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ORIGINAL ARTICLE

The Synergetic Effects of Chinpi Plus α-GPC for Proper Myelination in Aged Tg2576 Mouse Model of Alzheimer's Disease

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Abstract

Background：There is increasing evidence that myelin disruption is related to the cognitive decline in Alzheimer's disease（AD）. Previous studies established that the absence of myelin basic protein（MBP）reduces non-amyloidogenic processing of amyloid precursor protein（APP）. The aim of this study is to demonstrate the synergetic effects between Chinpi and α-GPC in aged Tg2576 mice（24 months）with regard to the APP processing and myelination status.

Methods：We have tested whether the treatment of Chinpi plus α-GPC can alter influence APP processing and expression levels of MBP in aged Tg2576 mice serving with mixed-feed contained Chinpi or α-GPC or Chinpi plus α-GPC, compared to control（non-treated）.

Results：We demonstrated that a non-amyloidogenic pathway, which is metabolized to produce a soluble fragment（sAPPα）and CTFα, significantly increased in aged Tg2576 mice after treatment with Chinpi plus α-GPC. Furthermore, the expression levels of the 21.5-KDa MBP isoform, which fluctuate according to myelination status,

Key words：Alzheimer's disease（AD）, myelin basic protein（MBP）, amyloid precursor protein（APP）, myelination, soluble APPα（sAPPα）, Chinpi plus α-GPC

increased exclusively in aged Tg2576 mice treated with Chinpi plus α-GPC suggesting sAPPα and MBP (especially the 21.5-KDa isoform) are closely related to maintaining myelination.

Conclusion：These findings suggest that synergetic effects of Chinpi and α-GPC in aged Tg2576 mice may provide new insight for a novel therapeutic approach of AD as well as neurodegenerative disease.

Introduction

Alzheimer's disease (AD) is a type of dementia that is associated with the development of β-amyloid peptide deposits, phosphorylated tau protein aggregates, synaptic loss and neuro-inflammation[1]. Although AD is a multifactorial illness, one of the most widely used working paradigms on the underlying-mechanisms is the amyloid cascade hypothesis[2].

Although the amyloid cascade hypothesis has become the dominant AD model, the recent failures of clinical trials based on it have led to a search for alternative hypotheses for AD in addition to the amyloid cascade[3)4)].

Neuroimaging studies implicated micro-and-macro structural abnormalities in white matter in the risk and progression of AD, suggesting that in addition to the neuronal loss characteristic of the disease, white matter degeneration and demyelination are important pathophysiological features. Myelin loss and the inability of the oligodendrocytes, the cells responsible for the production and maintenance of myelin, to repair myelin damage may be additional features of AD[5)6)]. This suggests that myelin repair can be considered as a potential treatment goal in the setting of progressive degenerative disease.

In a previous study, we demonstrated that oral administration of Ninjin's Yoeito (NYT), a kampo medicine, significantly ameliorated aging and cuprizone-induced demyelination via the promotion of remyelination, suggesting a possible therapeutic strategy for the treatment of multiple sclerosis and other demyelinating diseases[7]. Furthermore, demyelination was associated with aging and was prevented by the myelin protecting herbal medicine, citrus fruit Unshiu, a type of dried orange peel, whose constituents, hesperidin and narirutin, have therapeutic effects against aging-induced demyelination[8].

Myelin membrane integrity is essential for proper functioning of the nervous system and has a high lipid content；therefore, it is vulnerable to lipid metabolism disorders and makes lipid availability a rate-limiting step for myelination[9]. Lipid-rich myelin membranes are also the targets in many CNS diseases. For example, in human patients and rodent models of multiple sclerosis, myelin damage is associated with alterations in lipid composition[10)11)] such as in sulfatide

Abbreviations：APP, amyloid precursor protein；sAPPα, soluble APPα；sAPPβ, soluble APPβ；PAGE, polyacrylamide gel electrophoresis；PBS, phosphate-buffered saline；MBP, myelin basic protein；CNS, central nervous system

and phosphatidylcholine[12]. In addition, phospholipid metabolism is abnormal in AD brain, especially phosphatidylcholine and phosphatidylethanolamine and those precursor choline and ethanol amine were also decreased in AD brain[13].

APP-transgenic mice (Tg2576) generated by Hsiao, *et al.*[14], express human APP 695 that contains the Swedish FAD-APP mutation. In Tg2576 mice, as in other APP transgenic mice, there is evidence that amyloid-β (Aβ) is responsible for aging-related memory decline and mice do not develop Aβ plaques until an advanced age (~18 months) or exhibit neuronal death[14)15]. Of note the length of the myelinated fibers in the hippocampus of Tg2576 mice was significantly shorter than that of their wild type (WT) littermates[16]. It has also been reported that the fiber tract volume decreased in APP PS1 mice due to accelerated aging-related myelin loss[17] and white matter fiber density involving the splenium of the corpus callosum decreased in patients with late-onset AD[18].

Therefore, we selected the aged Tg2576 mice as AD progression model to investigate the myelination status after oral administration of hesperidin and narirutin, which are active constituents of dried peels of the citrus fruit Unshiu[10)19] and α-GPC as a novel and safe therapeutic supplemental drug.

