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Comparison of Presenilin 1 and Presenilin 2 γ-Secretase Activities Using a Yeast Reconstitution System*[S]

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γ-Secretase is composed of at least four proteins, presenilin (PS), nicastrin (NCT), Aph1, and Pen2. PS is the catalytic subunit of the γ-secretase complex, having aspartic protease activity. PS has two homologs, namely, PS1 and PS2. To compare the activity of these complexes containing different PSs, we reconstituted them in yeast, which lacks γ-secretase homologs. Yeast cells were transformed with PS1 or PS2, NCT, Pen2, Aph1, and artificial substrate C55-Gal4p. After substrate cleavage, Gal4p translocates to the nucleus and activates transcription of the reporter genes *ADE2*, *HIS3*, and *lacZ*. γ-Secretase activity was measured based on yeast growth on selective media and β-galactosidase activity. PS1 γ-secretase was ~24-fold more active than PS2 γ-secretase in the β-galactosidase assay. Using yeast microsomes containing γ-secretase and C55, we compared the concentration of Aβ generated by PS1 or PS2 γ-secretase. PS1 γ-secretase produced ~24-fold more Aβ than PS2 γ-secretase. We found the optimal pH of Aβ production by PS2 to be 7.0, as for PS1, and that the PS2 complex included immature NCT, unlike the PS1 complex, which included mature NCT. In this study, we compared the activity of PS1 or PS2 per one γ-secretase complex. Co-immunoprecipitation experiments using yeast microsomes showed that PS1 concentrations in the γ-secretase complex were ~28 times higher than that of PS2. Our data suggest that the PS1 complex is only marginally less active than the PS2 complex in Aβ production.

γ-Secretase consists of at least four subunits, presenilin (PS),[3] nicastrin (NCT), anterior pharynx defective 1 (Aph1), and presenilin enhancer 2 (Pen2) (1). PS is the catalytic subunit of γ-secretase with aspartic protease activity (2, 3). Amyloid-β (Aβ) peptide, which plays a causative role in Alzheimer disease (AD), is produced after sequential cleavage of amyloid-β precursor protein (APP) by β-secretase and γ-secretase. The Aβ mainly consists of Aβ40 and Aβ42 containing 40 and 42 amino acids, respectively. Aβ42 is more prone to aggregation (4) and more toxic to neuronal cells. Many studies have reported that familial AD (FAD) mutations in PS and APP result in increased ratios of Aβ42 to Aβ40. The high Aβ42 ratio is believed to lead to AD.

PS has two homologs, namely, PS1 and PS2 (67% identical at the amino acid level). Aph1 also has two homologs: Aph1a (with alternative splicing variants Aph1a-S and a-L) and Aph1b. Sato *et al.* (5) reported that γ-secretase contained only one of each subunit, and as such, six distinct γ-secretases exist. Indeed, both PS1 and PS2 form a γ-secretase complex with the other subunits, producing Aβ (6). γ-Secretase cleaves many type I transmembrane proteins including APP and Notch, but the mechanism by which the different γ-secretases select their substrates is unclear. These different γ-secretases may have different functions and substrate selectivity.

Ubiquitous expression of PS1 and PS2 mRNAs in many human and mouse tissues has been reported, with varying expression levels across their tissues and during brain development (7). For example, in human young adult and aged brains, PS1 and PS2 mRNAs expression was similar. The subcellular distribution of PSs are known to be predominantly in the endoplasmic reticulum and the Golgi compartment (8). Levitan *et al.* (9) showed that human PS1 and PS2 substituted for *Caenorhabditis elegans* sel-12, suggesting that PS1 and PS2 are functionally redundant.

Different phenotypes of PS1- and PS2-deficient mice have been reported. PS1 knock-out mice exhibit severe developmental defects and perinatal lethality (10, 11), whereas PS2 knock-out mice show only mild phenotypes (12). Over 160 FAD mutations in PS1, but only 10 in PS2, have been found. These findings suggest that PS1 and PS2 play distinct roles *in vivo*.

Lai *et al.* (13) indicated that Ps1 (Ps, mouse presenilin) γ-secretase produced 169 times more Aβ than Ps2 γ-secretase, using membrane fractions from Ps1-(+/−), Ps2-(−/−), and Ps1-(−/−), Ps2-(+/+) blastocyst-derived cells from knock-out mice. In their study, γ-secretase activity was calculated as follows: level of produced Aβ/total Ps. They did not use the calculation: level of produced Aβ/Ps in γ-secretase complex and thus did not evaluate the active γ-secretase content.

Yagishita *et al.* (14) developed a novel γ-secretase assay using yeast microsomes. Yeast lacks endogenous γ-secretase and

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[3] The abbreviations used are: PS, presenilin; APP, amyloid precursor protein; Aβ, amyloid β peptide; Aph1, anterior pharynx 1; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; CTF, carboxyl-terminal fragment; NCT, nicastrin; NTF, amino-terminal fragment; PC, phosphatidyl choline; Pen2, presenilin enhancer 2; FAD, familial Alzheimer disease; TM, transmembrane domain.

APP homologs, and one can reconstitute pure human γ-secretase in yeast and estimate the activity. Using this system, we compared the activity of PS1 and PS2 in γ-secretase complexes. Our data suggested that PS1-containing microsomes had much higher activity than PS2-containing microsomes. However, detailed analysis regarding the "active" γ-secretase complex revealed that the PS1 and PS2 complex produced similar levels of Aβ.

MATERIALS AND METHODS

Construction of γ-Secretase and Substrates—To reconstitute γ-secretase in yeast, human PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and substrates were cloned into the following vectors, as described previously (15). Briefly, PS1 or PS2 and NCT were ligated into KpnI and XbaI sites of the pBEVY-T vector (16). Aph1a-L-HA and FLAG-Pen2 were ligated into the XbaI and KpnI sites of pBEVY-L (16). C55-Gal4p, NotchTM-Gal4p, and C99 were fused to the *SUC2* signal sequence, facilitating translocation to the endoplasmic reticulum, and ligated into the BamHI and EcoRI sites of p426ADH (17). C55, C99, and NotchTM indicate amino acids 672–726 of the human APP770 isoform, 672–770 of the human APP770, 1703–1754 of the mouse Notch-1, respectively.

Myc-tagged PS1 and PS2 were PCR amplified and ligated into the KpnI site of pBEVY-T, using the following two pair of primers, respectively: mycPS1S, 5′-GGGGTACCAAAAATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGACAGAGTTACCTGCACCGTTG-3′ and PS1AS, 5′-GATCCGCTTATTTAGAAGTGTCGAATTCGACCTCGGTACCATGCTAGATATAAAATTGATGGAATGC-3′; mycPS2S, 5′-GGGGTACCAAAAATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGCTCACATTCATGGCCTCTGAC-3′ and PS2AS, 5′-GGGGTACCTCAGATGTAGAGCTGATGGGAGG-3′.

Yeast Transformation—Three plasmids were transformed into *Saccharomyces cerevisiae* strain PJ69–4A (*MATa, trp1–901, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ*) (18). The transformants were selected on SD media plate lacking Leu, Trp, and Ura (SD-LWU). In microsome assays, we used the yeast strain PJ69–4Apep4Δ(*MATa, trp1–901, leu2–3, 112, ura3–52, his3–200, gal4Δ, gal80Δ, LYS2::-GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ, pep4::kanMX*) (14) to avoid endogenous protease activity.

Reporter Gene Expression—Expression of *HIS3* (His) and *ADE2* (Ade) was estimated by transformant growth on SD-LWHUAde. β-Galactosidase assays were performed as described previously (15). Transformants were cultured in SD-LWU media until they reached an A_{600} of ~0.8. Cells were collected after centrifugation and suspended in lysis buffer (20 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol) including protease inhibitor mixture (Sigma), and lysed by glass beads. Protein concentration and β-galactosidase activity of the cell lysates were determined.

γ-Secretase Assay and Immunoblotting—Using yeast microsomes, we detected Aβ using an in vitro γ-secretase assay. *In vitro* γ-secretase assays were performed as described previously, with minor modifications (14). Microsomes (80 μg) were solubilized with γ-buffer (50 mM MES (pH 5.5) or 50 mM PIPES (pH 6.0, 6.5, 7.0, 7.5), or 50 mM HEPES (pH 8.0), 250 mM sucrose, 1 mM EGTA) containing 1% CHAPSO on ice for 60 min. Inhibitor mixture, thiorphan, *O*-phenanthroline, CHAPSO, and γ-buffer were added to the solubilized microsomes, as described previously (14). The mixture was incubated at 37 °C for 0 or 24 h. After incubation, the sample was extracted with chloroform/methanol (2:1) followed by addition of sample buffer, and boiled at 100 °C for 5 min. Aβ production was analyzed by Western blotting using the specific antibody, 82E1. Band signal was quantified using an LAS-3000 luminescent image analyzer (FujiFilm, Tokyo, Japan).

Immunoprecipitation of γ-Secretase—Microsomes (400 μg) were solubilized with IP buffer containing 1% CHAPSO and protease inhibitor mixture, on ice, for 60 min. Solubilized membranes were added to 40 μl of anti-FLAG affinity gel (50% slurry) (Sigma) and rotated at 4 °C for 2 h. Beads were washed with IP buffer and suspended in sample buffer containing 8 M urea to prepare the "IP sample" from 400 μg of microsomes. The "input sample" was prepared as follows: 100 μl of sample buffer containing 8 M urea was added to 80 μg of microsomes and incubated at 65 °C for 10 min. Microsomes (8 μg, 10–11 μl) were loaded as input.

Antibodies—The following antibodies were used for immunoblotting: monoclonal antibodies against Aβ, 82E1 (IBL, Fujioka, Japan), HA (12CA5; Sigma), FLAG (M2; Sigma), and polyclonal antibodies against NCT (AB5890; Chemicon, Temecula, CA), Myc, 2272 (Cell Signaling Technology, Beverly, MA), the PS1 loop region (G1L3) (19), and the PS2 loop region (G2L) (20).

