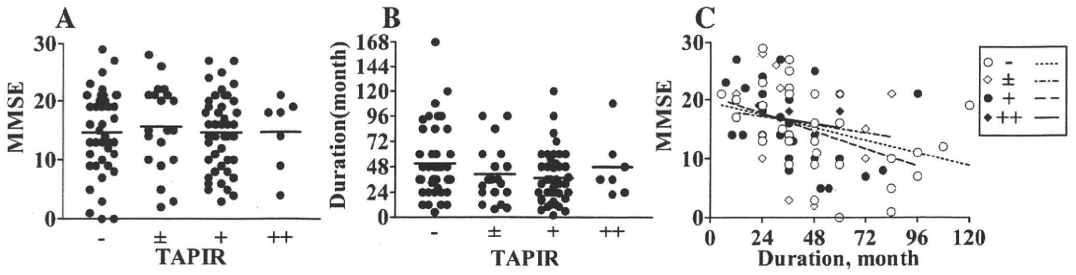

Fig. 2 – TAPIR was not correlated with clinical symptoms. There were no significant differences in MMS scores (A), disease duration (B) or decline of the clinical course of AD according to TAPIR grade. No significant difference in the decline of MMS scores according to duration was shown among AD subgroups (C). $Y=-0.09X+19.54$, $r^2=0.19$, $p=0.01$ in TAPIR – (○); $Y=-0.06X+18.50$, $r^2=18.50$, $p=0.52$ in TAPIR ± (◊); $Y=0.12X+20.59$, $r^2=0.17$, $p=0.02$ in TAPIR + (●); $Y=-0.06X+18.63$, $r^2=0.04$, $p=0.72$ in TAPIR ++ (♦).

amyloid cores in AD brains (Fig. 4G–I). The appearance rate was 1/3 at 4 months old (1 TAPIR +), 3/3 at 8 months old (1 TAPIR ++ and 2 TAPIR +), 1/1 at 16 months old (1 TAPIR ++) and 1/1 at 23 months old mice (1 TAPIR +).

Finally, we summarized age-dependent TAPIR-positive rates (TAPIR grading + and ++) in 10 year increments in both AD and tNC groups (Fig. 4J). TAPIR-positive rates were high in young subjects (1–20 years old), low during adulthood (21–50 years old) and then increased again after 50. No differences were observed between AD and tNC samples from 50 to 91 years old. Thus, the appearance of antibodies to Aβ preceded Aβ amyloid deposition in human and model mouse brains.

2.5. *Levels of plasma Aβ40 and Aβ42 were age-dependently regulated in the tNC group*

To examine the effect of antibodies to Aβ on plasma Aβ concentrations, we measured levels of Aβ40 and Aβ42 in 318 plasma samples by specific ELISA. In the tNC group, plasma Aβ40 levels increased after 40 years of age (Fig. 5A; $p<0.0001$). On the contrary, plasma Aβ42 levels increased between the teens and twenties, then gradually declined with age (Fig. 5B; $p=0.0158$). The Aβ ratio (Aβ40/Aβ42) was stable until ~30 years old and then gradually increased (Fig. 5C; $p<0.0001$).

2.6. *Plasma Aβ ratio is increased in AD*

Significantly increased levels of plasma Aβ40 were observed in the AD group (112±39.51 pmol/L) compared to the aNC group (95.38± 32.30; $p<0.0002$; Fig. 5D). Aβ42 levels were significantly decreased in the AD group (10.29±13.80 pmol/L) compared to the aNC group (12.13±12.29; $p<0.0001$; Fig. 5E). Based on these changes, the Aβ ratio (Aβ40/Aβ42) was more strongly increased in the AD group (14.42±10.00) than in the aNC group (8.34±3.83; $p<0.0001$; Fig. 5F). ROC analysis of the Aβ ratio indicated that the significant cut off value was 9.0, which provided high sensitivity (78.8%) and low specificity (30.3%) for clinical diagnosis of AD. When the mean+2 SD (15.9) of the aNC group was used as a cut off value, the sensitivity was 24% and the specificity was 96%. When AD was divided into 3 subgroups according to clinical stage, increasing Aβ40 levels and Aβ ratio, as well as decreasing Aβ42 levels progressed from the early stage to the advanced stage (Fig. 5G–I). Thus, the plasma Aβ ratio can be used as a specific biomarker for AD although the sensitivity and specificity are lower than those of CSF samples (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002).

2.7. *TAPIR did not modify Aβ concentration*

Finally, we examined whether antibodies to Aβ could affect levels of plasma Aβ40 and Aβ42. There were no significant differences in the concentrations of plasma Aβ40 or Aβ42, or in the Aβ ratio among AD and aNC classified by TAPIR score (Fig. 6A–C).

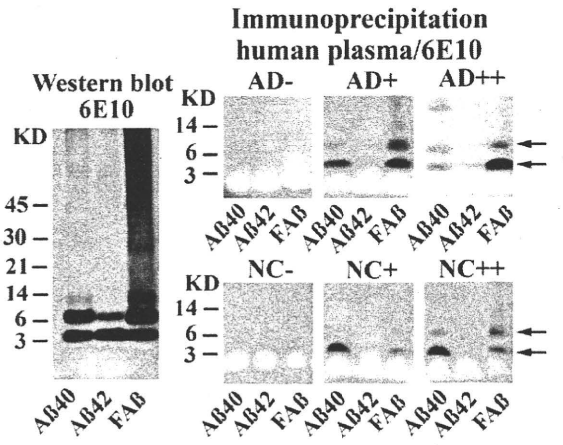

Fig. 3 – TAPIR-positive plasma immunoprecipitated Aβ40 and amyloid Aβ, but Aβ42 very weakly. On direct western blotting of synthetic Aβ40, Aβ42, and FAβ from the AD brain, antibody 6E10 detected monomers and dimers of Aβ40, Aβ42 and brain amyloid Aβ with smear aggregates (left panel). Immunoprecipitations of Aβ40, Aβ42, and FAβ using TAPIR –, +, and ++ plasma from the AD group (right upper panel, AD) or the aNC group (right lower panel, NC) were labeled by antibody 6E10, showing that monomers (arrow) and dimers (arrow) of Aβ40 were recognized by TAPIR-positive plasma (grading + and ++) in addition to Aβ42 monomers, and brain Aβ amyloid monomers and dimers with smear aggregates, which showed weak signals.

3. Discussion

In our study, a high positive rate of TAPIR was found in both AD (45.1%) and aNC (41.2%) groups, but no significant difference was found between these groups. Essentially the same findings were observed even in strongly positive (++) subgroups of AD (6.2%) and aNC (12.9%). Non-parametric analysis revealed that neither MMSE score nor disease duration correlated with TAPIR grade, indicating that the physiological impact of naturally occurring anti-Aβ antibodies is below clinical significance. This is consistent with previous reports describing frequent presence of low levels of antibodies to Aβ40 or Aβ42 peptides as detected by ELISA in plasma and CSF. A large scale study by Hyman et al. showed by ELISA that there were low and modest levels of anti-Aβ42 peptide antibodies in 52.3% and 4.7% of 365 plasma samples from AD and age-matched controls, respectively (Hyman et al., 2001). Neither the presence nor the amounts of anti-Aβ antibodies correlated with the likelihood of developing dementia or plasma levels of Aβ40 and Aβ42 (Hyman et al., 2001; Orgogozo et al., 2003; Moir et al., 2005; Li et al., 2007). Our study indicated that TAPIR-