I Materials & Methods

1. Transgenic Mice

Breeding pairs of the hAPP- transgenic mouse line Tg2576 were kindly provided by Dr. Chiba A (Keio University), which were originally obtained from Jackson Laboratories (Bar Harbor, ME). Tg2576 mice were maintained on a C57BL/6xSJL background in which transgene expression is driven by the hamster prion protein promoter[14], Tg2576 mice were examined at the postnatal age of 24 months as both an APP transgenic model and aging-induced demyelination model. Animals were housed in groups of 3-5 animals per cage and separated by sex, with food and water ad libitum, at 23℃, humidity 55% on a 12-hr day/12-hr night cycle.

All animal experimental procedures used in this study were in accordance with the guidelines of the institutional animal care and use committee of Keio University School of Medicine (Approval No. 08071).

2. Reagents and Drug Treatment

Chinpi extract made from dried peels of the citrus fruit Unshiu was reported previously[20]. Hesperidin and narirutin, two key components of Chinpi extracts, (Chinpi contains 2.1% hesperidin plus 1.3% narirutin), and L-Alpha-glyceryl phosphorylcholine (α-GPC), a widely used food supplement, were purchased from Nichiyu Chemical Industry, Ltd. (Tokyo, Japan). Other reagents were purchased from FUJIFILM Wako Pure Chemical Industry, Ltd. (Osaka, Japan) unless otherwise stated.

All experiments were performed using male mice. They were allocated cages/groups for experiments. Each mice (n=5) received Chinpi (19mg/day) or α-GPC (1mg/day) or Chinpi plus α-GPC (20mg/day) in food-paste (mixed-feed) and non-treated (control) food-paste only under controlled condition of temperature (23℃) humidity (55%) and lighting (12h light/dark cycles) for one month, from 24 to 25 months of age, depending on the mean amount in the previous experiments[19]. All supplemental

drugs were administered after approval for medical use by the Ministry of Health, Labour and Welfare of Japan.

3. Antibodies

The following antibodies were used for the experiments：mouse monoclonal anti-β-actin antibody（clone AC-15 ascites fluid）was purchased from Sigma-Aldrich（St.Louis, MO）.

Mouse monoclonal anti-β-amyloid（1-16）antibody（clone 6E10）was exclusively provided by COVANCE（Emeryville, CA）. Mouse monoclonal Anti-β-amyloid（17-24）antibody（clone 4G8）was exclusively provided by COVANCE（Emeryville, CA）. Mouse monoclonal anti-amyloid precursor protein（66-81 of the N-terminus）（clone 22C11）was provided by Chemicon International（Temecula, CA）. Rabbit polyclonal anti-oligomer antibody（A11）was provided by Thermo Fisher（Rockford, IL）. Mouse monoclonal anti-phospho-myelin basic protein（MBP）antibody（clone P12, Upstate, Lake placid, NY）, anti-MBP（rabbit polyclonal, prepared in our laboratory：Akiyama, *et al.*[21]）, mouse monoclonal anti-myelin proteolipid protein antibody（clone PLPC1：Merck Millipore, Temecula, CA）, mouse monoclonal anti-CNP antibody（clone11-5B, Sigma-Aldrich, St. Louis, MO）, rabbit polyclonal anti-α/β tubulin antibody（Cell Signaling Technology, Danvers, MA）, rabbit monoclonal to S100β antibody（ab52642, Abcam Japan, Tokyo）, and rabbit anti-cow glial fibrillary acidic protein（GFAP）antibody（DAKO Japan, Tokyo）were also used.

4. Preparation of Myelin

Mouse brain myelin was prepared as described previously[7)8)]. Briefly, the cerebrum（0.2 g）was homogenized using a teflon homogenizer in 20 volumes（w/v）of 0.32 M sucrose. After centrifugation at 25000 rpm. for 30 min, the crude myelin layers were resuspended in water by homogenization and then washed by repeated centrifugation. The homogenate was once again centrifuged on a 0.85 M sucrose bed and the purified myelin was removed.

5. Electron Microscopy

The cerebrum was fixed with 2.5% glutaraldehyde and then post-fixed with 1% OsO_4. After dehydration in ethanol, the specimens were embedded in Quetol 812（Nisshin EM, Tokyo, Japan）. Ultra-thin sections in 2% uranyl acetate and lead solution were observed using a JEOL 100C electron microscope（JEOL, Tokyo, Japan）, as described previously[8)]. For the G-ratio measurement（the ratio of the axon diameter to the diameter of the axon plus the surrounding myelin）, at least three mice per group were used. Eight to ten micrographs of the corpus callosum（coronal）at the midline and at a high magnification（×10000）were taken for each mouse, and the G-ratios of at least 50 axons were measured. Axons with aberrant morphology, such as myelin reduplication, abnormal splitting of the myelin sheath, vacuolization of the myelin lamellae, or myelin balloon formation, were excluded because such aberrant myelin morphology makes it impossible to evaluate the degree of myelination accurately.

For example, the abnormally large myelin sheath of axons with an abnormal morphology arising from the repetition of unaccomplished remyelination should not be estimated in the same manner as the myelin sheaths of normally developed myelinated axons. An

additional quantitative study was performed to assess the number of myelinated fibers per 400 μm^2 using three mice per group.