RESULTS

PS2 Was Less Active than PS1 in Growth and β-Galactosidase Assays—We constructed recombinant plasmids for γ-secretase and APP-based (C55-Gal4p) or Notch-based substrates (NotchTM-Gal4p) (15). We introduced the vectors into yeast strain PJ69, which expresses *HIS3*, *ADE2*, and *lacZ* under Gal4p control, and generated yeast transformants expressing the γ-secretase subunits (PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2) and an artificial substrate (C55-Gal4p or NotchTM-Gal4p). Gal4p released from C55-Gal4p or NotchTM-Gal4p by reconstituted γ-secretase activates *HIS3* and *ADE2* genes transcription. Therefore, γ-secretase activity was assessed by growth on media lacking histidine and adenine. As a result, yeast expressing PS1 γ-secretase and C55-Gal4p could replicate on the selection media. Yeast expressing PS2 γ-secretase could also grow, but was much slower than that of PS1-expressing yeast (Fig. 1*A*). PS1 L166P, G384A, and PS2 N141I are familial Alzheimer disease (FAD) mutations. Yeast carrying these mutations were unable to grow on media lacking histidine and adenine. After isolating these yeast cell lysates, we measured β-galactosidase activity to estimate γ-secretase activity. PS1 had ~24 times more β-galactosidase activity than PS2 (Fig. 1*B*). The results of the β-galactosidase assay were well correlated with the growth assay results (Fig. 1, *A* and *B*).

Next, we used NotchTM-Gal4p as a substrate instead of C55-Gal4p. The results were similar to those obtained when using

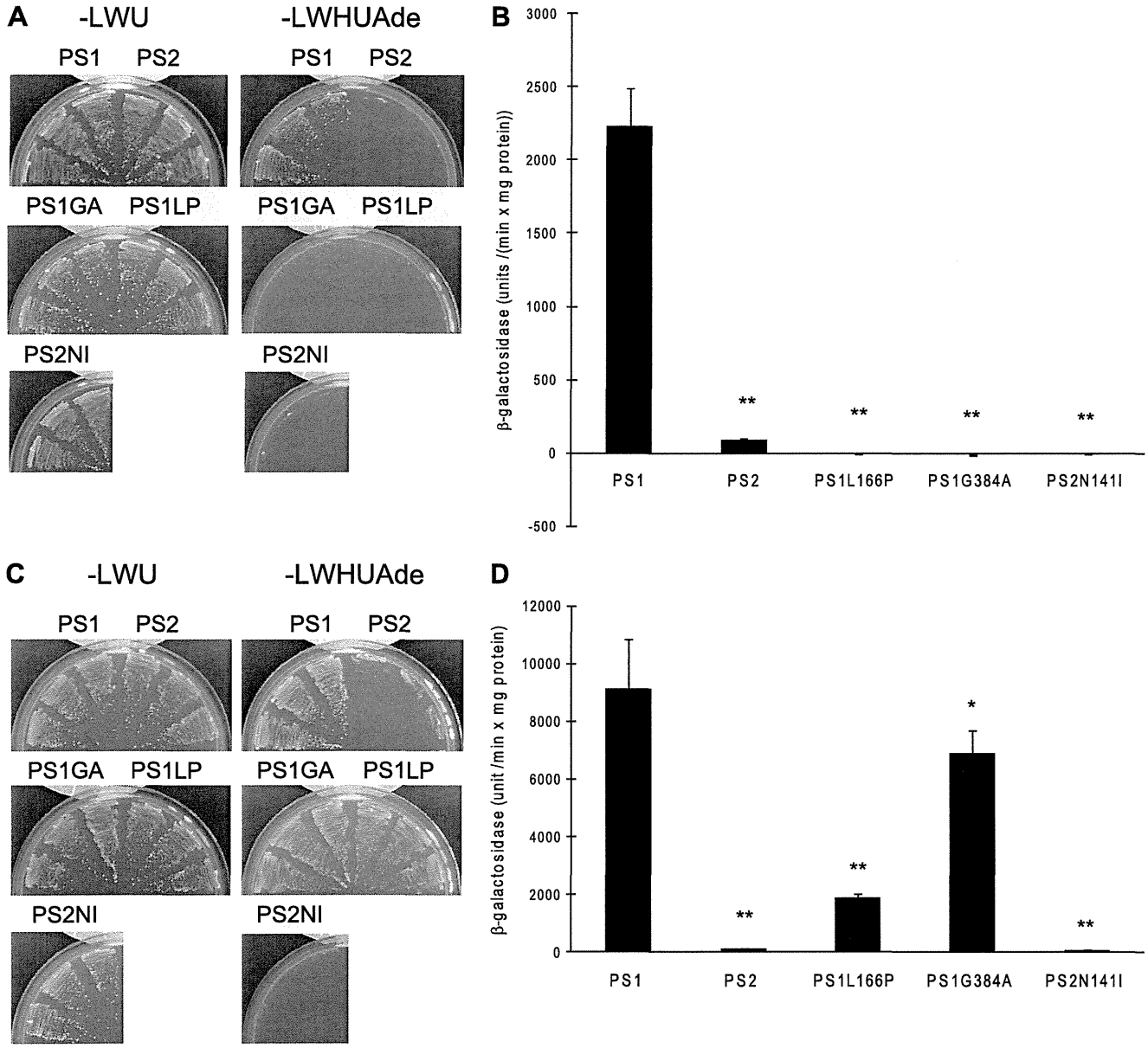

FIGURE 1. **Estimate of reconstituted PS1 or PS2 γ-secretase activity in yeast.** *A* and *C*, yeast cells were transformed with PSs (PS1 or PS2, or PS with FAD mutations), NCT, FLAG-Pen2, Aph1a-L-HA, and C55-gal4p (*A*), or NotchTM-gal4p (*C*). Three independent clones were cultured on non-selection media (SD-LWU) or selection media (SD-LWHUAde) at 30 °C for 3 days. Yeast cells not expressing PS did not grow on SD-LWHUAde. *B* and *D*, β-galactosidase activity was measured for each yeast lysate. Lysates were prepared from yeast cells using glass beads. One unit of β-galactosidase activity corresponds to 1 nmol of *O*-nitrophenyl β-d-galactopyranoside hydrolyzed per min, and activity was calculated as unit/(min × mg of protein in lysate). The activity was normalized by subtracting the activity in the absence of PS, 65 unit/(min × mg protein). Data are presented as mean value ± S.D., $n = 18$ (*A*), $n = 3$ (*C*) *, $p < 0.05$; **, $p < 0.01$ (analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test). Statistical analyses were performed with PRISM software.

the C55-Gal4p, with the following two exceptions. Notch1 was more likely to be cleaved by γ-secretase than C55 (APP) (Fig. 1, *B versus D*) and yeast cells expressing PS1 with FAD mutations (L166P and G384A) were able to grow on SD-LWHUAde, whereas cells expressing PS2 N141I were not (Fig. 1*D*). These results suggested that PS1 with the FAD mutations cannot cleave APP, whereas they can cleave Notch like wild-type γ-secretase.

Optimal pH for Aβ Production by the PS2 Complex—To study γ-secretase activity *in vitro*, we prepared yeast microsomes from yeast transformants expressing PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C55 (14). Three previous reports showed that γ-secretase with PS1 maximally produced Aβ at approximately pH 7.0 (14, 21, 22). The optimum pH of Aβ production by γ-secretase with PS2, however, remains unclear. Thus, we investigated the optimal pH of the PS2 complex to produce Aβ. When yeast microsomes prepared from three independent clones were incubated for 24 h at 37 °C with 0.25% CHAPSO and 0.1% PC, we found that the PS2 complex also maximally produced Aβ at approximately pH 7.0 in all three assays (Fig. 2, *A* and *B*), suggesting that the PS1 and PS2 complex have similar pH dependences for Aβ production.

Levels of Aβ Production by PS1 or PS2—We compared the level of Aβ produced by PS1 or PS2 using yeast microsomes. Each microsome was incubated at 37 °C for 24 h in the pres-

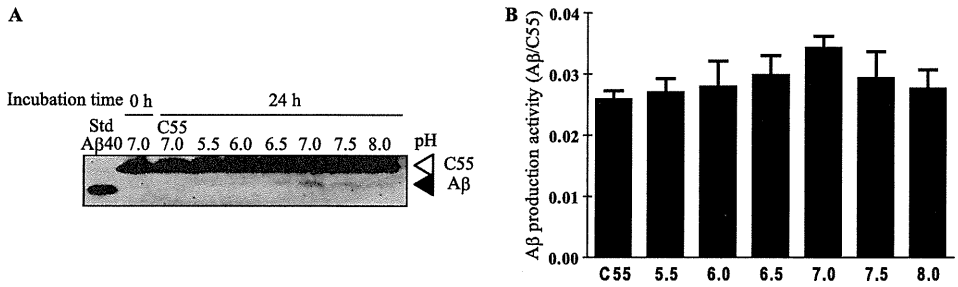

FIGURE 2. **Optimum pH of Aβ production by PS2.** *A*, microsomes (80 μg) prepared from three independent yeast cells transformed with PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C55, and from yeast expressing C55 were incubated with 0.25% CHAPSO and 0.1% PC at 37 °C for 0 or 24 h. Incubation samples were subjected to immunoblotting to compare Aβ production activity, Aβ/C55. Aβ was detected by 82E1. Synthetic Aβ40 (20 pg) was used as a positive control. Yeast expressing C55 and microsomes incubated for 0 h were loaded as a negative control. *B*, three independent assays were quantified using analyzing software (LAS-3000 luminescent image analyzer, Fuji Film, Tokyo, Japan). The column represents the mean ± S.D. ($n = 3$).

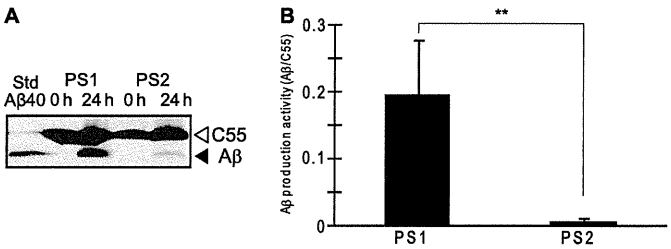

FIGURE 3. **Difference in Aβ production between PS1 and PS2.** *A*, yeast microsomes expressing PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C55 were subjected to *in vitro* γ-secretase assays at pH 7.0. Aβ produced by PS1 or PS2 γ-secretase was detected. Synthetic Aβ40 (30 pg) was loaded as a marker. *B*, the bands obtained in *A* were quantified to determine the ratio of Aβ to C55 using analyzing software (LAS-3000 luminescent image analyzer, Fuji Film, Tokyo, Japan). The column represents the mean ± S.D. ($n = 5$, **, $p < 0.01$). Data were analyzed by Student's *t* test.

ence of 0.25% CHAPSO and 0.1% PC. We found that the PS1 complex produced significantly more Aβ than PS2 (Fig. 3*A*). By quantifying the Western blotting signals, we calculated that PS1 produced ~24 times more Aβ than PS2 (Fig. 3*B*).

