O-Glycosylation in Sprouting Neurons in Alzheimer Disease, Indicating Reactive Plasticity

Blanca Espinosa, MS, Roberto Zenteno, PhD, Raúl Mená, MD, PhD, Yves Robitaille, MD, Edgar Zenteno, MD, PhD, and Jorge Guevara, PhD

Abstract. Reactive plasticity, including axonal and dendritic sprouting and reactive synaptogenesis, has been proposed to contribute to the pathogenesis of several neurological disorders. This work was aimed at identifying the possible role of protein glycosylation in the brain from patients with Alzheimer disease (AD), using lectin histochemistry, as determinants of reactive plasticity. Results indicate an increase in the production of cryptic O-glycosidically linked proteins (NeuAcα2,6 Galβ1,3GalNAc1,0 Ser/Thr or sialyl-T-antigen) in neuritic sprouting in AD brains as determined by positive labeling with Amaranthus leucocarpus (ALL, T-antigen-specific) and Macrobrachium rosenbergii (MRL, specific for NeuAcα5,9Ac2) lectins. Immunohistochemistry indicated that lectin staining was specific for the synaptic sprouting process (meganeurites) in AD. These results were confirmed using anti-synaptophysin and anti-GAP 43 antibodies, which recognized meganeurites and dystrophic neurites around amyloid-β deposits. In normal control brains, labeling with the aforementioned lectins was restricted to microvessels. Control experiments with neuraminidase-treated brain samples revealed positivity to the lectin from Arachis hypogaea (PNA), which is specific for galactose. Our results suggest specific O-glycosylation patterns of proteins closely related to neuronal plasticity in AD.

Key Words: Alzheimer disease; Lectins; O-glycosylation; Sialic acid-specific lectins; Sprouting neurons; T antigen.

INTRODUCTION

The neuropathological hallmarks of Alzheimer disease (AD) are neuritic plaques (NPs) and neurofibrillary tangles (NFTs). Although they are the most sensitive and specific criteria to establish a definite diagnosis of AD, it is still controversial whether the density or distribution of NPs or NFTs is directly related to cognitive deficits (1, 2). In recent years it has been shown that in addition to those lesions, abnormal synaptic immunoreactivity is present in the cortex and the hippocampus of AD brains (3, 4). Most of the studies addressing this synaptic pathology were focused on measuring synaptic densities and, interestingly, several reports have found good correlations between synaptic density decrease and cognitive impairment. It has been proposed that synaptic loss is an early phenomenon possibly preceding loss of neurons (5). Synaptic pathology has been considered particularly relevant in several neuropsychiatric illnesses. A synaptic failure may potentiate the cognitive impairment to deep dementia conditions (6, 7); additionally, abnormally dilated synaptic terminals have been observed in normally aging (8) and demented brains (9).

Glycosylation and other oxidative damage mechanisms have been associated with the earliest pathological change in AD (10–12); however, recent findings indicated that different proteins seem to participate in the major events of AD pathogenesis. These proteins seem to exist in several isoforms that arise (in many tissues) by alternative splicing of a single gene, but are subjected to post-translational modification such as glycosylation, particularly O-glycosylation and sialic acid transferase, during transit through the trans-Golgi and the intracellular protein secretory pathway (13). Because of their fine sugar specificity, lectins have been used to demonstrate post-translational modifications due to glycosylation of proteins in human brains from patients with AD (14–16), and they have also been used to identify microglial cells, neurons, microvessels, and cell types in different animal species (17–25). In this study we used lectins to identify glycan moieties in meganeuritic structures (a specific population of dystrophic neurites) in AD. Our findings suggest a differential glycan addition, indicative of protein processing differences in the synaptic sprouting process in AD but not in normal control brains, suggesting that these glycan modifications might be an early event in AD pathogenesis.