6. Western Blot and Immunoprecipitation

Experiments were carried out using aged (25 months) Tg2576 mice (male, n=3) and young (3 months) Tg2576 mice (male, n=3) as a negative control for the aging-induced demyelination. Western blot analysis was performed as previously described[7]. Briefly, mouse brain and myelin membrane lysates were separated by SDS-PAGE, transferred to Immobilon-P membranes (Merck Millipore, Bedford, MA), and probed with antibodies against β-amyloid (6E10) (diluted 1：600 in TBS with 5% skim milk), β-amyloid (4G8) (diluted 1：400 in TBS with 5% skim milk), amyloid precursor protein (22C11) (diluted 1：500 in TBS with 5% skim milk), MBP[22] (diluted 1：500 in TBS with 5% skim milk), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (diluted 1：500 in TBS with 5% skim milk), proteolipid protein (PLP1) (diluted 1：300 in TBS with 5% skim milk). As the protein loading control, homogenate samples were probed with anti-glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH, mouse, Abcam), and β-actin (mouse, Sigma) (diluted 1：1000 in TBS with 5% skim milk).

Bands were quantified by densitometry in Image J software. The difference in the amount of loaded protein among the lanes in a single experiment was normalized to the amount of actin loaded[7].

Immunoprecipitation (IP) was performed as described previously[7]. Briefly, the brain lysates were incubated overnight at 4°C with anti-MBP antibody[21], followed by incubation for 2hr at room temperature (RT) on a rotating shaker with protein A Sepharose (Sigma-Aldrich, St. Louis, MO). After washing, the immunoprecipitated materials were used for SDS-PAGE. The proteins were then electroblotted by semi-dry transfer onto an Immobilon PVDF membrane (Merck Millipore) and the blots were incubated with primary antibodies [anti-APP antibody (22C11) (diluted 1：500 in TBS with 5% skim milk), anti-MBP antibody non-immune rabbit IgG antibody] (diluted 1：500 in TBS with 5% skim milk). Next, the membranes were incubated with alkaline phosphate-conjugated anti-mouse or anti-rabbit IgG (diluted 1：1000 in TBS with 5% skim milk), and developed using NBT and BCIP solution (Sigma-Aldrich, St. Louis, MO).

7. Statistics

Morphological data are shown as the mean±SEM. Differences among groups were analyzed using the *t*-test with Bonferroni correction for multiple comparisons. A value of $p<0.05$ was considered significant. The statistical analysis of immunoblotting data was performed by the closed testing procedure with Dunnett's multiple comparison test and Welch's *t*-test with Bonferroni correction.

Ⅱ Results

1. Chinpi plus α-GPC increases the levels of 21.5 KDa P-MBP expression

Previous studies reported the effects of Chinpi extract on aging-induced demyelination in mice[7]. In this study, we performed additional analyses using aged Tg2576 mice, an APP transgenic model of AD, of Chinpi treatment with or without choline donor L-Alpha - GPC (α-GPC) (which is widely used as a food supplement and generally

considered safe for human use).

For our initial evaluation of the efficacy of the mixture of Chinpi plus α-GPC, immunoblotting analysis of phosphorylated MBP (P-MBP) was performed.

In a previous study, we demonstrated that myelination status is closely related to the levels of phosphorylated MBP, especially to that of the 21.5-KDa isoform, suggested by the specific increase and decrease in 21.5-KDa P-MBP in parallel with aging-induced demyelination[8].