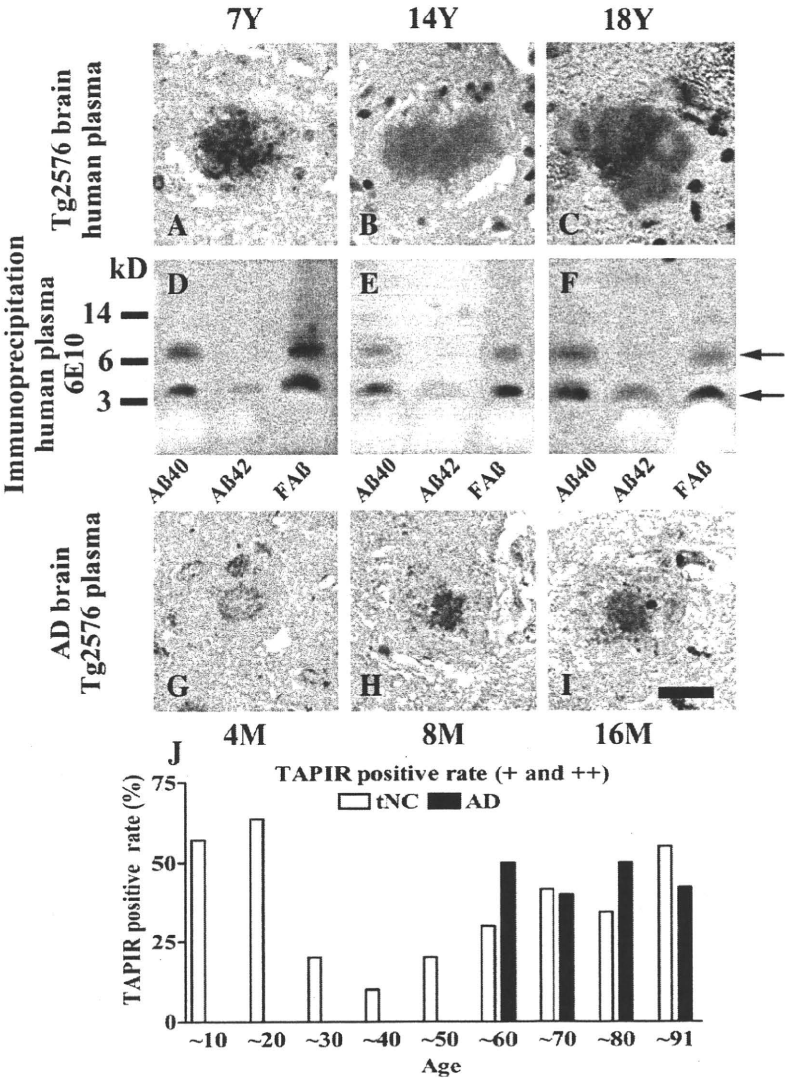

Fig. 4 – Antibodies to Aβ appeared before Aβ amyloid deposits in the brain. TAPIR was positive in 7 years old (TAPIR +; A, 7Y), 14 years old (TAPIR +; B, 14Y), and 18 years old young persons (TAPIR +, C, 18Y). TAPIR-positive plasma strongly immunoprecipitated monomers and dimers (arrow) of Aβ40 and FAβ, and weakly immunoprecipitated monomers of Aβ42 and Aβ amyloid (D, E and F; corresponding plasma of upper panels; D and A 7Y, E and B 14Y and F and C 18Y). Plasma from younger and older Tg2576 mice also labeled amyloid cores in AD brains (G: 4 months old TG; H: 8 months old Tg and I: 16 months old Tg). Bar scale=15 μm. J: TAPIR-positive rates in the tNC group according to age. Columns show the TAPIR-positive rate (TAPIR grading + and ++) for 10 year increases in the AD (black columns) and tNC (white columns) groups. TAPIR-positive rates were high in young subjects (1–20 years old), low during adulthood (21–50 years old) and then increased again after age 50. No differences were observed between AD and tNC groups in samples from subjects 50 to 91 years old.

positive antibodies to Aβ amyloid plaques also occur naturally and frequently in human plasma and that their titers are not sufficient to prevent development of dementia. High titer of antibodies are necessary to improve the Aβ burden as shown in AD patients treated with an Aβ vaccine (Hock et al., 2002) or an anti-Aβ antibody infusion therapy (Dodel et al., 2002).

TAPIR is a new method to detect anti-Aβ antibodies (Hock et al., 2002, 2003). The fact that cognitive impairment was improved in patients who generated anti-Aβ antibodies after Aβ vaccination leads us to hypothesize that TAPIR-positive anti-Aβ antibodies are substantially different from naturally occurring anti-Aβ peptides antibodies in their specificity for Aβ40 and Aβ42 species or conformational epitopes of Aβ oligomers (Mirra et al., 1991; Kayed et al., 2003). Antibodies labeling Aβ amyloid plaques were more effective for the clearance of the Aβ burden of transgenic mice in passive immunization experiments (Bard et al., 2000). Direct action of the anti-Aβ antibody through the blood–brain barrier without T-cell proliferation as well as microglial clearance via the Fc or non-Fc portion of the antibodies mediated disruption of the plaque structure (Bard et al., 2000; Bacskai et al., 2002; Lombardo et al., 2003). Binding of an IgG2a antibody to the special N-terminal region of Aβ correlated with a clearance response (Bard et al., 2003). Injected antibodies may bind and sequestrate blood Aβ completely and disturb the balance between CSF Aβ and blood Aβ, leading to increased clearance from the brain into the blood (DeMattos et al., 2001; DeMattos et al., 2002). Clearance of diffusible Aβ oligomers that impair cognitive function was considered to be another target for passive immunization (Kayed et al., 2003). Recently a 56-kDa soluble amyloid-β assembly termed Aβ*56 has been shown to disrupt memory (Lesné et al., 2006), and Aβ oligomers have been shown to be increased in CSF from AD patients (Georganopoulou et al., 2005).

These reports all support the hypothesis that naturally occurring TAPIR-positive antibodies to Aβ recognize special Aβ species. Our immunoprecipitation study suggested that

