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Protective effect of *N*-glycan bisecting GlcNAc residues on β-amyloid production in Alzheimer's disease

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Alteration of glycoprotein glycans often changes various properties of the target glycoprotein and contributes to a wide variety of diseases. Here, we focused on the *N*-glycans of amyloid precursor protein whose cleaved fragment, β-amyloid, is thought to cause much of the pathology of Alzheimer's disease (AD). We previously determined the *N*-glycan structures of normal and mutant amyloid precursor proteins (the Swedish type and the London type). In comparison with normal amyloid precursor protein, mutant amyloid precursor proteins had higher contents of bisecting GlcNAc residues. Because *N*-acetylglucosaminyltransferase III (GnT-III) is the glycosyltransferase responsible for synthesizing a bisecting GlcNAc residue, the current report measured GnT-III mRNA expression levels in the brains of AD patients. Interestingly, GnT-III mRNA expression was increased in AD brains. Furthermore, β-amyloid treatment increased GnT-III mRNA expression in Neuro2a mouse neuroblastoma cells. We then examined the influence of bisecting GlcNAc on the production of β-amyloid. Both β-amyloid 40 and β-amyloid 42 were significantly decreased in GnT-III-transfected cells. When secretase activities were analyzed in GnT-III transfectant cells, α-secretase activity was increased. Taken together, these results suggest that upregulation of GnT-III in AD brains may represent an adaptive response to protect them from additional β-amyloid production.

Keywords: Alzheimer's disease/amyloid precursor protein/bisecting GlcNAc/*N*-glycan

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by global cognitive decline involving memory, orientation, judgment, and reasoning. The presence of extracellular senile plaques is one of the classical characteristics of AD pathology. β-Amyloid (Aβ), the major component of senile plaques, is a cleaved fragment of a membrane-spanning glycoprotein, amyloid precursor protein (APP). APP requires cleavage by the β- and γ-secretases to release soluble Aβ. In contrast, α-secretase cleaves APP within the Aβ sequence and prevents the generation of Aβ. Indeed, α-secretase competes with β-secretase for APP processing in the trans-Golgi network (Skovronsky et al. 2000). According to the "amyloid cascade hypothesis," the abnormal accumulation of Aβ leads to neurodegenerative processes, finally resulting in neuronal death. Two types of Aβ are produced depending on the γ-secretase cleavage site: Aβ40 and Aβ42. Aβ42 is a minor form of Aβ but has a greater tendency to produce insoluble deposits and is a major component of senile plaques.

Glycoproteins glycans affect protein stability, conformation, cellular localization, and trafficking (Wang et al. 2005; Ohtsubo and Marth 2006). APP undergoes several posttranslational modifications including *N*- and *O*-linked glycosylation (Weidemann et al. 1989; Tomita et al. 1998; Sato et al. 1999). Core *N*-glycosylation and *N*-glycan processing modulate the synthesis and expression of APP (Pahlsson et al. 1992; Saito et al. 1995; Yazaki et al. 1996). In addition, sialylation of APP *N*-glycans enhanced secretion of its metabolites (Nakagawa et al. 2006). These studies suggest that *N*-glycosylation status may affect the APP metabolic pathway; however, much remains unknown.

We previously determined the *N*-glycan structures of normal and mutant APPs (i.e., the Swedish and London types) (Akasaka-Manya et al. 2008). The Swedish type mutation (Lys595/Met596 to Asn/Leu) increases Aβ42 secretion by 6- to 7-fold (Citron et al. 1992) and the London type mutation (Val642 to Phe) doubles the ratio of secreted Aβ42 to Aβ40 (Suzuki et al. 1994; Price et al. 1998; Sinha and Lieberburg 1999). When the *N*-glycan structures of these mutant APPs were analyzed, we found an increased content of bisecting GlcNAc residues. This prompted us to study the expression levels of β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) in the brains of AD patients because GnT-III is the glycosyltransferase responsible for adding bisecting GlcNAc during *N*-glycan processing (Nishikawa et al. 1992) (Figure 1). The presence of bisecting GlcNAc on individual *N*-glycans prevents the subsequent actions of several glycosyltransferases, including α-mannosidase II, GnT-II, GnT-IV, and GnT-V (Narasimhan 1982; Schachter et al. 1983; Schachter 1986). Thus, attachment of bisecting GlcNAc can significantly alter the types *N*-glycan structures that

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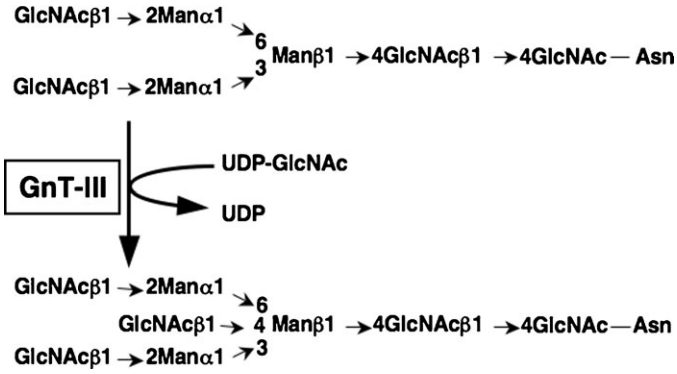

Fig. 1. Bisecting GlcNAc residues in *N*-glycans are synthesized by GnT-III.