MATERIALS AND METHODS

Tissue

Complete hippocampal formations from 7 autopsied brains, ascertained both clinically and morphologically for AD according to NINCDS-ADRDA criteria, were studied (1, 2). AD patients had a mean age of 74.6 ± 7.9 yr. Five nondemented control brains without any neurological impairment were also studied (age 74 ± 6.9 yr). All the tissues were sampled within 24 hours (h) after death. Samples were
fixed by immersion in 4% paraformaldehyde buffered solution at 4\(^\circ\)C. Samples were then embedded in paraffin and blocks were cut into 6-\(\mu\)m-thick sections and stained according to well-established histochemical and immunohistochemistry protocols (16).

**Lectins**

Biotinylated lectins: *Canavalia ensiformis* (ConA), *Lens culinaris* (LCA), *Limulus polyphemus* (LPA), *Maackia amurensis* (MAA), *Arachis hypogaea* (PNA), and *Sambucus nigra* (SNA) were obtained from EY Laboratories Inc. (San Mateo, CA). Sugars, reference glycoproteins, *V. cholerae* neuraminidase, and Extravidin-FITC were obtained from Sigma (St. Louis, MO). Lectins from *Macrobrachium rosenbergii* (MRL) and from *Amaranthus leucocarpus* (ALL) were purified by affinity chromatography as described previously. Briefly, ALL was purified from seeds by affinity chromatography on a column packed stroma from human erythrocytes type O (26). MRL was purified from hemolymph by affinity chromatography on a column containing stroma from rat erythrocytes-Sephadex G-25 (27).

**Lectin Histochemistry**

Lectin binding was indirectly recognized on paraffin-embedded samples with Extravidin-FITC-conjugated. Sections were washed in PBS for 5 min; lectins diluted in PBS (50 \(\mu\)g/ml) were applied for 2 h at 37\(^\circ\)C. Sections were thoroughly rinsed with PBS before incubating for 1 h at 37\(^\circ\)C in Extravidin-FITC (1:100) followed by PBS rinsing. Prior to mounting with Vectashield (Vector Laboratories, Burlingame, CA), samples were counterstained with 5 \(\mu\)M Propidium Iodide (PI) in an aqueous solution for 15 min (Sigma). Control experiments consisted of omitting the lectin. Specificity of lectin interaction was determined by hapten-inhibition assays: each lectin was pre-incubated with 200 mM of its specific sugar (Table). Ten \(\mu\)M bovine submaxillary gland mucin was used for MRL (27). Lectin interaction was also determined on slides previously incubated with 0.1 units of *V. cholerae* neuraminidase/100 \(\mu\)l of PBS at 37\(^\circ\)C for 30 min.
Fig. 2. Double labeled slides with lectins (green channel, a) and TR (red channel, b) in confocal microscopy. Lectin labeling was observed simultaneously in amyloid-β core of TR-positive NPs (arrowheads, b images). A: Double labeled meganeurites recognized by MRL (arrow, a) and TR, showing an amyloid-β deposit (arrowhead, b). B: Double labeled meganeurites recognized by SNA (arrow, a) and TR, showing an amyloid-β deposit (arrowhead, b). SNA labeling only recognized cytoplasmic membrane of meganeurites (arrow, a). C: Double labeling with LCA and TR. LCA recognized dystrophic neurites (arrow, a) and TR-positive neurites (arrow, b) but not meganeurites around the amyloid-β deposit (arrowhead, b).
**Immunohistochemistry**