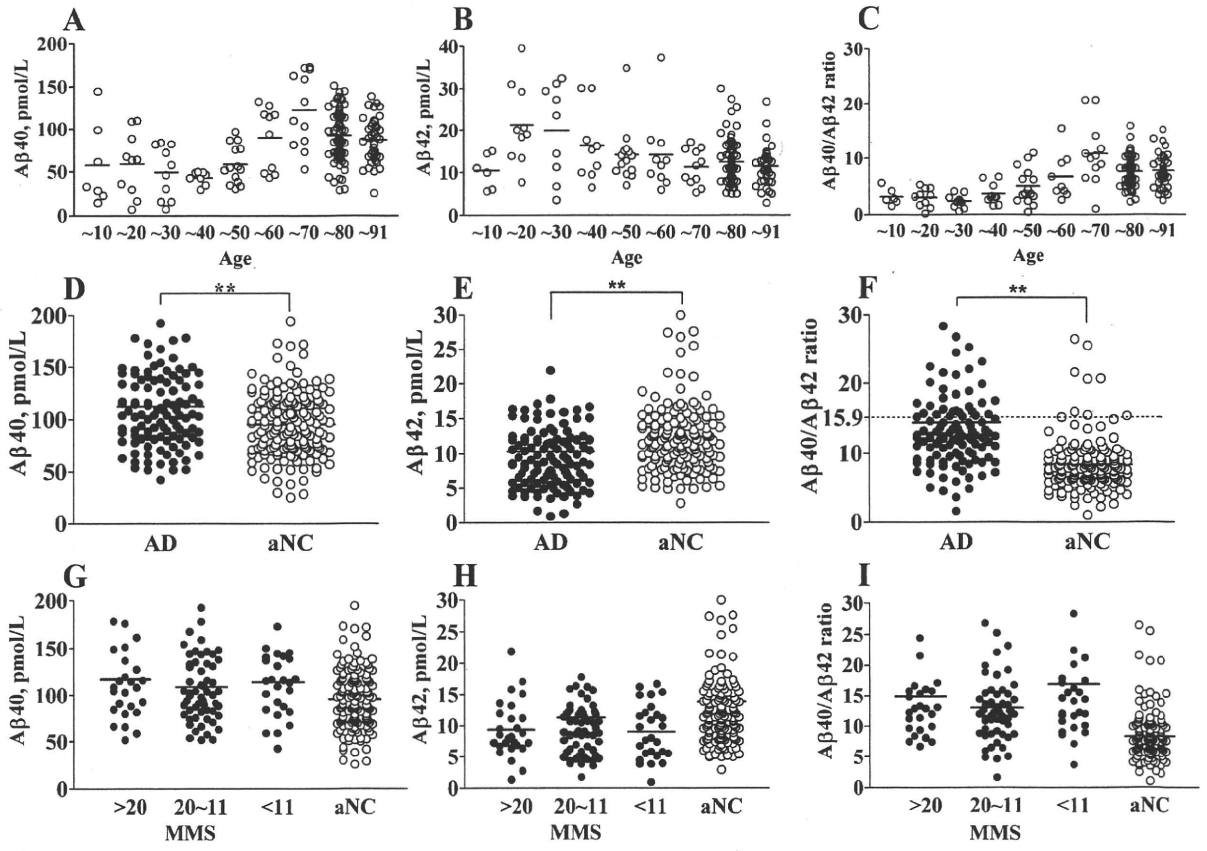

Fig. 5 – Age-dependent regulation of plasma Aβ levels in controls, and their alteration in AD. Plasma Aβ40 and Aβ42 levels showed different age-dependent alterations in the tNC group. Aβ40 levels increased from age 50 and decreased from age 70 (A). Aβ42 levels were high in the teens and twenties, then gradually decreased with age (B). Based on these different changes, the Aβ ratio (Aβ40/Aβ42) progressively increased from age 40 (C). Significantly increased levels of Aβ40 (D: $p=0.0002$) and increased Aβ ratio (F: $p<0.0001$) as well as decreased levels of Aβ42 (E: $p<0.0001$) were shown between the AD and aNC groups. When the mean +2SD of the Aβ ratio in the aNC group was used as a diagnostic marker for AD, the cut off value 15.9 (dot line) provided 24% sensitivity and 96% specificity (F). Constant alterations of plasma Aβ levels in AD were recognized at the early (MMS score >20), moderate (MMS score 20–11), and advanced stages (MMS score <11) (G–I). A, D, G: Aβ40; B, E, H: Aβ42; C, F, I: Aβ ratio. Bars show mean levels.

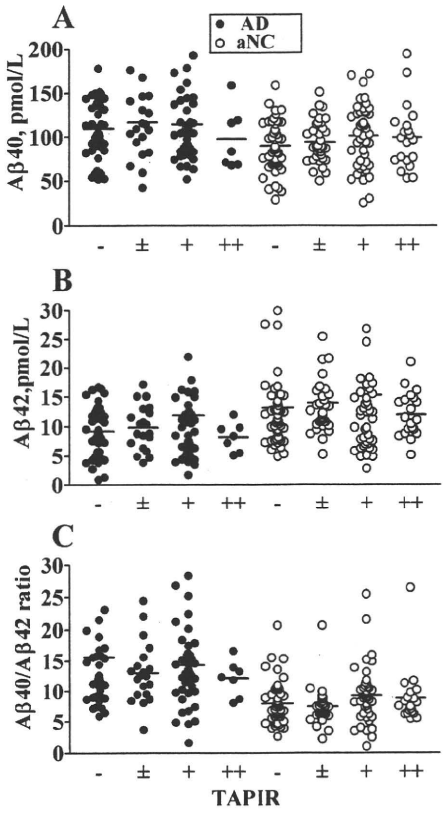

Fig. 6 – TAPIR did not modify Aβ concentration. No significant differences were found in Aβ40 and Aβ42 concentrations as well as Aβ ratios among all TAPIR grades (–, ±, + and ++) in AD (•) and aNC (○) group (A, B and C).

TAPIR ++/+ plasma obtained from AD and aNC subjects retrieved Aβ40 monomers and dimers as well as higher molecular mass polymers. Immunodetection of monomeric Aβ42 using 6E10 was very weak, whereas no dimeric form of Aβ42 was detected under our testing conditions. The absence of anti-Aβ42 dimer antibodies and the relatively low levels of anti-Aβ42 monomers were characteristic of naturally occurring antibodies to Aβ. These findings are considered to be another reason why naturally occurring antibodies to Aβ are not sufficient for prevention of development of dementia.

Our TAPIR assay also showed that anti-Aβ antibodies were naturally present throughout the entire human life span. It is relevant to note that naturally occurring anti-Aβ antibodies were unequivocally detected in young human subjects as well as young Tg2576 mice. In relative terms, the positive rates of anti-Aβ antibodies were highest in young individuals, lowest in those middle-aged and higher in the elderly. The presence of anti-Aβ antibodies in young human subjects was characterized by the subsequent immunoprecipitation study. Anti-Aβ antibodies retrieved Aβ40 monomers and dimers as well as high molecular mass oligomers in FAβ fractions, but they retrieved fewer Aβ42 dimers. To our knowledge, this is the first report showing the relatively selective presence of anti-Aβ40 antibodies, and reduced amounts of anti-Aβ42 antibodies in young individuals. We also found that this was the case in normal elderly as well as AD patients, suggesting that the immune response to Aβ was unchanged in the two groups tested. Impaired spontaneous production of anti-Aβ42 antibodies also took place in elderly human subjects as well as AD patients. It is unknown why these antibodies to Aβ appeared more frequently in the young and the elderly populations and how specific immune tolerance for Aβ42 monomers and oligomers could be present. However, it should be noted that naturally occurring antibodies to Aβ appear in young human subjects and young Tg2576 mice, which do not develop an Aβ burden in their brain. The appearance of naturally occurring antibodies to Aβ is not correlated with the Aβ burden in the brain.

The exact mechanism underlying spontaneous anti-Aβ antibody production remains unknown. Although increased Aβ42 levels have been detected in transgenic animal models (Kawarabayashi et al., 2001), immune hyporesponsiveness to Aβ42 was also shown (Monsonego et al., 2001). Increased T-cell reactivity to Aβ42 was shown to increase in elderly individuals and patients with AD (Monsonego et al., 2003). However, the previous findings and our results could not show increased titers of anti-Aβ42 antibodies in these groups. Thus, hyopoimmunue responses to Aβ42, especially to the Aβ42 oligomer, actually occurred in AD and healthy populations. Since Aβ42 is highly pathogenic and neurotoxic, Aβ42 may be sequestered and spontaneous immune responses to Aβ may be suppressed in human populations. For effective immunotherapy as shown in transgenic mice studies and Aβ vaccine trials (Orgogozo et al., 2003; Hock et al., 2003), it is necessary to further generate antibodies to Aβ42 oligomers as well as monomers and monitor their titers. Furthermore, in order to prevent unexpected adverse reaction as seen in the Phase II trials of AN1792, detection of these spontaneous antibodies to Aβ will be necessary before treatment.

Recent studies have shown that plasma concentrations of Aβ40 and Aβ42 are possible biomarkers (Ertekin-Taner et al., 2000; Fukumoto et al., 2003; Mayeux et al., 1999, 2003; van Oijen et al., 2006; Graff-Radford et al., 2007) and can be used to monitor the effects of special treatments for AD (Dodel et al., 2002; DeMattos et al., 2001, 2002). After administration of an antibody to Aβ, the rapid increase in plasma Aβ was highly correlated with the amyloid burden in the brain (DeMattos et al., 2002), suggesting the possibility that naturally occurring anti-Aβ antibodies may cause increases the plasma Aβ concentration. In order to clarify this effect, we first analyzed age-dependent levels of plasma Aβ40 and Aβ42, and then examined alterations of Aβ40 and Aβ42 levels according to the presence or absence of AD and antibodies to Aβ. In the tNC group, plasma Aβ40 levels increased from age 40. Plasma Aβ42 levels increased between age 10 and 20, then gradually declined with age. The Aβ ratio (Aβ40/Aβ42) was stable until about 30 years and then gradually increased. These natural time courses were identical to those of CSF Aβ40 levels, but completely different from those of CSF Aβ42 levels. CSF levels of Aβ40 and Aβ42 showed U-shaped age-dependent curves, suggesting their correlation with brain development and decline (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002). The correlation was prominent between the appearance of naturally occurring anti-Aβ antibodies and increased Aβ40 levels in

the CSF and plasma. Increased opportunities for immunological exposure to Aβ40 monomers and oligomers in immature or declining brains in young and elderly indivisuals may be sources for the naturally occurring immune response to Aβ40.