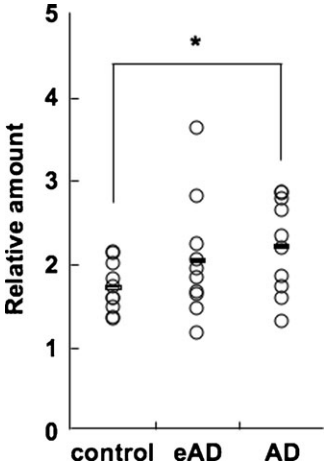

Fig. 2. Quantitative real-time RT-PCR analysis of *GnT-III* mRNA expression in brains of AD patients. Relative amounts of *GnT-III* mRNA were determined in 10 eAD patients, 10 AD patients, and 10 control subjects. All reactions were performed in triplicate and the open circles indicate average values for each individual brain sample. Each horizontal bar indicates the average value of the 10 subjects in that category. Statistically significant differences were identified using the Student's *t*-test ($P = 0.025$) and indicated with an asterisk.

are synthesized. Given the important biological functions of GnT-III (Gu and Taniguchi 2004), we examined the effects of the bisecting GlcNAc on Aβ production and on the activity of the various secretases responsible for Aβ production.

Results

GnT-III mRNA expression in the brains of AD patients

GnT-III catalyzes the transfer of GlcNAc to a core β-mannose residue, producing a bisecting GlcNAc (Wilson et al. 1976; Narasimhan 1982; Nishikawa et al. 1992). To investigate whether GnT-III levels are altered in AD, we measured the amount of *GnT-III* mRNA in the brains of AD patients by quantitative real-time RT-PCR. Preparation of total RNA from non-AD (control), early-stage AD (eAD), or AD brains and real-time RT-PCR analysis was performed as described in *Material and Methods*. As shown in Figure 2, the expression level of *GnT-III* mRNA was significantly increased in AD brains as compared to controls (mean relative amount of control, 1.74; standard deviation (SD), ± 0.28; mean relative amount of AD, 2.23; SD, ± 0.54; $P = 0.025$, Student's *t*-test). However, there was no statistically significant difference in *GnT-III* mRNA levels when comparing eAD brains to controls (mean relative amount of eAD, 2.06; SD, ± 0.68, $P = 0.21$, Student's *t*-test), or when comparing eAD brains to AD brains ($P = 0.56$, Student's *t*-test). Taken together, these results suggest that *GnT-III* mRNA expression increases with disease progression. Therefore, it is conceivable that the number of *N*-glycans having a bisecting GlcNAc residue is increased in AD brains.

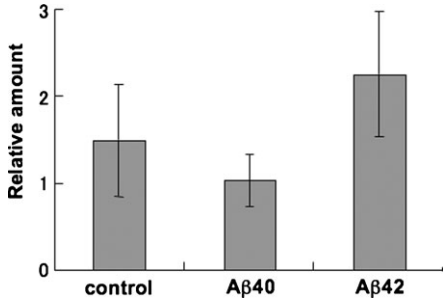

Fig. 3. Relative levels of *GnT-III* mRNA expression after incubation with Aβ. All reactions were performed in quadruplicate. Aβ40 or Aβ42 was added to Neuro2a cell culture medium at a final concentration of 2 μg/mL. After 48 h incubation, cells were harvested for RNA preparation followed by quantitative real-time RT-PCR. Average values ± 1 SD are shown.

Aβ42 exposure enhances GnT-III expression

We examined whether incubation with Aβ40 or Aβ42 affected *GnT-III* mRNA expression levels. Thus, after Aβ40 or Aβ42 was added to the culture media of Neuro2a cells, *GnT-III* expression level was analyzed by quantitative real-time RT-PCR (Figure 3). Compared to control cells (mean relative amount, 1.48; SD, ± 0.64), Aβ42 enhanced the *GnT-III* mRNA expression approximately 1.5-fold (mean relative amount, 2.24; SD, ± 0.72); in contrast, Aβ40 decreased the *GnT-III* expression (mean relative amount, 1.02; SD, ± 0.3). These results indicate that Aβ42, but not Aβ40, enhances GnT-III mRNA expression.

Effect of GnT-III on APP processing

According to our prior (Akasaka-Manya et al. 2008) and current (Figure 2) studies, it is likely that increased *GnT-III* mRNA levels increase the number of *N*-glycans having a bisecting GlcNAc residue. Therefore, we prepared stable transfectants of Neuro2a mouse neuroblastoma cells that express GnT-III by using an expression plasmid encoding *GnT-III*. The microsomal membrane fraction from the transfected cells was used as an enzyme source to measure GnT-III activity (Figure 4A). GnT-III activity was significantly increased in cells transfected with *GnT-III* (32.1 pmol/min/mg) as compared to cells transfected with the "empty" pCXN2 vector (mock transfectant, 0.1 pmol/min/mg). As expected, the intensity of staining by the *Phaseolus vulgaris* lectin E_4 ($PHA\text{-}E_4$), which specifically recognizes bisecting GlcNAc residues (Yamashita et al. 1983), was enhanced in cellular proteins prepared from *GnT-III*-transfected cells (Figure 4B), demonstrating that these proteins have a higher content of bisecting GlcNAc residues. There were no significant differences in the expression levels of membrane-bound APP and secreted APP (sAPP) (Figure 4C, upper-left panel and upper-right

