellum (lanes 5 and 11), and spinal cord (lanes 6 and 12) were analyzed by SDS-PAGE using 7.5% acrylamide gel as described in "Materials and methods". Samples in lanes 1–6 and lanes 7–12 were obtained from 9-week-old and 29-month-old rats, respectively. (A), The soluble fractions; (B), the membrane fractions. After electrophoresis, the proteins were transferred on a PVDF membrane, and then stained with Con A as described in "Materials and methods".

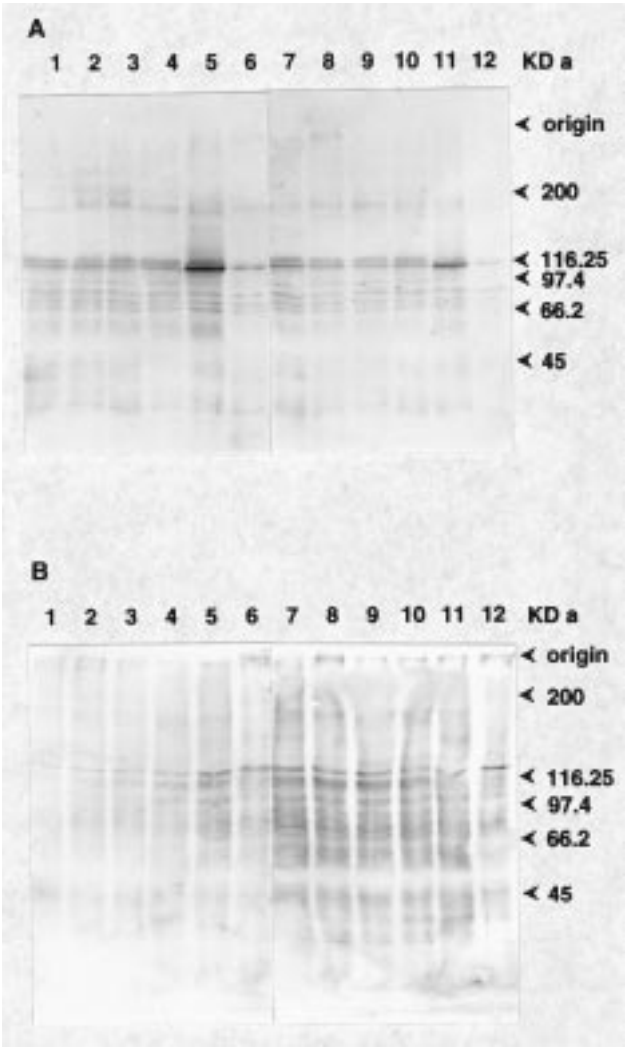


Figure 3. Western blot analysis of the membrane glycoproteins of various portions of rat brain and rat spinal cord using MAM and SSA. The membrane fractions of rat gray matter (lanes 1 and 7), white matter (lanes 2 and 8), basal ganglia (lanes 3 and 9), hippocampus (lanes 4 and 10), cerebellum (lanes 5 and 11), and spinal cord (lanes 6 and 12) were analyzed by SDS-PAGE using 7.5% acrylamide gel as described in "Materials and methods". Samples in lanes 1–6 and lanes 7–12 were obtained from 9-week-old and 29-month-old rats, respectively. (A), MAM; (B), SSA. After electrophoresis, the proteins were transferred on a PVDF membrane, and then stained with each lectin as described in "Materials and methods".