Paraffin-embedded blocks from AD and normal control brains were cut 6-μm thick and single labeled according to the indirect PAP method using a monoclonal antibody (mAb) against human Synaptophysin (MAB332, Chemicon International Inc. Temecula, CA. Dilution 1:1,000), PHF-tau hyperphosphorylated (mAb AD2. Dilution 1:500). Monoclonal antibody was kindly provided by Dr. Andre Delacourte, INSERM, Lille, France [28]). Secondary isospecifc antibody HRP-conjugated (Jackson Immunoresearch, Canada) was used to develop indirectly through the standard immunoperoxidase method with Diaminobenzidine (Sigma). Selected sections were double-labeled with the lectins and mAb AD2 and against Growth Associated Protein 43 (GAP-43), (MAB347, Chemicon International Inc., Dilution 1:500). Slides were pretreated in 80% formic acid for 5 min prior to immunostaining, washed, and incubated for 1 h at room temperature with the secondary isotype antibody lissamine-Rhodamine-conjugated (Jackson Immunoresearch). Lectin binding was indirectly recognized with Extravidin-FITC conjugated (Sigma). Selected slides were double-labeled to monitor amyloid-β and PHF-tau assembly (16, 29, 30). We performed double labeling with fluorescent dye thiazin red (TR) in aqueous solution at $2 \times 10^{-6}$% for 15 min (16, 29, 30) and PI (Sigma). Finally, the samples were washed and mounted with Vectashield (Vector, Burlingame, CA).

**Confocal Microscopy**

Double-labeled slides were viewed on an inverted ZEISS microscope equipped with epi-illumination and laser confocal system LSM-410. Consecutive images at 0.5- to 1-μm intervals in the z-axis were collected with a $60 \times$ oil-immersion objective lens and were simultaneously obtained in 2 channels. Images were projected on a 2-dimensional plane and merged using a pseudocolor display (green for FITC and red for TR and PI). In the merged image, a yellow color display is interpreted as co-localization between 2 markers. Images were stored on 1.2 GB optical disk cartridges, photographed on Kodak T-Max 100 or Ektachrome 100 films, and printed on a color video printer.

**RESULTS**

**Meganeuritic Sprouting in AD**

PNA preferentially recognized the cytoplasmic membrane of meganeuritic structures with a diameter bigger than 10 μm (Fig. 1A); these structures showed high tendency to
Fig. 4. Double labeling with GAP-43 and TR. A: Meganeurites are recognized by GAP-43 (arrows, a) immunohistochemistry around the amyloid-ß deposits (arrowhead, b). B: GAP-43 immunoreactivity was also localized in regular dystrophic neurites (arrow, a) around amyloid-ß deposits (arrowhead, b). Scale bar = 10 \mu m.

form clusters, sometimes associated with amyloid-ß deposits. ALL labeled the meganeurites as well as dystrophic neurites with TR-positive PHF-tau in filaments (Fig. 1B); interestingly, in these meganeurites, ALL also showed affinity for granular components. Figure 1C shows MRL-labeling of the intracytoplasmic and granular components of meganeurites. ConA (Fig. 1D), LPA, and SNA showed lower capacity than other tested lectins to label meganeurites (Fig. 1D). *Maackia amurensis* (MAA) and LCA lectins were negative (Table). Incubation of tissue slides with *V. cholerae* neuraminidase increased the positive labeling by PNA of granular components in meganeurites and rendered negative the labeling of structures by MRL.

Meganeuritic Sprouting Associated with Amyloid-ß Deposit in AD

TR was simultaneously visualized in the red channel through confocal microscopy, whereas FITC-labeled lectins were seen in the green channel. Control experiments indicated that SNA, LCA, LPA, MRL, ALL, and PNA positively recognized amyloid-ß deposits (Table). Double-labeled slides with lectins and TR from AD showed a differential pattern of meganeuritic labeling. We found that LPA, MRL, and ALL labeled the meganeurites associated with amyloid-ß deposits. Figure 2A illustrates the meganeurites recognized by MRL with characteristically ovoid structures intimately related to the amyloid-ß deposit. Figure 2B shows that SNA labeled only the cytoplasmic membrane of meganeurites around the amyloid-ß deposit. MRL- and SNA-labeled meganeurites are 10-\mu m thick.