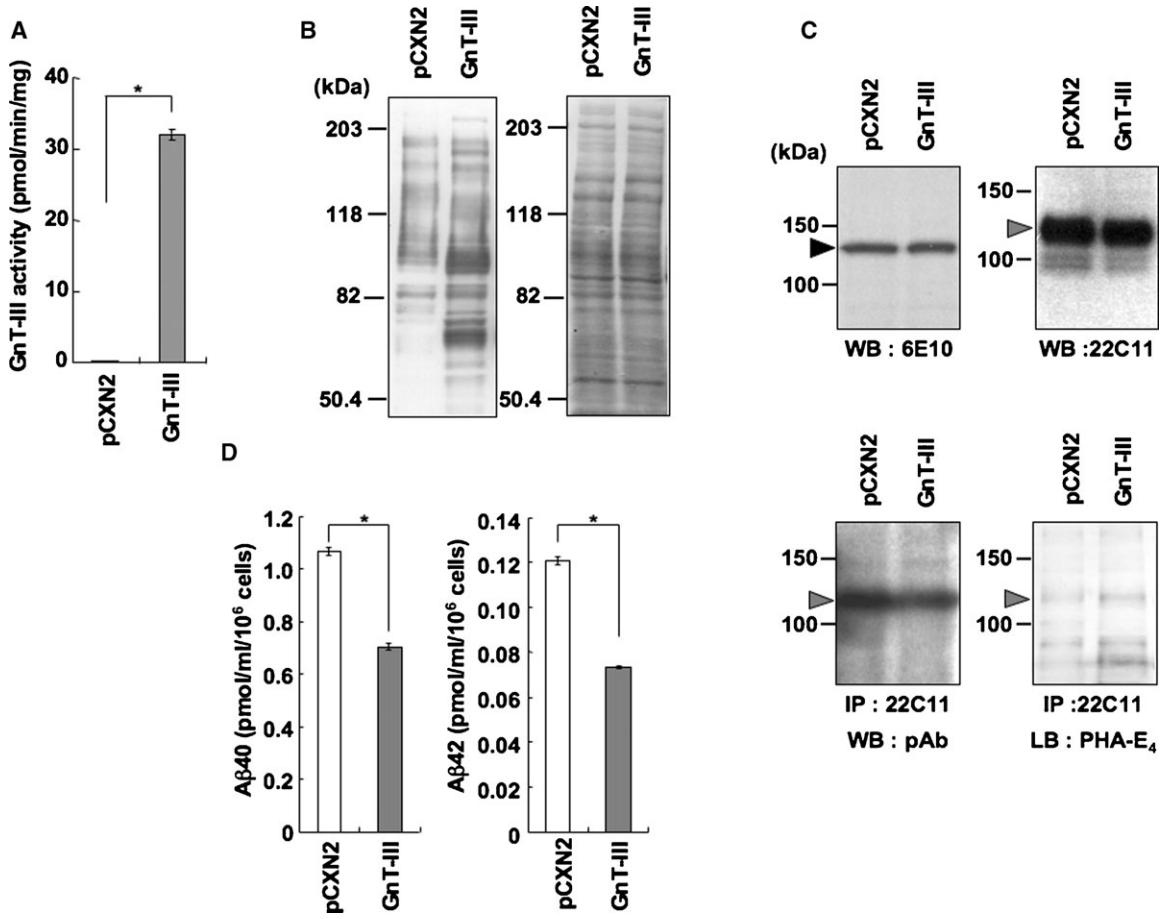

Fig. 4. Overexpression of *GnT-III* mRNA induces an increase in bisecting GlcNAc residues on cellular proteins and a decrease in Aβ secretion. (**A**) GnT-III activities of Neuro2a cells transfected with a *GnT-III* expression vector or an empty vector (pCXN2). Average values ± 1 SD of three independent experiments are shown. Asterisks indicate statistically significant differences ($P < 0.01$, Student's *t*-test). (**B**) Lectin (PHA-E_4) blot analysis of microsomal fraction of Neuro2a cells transfected with a *GnT-III* expression vector or an empty vector (pCXN2). Elevation of the bisecting GlcNAc modification was observed in *GnT-III*-transfected cells. Right panel indicates protein-staining patterns by Coomasie brilliant blue (CBB). Molecular weight standards are shown on the left. (**C**) Western blot analysis of membrane-bound APP or secreted APP (sAPP) in culture supernatants of Neuro2a cells transfected with a *GnT-III* expression vector or an empty vector (pCXN2). Membrane-bound APP was detected with an anti-APP monoclonal antibody (6E10) (upper-left panel) and sAPP in culture supernatant with an anti-APP monoclonal antibody (22C11) (upper-right panel). sAPP was immunoprecipitated from culture supernatant with an anti-APP monoclonal antibody (22C11), and then detected on blots by either an anti-APP polyclonal antibody (pAb, lower-left panel) or by the PHA-E_4 lectin (lower-right panel). Black triangle indicates membrane-bound APP and gray triangles indicate sAPP. Molecular weight standards are shown on the left. (**D**) The effect of GnT-III overexpression on Aβ production by transfected Neuro2a cells. Concentrations of Aβ40 (left) and Aβ42 (right) in culture supernatants were determined by ELISA. The average values ± 1 SD of three independent experiments are shown. Asterisks indicate statistically significant differences ($P < 0.01$, Student's *t*-test). pCXN2: stable mock transfectant of Neuro2a cells; GnT-III: stable transfectant of Neuro2a cells expressing GnT-III.