PS1 Complexes Were More Abundant than PS2 Complexes—To verify whether PS, NCT, Aph1a-L, and Pen2 form the γ-secretase complex, we isolated membrane fractions from yeast introduced with PS, NCT, Aph1a-L-HA, FLAG-Pen2, and C99, and performed co-immunoprecipitation experiments with the anti-FLAG M2 affinity gel. Both PS1 and PS2 were co-immunoprecipitated with FLAG-Pen2 (Fig. 4, *B* and *C*). NCT and Aph1a-L were also co-immunoprecipitated with FLAG-Pen2 (Fig. 4*A*), suggesting that PS1 and PS2 formed a γ-secretase complex. We also found that the PS2 complex predominantly included non-glycosylated immature NCT, whereas the PS1 complex contained highly glycosylated mature NCT (Fig. 4*A*).

Comparison of the PS1 and PS2 contents in γ-secretase is difficult due to the variable affinity of their specific antibodies. To estimate the amount of PS1 or PS2 in γ-secretase complexes, we constructed Myc-tagged PS1 and PS2. We introduced these constructs into yeast and reconstituted the γ-secretases. Preparing these microsomes, we immunoprecipitated γ-secretase complexes with anti-FLAG affinity gel. The immunoprecipitates were next subjected to immunoblotting. Aph1a-L levels in the PS1 or PS2 complex were similar (Fig. 5*A*). The Myc-tagged PS1 complex included mainly mature NCT, while Myc-tagged PS2 complexes contained immature NCT (Fig. 5*A*). The level of PS1 NTF in γ-secretase complexes (associated with FLAG Pen2) was ~28 times higher than that of PS2 NTF (Fig. 5*B*).

When calculating γ-secretase activity per one γ-secretase complex from these data, a significant difference between PS1 and PS2 does not exist. However, the PS1 complex was 24.15 more active in the β-galactosidase assay. *In vitro* Aβ production assays indicated that PS1 was 24.61 more active than PS2. Comparing PS1 and PS2 contents in γ-secretase in a co-immunoprecipitation experiment, we found that the amount of PS1NTF in the γ-secretase complex was 28.14 times higher than that of PS2NTF. These data suggested that the complete PS2 complex was 1.142 or 1.143 times more active than the PS1 complex.

DISCUSSION

γ-Secretase assays measuring released Aβ into conditioned media from cultured cells have been previously performed. These assays found that γ-secretase with PS FAD mutations increased the Aβ 40/42 ratio. However, very few *in vitro* assays have been reported. To accurately study γ-secretase activity, Yagishita *et al.* (14) established an *in vitro* assay system using yeast, which possesses no γ-secretase homologs. This system enabled us to directly compare activities between the PS1 and PS2 complex.

Yeast growth and β-galactosidase assays using C55-Gal4p or Notch-Gal4p as a substrate revealed that PS1 had a significantly higher activity than PS2. We also found that FAD mutations in PS abolished APP processing activity, and that PS1 L166P and G384A cleaved Notch with reduced activity compared with wild-type PS1. The assembly of PS1 FAD mutants (L166P or G384A) into γ-secretase complex was also assessed by immunoprecipitation (supplemental Fig. S1). The assembly of PS1 L166P mutant was similar to PS1 WT. On the other hand, ~36% of PS1 G384A (comparing to the WT) formed the γ-secretase complex. These results showed that PS1 L166P assembled normally with defective protease activity and PS1 G384A was defective both in the assembly and the protease activity, suggesting that loss of function of PS caused lower cleavage activity. These reductions in processing activity obtained in this report support PS loss of function hypothesis, which is believed to cause FAD (23). We evaluated the activity of other PS1 FAD mutations (A79V, M146L, A231V, M233T, and ΔExon9) in Notch cleavage (data not shown). Our Notch

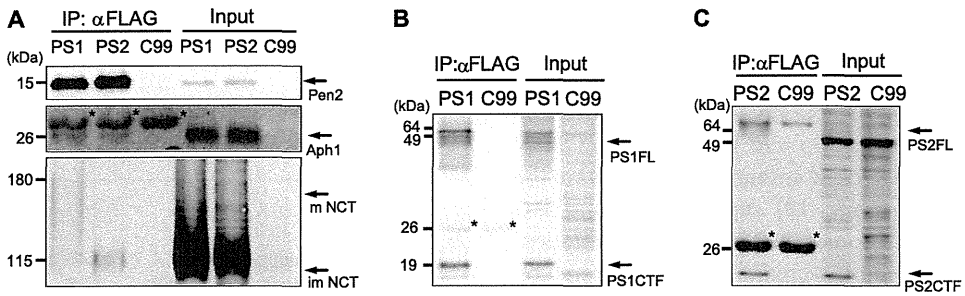

FIGURE 4. **Formation of PS1 and PS2 γ-secretase complexes.** Yeast microsomes expressing PS1 or PS2, NCT, Aph1a-L-HA, FLAG-Pen2, and C99, and microsomes expressing C99 were solubilized with IP buffer containing 1% CHAPSO and protease inhibitor mixture. γ-Secretase complexes were immunoprecipitated with anti-FLAG affinity gel (Sigma). The immunoprecipitates and input fraction were subjected to immunoblotting. NCT, Aph1, Pen2, and PS were detected by specific antibodies. The *asterisks* indicate nonspecific bands.

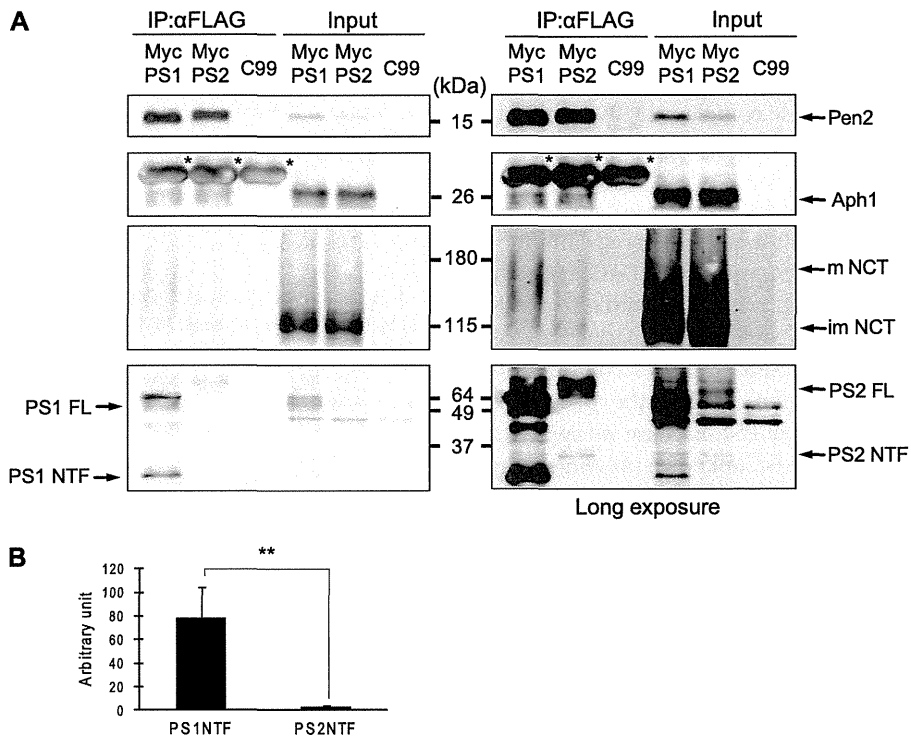

FIGURE 5. **Quantification of PS1 and PS2 in γ-secretase complexes.** *A*, yeast expressing Myc-tagged PS1 or PS2, the other secretase subunits, and C99, were incubated with anti-FLAG affinity gel. The immunoprecipitates were analyzed by immunoblotting. *B*, amount of Myc-tagged PS1NTF and Myc-tagged PS2NTF in the γ-secretase complexes were quantified using LAS-3000 luminescent image analyzer (Fuji Film, Tokyo, Japan). Data were analyzed by Student's *t* test. Error bar shows the mean ± S.D. $n = 4$, **, $p < 0.01$. The *asterisks* indicate nonspecific bands.

cleavage results with PS1 FAD mutations, PS1L166P and G384A, corroborated the findings of earlier studies (24, 25).

Based on the *in vitro* γ-secretase assay using yeast microsomes, we found that γ-secretase with PS2 optimally produced Aβ at approximately pH 7.0. Previous reports have shown that PS1 also maximally produced Aβ at pH 7.0 (14, 21, 22), suggesting that PS1 and PS2 make Aβ using a similar mechanism.

Our co-immunoprecipitation experiments using yeast microsomes containing PS1 or PS2, NCT, Aph1a-L-HA, and FLAG-Pen2 showed that PS2 bound to immature NCT, whereas PS1 bound to the mature NCT. Expression levels of immature or mature NCTs in cells transformed with PS1 or PS2 were similar, but the anti-FLAG affinity gel immunoprecipitates contained different levels of immature and mature NCT. Frånberg *et al.* (26) reported that Ps2 bound to immature NCT in Ps1-deficient (Ps1-(−/−), Ps2-(+/+)) MEF cells and Ps1 bound to mature NCT in Ps2 deficient (Ps1-(+/+), Ps2-(−/−)) MEF cells using affinity capture with an active site-directed γ-secretase inhibitor. This difference in NCT maturation in the complex may affect substrate affinity.

In this study, we used Aph1a-L as a γ-secretase subunit, which may facilitate PS2 binding to immature NCT. Also, Aph1a-S expression, or Aph1b as a γ-secretase subunit, may result in alternative binding patterns, such as PS2 binding to mature NCT or PS1 binding with immature NCT. In fact, we observed the PS1 complex with Aph1a-S containing more immature NCT than the PS1 complex with Aph1a-L (data not shown). To date, γ-secretase is known to target many substrates, but how γ-secretase selects its substrates is unclear. These variable γ-secretases may contribute to specific substrate selection.