Based on these natural time courses of plasma Aβ concentrations, a comparison between AD and aNC groups was performed that provided intriguing findings. Significantly increased levels of plasma Aβ40, increased Aβ ratio and decreased levels of Aβ42 were revealed in the AD group when compared to the aNC group. Since a clear separation was obtained in the Aβ ratio between the AD and aNC groups, we evaluated the value of the Aβ ratio as a diagnostic or monitor maker of AD. ROC analysis indicated high sensitivity (78.8%) and low specificity (30.3%) for diagnosis of AD. When the mean+2 SD (15.9) of the aNC group was used as a cut off value, the sensitivity was 24% and specificity was 96%. When AD was divided into 3 groups according to clinical stage, the Aβ ratio increased progressively from the early stage to the advanced stages of AD. These findings show that plasma Aβ ratio can be used as an easy, non-invasive, and useful biomarker for diagnosis and monitoring of clinical symptoms of AD, although the sensitivity and specificity are lower than those in CSF samples (Kanai et al., 1998; Shoji et al., 2001; Shoji, 2002). However, naturally occurring antibodies to Aβ did not affect plasma Aβ40 or Aβ42 levels, or the Aβ ratio. There was a possibility that our ELISA system could not detect increased levels of Aβ40 and Aβ42 oligomers. However, all results taken together, suggest that the titer and specificity of naturally occurring anti-Aβ antibodies were not sufficient to elevate plasma Aβ concentrations and increase Aβ clearance from the brain to the peripheral blood with subsequent improvement of clinical symptoms. Higher titers of antibodies to Aβ42 oligomers will likely be necessary to facilitate Aβ clearance from brain amyloid to peripheral blood for AD treatment.

4. Experimental procedures

4.1. *Patients and normal controls*

After informed consent was given, blood samples were collected into 0.1% EDTA from a total of 318 subjects including 113 patients with AD (AD group) and 205 normal controls (total normal control group: tNC group). As age-matched controls (aNC group), 115 samples from subjects over 43 years old were selected from the tNC group. The basic findings for the respective groups are summarized in Table 2. The clinical diagnosis of AD was based on NINCDS–ADRDA criteria (McKhann et al., 1984). Appropriate diagnostic studies including magnetic resonance imaging and single photon emission computed tomography were used to exclude other disorders of dementia. The clinical severity of AD was evaluated using the Mini-Mental State Examination (MMS) (Folstein et al., 1975). AD patients were divided into 3 subgroups according to clinical stages: early stage MMS score >20, moderate stage MMS score 10–20, advanced stage MMS score <10. Controls were judged to be normal based on their MMS score (>28 points) and follow-up with neurological evaluation. After separation of plasma from blood cells, plasma was stored frozen at −80 °C until use.

Table 2 – Summary of the study subjects

	No. of subjects	Gender (M/F)	Mean age (range), yr	Mean MMS Score (SD)	Mean duration (SD), mo
AD	113	32/81	75 (55–89)	14.9 (6.7)	44 (28)
tNC	205	84/121	64 (1–91)	29.8 (0.3)	–
aNC	155	59/96	76 (43–91)	29.7 (0.4)	–
Total	318	116/202	68 (1–91)		

AD: Alzheimer's disease patients; tNC: total normal controls; aNC: age-matched controls over 43 years old selected from the tNC group; M/F: male and female; yr: years old; MMS: Mini-Mental State Examination; SD: standard deviation; Duration: duration from onset, mo months.

4.2. *Tissue amyloid plaque immunoreactivity (TAPIR)*

Five micrometers serial paraffin sections of brains from Tg2576 mice (16–18 months old) or Alzheimer's patients were used. Sections were immersed in 0.5% periodic acid for blocking intrinsic peroxidase and treated with 99% formic acid for 3 min to increase Aβ staining. Sections were then immersed with blocking solution with 5% normal serum in 50 mM phosphate-buffered saline (PBS) containing 0.05% Tween20 and 4% Block Ace (Snow Brand Milk Products, Saporo, Japan) for 1 h; goat serum was used to stain human plasma, and horse serum was used to stain mouse plasma. Sections were incubated at 4 °C overnight with human or mouse plasma diluted with blocking solution (1:100). Sections were then incubated with biotinyzed second antibody (anti-human goat antibody or anti-mouse horse antibody), and horseradish peroxidase-conjugated avidin–biotin complex of Vectastain Elite ABC kit (Vector, Burlingame, CA). Immunoreactivity was visualized by incubation with 0.03% 3, 3′-diaminobenzidine, and 0.02% H_2O_2. Tissue sections were counterstained with hematoxylin. Immunostaining with Ab9204 (Saido et al., 1995) (1:1000, antibody to a synthetic Aβ peptide starting from the amino-terminus of normal L-aspartate) or without the primary antibody were used as positive and negative controls, respectively.

4.3. *Grading of TAPIR*

TAPIR findings were classified into 4 levels: negative –, no senile plaque core (Fig. 1F); weakly positive ±, senile plaque cores were stained weakly and less than 5 cores were stained in each brain section on a slide (Fig. 1G); positive +, ≥5 senile plaque cores were stained clearly in at least one brain section per slide (Fig. 1H); strongly positive ++, most senile plaque cores were strongly labeled when compared to Ab9204 immunostaining (Fig. 1I). Immunostaining findings of diffuse plaques, amyloid angiopathy, positive neurons, degenerative neurites and glial cells were excluded from this grading.

4.4. *Purification of amyloid Aβ (FAβ)*

An autopsy brain fulfilling the CERAD criteria for definite AD (Mirra et al., 1991) was selected. About 2 g of gray matter of the AD brain was homogenized with 4 volumes of TBS (10 mM Tris, 150 mM NaCl, pH 8) with protease inhibitors (1 μg/ml

Leupeptin, 1 μg/ml TLCK, 0.1 μg/ml Pepstain A, 1 mM phenylmethysulfonyl fluoride and 1 mM EDTA), and centrifuged at 100,000 ×*g* for 1 h. The resulting pellet was extracted with 10 ml of 10% sodium dodecyl sulfate (SDS) in TBS and then with 1 ml of 99% formic acid (FA). The final supernatant was lyophilized, dissolved with 20 μl of 99% dimethylsulfoxide (DMSO), and stored at −80 °C until use (formic acid soluble amyloid Aβ fraction: FAβ) (Harigaya et al., 1995; Matsubara et al., 1999).

4.5. *Immunoprecipation*

20 μl of protein G agarose (Roche diagnostic GmbH, Germany) was washed 3 times with 1 ml RIPA buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 0.5% cholic acid and 150 mM NaCl, pH 8.0). Prewashed protein G agarose was mixed with 600 ng synthetic Aβ40, 600 ng synthetic Aβ42 (Sigma, Mo) or 300 ng FAβ in 1 ml of RIPA buffer and incubated at room temperature for 30 min. After centrifugation, the resulting supernatant was mixed again with 20 μl of prewashed protein G agarose and 10 μl of plasma, incubated at room temperature for 3 h, and then centrifuged. The pellet was boiled with 1× NuPage LDS sample buffer containing 0.1 M dithiothreitol for 10 min at 70 °C and separated on a 4 to 12% NuPage Bis–Tris gel (Invitrogen, CA). After electro-transfer, the blot membrane was blocked with 10% skim milk (Snow Brand Milk Products, Saporo, Japan) in TBS with 0.05% Tween 20 (TBST), and incubated with monoclonal 6E10 (specific to Aβ1-16, 1:1000, Signet Lab. Inc. MA) at 4 °C overnight. After washing and incubation with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:2000, Amersham Biosci, Buckinghamshire, UK) at RT for 2 h, the signal was developed by SuperSignal west Dura extended duration substrate (Pierce Biotechology, CA), and quantified by a luminoimage analyzer (LAS 1000-mini, Fuji film, Japan).