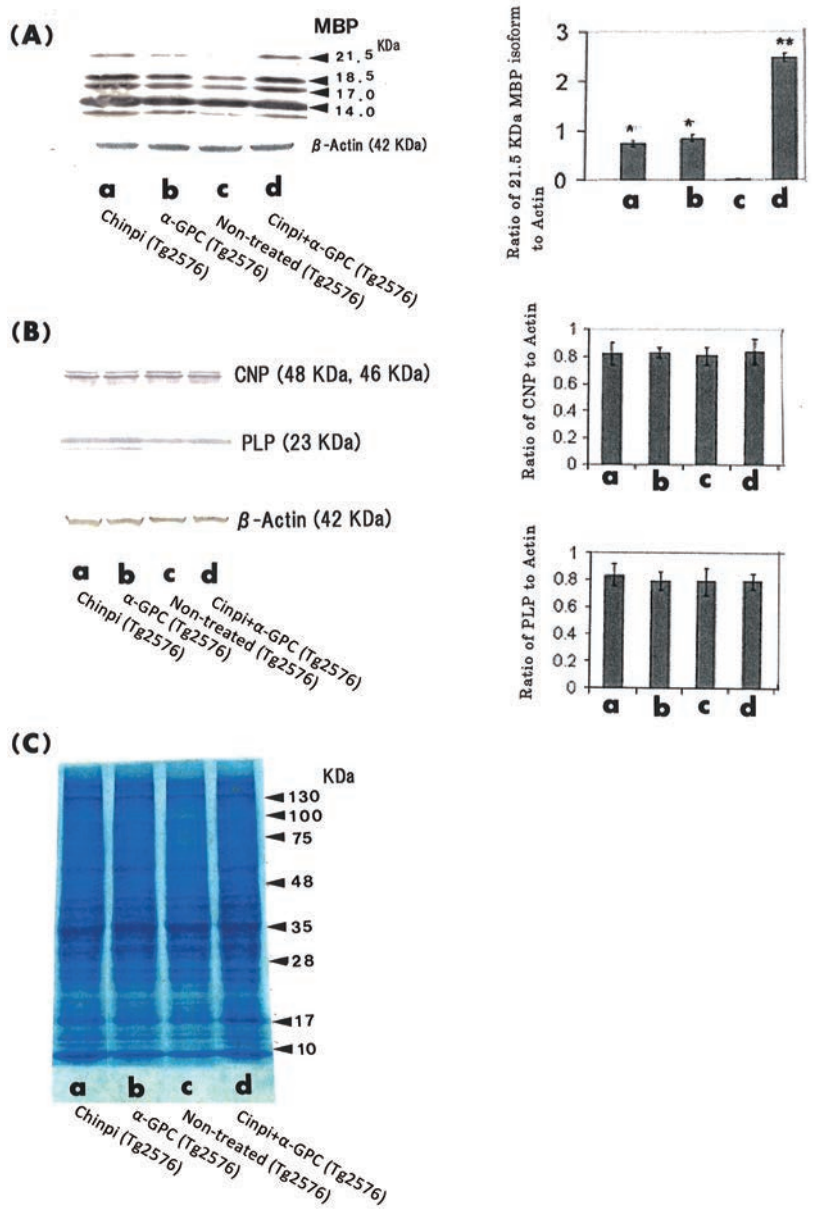
In the present study, the amount of 21.5-KDa P-MBP, which decreased as a result of aging-induced demyelination, increased after treatment with Chinpi (**Figure 1**Aa), α-GPC (**Figure 1**Ab), and the mixture of Chinpi and α-GPC (**Figure 1**Ad). Chinpi plus α-GPC was particularly effective for restoring the 21.5-KDa P-MBP level (**Figure 1**Ad). On the other hand, the 14.0-KDa, 17.0-KDa, and 18.5-KDa isoforms of MBP protein were detected in aged Tg2576 mouse brain myelin with no significant difference after supplement administration (**Figure 1**A). This is consistent with our previous report, which demonstrated that Chinpi promotes remyelination, as effected by the increase in 21.5-KDa P-MBP in aging-induced demyelination[8]. Other myelin component proteins, such as CNPase and PLP, were not affected by Chinpi plus α-GPC (**Figure 1**B). The densitometric quantitative analysis confirmed that Chinpi plus α-GPC strongly restored 21.5-KDa MBP compared with Chinpi or α-GPC treatment, although CNP and PLP were unaffected.

2. The expression amount of GFAP and S100β is not changed with Chinpi plus α-GPC treatment

To define whether astroglial markers such as GFAP which is an intermediate-filament protein[22] and S100β which is a small Ca^{2+}-binding protein member of the S100 family[23], Western blotting analysis were performed using mouse brain homogenates. The expression of GFAP and S100β proteins, enriched in astrocytes and implicated in proliferation morphogenesis of astrocytes did not change after treatment of Chinpi plus α-GPC compared to the single treatment of either Chinpi or α-GPC (**Figure 2**). These suggest that Chinpi and α-GPC may act on oligodendrocyte/myelination.

3. Chinpi plus α-GPC enhances the non-amyloidogenic APP processing pathway

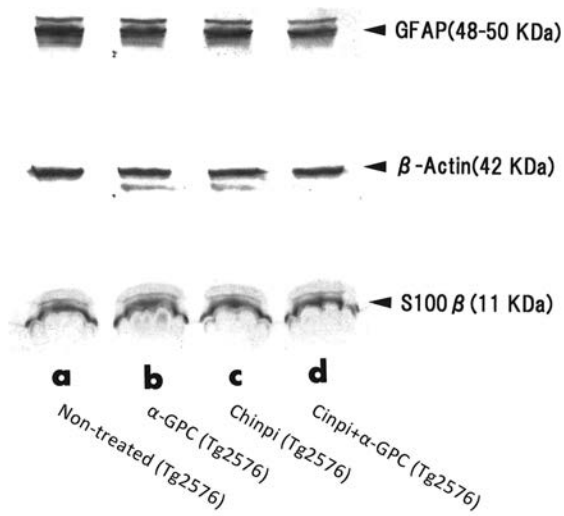
Western blotting analysis of brain homogenates after Chinpi plus α-GPC treatment using anti-Aβ (6E10) antibodies recognizing soluble α-secretase-cleaved brain APP ectodomains (sAPPα) and anti-Aβ (4G8) antibodies recognizing the APP C-terminal fragment (CTF) is shown in **Figure 3**A. The non-amyloidogenic APP processing pathway (sAPPα) was promoted by Chinpi plus α-GPC (**Figure 3**Ad). The N-terminal-specific APP antibody (22C11) recognized both full-length APP (FL-APP) and the soluble α- or β-secretase-cleaved APP ectodomains (sAPPα) (sAPPβ) (**Figure 3**B). Western blotting of the detergent-soluble supernatant of whole brain lysates of aged Tg2576 mice revealed no significant difference in the expression in full-length APP or sAPP after Chinpi plus α-GPC treatment (**Figure 3**B). We detected bands (96 KDa, 95 KDa) that migrated at the size



a：Chinpi, b：α-GPC, c：control, d：Chinpi plus α-GPC
**：P＜0.01, *：P＜0.05（with n＝3 for each condition） Welch's *t*-test

The signal intensity of the 21.5-KDa isoform of phosphorylated MBP was higher in Chinpi plus α-GPC-treated Tg2576 mice than in Chinpi-, α-GPC- or non-treated (control) Tg2576 mice (A). Other components of the myelin sheath proteins, such as proteolipid protein (PLP) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), were not affected by Chinpi plus α-GPC (B). Note that densitometry was performed and levels of each protein of interest were normalized to the β-actin internal control signal, as indicated in the panel on the right. Coomassie brilliant blue (CBB) staining for total proteins (C).