To compare the γ-secretase activity of PS1 and PS2 precisely, we employed two different approaches. First, we used C55(-Gal4p) or C99 as a substrate instead of C100Flag. NCT

plays a role in binding to the substrate by recognizing N terminus of C99 (27). So, natural N terminus of C99 or C55 is important to assess γ-secretase activity correctly. Using C100Flag as a substrate may result in inaccurate evaluation, because C100Flag possesses one extra amino acid, methionine, on the N terminus. Second, we estimated the amount of PS1 or PS2 in the γ-secretase complex. Lai *et al.* (13) reported Ps1 and Ps2 γ-secretase activity as a function of total protein concentration, but not all PS localizes to the γ-secretase complex. Therefore, γ-secretase activity should be calculated as follows: γ-secretase activity/concentration of PS in γ-secretase complex. γ-Secretase assembly is not a random process, but occurs sequentially. NCT and Aph1 form the NCT-Aph1 subcomplex in the initial step of complex formation. Two hypotheses have been proposed regarding the subsequent steps in γ-secretase complex assembly. One hypothesis is that PS binds to the NCT-Aph1 subcomplex, followed by Pen2, creating a γ-secretase complex (28, 29). Alternatively, the PS-Pen2 intermediate may bind to the preexisting NCT-Aph1 subcomplex to form the γ-secretase complex (30). To evaluate the construction process of the γ-secretase complex, we compared PS1 or PS2 in the γ-secretase complex by co-immunoprecipitating Myc-tagged PS1 or PS2 with anti-FLAG antibody (FLAG tag is on Pen2). Co-immunoprecipitation with other antibodies detecting NCT, Aph1, or PS could lead to inaccurate estimates regarding the amount of Myc-PS in the γ-secretase complex. We found that the concentration of PS2 in the γ-secretase complex was much lower than that of PS1. Because we applied a minimal reconstitution system in yeast, unknown protein(s) may stabilize PS2. This possibility is currently being explored.

In this study, we reconstituted human PS1 and PS2 γ-secretase complexes and compared their Aβ production (per γ-secretase complex). PS1 had 24.65 times and 24.61 times higher activity than PS2 in the β-galactosidase and *in vitro* Aβ production assay, respectively. Based on Co-IP experiments, the amount of PS1 in the γ-secretase complex was 28.14 times higher than that of PS2. Thus, our data suggest that PS1 did not have significantly higher activity than PS2, as has been reported (13). PS1 and PS2 were 67% identical at the amino acid level, suggesting that these two proteins have related functions in the γ-secretase complex. Our results suggest that the difference between PS1 and PS2 is their affinity to the other γ-secretase subunits. The contribution of PS1 on γ-secretase activity is more important than that of PS2 because PS1 knock-out mice exhibit severe phenotypes, whereas PS2 knock-out mice do not. We hypothesize that the differences in PS1 and PS2 knock-out mice phenotypes may result from different amounts of PS1 and PS2 γ-secretases, but not differences in their activity.

Currently, PS1 is believed to have a higher activity than PS2 in γ-secretases, while we showed that they have similar activities. In corroboration of our findings, recent reports have shown that PS2 γ-secretase cleaved more APP than PS1 γ-secretase in microglia cells, regardless of the presence of PS1 (31). Thus, when studying γ-secretase activity, we should consider the concentration of PS in the active γ-secretase complex, which may aid in clarifying the pathogenesis of FAD caused by PS loss-of-function FAD mutations.

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Communication

Production of Anti-Amyloid β Antibodies in Mice Fed Rice Expressing Amyloid β

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The main signs of Alzheimer's disease (AD) are cognitive impairment and senile plaques composed of amyloid beta (Aβ) observed in patients' brains. Therefore, therapy for AD focuses on the removal of Aβ. We developed an "edible vaccine" that employs intestinal immunity with little to no side effects. Rice was utilized as an edible vaccine. It expressed GFP-Aβ42. Aβ rice was administered orally to wild-type (WT) mice causing production of anti-Aβ antibodies. Since Aβ rice was mixed with the cholera toxin B subunit (CTB), antibody against the rice seed protein was also produced. Then, mice were caused to develop immune tolerance against the rice seed protein by oral administration of Aβ rice mixed with CTB. The results indicated that only anti-Aβ antibodies were produced.

Key words: Alzheimer's disease; amyloid beta; an edible vaccine; immune tolerance

The main signs of Alzheimer's disease (AD) are cognitive impairment, amyloid beta (Aβ)-containing senile plaques, and neurofibrillary tangles composed of tau observed in a patient's brain.[1] Aβ is generated by proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases. The main species of Aβ are Aβ40 and Aβ42. The longer Aβ42 has a greater propensity to aggregate than the shorter Aβ40.[2]

In AD model mice, cognitive impairment was improved by inhibiting senile plaque formation by means of antibody production stimulated by vaccination.[3–5] Vaccination is an antibody therapy that works by timely production of antibodies against the proper antigen. However, in a clinical trial in which Aβ42 peptide was administered to humans, meningoencephalitis was seen as a side effect and the clinical trial was discontinued.[6] Therefore, a therapy focusing on a mild antigen–antibody reaction is needed. We developed an edible vaccine that induced mild antigen–antibody reaction by oral administration of genetically modified plant-integrated Aβ.

Edible vaccines expressing various antigens have been developed. One benefit of edible vaccines is that they can be maintained at room temperature, whereas peptide vaccines must be preserved at cold temperatures. Furthermore, edible vaccines can eliminate the injection pain associated with traditional vaccines.[7] They act by stimulating the lymphatic immune system located in the intestines and suppressing the inflammatory Th1 response and enhancing the non-inflammatory Th2 response.

Previously, we expressed GFP-Aβ42 on green pepper leaves. Aβ antibodies were produced when the leaves were administered orally to AD model mice, Tg2576, successfully reducing the Aβ accumulating in their brains.[8,9] However, new edible vaccines must be developed because green pepper leaves are not easy to digest, and mass production of the antigen is limited.

Rice (cultivar Hayayuki) was chosen as the edible vaccine and, Aβ was expressed as a GFP-fusion protein. GFP-Aβ42 was introduced into the rice by the *Agrobacterium* method (Yoshida, submitted). We performed SDS–PAGE and Western blotting on transgenic rice extracts in urea buffer to detect Aβ expression in the rice. Rice seeds were ground to a fine powder with a IFM-650D Millser (Iwatani International, Tokyo), and total proteins were extracted in urea–SDS buffer (50 mM Tris–HCl, pH 6.8, 8 M urea, 2% SDS, 5% 2-mercaptoethanol, and 20% glycerol) and centrifuged. We used mouse monoclonal antibody 6E10 as the primary antibody to detect Aβ.[9] Then we quantified the expression of Aβ using synthesized mouse IgG as the protein standard. The Aβ concentration was calculated as 120 μg for 1 g of transgenic rice.

To study antibody production after vaccination and the precise immunological response, subcutaneous and oral immunization of wild-type (WT) B6 mice was performed starting at 6 weeks of age, and was continued for 6 weeks. All the animal experiments followed the guidelines for the regulation of animal experiments of The University of Tokyo. For subcutaneous immunization, each mouse was anesthetized with diethyl ether and injected with 100–200 μL of an emulsion of rice powder dissolved in PBS mixed with Freund's adjuvant (Wako Pure Chemical Industries, Osaka) at several points using a 21-gauge needle. For oral immunization, the mixture of Aβ rice powder and CTB (Sigma-Aldrich, Tokyo) in 5% sucrose was delivered orally by syringe. We examined four groups of WT B6 mice. Five WT mice

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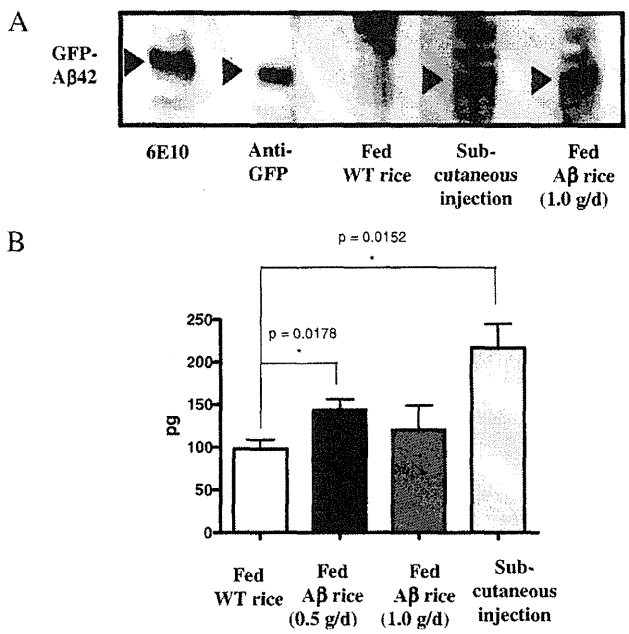

Fig. 1. Detection of Anti-Aβ Antibody in Mice.

A, GFP-Aβ42 was expressed in COS-7 cells and its lysate was separated by SDS–PAGE. Serum was applied to detect anti-Aβ antibody. GFP-Aβ42 was detected in serum from mice fed Aβ rice (1.0 g/d) and injected with the lysate of Aβ rice. For controls, 6E10 and anti-GFP antibody were used. These antibodies also detected GFP-Aβ. B, The amount of anti-Aβ antibody in the serum was calculated ELISA. Serum samples were diluted 100× in this assay. Mean ± SE. $^*p < 0.05$.

vaccinated orally with non-transgenic rice served as negative controls. The rest of the WT mice were immunized with GFP–Aβ42 either subcutaneously (n = 6) or orally (0.5 g/d (n = 5) or 1.0 g/d (n = 4). The mice in the orally administered group received an edible antigen once every week from 6 to 12 weeks of age, and the subcutaneous-injected mice received doses of vaccine biweekly. Two weeks after an Aβ booster given at 12 weeks, whole blood was collected from the heart. GFP–Aβ42 expressed in COS-7 cells was detected in serum from the mice immunized orally (1.0 g/d) and subcutaneously (Fig. 1A). We quantified by ELISA and calculated the amounts of serum anti-Aβ antibody (Fig. 1B).[9] Compared to the WT rice control, the amount of serum anti-Aβ antibody was significantly elevated in the groups receiving GFP–Aβ.