4.6. *Quantification of plasma Aβ40 and Aβ42 concentrations by ELISA*

Sandwich ELISA was used to specifically quantify whole plasma Aβ, as previously described (Matsubara et al., 1999). Microplates were pre-coated with monoclonal BNT77 (IgA, anti-Aβ11-28, specific Aβ11-16) and sequentially incubated with 100 μl of samples followed by horseradish-peroxidase-conjugated BA27 (anti-Aβ1-40, specific Aβ40) or BC05 (anti-Aβ35-43, specific Aβ42 and Aβ43) (Kawarabayashi et al., 2001). Synthetic Aβ40 (peptide content: 79.95%, Sigma, MO) and Aβ42 (peptide content:76.58%, Sigma, MO) were used for standard Aβ. The sensitivity was 40 fmol/ml in the Aβ40 assay and 10 fmol/ml in the Aβ42 assay. Both intra-assay coefficients of variation were less than 10% (Matsubara et al., 1999).

4.7. *Statistical analysis*

Comparisons among the groups using Student's *t*-test, one-way analysis of variance or a non-parametric test with post hoc tests, a receiver-operating characteristic (ROC) curve analysis to determine the cut off value, Mann–Whitney *U* test for appearance rates, and 1st order regression analysis of the relationship between MMS score and AD duration were all performed using SPSS 11.0 (SSPS Inc., IL) and GraphPad Prism, Version 4 (GraphPad Software, San Diego, CA).

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アルツハイマー病

松原悦朗　柳澤勝彦

はじめに

アルツハイマー病は，細胞内外への線維性構造物の蓄積を認める，いわゆる“原因蛋白の蓄積病”をその基本病態とする．このアルツハイマー病の病理過程でみられる最も早期の変化は細胞外に認められるβアミロイド(Aβ)を主要構成成分とする斑状の嗜銀性構造物(老人斑)である．アルツハイマー病患者脳においては，この本来可溶型の生理的Aβの産生・分解・クリアランスの代謝機構が何らかの原因で破綻し，不溶性の高まった病的Aβが脳実質に蓄積し，老人斑としての脳アミロイド沈着を形成すると考えられている．しかし最近，老人斑Aβアミロイド線維自体の発症病態への関与は低く，アミロイド線維形成して沈着・蓄積する前の中間分子(Aβオリゴマー)こそがその本態(いわゆるシナプス機能障害を引きおこす病態惹起性神経毒性分子)で，アルツハイマー病の治療標的であることが認識されてきている．こうしたAβオリゴマーの蓄積が引き金となり，二次的に神経細胞内にタウを主要構成成分とする嗜銀性構造物(神経原線維変化)蓄積をきたす神経変性(タウオパチー)・神経細胞死が引きおこされアルツハイマー病の病像が完成すると考えられている(アミロイドカスケード仮説)が，in vivoにおいてその直接的連関を示す証拠は現時点でまだ得られていない．神経変性疾患の代表格であるアルツハイマー病において，その中枢神経系の神経細胞は非分裂細胞であるため，傷害を受けた脳を再生させることは全くの夢物語と考えられ，本特集のトッピックスである神経新生の問題はほとんど蚊帳の外であった．ところが，哺乳類の成体脳における神経新生が海馬[1]や側脳室周囲[2]の限られた部位でおこっていることが確認され，実際に海馬での神経新生は，学習や記憶など成体脳の可塑性に重要な機能を有していること[3,4]，アルツハイマー病のシナプス機能障害の発症分子基盤がAβオリゴマーであるとの知見から，シナプス機能における神経回路の観点から両者の接点が見えてきた．本稿においては，このアミロイドカスケード仮説における神経新生の病態形成への関与や，神経新生を標的とした再生医療の試みにつき，これまで報告されている現況を整理しつつ概説したい．

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アルツハイマー病患者脳での神経新生

アルツハイマー病の報告後100年以上が経過したが，アルツハイマー病患者脳における神経新生の報告はわずか2報に留まっている．最初の報告は2004年Jinら[5]によってなされた．驚くことにアルツハイマー病患者脳海馬では神経細胞新生の程度が高まっていたのである．しかしながらアルツハイマー病の確固たる病理所見は神経細胞死であり，この神経新生亢進は結果的にこの神経細胞死を置換できるレベルには至っていない訳である．剖検脳の段階では神経細胞死が神経新生を圧倒している可能性，新生神経細胞が成熟し機能的な神経細胞に成り得ない可能性，アルツハイマー病患者脳の微少環境が新生神経細胞にとって毒性を持っている可能性が推測されていた．2008年，ようやくLiら[6]により，アルツハイマー病患者脳において，これらの推測の一つである海馬新生神経細胞の機能的にも成熟した神経細胞への分化が障害されていることが証明された．この結果は，海馬新生神経細胞が，傷害を受けたアルツハイマー病患者脳において，正常脳と同じような機能的な神経回路ネットワークを再構築することが如何に困難であるかを改めて示した例とも考えられると同時に，如何に新生神経細胞がおかれる微少環境が重要かを示している．

アルツハイマー病モデルマウス脳での神経新生

アルツハイマー病のモデルマウスの登場は，アルツハイマー病の病態解析や治療法開発の環境を一変させ，Aβオリゴマーがアルツハイマー病発症の分子基盤であることやAβワクチン療法などの根本治療法の可能性提示など，そのアルツハイマー病への貢献度は計り知れないものがある．ところが，アルツハイマー病モデルマウスにおいてこれまでなされた神経新生の検討では統一見解が得られていないのが現状である．新生神経細胞の増殖・生存・移動・

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分化の見地からこれまで報告された結果を整理すると，神経新生亢進・低下・著変なしの如くその報告結果は混乱を招いている．アルツハイマー病患者脳での神経新生増加を報告したJinら[7]はモデルマウスにおいても同様の神経新生増加を報告している．逆に，Haugheyら[8]は新生神経細胞増殖抑制とアポトシーシス誘導による神経細胞死増加を，Dongら[9]は老人斑アミロイド出現以前からの新生神経細胞増殖抑制，またDonovanら[10]は老人斑アミロイド出現以後からの新生神経細胞増殖抑制を報告している．著変なしの報告はZhangら[11]によりなされているが，この報告ではAPP変異とPS1変異を共発現させたノックインマウスでは老人斑アミロイド形成に依存した新生神経細胞増殖抑制(神経芽細胞の形成障害)が認められるとしている．またVerretら[12]は比較的選択的な成熟神経細胞死を報告している．最近nestin enhancer制御下LacZ発現マウスとAPP発現マウスの掛け合わせ結果から，老人斑アミロイド形成により神経新生，特に神経移動・分化が亢進されるが，グリオーシスは影響を受けぬことが報告された[13]．以上の如く報告された知見のばらつきは，使用したモデル動物や神経新生評価法の相違に由来していると考えられるが，図らずともアルツハイマー病患者脳の検証からも示唆されたように，アルツハイマー病モデルマウス脳でも新生神経細胞がおかれる微少環境が果たす役割が十分検討されていないことにも由来していると考えられる．