panel, respectively), but the intensity of PHA-E_4 staining of sAPP was enhanced in cells transfected with *GnT-III* (Figure 4C, lower-right panel). These results demonstrate that APP secreted from *GnT-III*-transfected cells has a higher content of bisecting GlcNAc residues.

We then measured levels of Aβ secreted by Neuro2a cells expressing recombinant GnT-III (Figure 4D). The concentrations of Aβ40 and Aβ42 secreted from the mock transfectant were 1.08 pmol/mL/10^6cells and 0.12 pmol/mL/10^6cells, respectively. For the *GnT-III* transfectant, the concentrations of Aβ40 and Aβ42 were 0.69 pmol/mL/10^6cells and 0.07 pmol/mL/10^6cells, respectively; these were 36.2% and 42.7% lower than those from the mock transfectant. These statistically significant results indicate that increased cellular expression of GnT-III significantly downregulates the secretion of Aβ peptides.

Western blot analysis of secretases

Contrary to our expectations, increased modification of *N*-glycans by bisecting GlcNAc downregulated Aβ secretion (Figure 4D). At least two mechanisms by which increased bisecting GlcNAc could reduce Aβ production should be considered. One possibility is that increasing bisecting GlcNAc expression on APP affects the conformation of APP, changing its susceptibility to α-, β-, and/or γ-secretase, and/or the intracellular localization of APP. Another possibility is that increasing the bisecting GlcNAc content of the secretases affects their enzymatic activity. α-Secretase activity is encoded by two proteins: ADAM 10 (a disintegrin and metalloproteinase 10) and tumor necrosis factor-α converting enzyme (TACE or, equivalently, ADAM 17). TACE has six potential *N*-glycosylation sites (Moss et al. 1997). ADAM 10 has four potential *N*-glycosylation sites, and their *N*-glycans are crucial for processing, localization, and

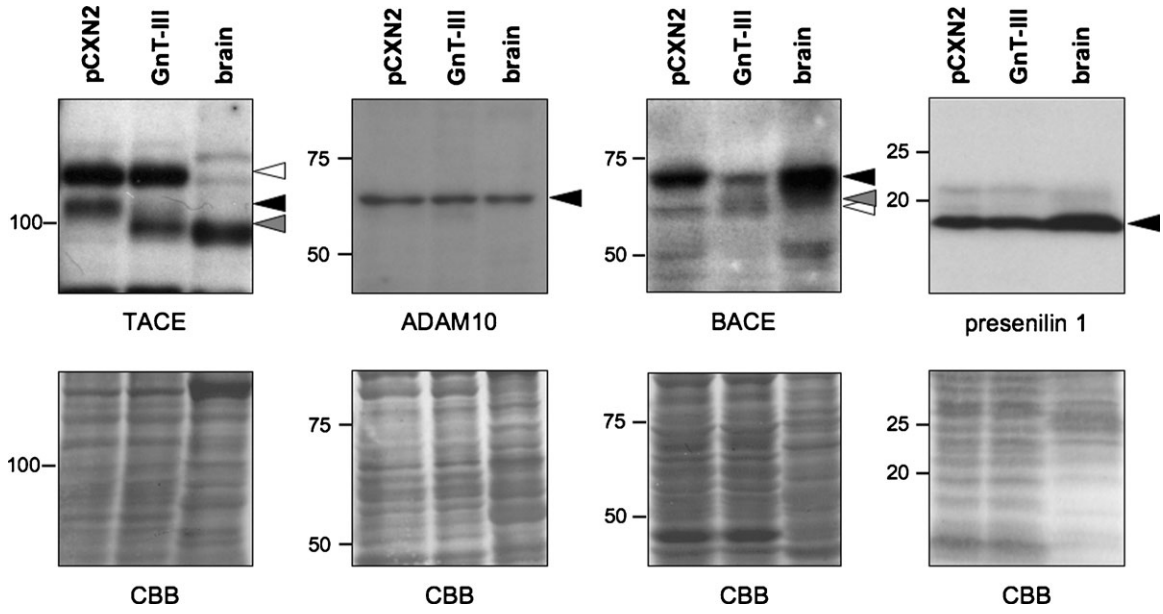

Fig. 5. Western blot analysis of various secretases (TACE, ADAM10, BACE, and presenilin) in *GnT-III*-transfected Neuro2a cells. For TACE and BACE, black and gray triangles indicate mature forms and white triangles indicate immature forms. For ADAM10 and presenilin 1, black triangles indicate the migration positions of ADAM10 and the C-terminal fragment of presenilin 1, respectively. Molecular weight standards are shown on the left. pCXN2: stable mock transfectant of Neuro2a cells; GnT-III: stable transfectant of Neuro2a cells expressing recombinant GnT-III; brain: mouse brain membrane fraction. Bottom figures indicate protein-staining patterns by CBB corresponding to each upper panel.