Figure 1 Western blot analysis of brain homogenates focusing on myelin component proteins from Chinpi plus α-GPC-treated aged Tg2576 mice



Note that the signal bands of both GFAP and S100β protein which are used as astrocytes marker proteins were not affected by Chinpi plus α-GPC.

Figure 2 Western blotting of brain homogenates focusing on astrocytes component proteins such as glial fibrillary acid protein and S100β protein from Chinpi plus α-GPC-treated aged Tg2576 mice

expected for sAPP pool (such as sAPPα, sAPPβ) and bands (12KDa, 10KDa) that migrated at the size expected for the fragment, of the APP intracellular domain on immunoblotting with the C-terminal APP antibody (mAb 4G8) recognizing α-CTF (CTFα) and β-CTF (CTFβ) (Melnikova, *et al*. 2013) using lysates of the brain of aged Tg2576 mice (**Figure 3**A, **3**B).

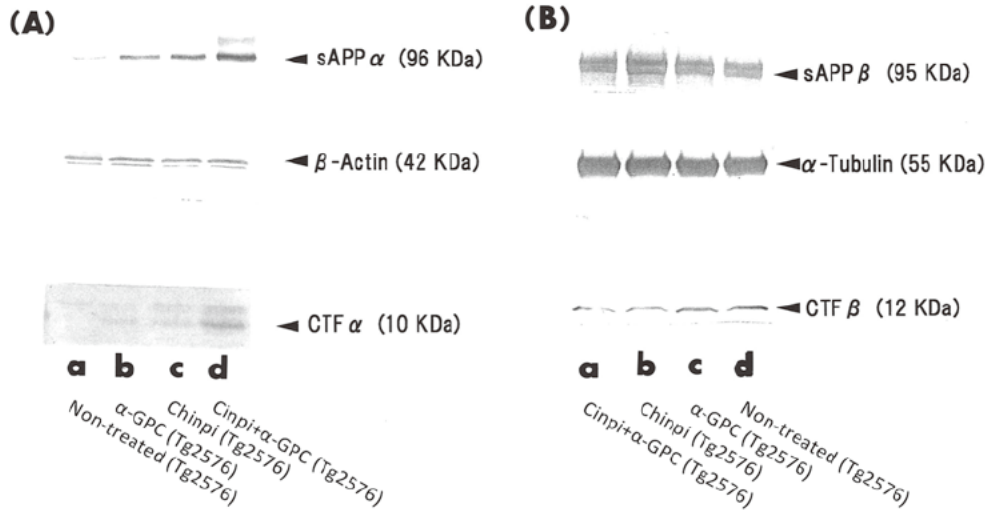
Of note, the non-amyloidogenic APP processing pathway (sAPPα), was promoted and the fragments of the APP intracellular domain and CTFα increased after treatment with Chinpi plus α-GPC (**Figure 3**A), but the expression levels of sAPPβ and CTFβ were not affected by treatment with Chinpi plus α-GPC (**Figure 3**B).

4. 21.5-KDa MBP is a particularly influential protein isoform for APP functions

To investigate the interaction MBP and APP, immunoprecipitation was performed using mouse brain homogenates. MBP (the 21.5-KDa and 18.5-KDa isoforms) coprecipitated with APP in young mice (d), but not in aged mice (c) (**Figure 4**B), and APP of full length was also immunoprecipitated with MBP in young mice (d), but not in aged mice (c) (**Figure 4**A). This suggests that at least two MBP isoforms can bind APP and affect APP processing in the young Tg2576 mouse brain, but not in the aged mouse brain because the exon Ⅱ-containing 21.5-KDa isoform was almost absent[24] (**Figure 1**A).

5. Chinpi plus α-GPC ameliorate the levels of demyelination in aged Tg2576 mice

As shown in electron micrography study



a : control, b : α-GPC, c : Chinpi, d : Chinpi plus α-GPC

a : Chinpi plus α-GPC, b : Chinpi, c : α-GPC, d : control

(A) The signal intensity of both sAPPα and CTFα was higher in Chinpi plus α-GPC-treated mice than in each biologically active ingredient (Chinpi or α-GPC-) or non-treated control mice using anti-Aβ (6E10) antibodies recognizing the soluble α-secretase-cleaved APP ectodomains (sAPPα). Anti-amyloidβ (4G8) antibodies recognizing the C-terminal fragment generated by α-secretase cleavage CTFα (C89).

(B) The expression levels of sAPPβ and CTFβ did not significant change in Chinpi plus α-GPC-treated mice using the anti-APP antibody (22C11), which recognizes the 66-81 amino acid sequence of APP (N-terminus). This epitope is present in all sAPP forms, such as sAPPα and sAPPβ, and their post-translational modification enables defection of the sAPP pool. Anti-Aβ (6E10) antibodies recognizing the C-terminal fragment generated by β-cleavage (CTFβ) and anti-amyloidβ (4G8) antibodies were used.