Antibodies against rice seed protein can also be produced by intestinal immunity. That means that food allergy can occur in oral vaccination. We found that an antibody against rice seed protein, possibly prolamin or glutelin, was produced by induction with CTB (Fig. 2A, B). To detect the antibody against rice seed protein in the serum, the serum was diluted 50 times from mice orally administrated Aβ, and was diluted 100 times from mice subcutaneously injected. Rice seed protein-specific IgG responses were measured by ELISA with 20 μg/mL the rice storage protein extracted with 0.01% Triton X-100.[10] Verhasselt *et al.* reported that an antigen can be transferred from lactating mice to their progeny through breast milk.[11] We assumed that the B6 mice acquired immune tolerance through breast milk from lactating mice to which WT rice was administrated orally. In addition, we thought it more efficient to induce immune tolerance through breast-feeding rather than direct administration of WT rice to young mice. Per one lactating mother, six progeny (three male, three female) were prepared. We used two mother mice from the oral administration group and one from the subcutaneously injected group. The WT mice received an edible vaccine orally every week from 6 to 15 weeks of age, and other WT mice received doses of vaccine subcutaneously every 2 weeks. Two weeks after an Aβ booster was given at 15 weeks, whole blood was collected from the heart. The experimental details are schematically shown in Fig. 2C. We found that the amount of antibody against rice proteins tends to be suppressed by oral administration of WT rice to lactating mothers (Fig. 2D, E). The T cell response could be tolerated because the production of the antibody against the rice seed protein is suppressed, but rice seed protein antibody production was not suppressed in mice without pretreatment. Though we tried to quantify the amount of rice protein-induced release of IL-4, -5, and -10 from spleen cells, we were not able to detect these by sandwich ELISA (data not shown).

After inducing immune tolerance by oral administration of WT rice to lactating mice, we investigated the production of anti-Aβ antibodies. We found that they were significantly elevated in the group receiving Aβ rice orally as compared to the group given WT rice (Fig. 3A). Next we investigated which sites of Aβ42 peptide were recognized. Using Aβ1-16 (Peptide Institute, Osaka, Japan), Aβ11-28 (Immuno-Biological Laboratories, Takasaki, Japan) and Aβ25-35 peptide (Peptide Institute), epitope mapping of anti-Aβ antibody was done by ELISA (Fig. 3B). The results suggested that the antibodies produced by oral administration or subcutaneous injection recognized a wide variety of Aβ epitopes, because no differences were observed among Aβ1-16, Aβ11-28, Aβ25-35, and Aβ1-42.

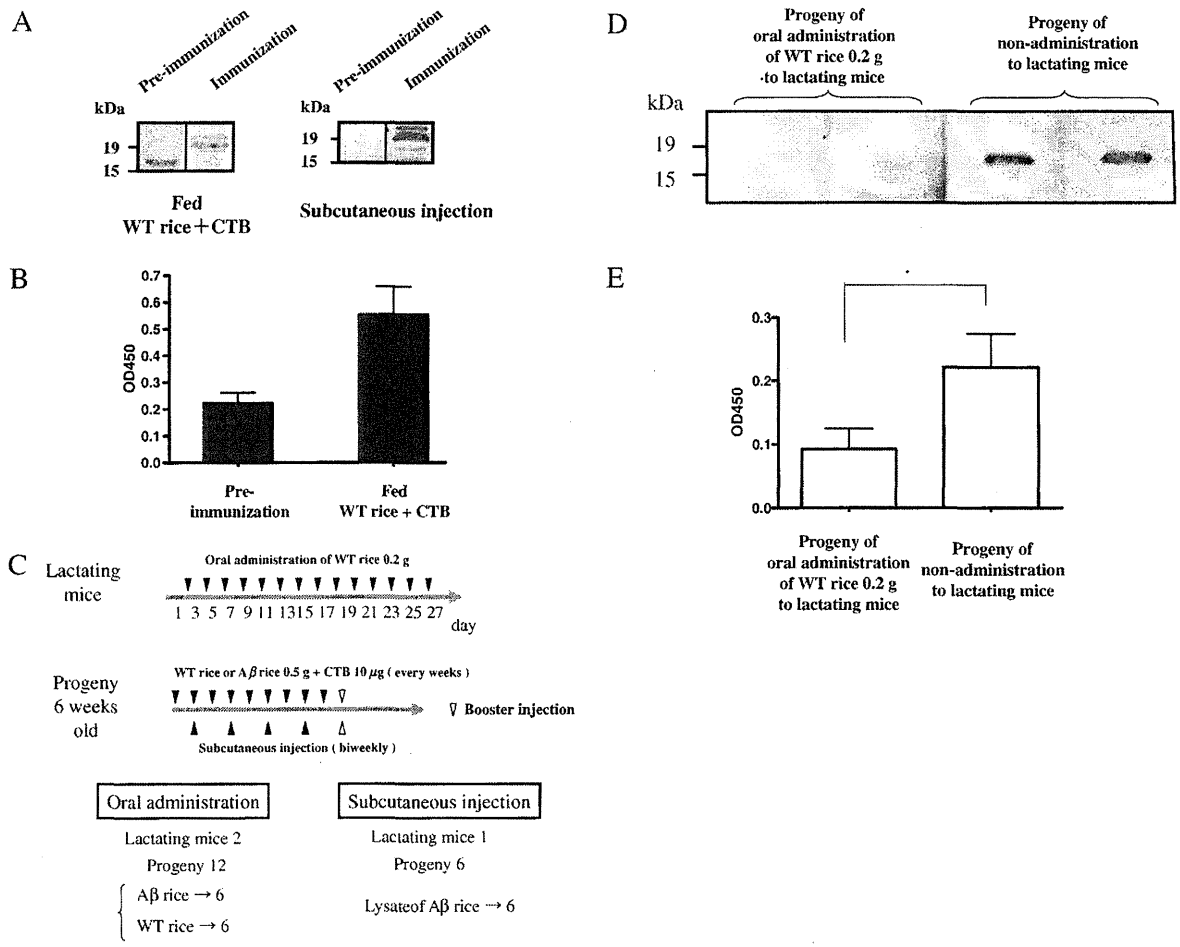

Fig. 2. Production of Anti-Rice Storage Protein Antibodies in Mice.

A, The difference in the production of the antibody against the rice seed protein before and after immunization was investigated SDS–PAGE. After immunization, anti-rice seed proteins were observed in both oral (left) and subcutaneous injection (right). B, The amount of the antibody against the whole rice seed protein was calculated ELISA. C, Experimental protocol. Lactating mothers were orally administrated wild rice (0.2 g) every other day from delivery until weaning. D, Rice extract in urea–SDS buffer was applied to SDS gels, and the various sera was used to detect anti-rice seed protein antibody. The rice storage protein (15–19 kDa) was not detected in the serum from progeny of oral administration of WT rice to lactating mice (left). High antibody production was observed in the mice without prior administration of rice (right). Serum samples were diluted to 50× in this assay. E, Amount of antibody against rice whole protein. The value of the newly developed anti-rice protein antibody due to Aβ-rice is shown. Production against the rice protein was suppressed in mice whose mothers were orally administrated rice at the lactating stage. Mean ± SE. $^{*}p < 0.05$.

To check the safety of the edible vaccine, we examined the isotype of anti-seed protein antibody in the serum and looked for inflammatory reactions in mice vaccinated orally and subcutaneously. IgG1 is known to be the non-inflammatory Th2 isotype IgG, and IgG2a is the inflammatory Th1 isotype IgG. If the ratio of IgG1/IgG2a exceeds 1, the humoral immune responses of the non-inflammatory Th2 act dominantly. Therefore, to quantify rice seed protein specific immunoglobulin subclasses produced by intestinal immunity (orally administration) and systemic immunity (subcutaneous injection), ELISA with IgG1 and IgG2a was done. The ratio of IgG1 to IgG2a (IgG1/IgG2a) was calculated. In all the groups, the ratio of IgG1/IgG2a exceeded 1, suggesting that feeding of Aβ rice induced the Th2 response (data not shown).

The number of patients with AD continues to increase and this is predicted to become a major problem, but no effective therapy exists.[12,13] According to the amyloid hypothesis, AD is triggered by an accumulation of Aβ, and peculiar pathogenic changes, such as neurologic deficit, are seen in AD brains.[1] Therefore, the focus for therapy of AD is to suppress the production of Aβ or to inhibit the deposition of Aβ in the brain.[14] To remove Aβ from the brain, antibody therapy *via* injection of Aβ into the body was performed at the beginning of 2000, but meningoencephalitis appeared as a side effect.[6] Therefore, the conclusion was drawn that the therapy needs a mild antigen–antibody reaction with a non-inflammatory response toward Th2.

Hence we started a study of edible vaccines.[8] Because edible vaccines work through intestinal immunity, which induces the Th2 reaction and produces antibodies, it was thought to have few side effects. We expressed GFP-Aβ42 in green pepper leaves with *Tobamovirus*, and these lysates mixed with CTB were orally administrated to AD model mice, Tg2576. We succeeded in producing anti-Aβ antibodies and decreased Aβ in the mouse brains. We also observed that the edible vaccine induced a noninflammatory response toward Th2.[9]