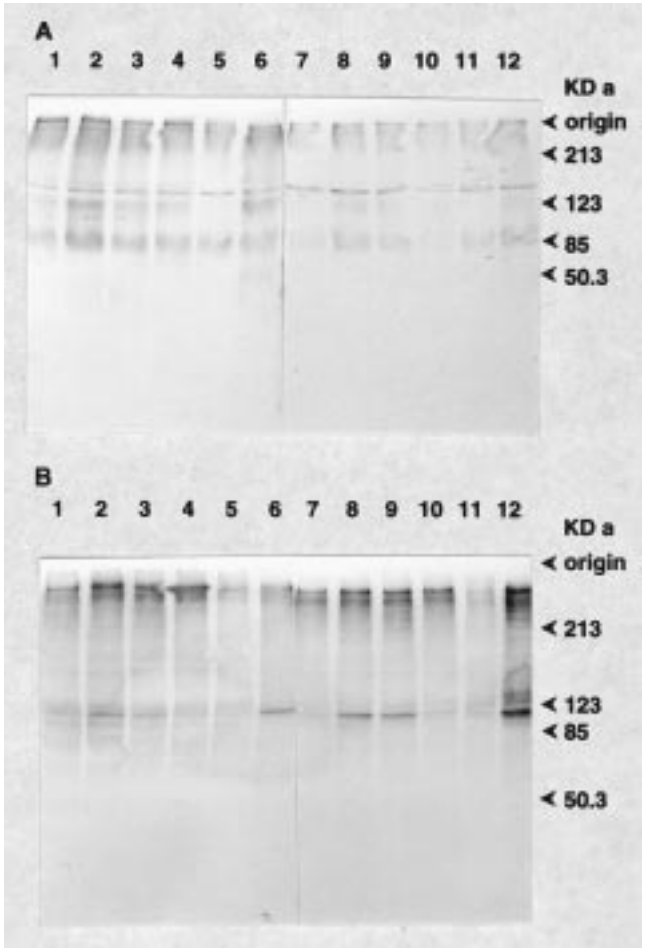
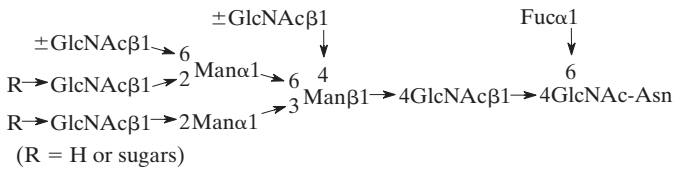


Figure 4. Western blot analysis of the soluble glycoproteins of various portions of rat brain and rat spinal cord using PNA. The soluble fractions of rat gray matter (lanes 1 and 7), white matter (lanes 2 and 8), basal ganglia (lanes 3 and 9), hippocampus (lanes 4 and 10), cerebellum (lanes 5 and 11), and spinal cord (lanes 6 and 12) were analyzed by SDS-PAGE using 7.5% acrylamide gel as described in "Materials and methods". Samples in lanes 1-6 and lanes 7-12 were obtained from 9-week-old and 29-month-old rats, respectively. After electrophoresis, the proteins were transferred on a PVDF membrane, and then stained with PNA as described in "Materials and methods". (A), Before sialidase digestion; (B), after sialidase digestion.

AAL binds with α-fucosyl residue on the oligosaccharides [16] and LCA showed to bind specifically to the following glycan structures [17,18]:



As shown in Fig. 6A, each brain portion from the young adult and the aged rats showed very similar staining patterns with a heavily stained band at 80K, but a slight but significant decrease in intensity around 133K and 125K bands in the membrane of white matter and basal ganglia of aged rats. In addition, reactivity of glycoprotein band, which moved to the front of gel under this condition with LCA and AAL, has increased in the aged rats (Fig. 6A and Fig. 6B, lanes 12 and 6, respectively). Reactivity of AAL with 130K band decreased in spinal cord from the aged rat (Fig. 6B, lanes 6 and 12). It is known [16] that AAL interacts with various fucosyl residues [type-2 H:Fucα1→2Galβ1→4GlcNAc, Le^x antigenic determinant: Galβ1→4(Fucα1→3)GlcNAc,