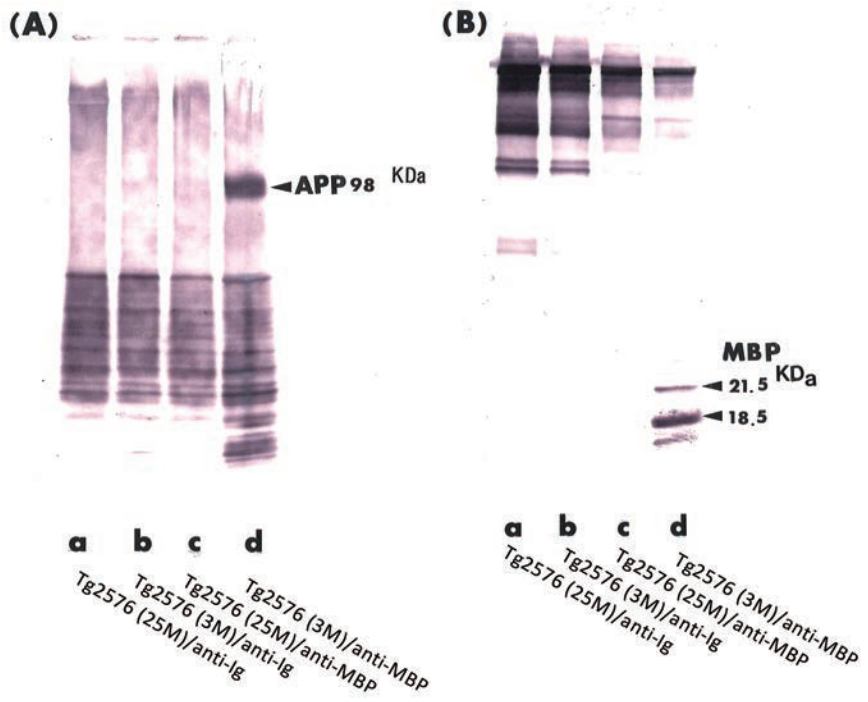
Figure 3 Western blotting of brain homogenates from Chinpi plus α-GPC-treated aged Tg2576 mice (at 25-month-old)

(**Figure 5**), Chinpi plus α-GPC ameliorated the abnormal morphology of myelin sheathes, such as myelin balloon formation, splitting of the myelin sheath, and vacuolization of myelin lamellae, indicative of aging-induced demyelination in aged Tg2576 mice.

Based on quantitative analyses of the G-ratio, the thickness of the myelin sheath increased more after Chinpi plus α-GPC treatment (d) than after a single treatment of Chinpi (b) or α-GPC (c), presumably because of increased remyelination via the synergistic effects of Chinpi and α-GPC, and a marked reduction in abnormal histological findings was observed in the brain of Chinpi plus α-GPC-treated mice, as shown in **Figure 5**.

Ⅲ Discussion

There is increasing evidence supporting the role of oligodendrocytes and myelin in the cellular phase of AD[25]. Transgenic Tg2576 mice are a well-known AD mouse model and are broadly available to the scientific community. Therefore, we selected this model to investigate the effects of MBP on the amyloidogenic pathway in aged Tg2576 mice. Of note the 21.5-KDa isoform was abundant after Chinpi plus α-GPC treatment.



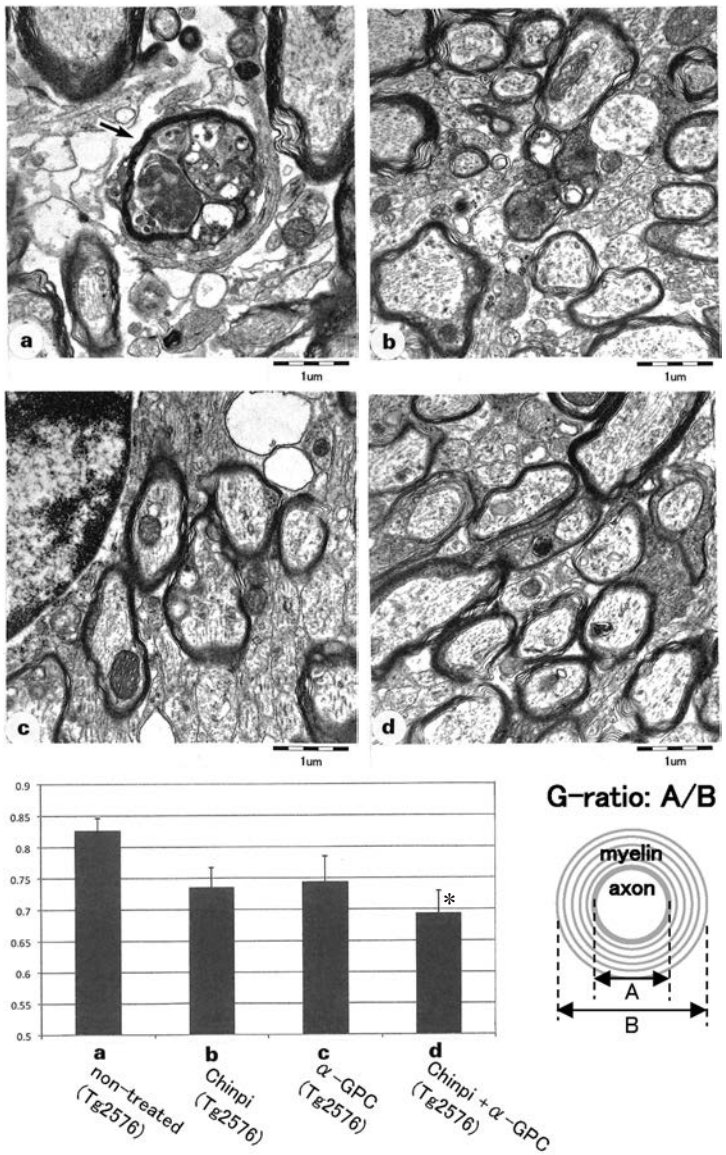
Immunoprecipitation (IP) of young (3 months) (b, d) and aged (25 months) Tg2576 mice brain homogenates (a, c) was performed using the anti-MBP antibody (c, d) or rabbit immunoglobulin antibody (a, b) as a negative control on. WB of IP products with anti-APP antibody (22C11) (A) or anti-MBP antibody (B). Note that the 21.5-KDa and 18.5-KDa MBP isoform bands were immunoprecipitated to a higher degree using the specific antibody against the 21.5-KDa and 18.5-KDa MBP isoforms (mouse monoclonal IgG ; Boehringer Mannheim, Mannheim, Germany) (B-d), and full-length APP was also immunoprecipitated (arrow) (A-d) in young Tg2576 mice. However, APP in aged mice did not bind MBP (21.5-KDa, 18.5-KDa) (A-c, B-c), suggesting no interaction of MBP and APP.