Regular dystrophic neurites (approximately 1-\mu m thick) were recognized by LCA (Fig. 2C), ALL, and MRL. MAA, PNA, and ConA lectins were negative for dystrophic neurites (Table). Incubation of tissue slides with *V. cholerae* neuraminidase did not modify ALL labeling of
Lectin Labeling in AD Tissue Sections

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Meganeurites</th>
<th>Dystrophic neurites</th>
<th>Amyloid-β deposit</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNA</td>
<td>±</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>LCA</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ConA</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>LPA</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MAA</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MRL</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>ALL</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>PNA</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Histochemistry in AD tissue sections was performed using biotin-conjugated lectins and the biotinyltyramide amplification technique (44). The lectins were Canavalia ensiformis (ConA, specific for α-Man; α-Glc), Lens culinaris (LCA, α-Man), Limulus polyphemus (LPA, Neu5Ac); Maackia amurensis (MAA, Neu5Acα2,3Gal), Sambucus nigra (SNA, Neu5Acα2,6Gal/GalNAc), Macrophomium rosenbergii (MRL; Neu5,9Ac2); Peanut agglutinin (PNA, specific for T antigen: Galβ1,3GalNAcα1,0Ser/Thr) and Amaranthus leucocarpus (ALL, specific for Tn antigen: GalNAcα1,0Ser/Thr). Lectin binding was evaluated as follows: −, absent; ±, low reaction, observed in dispersed cells; +, high intensity, observed homogeneously in a tissue section.

dystrophic neurites but rendered positive PNA-labeling, whereas SNA and MRL labeling became negative.

Other Markers

Figure 3 illustrates the labeling of meganeurites with different markers. Anti-human synaptophysin antibody recognized meganeurites in clusters (Fig. 3A). Meganeurites immunoreactive to human synaptophysin were found only in AD brains (mean density 2.4 ± 0.3/mm²). These meganeurites were also recognized by the anti-hyperphosphorylated PHF tau antibody (Fig. 3B). In contrast to this labeling, slides double-labeled with the lectins and counterstained with TR revealed NFTs, but meganeurites were not labeled (Fig. 3C). Furthermore, slides double-labeled with the lectins and counterstained with PI revealed meganeurites labeled by the lectins but negative to PI labeling (Fig. 3D). Anti-GAP 43 antibody recognized meganeurites in amyloid-β deposits stained by TR in AD (Fig. 4A). Also, anti-GAP 43 antibody recognized the regular dystrophic neurites around amyloid-β deposits (Fig. 4B) as well as neurons. In control brains, anti-GAP 43 label appeared as very small dots and in few neurites (not illustrated).

Lipofuscin Autofluorescence

Lipofuscin deposits were autofluorescent in the red channel and were not labeled by TR (Fig. 1A, D). In these slides, lipofuscin autofluorescence was monitored permanently to avoid any kind of interference with the lectin labeling on meganeurites. Lipofuscin appeared as intracellular granular deposits clearly distinguished by their brightness from the lectin labeling in the green channel (Fig. 1A, D).

**DISCUSSION**

Alzheimer disease is characterized by extracellular amyloid-β deposits without neuritic elements and by intraneuronal changes (1, 2). These changes involve NFTs and synaptic loss (6). Reactive plasticity, including axonal and dendritic sprouting as well as reactive synaptogenesis, has been proposed to contribute to the pathogenesis of several neurological disorders. Results from a previous study in AD suggest altered glycosylation and sialylation of glycoproteins (13). In the present study, the use of lectins specific for sialic acid and O-glycans allowed us to find a particular pattern of glycosylation in sprouting mechanisms, specifically of meganeurites and dystrophic neurites in AD. The nature of the glycosylated megalaneurites was also confirmed by double-staining assays with lectins and TR, PI, synaptophysin, or GAP-43. TR specifically identifies tau and amyloid-β in a fibrillar state (29, 30). In our experimental conditions, lipofuscin autofluorescence was monitored permanently to avoid any kind of interference with the lectin labeling. It is interesting from a technical point of view for the correct interpretation of lectin-labeled meganeurites (Fig. 1A, D).