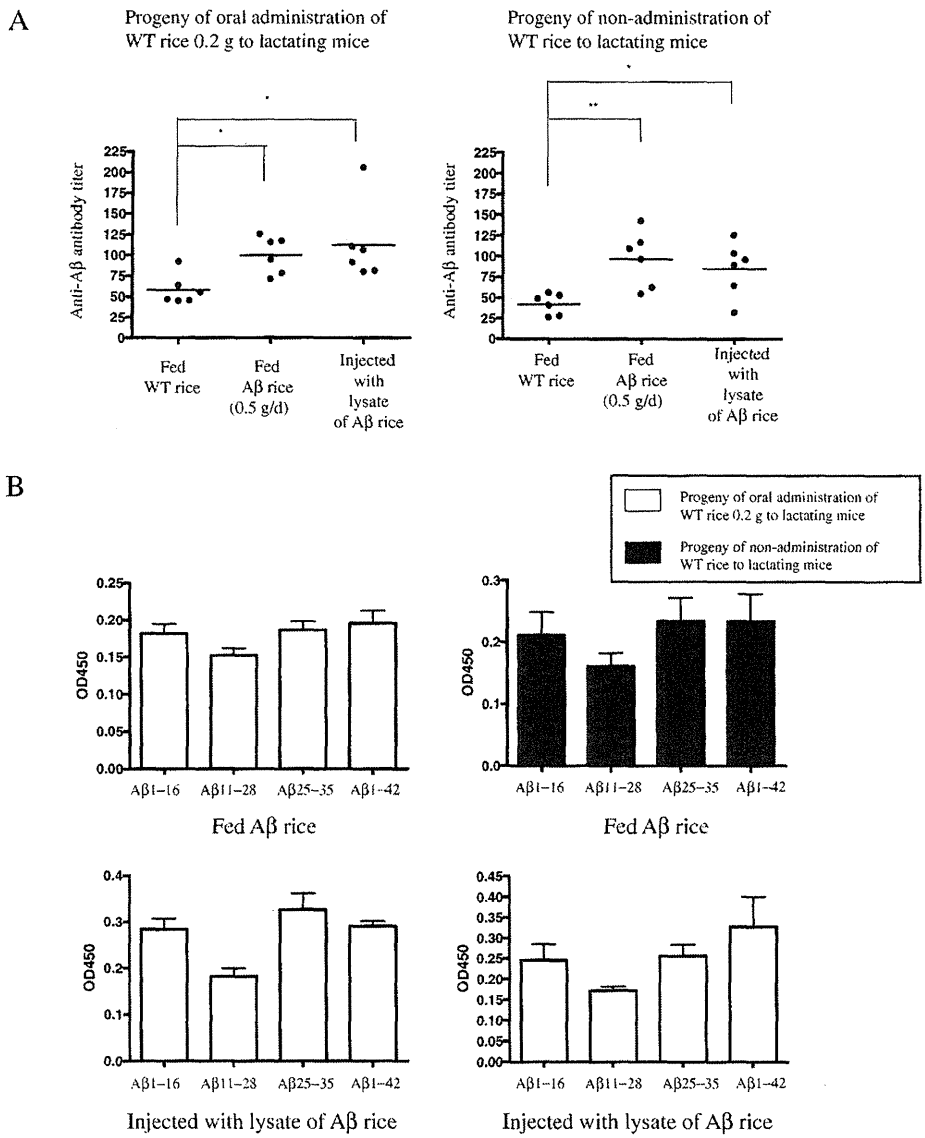

Fig. 3. Detection of Anti-Aβ Antibody in Mice and Epitope Mapping.
A, The Aβ-antibody titers of the mice are shown ($^*p < 0.05$, $^{**}p < 0.01$). B, The epitope mapping of Aβ. The peptides of Aβ1-16, Aβ11-28, and Aβ25-35 were used as antigens. Sera from mice fed Aβ rice or injected with the lysate of Aβ rice were used in the assay.

We investigated to determine whether the antibody against Aβ would be produced by oral administration of Aβ rice to B6 mice, as in the case of green pepper leaves. Compared to the group with oral administration of WT rice, the amount of anti-Aβ antibody increased significantly in the group subjected to oral administration of Aβ rice and in that receiving subcutaneous injections (Fig. 1A, B), but the antibody titer against Aβ did not increase greatly. We found that the anti-Aβ antibody titer was inversely correlated with the level of soluble intracerebral Aβ42 in Tg2576 mice in the green pepper experiment.[9] The frequency of immunization was different in this rice experiment, and the possibility exists that the effect of CTB attenuates it, because the amounts of proteins in rice are larger than in green pepper leaves. In the case of transgenic rice expressing *Chlamydophila psittaci* (*Cp. Psittaci*) antigen (MOMP)-fused LTB, mice were fed large amounts of rice in order to produce an antibody against MOMP.[15] Additionally, immunizing the AD model mice for a long time is important to decrease senile plaques, so one can remove Aβ in Tg2576 mice even if an antibody is produced in modest amounts.[16]

Because we administered CTB orally, antibody against rice seed protein was produced. However, as for humans, some believe that one can suppress antibody production due to immune tolerance against rice seed proteins by eating rice frequently. Since it has been reported to induce immune tolerance through lactation,[11] we tried to produce an antibody against Aβ by suppressing antibody production against rice seed proteins by administering lactating mice WT rice. Even if humans use an edible vaccine with CTB, the antibody against the rice seed proteins may be suppressed. However, because IgE rather than IgG is the main immunoglobulin produced in food allergy, the production of IgE must also be also investigated in the future. Although the antibody against CTB was reportedly

produced by the rice that expressed CTB, the antibody against the rice seed protein was not produced.[10] Therefore, rice expressing Aβ with CTB might not produce antibody against rice seed protein, when immune tolerance is induced.

We performed epitope mapping and found that various antibodies were produced. The antibodies recognizing the N-terminus, such as Aβ1-6, are efficient at inhibiting the formation of senile plaques, and the antibody used in the clinical trials has tandem repeats recognizing the N-terminus.[17–19] This food vaccine must improve a DNA construct to produce the antibody recognizing the N-terminus effectively and to fuse oral adjuvants such as CTB, instead of GFP for clinical use. Furthermore, we examined the T-cell response by calculating IgG1/IgG2a. In our system, intestinal immunity is thought to act dominantly on the Th2 immune response.

In this study, we were able to confirm the possibility of a vaccine treatment for AD. Rice is valuable as an edible vaccine because the effect of the vaccine reportedly is not lost even if the rice is preserved at room temperature for more than 1 year.[10] However, we must use AD model mice in future studies and examine the utility of the vaccine in AD therapy.

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Short Research Communication

Transgenic Rice Expressing Amyloid β-peptide for Oral Immunization

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Abstract

Various vaccine therapies for Alzheimer's disease (AD) have been investigated. Here we report transgenic rice expressing amyloid β-peptide (Aβ). The Aβ42 gene fused with a green fluorescent protein gene was introduced into rice using the *Agrobacterium* method. When transgenic brown rice expressing Aβ was orally administered to mice, serum anti-Aβ antibody titers were elevated. The same results were observed when mice were fed boiled, transgenic brown rice. The results indicate that an edible vaccine against AD using rice may be feasible. A vaccine derived from rice would be far cheaper than existing medical vaccines.

Key words: Alzheimer's disease, amyloid β-peptide, edible vaccine, Oryza sativa, brown rice

Introduction

Alzheimer's disease (AD) causes cognitive impairment and while symptomatic therapies such as donepezil hydrochloride are available, no existing therapeutic medication offers complete recovery from AD. The development of new AD therapies would lessen the social and economic burden of the disease.

The onset of AD is thought to be due to amyloid β-peptide (Aβ) deposition in the cerebral cortex [1,2]. As Aβ is a protein, vaccines for AD are potential means of therapy or prevention [3-5]. In trials using mouse models of AD, injection with Aβ as an antigen reduced the level of Aβ accumulation in the brain notably, reduced memory defects and improved behavioral disorders [6,7]. A phase I clinical trial of vaccine therapy by intramuscular injection of Aβ was completed without problems but the phase II clinical trial was terminated because some patients developed meningoencephalitis [8]. In a 6-year follow-up of patients in the trial of AD immunization [9], cortical Aβ loads were lower in immunized patients compared to the control group. Patients with higher antibody responses had more extensive Aβ removal. However, this study did not find survival or time to severe dementia improved in immunized patients versus the control group.

A vaccine therapy with no side effects is needed. Oral or other mucosal vaccinations appear to have fewer side effects than vaccines administered by injection [10,11]. A potential method of oral vaccination is to express a target protein in an edible plant. Increased serum anti-Aβ antibody titer and suppressed Aβ deposition in the brain were observed when green pepper or potato containing Aβ was fed to a mouse model of AD [11,12,13,14]. Edible vaccine might also be produced in genetically modified food plants such as rice or soybeans that accumulate Aβ in seeds. Ce-

real seeds are better suited for edible vaccines than fruit or vegetables as many have high protein content and can be stored for long periods at room temperature.

In the present study, we introduced the Aβ gene conjugated with green fluorescent protein (GFP) into rice, and orally administered modified rice to mice in order to investigate the effects of Aβ on serum anti-Aβ antibody titer.

Materials and Methods

Plant materials

Oryza sativa L. cultivar Hayayuki (Japonica rice in Japan) was used in this study. Mature seeds (brown rice) were sterilized in 70% ethanol for 10 s and 1% sodium hypochlorite for 15 min, and rinsed in sterile distilled water. Seeds were placed on N6D medium [15,16] for callus formation. Cultures were incubated at 25°C under a 16-h photoperiod using cool-white fluorescent light at 40 μmol/m²/s. Calli were isolated from seed scutella 10-30 days later, and used for Aβ gene induction.

Plasmid construction

The nucleoside sequence coding Aβ42 was amplified by PCR using primers Aβ-5'-XhoI (5'-GAAGTCTCGAGTGATGCAGAAT-3') and Aβ-3'-HindIII (5'-GAACGAAGCTTTTACGCTATGACA-3'). The gene for the APP695 protein was used as a template. The product was digested with XhoI and HindIII, and inserted into pEGFP-C2 (Clontech) at restriction sites resulting in pEGFP-Aβ-C2.

The nucleoside sequence coding sGFP(S65T) [17] was amplified by PCR using primers sGFP-5'-AgeI (5'-ATACCGGTCGCCACCATGGTGAGCAAGGGCGAG-3') and sGFP-3'-BglII (5'-TCAGATCTGAGTCCGGCCGGACTTGTACAGCTCGTCCAA-3'). The product was digested with AgeI and BglII, and ligated to the pEGFP-Aβ-C2 at the restriction sites to produce psGFP-Aβ-C2. The GFP-Aβ fused gene was produced by amplifying psGFP-Aβ-C2 by PCR using primers sGFP-5'-XbaI (5'-TTTCTAGAATGGTGAGCAAGGGCGAGGAG-3') and Aβ-3'-SacII (5'-TTGAGCTCGACTGCAGAATTCGAAGCTT-3'), followed by digestion with XbaI and SacII.

The binary vector pIG121-Hm [18] was digested with XbaI and SacII to remove the Intron-Gus gene. The GFP-Aβ fused gene was then ligated to pIG121-Hm to produce pIG121-Hm (sGFP+Aβ).

The binary vector pIG121-Hm (sGFP+Aβ) was electroporated into *Agrobacterium tumefaciens* strain EHA101 using an *Escherichia coli* pulser (Bio-Rad). Transformants EHA101 (pIG121-Hm (sGFP+Aβ)) were selected on LB medium containing 50 mg/L kanamycin, 50 mg/L hygromycin B and 1.2% Bacto-agar.

Transformation

EHA101 (pIG121-Hm (sGFP+Aβ)) was grown overnight on LB medium with 50 mg/L kanamycin, 50 mg/L hygromycin, and 1.2 % Bacto-agar at 25°C. Bacteria were suspended in AAM medium [19]. Rice calli were immersed in AAM medium containing the bacteria for 15 min.

Calli were transferred to N6D medium, modified to pH 5.2, containing 100 μM acetosyringone, and incubated in the dark at 22°C for 3 days. After co-cultivation, calli were washed with the medium (N6 salts, N6 vitamins, 2 mg/L 2,4-D, 30 g/L sucrose, 400 mg/L carbenicillin, pH 5.8).