Figure 4 Immunoprecipitation and Western blotting (IP/WB)

We previously reported that the 21.5-KDa isoform is associated with the myelination status[9]. Furthermore, MBP inhibits amyloid fibril formation via its direct binding to and degradation of amyloid through intrinsic protease activity[26], and it promotes endogenous sAPPα release, which has therapeutic potential for remyelination to promote myelin repair following demyelination[27].

sAPPα may also rescue of spatial learning and synaptic plasticity deficits[28][29]. Recently, we reported a significant reduction of sAPPα in the Shiverer (shi/shi) mice brain, although the levels of total APP and sAPPβ both remained unchanged, suggesting that MBP loss inhibited sAPPα release, subsequently causing soluble Aβ oligomers in the brain[30].

Although the role of sAPPα in remyelination has not been fully investigated, myelin is associated with AD because the vulnerability of oligodendrocytes under Alzheimer's pathology easily induces myelin breakdown and loss of the myelin sheath, which may



a：control, b：Chinpi, c：α-GPC, d：Chinpi plus α-GPC

The myelination status improved after one month of Chinpi plus α-GPC treatment. Scale bar＝1μm.

The G-ratio, an indicator of demyelination, which is defined as the ratio of the axon diameter to that of the axon diameter plus the surrounding myelin sheathes, was calculated. The increased G-ratio in the elderly Tg2576 mice was improved by Chinpi plus α-GPC treatment. The number of measured axons was 100, using nine election micrographs from three mice. Data represent the mean±SEM. *：P＜0.01 versus non-treated control. The analyses were performed using 25-month-old Tg2576 mice. Note that myelin reduplication as demyelinating component was observed in aged mice（a, arrow）.

Figure 5 Electron micrographs of-cross sections of the corpus callosum show aging-induced demyelination in aged Tg2576 mice

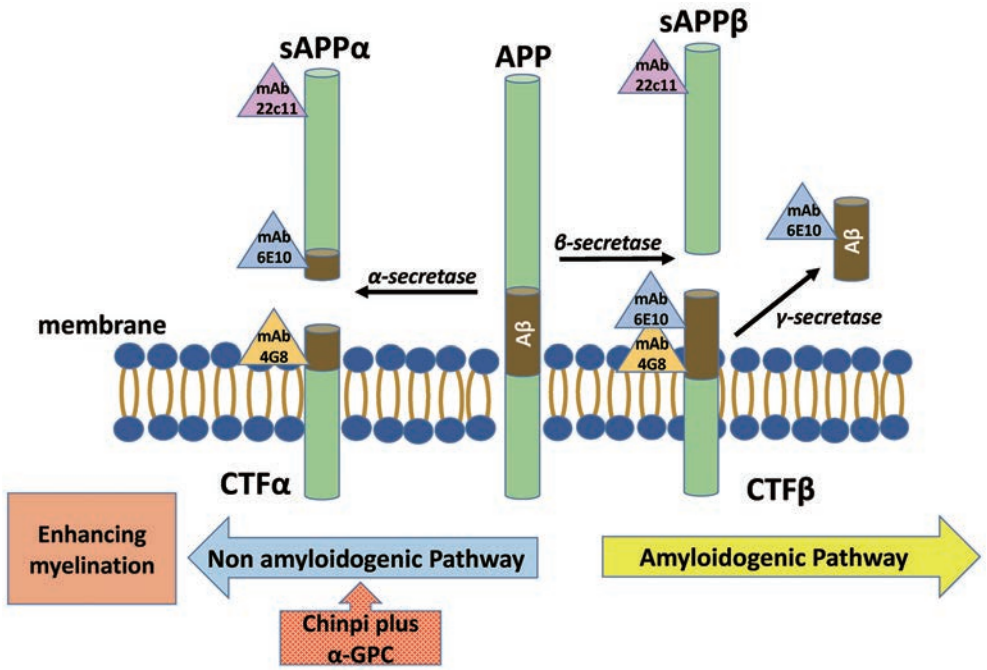
be the initiating step in the earliest stage of AD prior to the appearance of amyloid and tau pathologies[31].