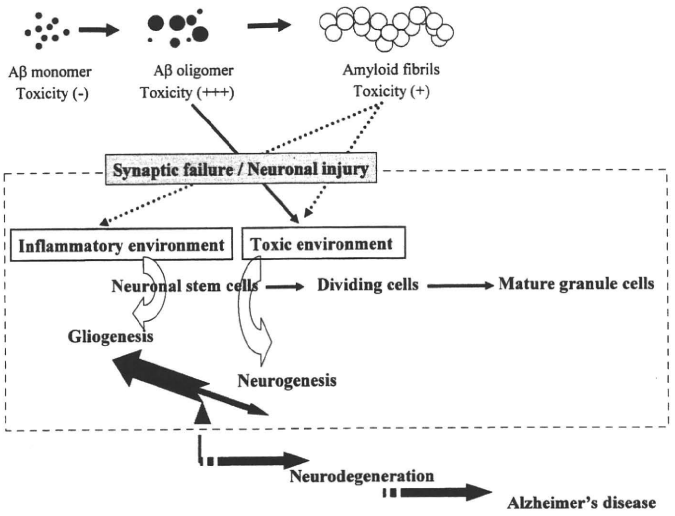
新生神経細胞がおかれる微少環境

では，神経新生を左右するアルツハイマー病の微少環境の特徴は何であろうか．アルツハイマー病患者脳に沈着する典型的老人斑周囲にはミクログリア活性化などの慢性炎症反応が認められ，多くの疫学調査が非ステロイド系抗炎症薬(NSAIDs)の長期服用でアルツハイマー病の発症が減ることを報告している．さらにアルツハイマー病患者脳ではミクログリア活性化に加え，IL6発現増強[14]も報告されている．またこうしたミクログリア活性化による炎症反応環境の存在がアルツハイマー病モデル動物でも示されている[15,16]．興味深いことに慢性炎症やミクログリア活性化が生体脳における海馬神経新生を抑制し，抗炎症薬(イブプロフェン)や抗IL6薬(ミノサイクリン)投与でその回復が図られることがわかっている[17,18]．一方，IL6にはグリオーシス，オリゴデンドロサイトーシスを促進させる作用が報告されている．従って，ミクログリア活性化された慢性炎症反応下では神経新生自体の抑制とアストログリオーシス[19]やオリゴデンドログリオーシス[20]への誘導による間接的な神経細胞新生が抑制される微少環境にあると考えられる．

アルツハイマー病患者脳においては前脳基底部におけるアセチルコリン合成酵素活性が低下しており，この補充目的に現在アセチルコリン分解酵素阻害薬が広く使用されているのは周知の如くである．アルツハイマー病患者脳やモデルマウス脳では成熟新生神経細胞への分化に重要なGABA作動性神経細胞は比較的保たれた環境にあるが，最近，グルタミン酸作動性神経細胞からなる腫大変性神経突起の存在が両脳で明らかとなった．従って，アルツハイマー病患者脳やモデルマウス脳では結果的にGABAやグルタミン酸の入力低下を招き，神経新生にとり抑制的な微少環境が形成されていると推測される[21]．

最近，孤発性アルツハイマー病患者脳においてBACE1活性が上昇していることが明らかとされ，相対的な分泌型APPの減少がアルツハイマー病の病態形成に重要である可能性が推測されている．前述したIL6と同様にこの分泌型APP自体に神経幹細胞からアストロサイトへの分化促進作用があることがin vivoとin vitroにて確認されており[22]，アルツハイマー病患者脳では，その相対的活性低下は神経新生にとり保護的な環境に貢献していると考えられる．

アルツハイマー病患者脳の発症基盤を担う本質的な分子変化はAβやAβオリゴマーの蓄積である．前述したアルツハイマー病モデルマウスやアルツハイマー病患者脳では統一的な見解は得られていないが，Aβ自体の直接的な神経新生への効果を検証したin vitro研究においてHaugheyら[23]は，① 脳室内投与したAβが成体マウス脳室下層の神経新生を障害すること，② Aβ自体が培養神経前駆細胞増殖・分化抑制とアポトーシス誘導による神経細胞死効果を持つことを報告した．逆にLópez-Toledanoら[24]はAβ，特にAβオリゴマー[25]が神経前駆細胞へ作用し神経細胞への分化を促進し神経細胞数を増加させることを報告した．Calafioreら[26]も脳室内投与したAβの成体マウス脳室下層の神経前駆細胞の神経細胞への分化促進効果を報告している．相反する結果でいずれが正しいのかの結論は得られていないが，Aβによる神経細胞分化誘導は成熟神経細胞まで至らぬ，仮に至ったとしてもその成熟神経細胞で神経毒性を示し死滅させてしまう，もしくはその神経回路に組み込みを障害するなど負の側面が大きいと推測される．いずれにしても，細胞死が優位な状況にあるアルツハイマー病では神経前駆細胞にとり有害な微少環境にある，もしくは成熟神経細胞にとり有害な微少環境が優位に立っている



神経新生とアルツハイマー病の病態

と考えられ，この微少環境の改善は神経新生の側面から極めて重要な問題と考えられる．

アルツハイマー病における再生医療の可能性

Magavi ら[27]は神経変性疾患における脳内在性の神経前駆細胞をその場で操作し，神経細胞への置換治療の可能性を報告している．Koketsu ら[28]は Magavi らが報告した障害後の特殊な条件下では大脳皮質でも神経細胞再生がおこるが，健常脳では大脳皮質における神経細胞新生は極めて稀であることを明らかとした．Nakatomi ら[29]は，一過性の脳虚血後認められる死滅海馬錐体細胞の，脳室内への神経成長因子投与による内在性神経幹細胞からの神経新生による海馬錐体細胞補充と，その機能回復が確認されている．以上の知見を総合し神経新生とアルツハイマー病における病態を整理すると，一過性でなく，慢性持続型の，どちらかというと神経新生にとり不利益な微少環境でのアルツハイマー病における神経新生の側面が浮き彫りとなる(図)．現時点においてはアルツハイマー病における再生医療への道程はまだまだ長いようである．

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アルツハイマー病の抗体治療

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〔SUMMARY〕 アルツハイマー病の抗体治療は，その根本治療の可能性を秘めた魅力的な治療法である．現在世界中で熾烈な競争下にあり，臨床の場に登場してくる日も近いと考えられている．〔臨床検査 52：297-301，2008〕

〔KEYWORDS〕 アルツハイマー病，抗体治療，受動免疫療法

はじめに

アルツハイマー病は，細胞内外への線維性構造物の蓄積を認める，いわゆる“原因蛋白の蓄積病”をその基本病態とする．このアルツハイマー病の病理過程でみられる最も早期の変化は細胞外に認められ，βアミロイド(amyloid β；Aβ)を主要構成成分とする斑状の嗜銀性構造物(老人斑)として知られている．アルツハイマー病患者脳ではなんらかの原因でこの本来可溶型の生理的Aβの産生・分解・クリアランスの代謝機構が破綻し，不溶性の高まった病的Aβが脳実質に蓄積し，老人斑としての脳アミロイド沈着を形成すると考えられている．生体の免疫系を駆使しいったん沈着した脳アミロイド除去を標的とした治療がAβワクチン療法である．いわゆる老人斑除去を抗体で行う治療法である．アルツハイマー病の免疫療法(Aβワクチン療法)には，Aβペプチドをadjuvantとともに投与し抗体産生を誘導する能動免疫と直接抗体を投与する受動免疫，全身性免疫ではなく粘膜免疫を用いた免疫療法の3種が試みられているが，本稿では能動・受動免疫につき概説する．

Aβワクチン療法(能動免疫療法)から学んだこと

1999年Schenkらは，革新的なAβワクチン療法を開発し報告した．アルツハイマー病患者脳の老人斑に最初に蓄積を開始するAβ42ペプチドを，試験管内で重合させ老人斑に蓄積しているアミロイド線維様にした後，アルツハイマー病の老人斑を再現するアルツハイマー病モデルマウスに免疫を行った．驚いたことにワクチンを受けたマウス脳内の老人斑(Aβ沈着)は劇的に除去されていたのである[1)]．翌年の2000年になると同グループは，抗Aβ抗体が血液脳関門(blood-brain barrier；BBB)を通過して老人斑をピンポイント攻撃後，ミクログリアを活性化させてアミロイド沈着を貪食・消化させるAβ除去メカニズムを明らかとした[2)]．元来，抗体はBBBを通過しないと考えられており，このメカニズムもまた驚きであった．このAβワクチン療法は，異なる3施設により追試され，ほぼ同様のAβワクチン療法で記憶障害も改善されることや[3,4)]，鼻粘膜投与(粘膜免疫)でも脳アミロイド蓄積改善効果[5)]があることが相次いで報告された．こうしたモデル動物での前臨床試験の結果を受け，2001年ヒトを対象としたAβワクチン療法が開始されたが，残念なことに2002年第Ⅱ相試験中に15例の脳炎患者が発症したためこれらの治験は中止となった．こ