activity (Escrevente et al. 2008). BACE (β-site APP cleaving enzyme), which possesses β-secretase activity, has four potential *N*-glycosylation sites, three of them appear to be glycosylated (Charlwood et al. 2001). γ-Secretase is a protein complex consisting of presenilin, nicastrin, APH-1, and PEN-2. Nicastrin has 16 potential *N*-glycosylation sites, although inhibition of complex *N*-glycan processing does not affect γ-secretase activity (Herreman et al. 2003).

To clarify the mechanism(s) responsible for downregulating Aβ secretion, the expression levels of the secretases were measured. TACE is reported to change from an immature to a mature form (Milla et al. 1999; Schlondorff et al. 2000; Peiretti et al. 2003). Our Western blot analysis of TACE expressed by Neuro2a cells showed two major bands (Figure 5, left lane); results with proteins isolated from normal mouse brain are shown for comparison. The upper band (white triangle) corresponds to immature TACE bearing high-mannose *N*-glycans; the lower band corresponds to mature TACE (black triangle). Although two TACE bands were also observed in *GnT-III*-transfected Neuro2a cells (Figure 5, right lane), the mobility of mature TACE (gray triangle) from *GnT-III*-transfected cells was faster than that from the mock transfectant. As reported previously, this type of finding is a unique feature seen by introducing bisecting GlcNAc into glycoprotein *N*-glycans (Shigeta et al. 2006). In addition, the expression level of TACE in *GnT-III*-transfected cells was nearly the same as compared with mock transfectant. BACE is also reported to change from an immature form to a mature form (Benjannet et al. 2001; Schmechel et al. 2004). Our Western blot analysis of Neuro2a cells showed two BACE bands (Figure 5, left lane). The upper band corresponds to mature BACE (black triangle) and the lower to immature BACE (white triangle). An additional new band of intermediate mobility appeared in the *GnT-III*-transfected cells (Figure 5, gray triangle in the right lane). Interestingly, in the *GnT-III* transfectant, the

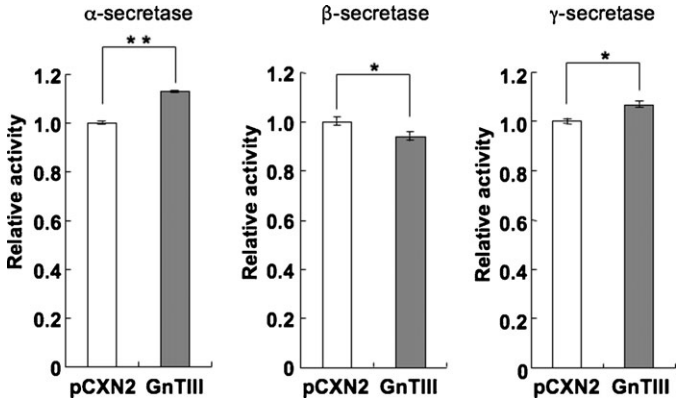

Fig. 6. Secretase activities in *GnT-III*-transfected Neuro2a cells. α-, β-, and γ-secretase activities (left, middle and right panels, respectively) were determined. For comparison, the fluorescence intensity of the pCXN2 transfectant was set to 1.0. The average percentages ± 1 SD of three independent experiments are shown. Asterisks indicate statistically significant differences ($^{*}P < 0.01$, $^{**}P = 0.0001$, Student's *t*-test).

molecular size and expression of BACE both decreased. In contrast, when comparing the mock transfectant with the *GnT-III* transfectant, no differences in the expression level or molecular size of ADAM 10 or the C-terminal fragment of presenilin 1 were seen (Thinakaran et al. 1996) (Figure 5). Taken together, these results suggest that changing the *N*-glycans of TACE and BACE may affect α- and β-secretase activities.

Secretase assays

To examine the effect of *N*-glycan changes of TACE and BACE on enzymatic activity, we measured α- and β-secretase activities in *GnT-III* transfectants of Neuro2a cells. As shown in Figure 6, in the *GnT-III* transfectant, α-secretase activity (113% of the

activity of the control pCXN2 transfectant, $P = 0.0001$) was slightly upregulated, but β-secretase activity (97% of the pCXN2 transfectant, $P = 0.042$) was modestly downregulated. Because changes in γ-secretase activity may also affect Aβ production, its activity in *GnT-III*-transfected cells was measured; modest upregulation was observed (107% of the pCXN2 transfectant, $P = 0.015$). Taken together, the increased α-secretase activity and decreased β-secretase activity in the *GnT-III* transfectant were the most probable cause of the reduction in Aβ production shown in Figure 4D. Thus, these results suggest that changes in *N*-glycan of TACE and BACE affect their enzymatic activities and lead to downregulation of Aβ production.