The presence or prevalence of specific saccharidic sequences has been considered an indicator of maturity or of cell transformation; for example, the Thomsen-Friedenreich (T) antigen and the Tn antigen (Galβ1,3GalNAcα1–0Ser/Thr and GalNAcα1–0Ser/Thr, respectively) have been identified as specific structures for immature or transformed cells (31). In this study, we performed a screening of glycosylated structures from AD brains with the aid of lectins with well-known sugar specificity. T and Tn antigens were evaluated with PNA and ALL. PNA is a Gal-specific lectin that shows T-antigen reactivity; capping off the disaccharide by sialic acid abolishes binding of PNA (32). PNA recognized megalaneuritic structures; however, the presence of sialylated T antigens in meganeurites and amyloid-β deposits was confirmed by incubation of tissue slides with V. cholerae neuraminidase, which increased the positive labeling by PNA on meganeurites and rendered positive the amyloid-β deposits. ALL is a GalNAc-specific lectin that shows great affinity for the T antigen; interaction with its receptor is not modified by the presence of sialic acid (33, 34). ALL recognized megalaneurites, dystrophic neurites, as well as amyloid-β deposits; furthermore, neuraminidase treatment did not modify the structures recognized by this lectin, indicating that the T antigen is a constitutive component in sprouting neurons in AD.

Our results also indicated relevant differences in the degree of sialylation of the samples tested. Diversity in sialic acids is generated by the glycosidic linkage at carbon 2, resulting in NeuAcα2,3 or 2,6 linkages of hydroxyl groups of galactose (35). The lectin from LPA...
recognizes sialic acid in spite of the glycosidic linkage (36) and, as indicated, this lectin recognized amyloid-β deposits and, to a lower extent, meganeurites. *Sambucus nigra* agglutinin recognized the same structures as LPA lectin. However, recognition of the lectin from MAA with specificity for Neu5Ac in α2,3 bond was absent in all tested samples, indicating that sialic acid residues present in these structures were mainly linked to galactose by an α2,6 bond (37), suggesting that α2,3 sialyl-transferverses are ineffective in AD. O-acetyl sialic acid (Neu5,9Ac2) is identified with MRL (27). MRL labeling indicates that neuritic sprouting might be associated with the addition of O-acetyl-sialic acid (Neu5,9Ac2) on O-glycosylically linked proteins (38). These changes in the sialylation pattern might contribute to the early neurofibrillary degeneration (39), but would also make the glycosylated structure more rigid, possibly inducing modifications in cell-cell communication; for example, glycosylation may not only modulate tau function but also play a role in microtubule depolymerization (39). Sialylated gangliosides have been implicated in numerous cellular functions in developing nervous systems and it has been suggested that altered glycosylation correlates with periods of cell migration and axonal pathfinding, suggesting a modulatory role of sialic acid derivatives on growth cone motility and adhesion (39).

LCA and ConA assays indicated that LCA recognizes only amyloid-β deposits, whereas, ConA stained meganeurites. Both lectins react with N-glycosylated proteins; although, in contrast to ConA, LCA tolerates substitutions with N-acetyllactosaminic residues in the same structures, suggesting that oligomannosidic and N-acetyllactosaminic type chains occur in minor proportion than O-glycosylically linked glycans in amyloid-β deposits and meganeurites.

Structurally, meganeurites recognized by PNA, ALL, and MRL lectins might be atrophic dendritic structures. Synaptophysin and GAP-43 accumulation in these structures indicates an abnormal synaptic sprouting in AD (Figs. 3A, 4A, B). Furthermore, it has been reported that GAP-43 is a growth-associated phosphoprotein expressed at high levels in neurons during development, axonal regeneration, and neuritic sprouting (40–43). Although the lack of suitable markers for sprouting in human brain makes it difficult to examine changes in neurodegenerative diseases, or to pinpoint the biochemical events that underlie the sprouting response, our results indicate that meganeurites may represent brain plasticity in AD. In summary, our results strongly suggest that alterations in brain plasticity could be due to differential participation of O-glycosyl- and sialyl-transferverses through the trans-Golgi processing of proteins that participate in reactive synaptogenesis, and could be an early event in AD-type neuronal degeneration.

**REFERENCES**


Received October 3, 2000
Revision received January 3, 2001 and February 5, 2001
Accepted February 6, 2001