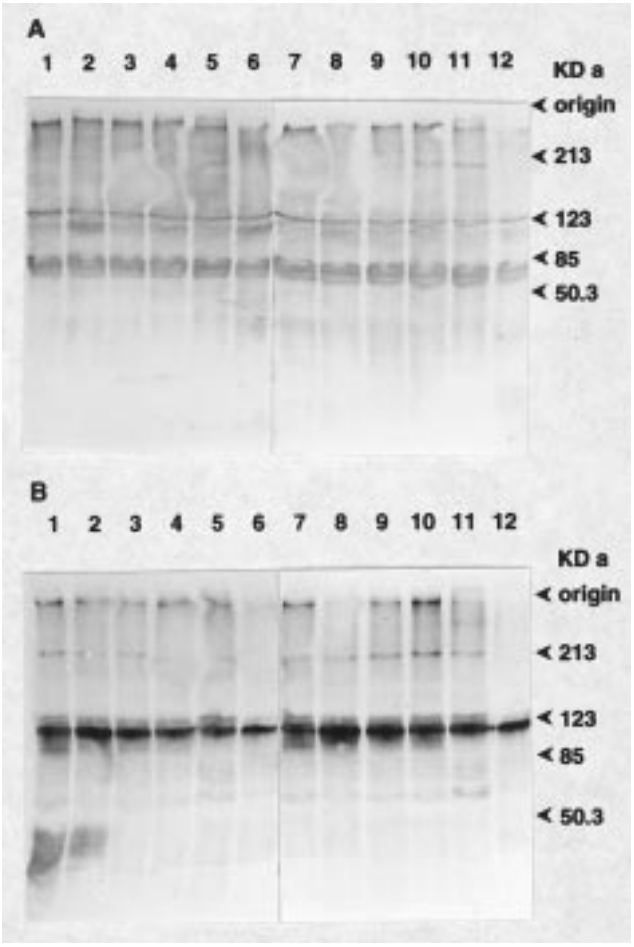


Figure 5. Western blot analysis of the membrane glycoproteins of various portions of rat brain and rat spinal cord using PNA. The membrane fractions of rat gray matter (lanes 1 and 7), white matter (lanes 2 and 8), basal ganglia (lanes 3 and 9), hippocampus (lanes 4 and 10), cerebellum (lanes 5 and 11), and spinal cord (lanes 6 and 12) were analyzed by SDS-PAGE using 7.5% acrylamide gel as described in "Materials and methods". Samples in lanes 1–6 and lanes 7–12 were obtained from 9-week-old and 29-month-old rats, respectively. After electrophoresis, the proteins were transferred on a PVDF membrane, and then stained with PNA as described in "Materials and methods". (A), Before sialidase digestion; (B), after sialidase digestion.