Discussion

In previous studies, we described the *N*-glycan structures of APP695 produced by Chinese hamster ovary cells (Sato et al. 1999) and the C17.2 mouse neural stem cell line (Akasaka-Manya et al. 2008). Recombinant APP695 in both cell lines had sialylated bi- and triantennary complex-type *N*-glycans with fucosylated and nonfucosylated trimannosyl cores. However, only APP695 produced by C17.2 cells had *N*-glycans containing bisecting GlcNAc. This may be due to cell-type-specific differences in *N*-glycan processing that can be found with various recombinant glycoproteins (Kagawa et al. 1988; Cumming 1991). To determine whether mutations in the *APP* gene alter the structures of processed *N*-glycans, we expressed two mutant recombinant APPs (i.e., the Swedish type and the London type) in transfected C17.2 cells. Structural analysis of these *N*-glycans revealed that the two mutant APPs had higher contents of bisecting GlcNAc and core-fucose residues as compared to wild-type APP. These results clearly showed that these slight changes in amino acid sequence affected *N*-glycan processing.

The glycosyltransferase responsible for adding the bisecting GlcNAc residue is GnT-III (Wilson et al. 1976; Narasimhan 1982; Nishikawa et al. 1992). To examine whether *GnT-III* mRNA levels are related to the pathogenesis of sporadic AD, we examined this issue by quantitative real-time RT-PCR using brains of normal individuals and AD patients. As shown in Figure 2, *GnT-III* mRNA levels were significantly increased in the brains of AD patients. This upregulation may affect AD pathogenesis because significant differences were found in patients with an advanced stage of AD. Interestingly, incubation of Neuro2a cells with Aβ42 increased *GnT-III* gene expression levels (Figure 3). In a recent report (Fiala et al. 2007), exposure of normal peripheral blood mononuclear cells to Aβ peptide upregulated transcription of *GnT-III* and led to increased Aβ clearance by phagocytosis; interestingly, mononuclear cells isolated from AD patients exhibited downregulated *GnT-III* gene expression and were defective in phagocytosis of Aβ. Since upregulation of GnT-III expression was associated with enhanced phagocytosis of Aβ, an increment of GnT-III levels in mononuclear cells may lead to improved Aβ clearance. In contrast, as reported here, increased expression of GnT-III in Neuro2a cells downregulated Aβ production (Figure 4D), and *GnT-III* mRNA levels were increased in AD brains (Figure 2). Taken together, these results suggest that upregulation of GnT-III in neuronal cells may diminish Aβ production in AD brains. In addition, expression of GnT-III in neurons and monocytes may modulate Aβ accumulation by different mechanisms. That is, upregulation of GnT-III expression in monocytes may enhance Aβ clearance, and increased GnT-III expression in neuronal cells may inhibit Aβ production. Taken together, both responses may be adaptive, protective responses that inhibit the further progression of AD.

To evaluate the mechanism by which an increased number of bisecting GlcNAc residues could reduce Aβ production, several possibilities should be considered. As reported here, the APP secreted by *GnT-III*-transfected Neuro2a cells has a higher content of bisecting GlcNAc than that secreted by control cells (Figure 4C). The addition of bisecting GlcNAc may affect the conformation of APP, thereby leading to a change in its susceptibility to α-, β-, and/or γ-secretases. Alteration of glycoprotein glycans is known to affect various properties of a given protein including its susceptibility to various modifying enzymes. For example, organ-specific differential glycosylation of low-density lipoprotein receptor-related protein 1 (LRP1) alters its proteolytic cleavage by γ-secretase (May et al. 2003). In addition, increased sialylation of APP enhanced Aβ secretion (Nakagawa et al. 2006). Bisecting GlcNAc residues are also known to affect the branching and elongation of various *N*-glycans antennae (Narasimhan 1982; Schachter et al. 1983; Schachter 1986). Therefore, it is possible that increasing bisecting GlcNAc expression on APP leads to changes in the APP *N*-glycan structure, including less sialylation, which may alter it susceptibility to cleavage by individual secretases (Fukuta et al. 2000; Koyota et al. 2001). Furthermore, because changing the *N*-glycan structure can alter intracellular glycoprotein localization, it is possible that bisecting GlcNAc affects APP trafficking and, thereby, its susceptibility to secretases. For example, in cells that overexpress GnT-III, cell surface turnover of E-cadherin is delayed (Yoshimura et al. 1996). In contrast, the cell surface expression of epidermal growth factor receptor is reduced in GnT-III overexpressing cells (Rebbaa et al. 1997). In addition, APP localization and trafficking vary according to its glycan modifications (McFarlane et al. 1999).