Washed calli were cultured on selective media (N6D medium with 50 mg/L hygromycin B and 400 mg/L carbenicillin) at 25°C under a 16-h photoperiod. White or yellow calli were transferred every 10 days to the same medium.

Calli were transferred to plant regeneration medium [20] modified with 100 mg/L carbenicillin. Cultures were incubated at 25°C under a 16-h photoperiod. Calli with green spots or plantlets were transferred every 5-10 days, until the plantlets grew to more than 1 cm in height. Plantlets were transferred into hormone-free MS medium, and fully grown plantlets were planted in soil.

Southern blot analysis

Extracted leaf DNA was digested with XbaI. Electrophoresis was performed on 1.0% agarose gels, and DNAs were blotted onto a Hybond-N+ membrane (GE Healthcare) and were subjected to Southern hybridization. GFP sequence containing the whole coding region was used as a probe. Probe labeling and southern hybridization procedures were performed using the AlkPhos direct labeling and detection system with CDP-Star (GE Healthcare).

Quantification of expression levels in seeds

Frozen seeds were crushed. Total protein in one seed (approximately 20 mg) was extracted for 1 h with 400 μL protein extraction buffer (20 mM Tris-HCl (pH 6.5), 8 M urea, 5% 2-mercaptoethanol, 20% glycerol, 4% SDS), and was centrifuged for 10 min at 20,000 × *g*. Three microliters of each supernatant was applied to Tris-Tricine SDS-PAGE (12% T, 3% C) together with Aβ (human, 1-42) (Peptide Institute) as standards, and the separated protein were transferred to a Hybond-P PVDF membrane (GE Healthcare). The membrane was first incubated in blocking buffer (5% skim milk, T-PBS), and treated with anti-Aβ antibody 6E10 (Sig-

net). The GFP-Aβ fusion protein bound to the membrane was detected using the HRP-conjugated secondary antibody (GE Healthcare) and the ECL plus Western blotting detection system (GE Healthcare).

Mouse immunization

The quantity of brown rice administered to each mouse was adjusted to deliver 10 μg of Aβ. Crushed brown rice was mixed with cholera toxin B (CTB, List Biological Laboratories) (5 μg per mouse) in PBS. C57BL/6J mice (Charles River) were divided into three groups (eight mice per group) and were orally fed with a feeding needle (non-transgenic brown rice, Aβ-containing brown rice (Aβ rice), or boiled Aβ rice). Mice received doses of rice once a week from 8 to 11 weeks of age. As a booster, 0.5 μg of Aβ mixed with Freund's incomplete adjuvant was injected subcutaneously into all mice at 14 weeks of age. Blood serum was collected at 8, 12, 14, and 16 weeks of age. Mice were housed at 25°C with a 12-h light/dark cycle. All animal procedures were approved by the Animal Care and Use Committee of the National Agricultural Research Center for the Tohoku Region.

Quantification of the anti-Aβ antibody titer with ELISA

Micro plate wells were coated with Aβ42 dissolved in 0.15 M ammonium. After washing with PBS-T, wells were blocked with blocking buffer (3% skim milk, PBS), and were washed. Blood serum samples were diluted 10-50 times. Anti-Aβ antibody 6E10 (1 mg/mL) was diluted 10,000-160,000 times as a positive control. Each sample was applied to a well and incubated at 37°C for 1 h. After washing, each well was blocked at room temperature for 30 min with 3% skim milk and was washed again. Wells were incubated with HRP-conjugated secondary antibody at 37°C for 1 h and were washed. Wells were incubated with a TMB kit (Pierce) at room temperature in the dark. The reaction was stopped with 2 M sulfuric acid. Absorbance at 450 nm was measured with a spectrophotometer (Infinite F300, Tecan), and antibody titers were calculated (0.1 μg/mL 6E10 = 100 units/mL antibody titer).

Detection of the anti-Aβ antibody production by Western blot analysis

Aβ (240 ng) was applied to 12% polyacrylamide gels containing 0.1% SDS; separated proteins were transferred to a Hybond-P PVDF membrane. The membrane was first incubated in blocking buffer (ECL Advance blocking agent, GE Healthcare), and treated with serum samples (10 times dilution). Anti-Aβ production was detected by HRP-conjugated secondary antibody and the ECL advance Western blotting detection kit (GE Healthcare).

Results and Discussion

Initially, the luminescence of transgenic rice expressing the EGFP-Aβ fusion gene was weak. The sGFP(S65T) gene is designed for plant use and gives brighter light in the plant than the original GFP [17]. We therefore expressed sGFP instead of EGFP in rice, and the resulting transgenic rice seeds exhibited stronger luminescence with many fluorescent spots (Fig. 1A, B). Fluorescence was localized mainly in the aleurone layer of brown rice (Fig. 1C). Polished rice had a little fluorescence (Fig. 1D).

These results show that Aβ accumulates in aleurone layer, and if brown rice is polished, a proportion of Aβ is removed from the rice. Most rice eaten in Japan is polished, and brown (non-polished) rice must be eaten for AD vaccination to succeed.

The presence of the Aβ-GFP gene in the leaves of primary transgenic rice plants (R_0 rice plants) was investigated by PCR analysis using primers sGFP-5'-*Xba*I and Aβ-3'-*Sac*II (data not shown). In samples where PCR analysis indicated the presence of the Aβ-GFP gene, Southern blot analysis was used to confirm transformation in individual plants (Fig. 2). The Aβ-GFP gene was introduced into R_0 plants except line No. 32. The positive PCR result in the Line No. 32 plant may have been due to residual *Agrobacterium* in plant tissue.

Western blotting was used to investigate the accumulation of the Aβ-GFP fusion protein in Aβ transgenic rice (Fig. 3). The signal intensity of the band was compared against the signal intensity of Aβ42 as a control, and differences were observed among lines. The highest concentration, 8 μg of Aβ in a single grain of brown rice (400 μg/g brown rice) was found in samples from line 29, compared with 18-50 μg Aβ [13] and 77 μg Aβ [14] per gram of soluble protein found in potato in previous studies.

Immunogenicity of Aβ rice was assessed by feeding brown Aβ rice to C57BL/6J mice, from 8 to 11 weeks of age, and assessing serum anti-Aβ antibody titer by ELISA (Fig. 4). At 12 weeks age, we observed a significant increase in serum anti-Aβ antibody titer in mice fed boiled Aβ rice; the increase was not significant in mice fed uncooked Aβ rice. Anti-Aβ antibody was also detected by Western blot analysis in mice fed Aβ rice (Fig. 4B). No increase of anti-Aβ antibody titer was detected in mice at 14 weeks of age, three weeks after the last oral administration (Fig. 4C); but the anti-Aβ antibody titer significantly increased again at 16 weeks of age, after a subcutaneous booster injection (Fig. 4D).

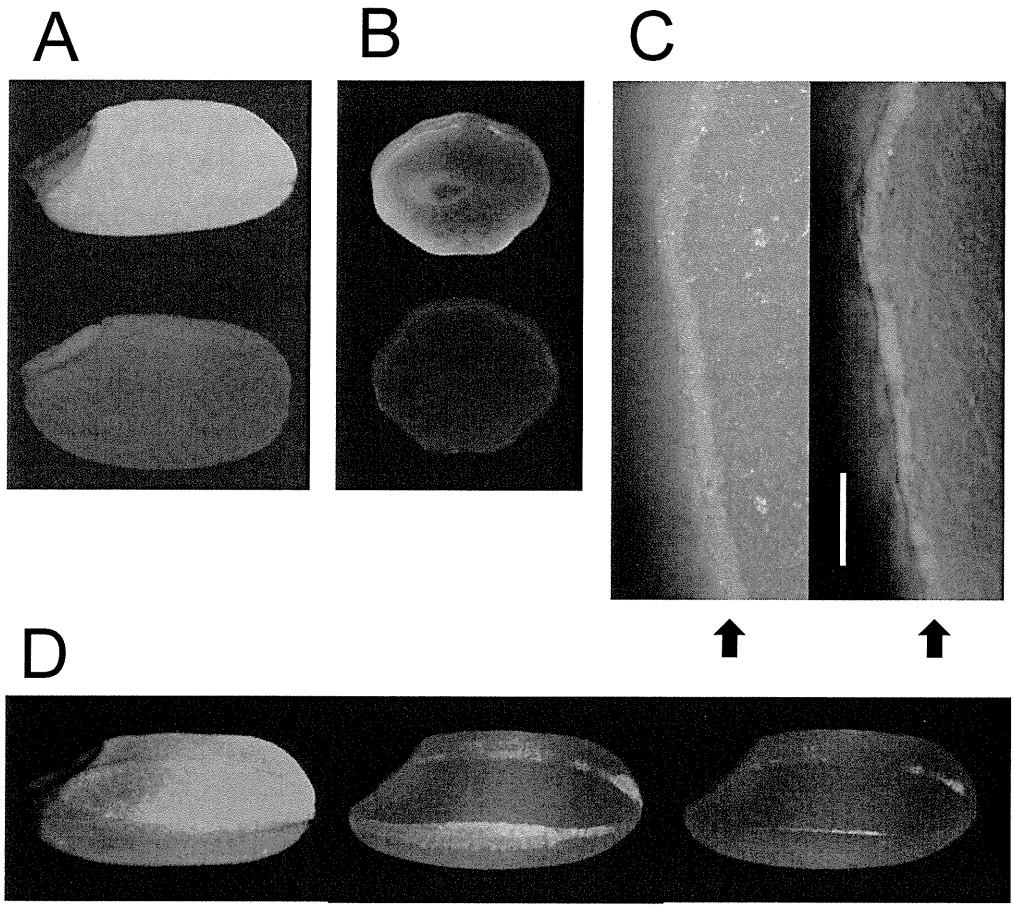

Fig. 1. Detection of GFP fluorescence in seeds (brown rice). (A) Transgenic brown rice (upper seed) and non-transgenic brown rice (lower seed). (B) Transverse sections of transgenic brown rice (upper seed) and non-transgenic brown rice (lower seed). (C) Aleurone layer in transverse section of transgenic brown rice. Right panel shows fluorescence view. Bar indicates 200 μm. Arrows show aleurone layer. (D) Effects of polishing transgenic brown rice: non-polished rice (left seed, weight: 21.3 mg), the roughly polished rice (middle seed), and the polished rice (right seed, weight: 19.1 mg, rice-polishing rate: 89.3%). The yellow region of roughly polished rice is bran layer. A fluorescence stereo-microscope (Nikon SMZ800) was used to observe GFP fluorescence.