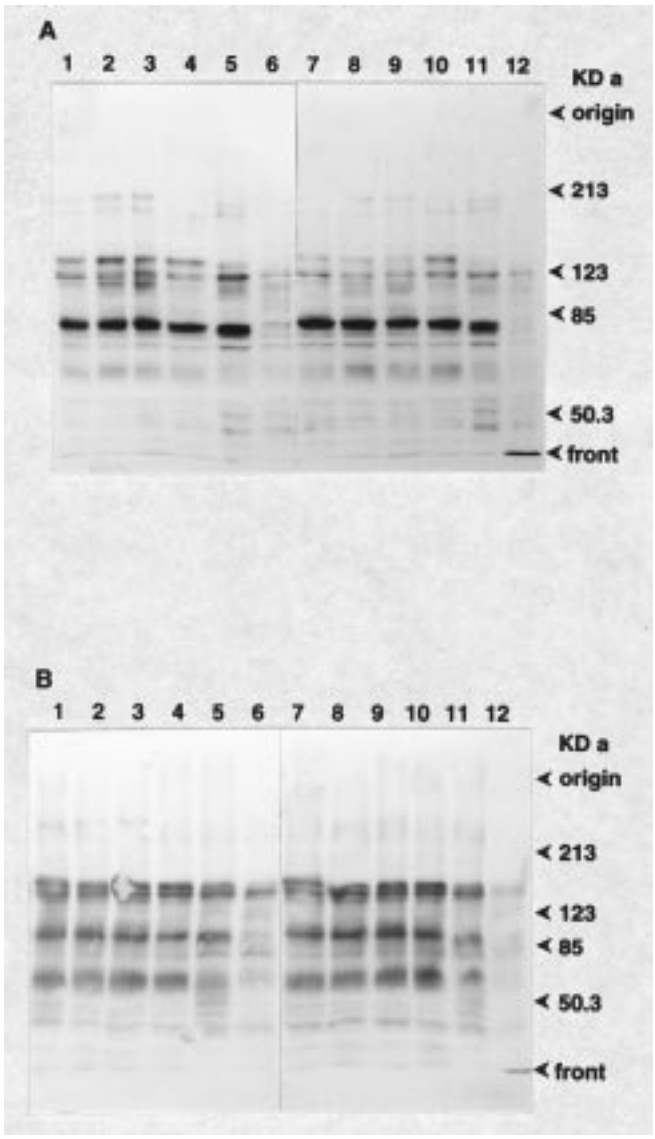


Figure 6. Western blot analysis of the membrane glycoproteins of various portions of rat brain and rat spinal cord using LCA and AAL. The membrane fractions of rat gray matter (lanes 1 and 7), white matter (lanes 2 and 8), basal ganglia (lanes 3 and 9), hippocampus (lanes 4 and 10), cerebellum (lanes 5 and 11), and spinal cord (lanes 6 and 12) were analyzed by SDS-PAGE using 7.5% acrylamide gel as described in "Materials and methods". Samples in lanes 1–6 and lanes 7–12 were obtained from 9-week-old and 29-month-old rats, respectively. (A), LCA; (B), AAL. After electrophoresis, the proteins were transferred on a PVDF membrane, and then stained with each lectin as described in "Materials and methods".

Le^a antigenic determinant: Galβ1→3(Fucα1→4)GlcNAc] in addition to core fucosyl residue as shown in above structures. Therefore, the results indicated that the fucosylation of outer chain moiety of glycan of 130K band in spinal cord may have been changed, because the 130K band was not stained by LCA at all (Fig. 6A, lanes 6 and 12). However, another possibility, a change of the outer chain branching, should be considered because LCA could not bind to 2,4-branched tri- or tetra-antennary glycans with a core fucosyl residue [17,18]. It should be noted that other lectins such as E-PHA (bisecting GlcNAc residue), DSA (2, 6-branched and poly-*N*-acetyllactosamine residues), RCA-I (Galβ1→4GlcNAc group) and TL (poly-*N*-acetyllactosamine residues) also showed several positive bands stained but no differences in binding patterns were observed. Therefore, the results will not be shown here.

To examine that the change of glycoprotein staining by the lectins is not the phenomenon of individual difference analyses were made on the samples from additional five young adult and aged rats, respectively. These rats showed

the same staining results by each lectin as described (data not shown). Therefore, it was concluded that the occurrence of glycoprotein change in the rat brain including spinal cord is the age-related phenomenon and the summarized data were shown in Fig. 7.

In a previous study [11], Brunngraber and Javaid examined subcellular distribution of rat brain glycoproteins that contain mannose-rich oligosaccharides, and they reported that the mannose-rich glycopeptides were localized in the microsomal (29%), synaptosomal fractions (24%) and myelin (12%) and that the complex-type tri- and tetraantennary glycopeptides were in the microsomal (38%), synaptosomal fractions (26%) and myelin (6%) on the basis of Con A-fractionation patterns. Additionally, Brunngraber's group reported about changes in glycoprotein carbohydrate content in the aging human brain from 25 to 85 years [19,20] and the following results were obtained: (i), no change in the concentration of the total carbohydrate associated with glycoproteins; (ii), increase of *O*-linked oligosaccharides, high-mannose oligosaccharides and complex-type biantennary oligosaccharides; (iii), decrease of complex-type tri- and tetra-antennary oligosaccharides; (iv), increase of sulfation and decrease of phosphorylation of oligosaccharides. These data suggested that carbohydrate moieties of glycoproteins were different at each portion of the brain and were changed during aging. It is not clear, however, whether all glycoproteins or some specific glycoproteins in the brain are responsible for inducing these changes.