Another possibility is that increasing the bisecting GlcNAc content of the secretases affects their enzymatic activity. For example, glycosylation is known to play a critical role in maintaining the enzymatic activity of β-secretase (Charlwood et al. 2001). In that study, baculovirus-expressed β-secretase, which only has high-mannose-type *N*-glycans, exhibits only ∼50% of the activity found when the enzyme is expressed by mammalian cells, when it has complex-type *N*-glycans (Charlwood et al. 2001). To investigate this issue, we measured secretase activities in *GnT-III*-transfected cells; α- and β-secretase activities were significantly increased and decreased, respectively (Figure 6). By Western blot analysis, the *N*-glycan structures of TACE and BACE are altered (Figure 5), perhaps explaining the changes in their enzymatic activities. In a previous study (Skovronsky et al. 2001), TACE-expressing neurons often colocalized with Aβ plaques. Our results showed that GnT-III expression was increased in AD brains (Figure 2) and that increases in GnT-III might decrease BACE expression (Figure 6). Taken together, it is likely that upregulation of GnT-III in AD brains induces changes in the APP processing enzymes, TACE and BACE, which may inhibit Aβ formation. Although the detailed mechanisms are not yet clear, this increased expression of GnT-III may homeostatically partially protect AD brains from further Aβ production.

Bisected *N*-glycans play important roles in neurological function in vitro and in vivo. For example, bisecting GlcNAc

regulated serum depletion-induced neuritogenesis (Shigeta et al. 2006). In addition, truncated, inactive GnT-III induced abnormal neurological phenotypes in mice (Bhattacharyya et al. 2002). As another example, changes in bisected *N*-glycans may be related to the pathogenesis of prion disease (Rudd et al. 1999). Therefore, further studies are required to understand the precise physiological and pathological roles of bisecting GlcNAc in brain development and function.

In summary, based on the current results, we propose that high expression of GnT-III in human AD brains reduces Aβ production and protects against further deterioration of neurological function during this disease process. Therefore, compounds that upregulate the expression of bisecting *N*-glycans may provide a novel therapeutic approach toward preventing or ameliorating AD.

Material and methods

Patients and controls

Human brain tissues were obtained from the Brain Bank for Aging Research (BBAR), which consists of consecutive autopsy cases from a general geriatric hospital with informed consent obtained from the relatives for each autopsy. The brains were handled using the BBAR protocol described previously (Fumimura et al. 2007). In brief, half of the brain was serially sections into 7 mm slices, snap-frozen using powdered dry ice, and stored at −80°C. To minimize RNA degradation, samples with the shortest postmortem intervals were selected for study. Two grams of frozen gray matter were sampled from the temporal pole of 10 cases each with AD, eAD, and age-matched normal controls. The diagnosis of AD was based on the BBAR criteria (Hughes et al. 1982; Murayama and Saito 2004), as follows: (1) clinical dementia rating (Hughes et al. 1982) $\geq$ 1; (2) Braak's senile plaque stage equal to C; and (3) the Braak's neurofibrillary tangle stage $\geq$ IV. The diagnosis of eAD was based on the following criteria: (1) clinical dementia rating, either 0 or 0.5; (2) Braak's senile plaque stage $\geq$ B; and (3) Braak's neurofibirillary tangle stage $\geq$ III. The criteria for designating brains as coming from normal controls included a clinical dementia rating of 0, Braak's senile plaque stage 0, and Braak's neurofibrillary tangle stage $\leq$ II. The age of the selected AD cases ranged from 79 to 98 years old (average of 88.2 years), and the postmortem interval from 1.8 to 17.7 h (average of 7.1 h). The age of the eAD cases ranged between 76 and 96 years (average of 90.3 years), and the postmortem interval between 1.2 and 39.9 h (average of 9.6 h). The age of the normal controls ranged from 68 to 86 years (average of 75.8 years), and the postmortem interval ranged from 1.5 to 29.1 h (average of 7.4 h). This study was approved by the Internal Review Board of Tokyo Metropolitan Institute of Gerontology and of Tokyo Metropolitan Geriatric Hospital.

Real-time RT-PCR analysis

Total RNA was isolated from a portion of each patient's brain using the guanidinium thiocyanate method with TRIzol (Invitrogen Corp., Carlsbad, CA), following the manufacturer's instructions. The integrity of the isolated total RNA was confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Total RNA from Neuro2a cells was isolated using ISOGEN (Nippon Gene Co., Ltd, Tokyo, Japan), following the manufacturer's instructions. First-strand cDNAs were synthesized using 5 μg of total RNA, SuperScript II RNase H^- Reverse Transcriptase, and random primers (Invitrogen). The relative quantification of target mRNA was determined using a TaqMan real-time RT-PCR assay on a 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), following the manufacturer's instructions using the TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (i.e., a mixture of designed primers and TaqMan probes, Applied Biosystems): *GnT-III*, Hs02379589_s1; endogenous control, the TaqMan Ribosomal RNA Control Reagents VIC Probe. 18S rRNA was used as normalization control.

Cell culture and expression of GnT-III

Neuro2a mouse neuroblastoma cells were maintained in a mixture of Dulbecco's modified Eagle's medium and OptiMEM (1:1, v/v, Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 units/mL penicillin, and 50 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. The pCXN2-rat-*GnT-III* expression plasmid was described previously (Kitada et al. 2001). This plasmid was transfected into Neuro2a cells using Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected with G418 (Invitrogen) at 1 mg/mL. The culture supernatants of these transfectants were collected after 24 h incubation in Dulbecco's modified Eagle's medium:OptiMEM (1:1, v/v) supplemented with 0.2% fetal bovine serum. The cells were homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, with protease inhibitor mixture (3 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 mM benzamidine-HCl, 1 mM PMSF). After centrifugation at 900 × *g* for 10 min, the supernatant was centrifuged at 100,000 × *g* for 1 h; the pellet was used as the microsomal fraction. Protein concentration was determined by BCA assay (Thermo Fisher Scientific Inc., Waltham, MA).