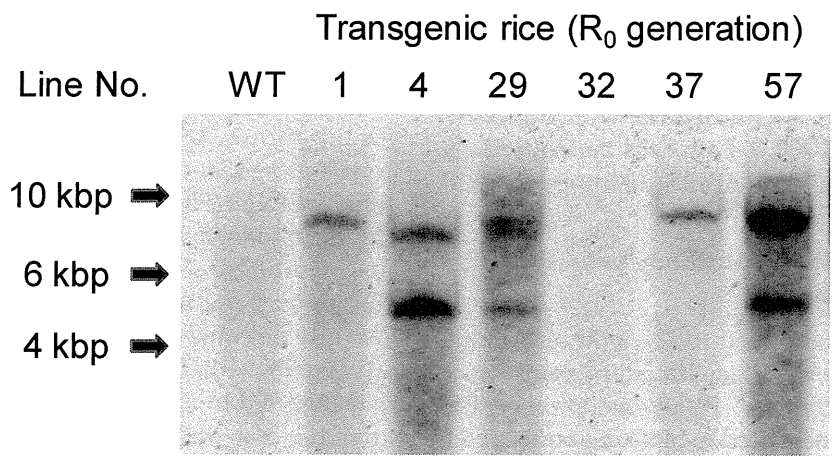

Fig. 2. Southern blot analysis of Xba I-digested total DNA probed for GFP-specific genes. WT, non-transgenic rice plant: R_0, primary transgenic rice plant.

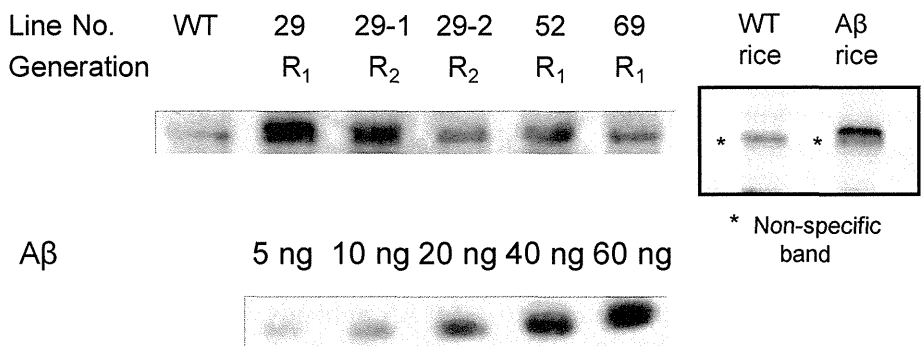

Fig. 3. Determination of Aβ42 expression levels. Brown rice samples were subjected to SDS-PAGE with Aβ42 at increasing concentration (5, 10, 20, 40 and 60 ng). Approximately 0.15 mg of crushed seeds was applied to each lane. R_0 seeds (R_1 generation) and R_1 seeds (R_2 generation) were used. WT; non-transgenic rice; R_1, R_0 progeny; R_2, R_1 progeny. Faint band in WT is non-specific band just below Aβ42 band.

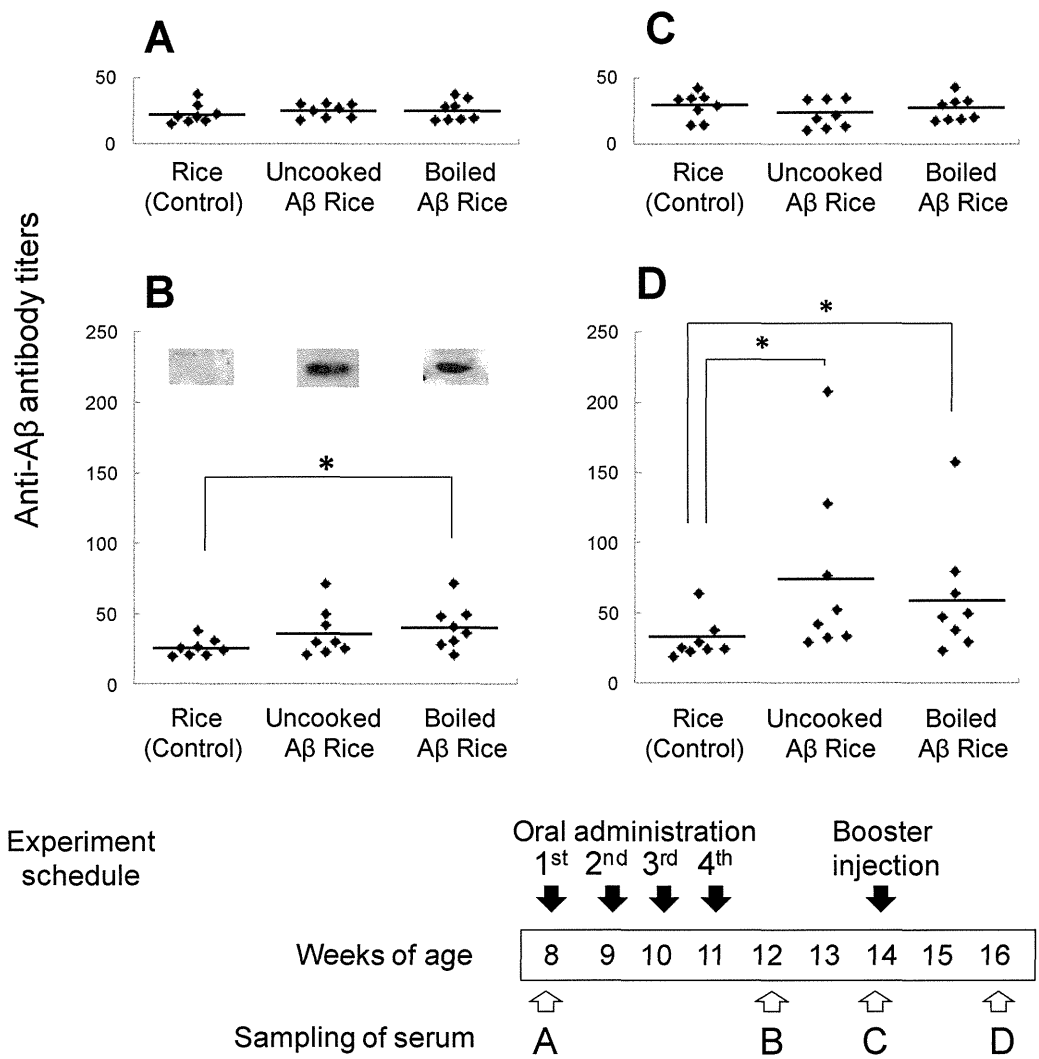

Fig. 4. Titers of antibodies against Aβ in serum from 8-week-old mice before immunization (A), 12-week-old and 14-week-old mice after immunization (B, C, each), and 16-week-old mice after booster injection (D). Anti-Aβ antibody titers for each mouse are shown. Horizontal lines show average.* $P < 0.05$ vs. control. Data were compared by *t*-test after logarithmic conversion. Antibody production evaluated by Western blot analysis (B). For each group, four serum samples with highest anti-Aβ antibody titers were mixed and used for Western blot analysis.

The increase of anti-Aβ antibody titer after booster injection shows the presence of anti-Aβ antibody response in mice fed uncooked Aβ rice or boiled Aβ rice. However, the increase in anti-Aβ antibody titer at 12 weeks of age shows the booster injection was not necessary for Aβ oral immunization.

In a previous study using green pepper containing Aβ [11], we examined the effect on anti-Aβ antibody titers of orally immunized mice and subcutaneously immunized mice over a long-term (12.5 months) trial. The increases in anti-Aβ antibody titer of orally immunized mice were similar to increases in injected mice. In the present study, the increases in anti-Aβ antibody titers of mice fed Aβ rice were not as great as those observed in mice given a booster injection. The difference between the studies may arise from the differences in the period of antigen administration. Further, even if an antibody response is weak, Aβ in mice brains may be removed by long-term immunization of the AD mouse models [21]. Taken together, we conclude that long-term oral administration of Aβ rice without Aβ injection can prevent and treat AD in mice.

In general, the immunological effect after oral-intestinal mucosal immunization tends to be weak, and this method may induce immunological tolerance. Oral immunological tolerance can be suppressed by the use of specific adjuvants. Bacterial toxin, such as CTB, is often used as an adjuvant in oral immunization of mice. Although CTB may not be highly toxic, there may be some clinical side effects. A safer adjuvant might be developed from plants that produce compounds such as saponin [22] and it may be feasible to develop adjuvant-free oral vaccine from plants. Further animal study is necessary to determine the effectiveness of adjuvant-free Aβ rice for AD.

In a previous study, we developed a technique in which a plant (green pepper) was infected with a plant virus, causing Aβ to accumulate within the plant [12]. Mice that were orally administered Aβ-containing plant tissue showed lower levels of serum IgG2a, an inflammatory Th1 immunological globulin, than mice in which the vaccine was administered by injection [11]. These results indicate that the plant-derived vaccine is safe and effective. In addition, vaccines made using plants are far safer than vaccines from animal cells or microbes as there is less danger of the vaccine being adulterated with prion proteins, pathogenic viruses, or bacterial toxins. Thus, plant-derived vaccines require less purification, and may be produced cheaply.

The rice cultivar 'Hayayuki' used in this study is an early-ripening variety that can be harvested approximately 3 months after planting. Moreover, its compact form allows year-round production in a greenhouse or plant factory so that transgenic rice would be easily contained. The additional cost of contained production is likely to be justified by a high added-value product, such as a remedy for AD.

In the present study, we showed that oral administration of Aβ rice to mice elevated serum Anti-Aβ antibody titer. We previously found oral administration of Aβ green pepper to Tg2576 mouse models elevated serum Anti-Aβ antibody titer and reduced senile plaques; and that there was an inverse correlation between anti-Aβ antibody titers and soluble intracerebral Aβ [11]. It is likely that accumulation of Aβ in the brain can be suppressed by administering Aβ rice. We plan a further experiment with AD mouse models to investigate whether oral immunization by long-term administration of Aβ rice decreases senile plaques.

Rice is commonly eaten in grain form without first being pulverized. This would make it easy to control intake, as with medicines in pill form. In addition, where rice is eaten as a staple, it is possible to ensure regular intake. In the present study, we showed that boiled Aβ rice does not reduce the efficacy of the vaccine, thereby allowing its use as an edible vaccine. The ease of use of an Aβ rice vaccine for AD makes this the most attractive vaccine for preventing and treating the disease.

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Conflict of interests

The authors have declared that no conflict of interest exists.

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