In this study, we elucidated that the glycosylation state of some proteins but not all ones changed by aging: some were increment and others were decrement. In addition, these changes could be found in specific regions of the central nervous system. It is quite interesting because regions of the central nervous system were specialized for different functions [21]. Although the specific role and mechanism of the glycosylation change in particular glycoproteins during aging remains to be elucidated, at least two possibilities can be considered to explain this interesting phenomenon. First, the glycan structure of young adult glycoproteins is different from that of aged rats. If this were the case, it may indicate that the glycan structure of mammalian brain glycoproteins

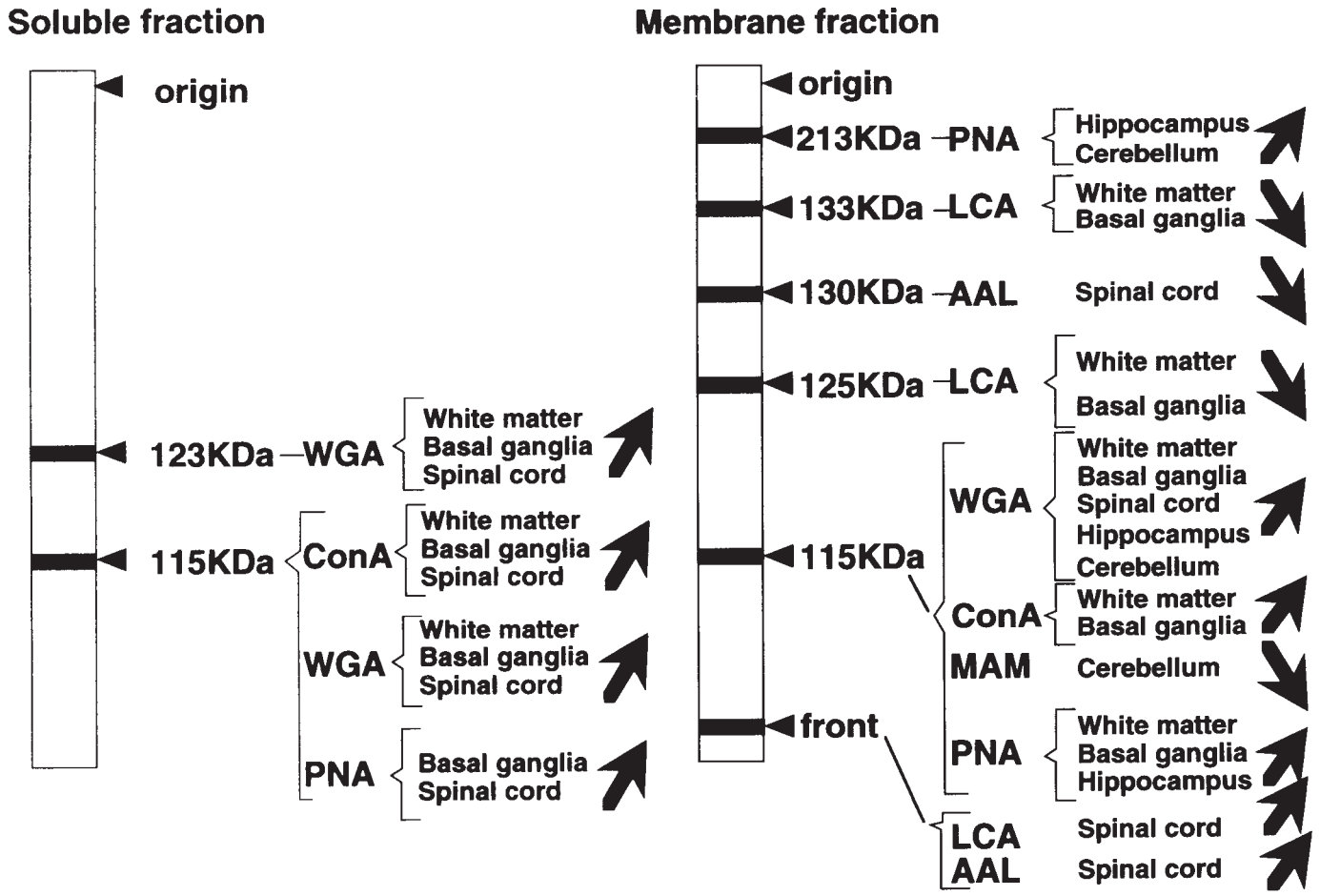


Figure 7. Summary of the change of glycoproteins of various portions from rat brain and rat spinal cord. Upward and downward arrows, respectively, indicated the increase and decrease of glycoprotein expression detected by various lectins in the aged sample.

changes during aging. The second possibility is that the expression of glycoproteins molecule themselves differs in aged rats. Identification of glycoproteins showing different expression during aging still remains to be revealed.

Detailed structural information of glycan on glycoprotein is accumulating [22,23], but still only a limited number of reports are available in the aspect of brain glycoprotein [24–30]. Based on the data obtained, the "brain-type" *N*-glycans were characterized by the presence of the Le^x [Galβ1→4(Fucα1→3)GlcNAc)] determinant, core fucosylation (Fucα1→6GlcNAc) and the presence of a bisecting GlcNAc residue. It is also reported that brain glycoproteins contain a relatively high proportion of non-substituted terminal GlcNAc residues, consistent with truncation and bisecting GlcNAc residues. It is noteworthy that only a few proteins reacted with RCA-I even after sialidase treatment (data not shown). This suggests the presence of a limited amounts of galactosyl residue in brain glycoproteins, which may be related