Aβ treatment of Neuro2a cells was performed as follows: Aβ40 and Aβ42 were each purchased from PEPTIDE INSTITUTE, INC. (Osaka, Japan) and dissolved in H_2O. Aβ40 or Aβ42 were added to culture medium at a final concentration of 2 μg/mL. Cells were cultured for 48 h and harvested for RNA preparation followed by real-time RT-PCR.

Preparation of mouse brain membrane fraction

Brains were obtained from 4-week-old C57BL/6 mice, and homogenized with 9 volumes (weight/volume) of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose. After centrifugation at 900 × *g* for 10 min, the supernatant was centrifuged at 100,000 × *g* for 1 h; the pellet was used as the microsomal membrane fraction. Protein concentration was determined by BCA assay. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology.

Assay for GnT-III activity

GnT-III activity was measured using a modification of a previously reported method (Taniguchi et al. 1989). The enzyme assay mixture, containing 125 mM MES buffer (pH 6.25), 200 mM GlcNAc, 10 mM $MnCl_2$, 20 mM UDP-GlcNAc, 0.5% Triton X-100, 10 μM of 2-aminobenzamide-labeled [GlcNAcβ1-2Manα1-6 (GlcNAcβ1-2Manα1-3) Manβ1-4Glc

NAcβ1-4GlcNAc] (ProZyme, Leandro, CA), and cell homogenate were incubated at 37°C for 1 h. After boiling for 3 min to stop the reaction, the mixture was subjected to reversed-phase HPLC using a Cosmosil 5C18-AR column (Nacalai Tesque, Kyoto, Japan), which was equilibrated with the 100 mM ammonium acetate buffer, pH 4.0, and eluted with a gradient of 1-butanol (0.25–1% butanol) over 120 min at a flow rate of 1 mL/min at 55°C.

Immunoprecipitation

For APP immunoprecipitation, culture supernatants were mixed with an anti-APP monoclonal antibody (22C11, Millipore, Billerica, MA). After incubation at 4°C for 2 h, Protein G-coupled Sepharose-4B beads (GE Healthcare UK Ltd., Buckinghamshire, England) were added and the mixture rotated at 4°C for 2 h. The beads were washed three times with PBS and suspended in the sample buffer. Immunoprecipitated proteins were recovered by boiling for 3 min and then subjected to Western blot and lectin blot analyses.

Western blot analysis

Proteins were separated by SDS–PAGE (for TACE, a 5–10% gradient gel; for APP, BACE, and ADAM 10, a 7.5% gel; for presenilin 1, a 12.5% gel) and transferred to a PVDF membrane. The membrane, after blocking in PBS containing 5% skim milk and 0.05% Tween 20, was incubated with an anti-APP polyclonal antibody (Millipore, Billerica, MA) or an anti-APP monoclonal antibody (6E10, Signet laboratories, Dedham, MA). The membrane was then incubated with anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare). Antibody-bound proteins were visualized using an ECL kit (GE Healthcare).

Secretases in the microsomal fractions were visualized after separation by SDS–PAGE using anti-TACE polyclonal antibody (Thermo Fisher Scientific), anti-ADAM10 antibody, anti-presenilin 1 antibody, and anti-BACE antibody (Abcam, Cambridge, England).

Lectin blot analysis

Immunoprecipitated proteins were separated by SDS–PAGE and transferred to a PVDF membrane. After blocking with 3% bovine serum albumin (BSA, Nacalai Tesque) in 10 mM Tris-HCl (pH 7.4) containing 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.05% Tween 20 (TBS-T), the membrane was incubated with biotin-conjugated PHA-E_4 (Seikagaku Corporation, Tokyo, Japan) in TBS-T containing 1% BSA. After treating the membrane with the Vectastain ABC kit (Vector, Burlingame, CA), lectin-bound proteins were visualized with an ECL kit.

Quantification of soluble Aβ by sandwich ELISA

Culture supernatants were subjected to enzyme-linked immunosorbent assay (ELISA) using the Human/Rat β-Amyloid 40 ELISA kit II and the Human/Rat β-Amyloid 42 ELISA kit High Sensitive (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to manufacturer's instructions.

Secretase assays

Secretase enzymatic assays were performed using the α-secretase assay kit, β-secretase assay kit, and γ-secretase assay kit (R & D Systems, Inc., Minneapolis, MN), according to manufacturer's instructions. Briefly, cultured Neuro2a cells were harvested and cell numbers counted. Cells were lysed with the extraction buffer and used as an enzyme source for the assay. An APP peptide conjugated to fluorescent reporter and quencher was used as the substrate. The protein content of cell lysates was determined by BCA assay and secretase activities were normalized to protein concentration.

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Conflict of interest statement

None declared.

Abbreviations

Aβ, β-amyloid; AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; eAD, early-stage AD; GnT, *N*-acetylglucosaminyltransferase; TACE, tumor necrosis factor-α-converting enzyme.

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