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のワクチン療法の反応者(抗体上昇群)は約20%にとどまったが，興味深いことに得られた抗体は脳内および合成の可溶性Aβやその前駆体分子(amyloid precursor protein；APP)は認識せず，脳内に沈着した病的アミロイドのみを特異的に認識する極めて選択性の高いものであり，抗体の脳脊髄液中移行も確認された[6]．一方，不幸にして亡くなられた2剖検脳(髄膜脳炎合併例[7]と非合併例の各1症例)においては，ワクチン治療で確かに老人斑アミロイドが消失していることも確かめられた．老人斑が消失している部位では，Aβを貪食したミクログリア像が認められたことから，この所見は，脳内移行し老人斑アミロイドに結合した抗体がFc受容体を介してミクログリアに貪食されている像と考えられた．以上のAβワクチン療法の結果から，抗体を血液中で作らせることができれば脳内のアルツハイマー病変を治療可能であるとの大きなヒントが得られた訳である．しかしながら残念なことに，ワクチン後接種後1年の患者追跡結果報告[7]では，Aβワクチンで認知機能の顕著な改善効果は得られていない．この結果を好意的に捉えると，Aβワクチン療法も中等症以上にまで進行した患者においてはその認知機能改善効果が望めず，このワクチン療法はやはり予防的な効果を期待すべき治療法なのではないかとも考えられる．一方，厳しい見方をすれば，老人斑アミロイドを標的とした抗体では認知機能改善効果は望めないということを，本ワクチン治療は実証した治験と捉えることも可能である．ではいかなる抗体が最も効果的なのか？　効果的な抗体を直接投与したほうがいいのではないか？　抗体治療の発想の原点でもある．

受動免疫療法(Aβ抗体治療)と抗体の作用機序

では，受動免疫療法(Aβ抗体治療)とは，このワクチン療法と比較して，どんな利点があり，またどのようにして治療効果を発揮するのかを考えてみたい．Aβワクチン療法は，対象を高齢であるアルツハイマー病患者に設定しており，抗体誘導が投与された個体に依存的で不確定であるとの克服困難な弱点を抱えていた．実際に，ヒトの治験でもワクチン反応群，いわゆる抗体産生群は約20%にとどまり，この問題の深刻さを露呈するかたちとなった．抗体の直接投与はこの弱点を見事に克服可能であるが，アルツハイマー病はあくまで脳に病理変化をきたす中枢性疾患であることから，原則的に血液脳関門(BBB)を通過しない抗体を治療として用いる発想は，抗体治療の致命的弱点であった．ところが，ヒトおよびアルツハイマー病モデルマウスにおけるAβワクチン療法効果発現機序の考察から，①これまで，BBBを容易に通過しないと考えられてきた抗体のわずかがこのバリアーを突破し，脳実質内に異常沈着したAβ凝集塊と結合することがシグナルとなり抗原抗体反応によるオプソニン化[1,2]，ミクログリアによる貪食が促進され老人斑除去がなされていることが実際に確認され，抗体治療にも整合性が得られたわけである．老人斑に親和性の高い抗体が可溶性Aβに親和性の高い抗体より，受動免疫療法では効果的であるとの報告[8]もこの機序を支持し，その追い風となった．一方，AβのN末部に対する抗体は，Aβの凝集を抑制し，さらにこれを溶解し細胞毒性を中和することから，②脳内移行した抗体が直接Aβと結合し，その重合抑制・線維溶解・毒性中和をするとの作用機序も想定されるに至った．Fcγ受容体受容体ノックアウトマウスとアルツハイマー病モデルマウスをかけ合わせたマウスでもAβワクチン効果が認められたこと[9]，また抗Aβ抗体F(ab')2断片でも治療効果が発揮されるとの報告[10]は，この作用機序を支持している．また抗体治療が記憶障害を可逆的に回復させるとの報告は，脳内に移行した抗体がAβ重合体を標的として効果を発揮している可能性を示唆している[11,12]．しかしながらBBBを通過する抗体量はたかだか0.02%との報告もあり[13]，抗体治療をアルツハイマー病において積極的に推進するにはまだハードルが高い状況であったのもまた事実である．こうした抗体治療の常識を一変させる非常に有用な治療概念として登場したのがDeMattosら[14]により提唱されたperipheral sink仮説である．③老人斑除去のために脳内に抗Aβ抗体が入る必要がなく，末梢投与した抗Aβ抗体が血液中に存在する可溶性Aβと結合するだけで，もともと末梢血液と脳内のAβ間に

存在した平衡関係をくずし，脳内から血液中へAβを引き出すとの概念である．脳内に抗体が入ることで惹起されるミクログリア誘発性炎症反応の心配がなく，Aβワクチンにみられる恒常的な自己免疫性の副作用がない点，より安全な脳アミロイド除去療法になるという魅力的な作用機序である．また，Aβに高親和性を持ち結合するgelsolinやGM1を投与し血液中のAβを吸着・除去することで治療効果が上がるとの報告もこの作用機序の整合性を支持している[10]．では，このうちどの機序が抗体治療効果発揮に重要なのか？　おそらく以上述べた3機序は互いに独立排他的なものではなく，それぞれ複合的に作用しているものと考えられる．事実，Wilkock等は受動免疫療法により，ミクログリアによるアミロイド除去と脳から血液へのAβ引き出しによる血中Aβ上昇の両者が起こっていることを報告している[15]．

受動免疫療法の前臨床試験と問題点

この受動免疫療法が最も注目を集めたゆえんは，この抗体投与によりアルツハイマー病モデルマウスで発症した記憶障害を回復できるとの報告[11,12]に端を発している．アルツハイマー病の病態解明に対する貢献度も大きく，現在最も広く信じられている“Aβオリゴマー仮説”の根拠となった報告の一つである．この発見により，実は老人斑Aβアミロイド線維自体の発症病態への関与は低く，アミロイド線維を形成して沈着・蓄積する前の中間分子(Aβオリゴマー)こそがその本態(いわゆる病態惹起性神経毒性分子)で，アルツハイマー病の治療標的であることが認識されたわけである．