to low expression of β1→4:galactosyltransferase in the brain [31]. It must to stress here that characters of "brain-type" oligosaccharides [27,28,30] could not be applied to all glycoproteins from brain. For example, bovine brain ribonuclease contained the Galα1→3Galβ1→4GlcNAc group instead of the presence of truncated outer chain, bisecting GlcNAc, nor the Le^x determinant [26]. These structural variations of glycan on each glycoprotein may be produced by at least two factors: cell-type and protein specific glycosylation. The cell-type specific glycosylation is occurred because the sets of glycosylation machinery including the glycosyltransferases and glycosidases which act on glycoprotein biosynthetic intermediates are considered to be different in cell-type [22,23]. Therefore, it will be an important task to determine which cell-type, nerve and/or glial cells, contributes to the change of expression of glycoproteins during brain aging. Another aspect is the protein specific glycosylation. It is well known that glycoproteins carried distinctly different structure of sugar chains even these were synthesized in the same cell [32,33]. For instance, in brain, the polysialic acid is almost exclusively associated with the neural cell adhesion molecule (NCAM) and sodium channels in adult rat brain [34]. However, regulatory mechanism of glycosylation on each protein is at present unclear, and undoubtedly complex. Further studies, therefore, are necessary for understanding the formation of distinctly different sugar chains in glycoproteins of the same cell. It will be another task to determine whether change of such regulatory mechanism may be related to change of expression of glycoproteins during brain aging.

In this study, we report that the glycosylation state of some proteins but not all ones has changed by aging. Elucidation of the background of this phenomenon will provide many useful data not only for finding out the functional roles of glycan, but also for the understanding of the molecular events which occur during aging.

Acknowledgments

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