The cognitive decline associated with normal aging was believed to be due primarily to neuron loss. However, recent studies demonstrated that aging-related cognitive decline is associated with changes in the white matter, which may be caused by marked demyelination and loss of oligodendrocytes[32]. Of note, Chen, *et al.*[33] demonstrated that new myelin formation rescues cognitive deficits and hippocampal physiology in a murine model of AD, and MBP-positive areas in the frontal lobe and hippocampus of AD patients significantly decreased compared with age-matched controls. sAPPα levels decrease in many disease states and neurological disorders, including AD, which is involved in impaired or altered plasticity[34][35]. Therefore, the regulation of sAPPα is a key step in many cognitive diseases such as AD[36]. In previous study, we present that the 21.5-KDa MBP isoform may influence α-secretase activity by binding ADAM9 protease[30]. We found in present study that two MBP isoform (21.5-KDa, 18.5-KDa) in young mice, but not old, can bind APP (**Figure 4**). Aging is the most risk factor for AD. MBP dysfunction in aged brain may deeply correlate with pathogenesis AD. Intriguingly, the expression bands of both GFAP and S100β, a marker for astrocytes were not changed by immunoblot analysis after treatment with α-GPC, Chinpi and plus α-GPC (**Figure 2**). These suggest that Chinpi plus α-GPC may have effects for oligodendrocyte lineage.

Although the mechanism of action of Chinpi plus α-GPC in AD patients is not completely understood. Our previous studies demonstrated that hesperidin and narirutin (two of the active constitutions of Chinpi) revealed the significant increase in the ratio of 21.5-KDa isoform of phosphorylated MBP, which was closely related to myelination status, in aging-induced demyelination[21]. Therefore, hesperidin and narirutin may have therapeutic and physically potent effects on aged Tg2576 mice. The present study also supports this notion, as the levels of 21.5-KDa P-MBP increased after treatment with Chinpi plus α-GPC, suggesting a candidate mechanism underlying myelin formation and/or remodeling (**Figure 3, 5, 6**). In addition to the specific decrease and increase in 21.5-KDa P-MBP expression, correlated with the non-amyloidogenic APP processing pathway (sAPPα) and the fragments of APP intracellular domain CTFα, also increased after treatment with Chinpi plus α-GPC (**Figure 3, 6**). Taken together, our study demonstrated that myelination occurs in response to Chinpi plus α-GPC, and Chinpi plus α-GPC may have therapeutic effects against degenerative disorders in which demyelination plays a key role such as AD.

Limitation

The data in the present study is quite limited in the point of aged Tg2576 mice (24-month-old). To clarify this point, the studies in different developmental stages (aged mice), also in some other aged mice model of AD are necessary. In addition, the number of animals is small and analysis of molecular events are in sufficient. We lacked data on clinical studies using supplement drugs. To elucidate the



△ represents recognition site of monoclonal antibodies. 6E10：β-amyloid（1-16), 22C11：APP-N-terminus（66-81), 4G8：β-amyloid（17-24）
CTFα ≒10-KDa, CTFβ ≒12-KDa

The stimulation of non-amyloidogenic processing of amyloid precursor protein by elevated sAPPα after Chinpi plusα-GPC treatment. Elevated sAPPα would then may contribute to myelination correlated with 21.5-KDa P-MBP which would lead to white matter growth.

Figure 6 Proposal diagram of APP processing by the treatment of Chinpi plus α-GPC

therapeutic effect against AD, extensive studies in cooperation with clinical researchers of AD and other myelin disease are necessary. Such studies are currently underway.

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Conflicts of Interest

The authors declare no conflicts of interest.

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原著

老齢アルツハイマー病モデルマウスにおける陳皮とα-GPCの相乗効果

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要　旨

背景：近年アルツハイマー型認知症での認知機能低下にミエリンの崩壊がかかわっているという報告が多くみられるようになってきており，私たちは以前の研究でミエリン膜を構成するミエリン塩基性タンパク質（MBP）を欠損したマウスでは非アミロイド生産系が減少することを報告した。本研究では24カ月齢の老齢アルツハイマー病モデルマウス（aged Tg2576）を用い非アミロイド生産系とミエリン形成に及ぼす陳皮とα-GPC

の相乗効果作用について検討した。

方法：Aged Tg2576マウスに陳皮，α-GPC，陳皮＋α-GPCを混入したねり餌を1カ月間食べさせた後，対照群（non-treated）とあわせ，脳を採取して非アミロイド生産系とミエリン形成に関与するMBPについて調べた。

結果：陳皮にα-GPCを加えた方が陳皮またはα-GPCをそれぞれ単独で投与したときよりも非アミロイド生産系（sAPPα・CTFα）をより強く亢進した。さらにミエリン形成に必須であるMBP 21.5-KDaアイソフォームを増加させたことから非アミロイド生産系には特に21.5-KDa MBPが密接に関与していることが示唆され，これらは陳皮とα-GPCの相乗作用によって一層効果が強くなると考えられた。

結語：Aged Tg2576を用いた今回の結果から，陳皮とα-GPCを組み合わせて投与することでミエリンの再生にさらに大きな効果があることがわかった。この組み合わせで投与することでアルツハイマー病などの“ミエリンロス”がかかわる疾患に有効な治療法になると期待できる。

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