ここで，これまで進められてきた抗体治療の前臨床試験を整理してみたい．様々な抗体による受動免疫療法がアルツハイマー病モデル動物で施行され，その有用性が確認されてきた．老人斑除去を標的とした抗体がいわゆる第一世代に分類される．老人斑においてはAβのN末の3～10番目位までのアミノ酸が顔を出しているとの考えに基づき，この部分を認識する抗体が多く属する[2]．一方，AβのC末断端特異的な抗体でも予防的治療効果[16]や選択的老人斑除去効果[13]が認められると報告されている．いずれの抗体においても脳から血液中へAβを引き出して治療効果を上げているとも報告されており，前述のごとく複合的作用を有している．また脳アミロイドを認識せず可溶性Aβのみを認識する抗体であるにもかかわらず脳アミロイド除去が可能な抗体として，Aβの中間部位を認識する抗体m266(Aβ16-24)も報告されている[14]．この抗体は脳アミロイド除去とは無関係に記憶障害改善効果をも発揮する抗体[11,12]であることが示され，第一世代と次に述べる第二世代の抗体の中間的存在である．一方，老人斑除去のみでなく，Aβのアミロイド線維形成や毒性などの抑制活性を念頭に考案された第二世代の抗体治療に注目が集まっている．Aβオリゴマー[17]やシード[18]に対する抗体がこの範疇に分類される．では，Aβワクチンと比較してその安全性の評価はいかなるものであろうか？　これまで沈着した脳アミロイドに結合性を示す抗体を使用した受動免疫療法で，微少出血[19,20]と頻度は低いものの髄膜脳炎[21]の副作用発生がアルツハイマー病モデルマウスで報告され，使用抗体選択の重要性が改めて浮き彫りになってきた．と同時に副作用を未然に防ぐための教訓として，いかにアミロイドが相当量沈着する前に治療を開始することができるか，またそうした対象をいかにして知りうる術をもつか，が大事なポイントであることが明らかとなった．先に述べたAβのC末端部特異的な抗体，ミクログリア誘発性炎症反応を誘発せぬようFc領域を含まぬF(ab')2抗体などの工夫もその一つである．抗体のアイソタイプも老人斑除去機能や副作用発現機序に深く関与しており，治療抗体選択時に注意すべきポイントである．しかしながら，最も重要な試みは，いわゆる病態発症を特異的に制御する目的で，生理的な分子には反応しない，病態惹起分子特異的な抗体による治療法の開発であろう．アルツハイマー病においてはAβオリゴマーがまさにこの標的となり，これまでいくつか報告がなされてきている．われわれもすでに前臨床試験まで終了し，良好な結果が得られてきている．

ヒトにおける受動免疫療法の現況

現在，Wyeth社とElan社により，ヒト化モノクローナル抗Aβ抗体による受動免疫療法の第Ⅱ相臨床治験が進行中である．まだこの結果は出ていないが，第Ⅲ相臨床治験への移行が2007年5月にアナウンスされた．その効果が注目されるところである．一方，市販されている免疫グロブリン製剤にも抗Aβ抗体が含有されていることが知られており[22]，この利点を活用した点滴静脈注射療法も第Ⅱ相臨床治験まで進行中である．

おわりに

アルツハイマー病における抗体医療について解説した．アルツハイマー病を“治療可能な認知症”とするため，今後よりいっそう研きをかけ抗体治療を世の中に送り出したいものである．

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Novel Role of Presenilins in Maturation and Transport of Integrin $\beta1$[†]

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ABSTRACT: Presenilins (PSs) play important roles in modulating the trafficking and maturation of several membrane proteins. However, the target membrane proteins whose trafficking and maturation are regulated by PS are largely unknown. By characterizing PS-deficient fibroblasts, we found that integrin $\beta1$ maturation is promoted markedly in PS1 and PS2 double-deficient fibroblasts and moderately in PS1- or PS2-deficient fibroblasts; in contrast, nicastrin maturation is completely inhibited in PS1 and PS2 double-deficient fibroblasts. Subcellular fractionation analysis demonstrated that integrin $\beta1$ maturation is promoted in the Golgi apparatus. The mature integrin $\beta1$ with an increased expression level was delivered to the cell surface, which resulted in an increased cell surface expression level of mature integrin $\beta1$ in PS1 and PS2 double-deficient fibroblasts. PS1 and PS2 double-deficient fibroblasts exhibited an enhanced ability to adhere to culture dishes coated with integrin $\beta1$ ligands, namely, fibronectin and laminin. The inhibition of γ-secretase activity enhances neither integrin $\beta1$ maturation nor the adhesion of wild-type cells. Moreover, PS deficiency also promoted the maturation of integrins $\alpha3$ and $\alpha5$ and the cell surface expression of integrin $\alpha3$. Integrins $\alpha3$ and $\alpha5$ were coimmunoprecipitated with integrin $\beta1$, suggesting the formation of the functional heterodimers integrins $\alpha3\beta1$ and $\alpha5\beta1$. Note that integrin $\beta1$ exhibited features opposite those of nicastrin in terms of maturation and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus in PS1 and PS2 double-deficient fibroblasts. Our results therefore suggest that PS regulates the maturation of membrane proteins in opposite directions and cell adhesion by modulating integrin maturation.

Mutations in the genes encoding presenilin-1 (PS1[1]) and PS2 account for most cases of familial early onset Alzheimer's disease (FAD) (*1, 2*). PS1 and PS2 most likely provide the catalytic subunit of the γ-secretase complex (*3*). FAD-linked mutant PS proteins increase the level of highly amyloidogenic Aβ42, which is generated by the proteolytic processing of the amyloid precursor protein (APP) and deposited early as senile plaques in the brains of aged individuals and AD patients (*4–6*). PS-mediated cleavage occurs within the transmembrane domain of several type I membrane proteins such as Notch, APP, the APP homologues APLP1 and APLP2, ErbB-4, CD44, N- and E-cadherins, the low-density lipoprotein receptor-related protein (LRP), Syndecan, Delta, Jagged, and Nectin1α (*7*).

PS1 and PS2 may also have other functions, in addition to their central role as catalytic subunits of the γ-secretase complex. Previous studies have shown their involvement in β-catenin turnover, apoptosis, Ca^{2+} homeostasis, and protein trafficking (*8, 9*). PS proteins have also been shown to function as endoplasmic-reticulum (ER)-resident chaperones affecting the maturation of nicastrin (*10–12*), APP (*13–15*), TrkB (*16*), N-cadherin (*17*), and the neurotrophin receptor-like death domain (NRADD) protein (*18*). Nicastrin maturation and cell-surface delivery are completely inhibited in the absence of PS1 and PS2 (*10–12*). PS1 aspartic acid mutants expressed in a PS-null background restore nicastrin maturation but not γ-secretase activity, suggesting a γ-secretase-independent function of PS in the maturation and trafficking of nicastrin (*19*). PS1-null neurons exhibit compromised TrkB maturation (*16*). The transfection of dominant-negative PS1 D385A in SH-SY5Y cells leads to disrupted maturation and a decreased cell-surface expression level of N-cadherin (*17*). In addition, the absence of PS1 and PS2 results in the intracellular retention of caveolin 1, the loss of caveolae (*20*),

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[1] Abbreviations: PS, presenilin; PS1, presenilin-1; PS2, presenilin-2; wt, wild-type; PS-ko, presenilin-1 and -2 double knockout; Aβ, amyloid β-protein; APP, amyloid precursor protein; PNGase F, peptide: *N*-glycosidase F; ER, endoplasmic reticulum; NTF, N-terminal fragment; CTF, C-terminal fragment.

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and an abnormal accumulation of telencephalin/ICAM in intracellular compartments (*21*). These suggest that PS deficiency disrupts the ER-to-Golgi apparatus trafficking of a set of membrane proteins. In contrast to these membrane proteins, APP exhibits enhanced expression and cell surface accumulation in PS1- and PS2-deficient cells. The expression of dominant-negative PS1 D385A or treatment with a γ-secretase inhibitor, DAPT, also leads to an enhanced cell surface accumulation of APP, via the acceleration of APP trafficking (*13*) or the delay of APP endocytosis (*14*).

We determined whether PS deficiency affects the maturation of other membrane proteins and whether ER-to-Golgi apparatus trafficking is generally disrupted in PS-deficient cells. We examined several type I membrane proteins in PS1 and PS2 double-deficient cells and found that the loss of PS1 and PS2 results in an enhanced maturation of integrin β1 and an enhanced cell-surface delivery of mature integrin β1.

MATERIALS AND METHODS

Cell Culture and Antibodies. Wild-type (wt), PS1- and PS2 double-knockout (PS-ko), PS1-deficient (PS1 −/−), and PS2-deficient (PS2 −/−) mouse embryonic fibroblast (MEF) cell lines were kindly provided by Dr. Bart De Strooper (*22*). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS). The cells were lysed in RIPA buffer [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.2% sodium deoxycholate, containing a protease inhibitor cocktail (Roche, Mannheim, Germany)] at a point before or after confluence. Monoclonal antibodies against integrins β1, α3, αV, syntaxin 6, BiP/GRP78, and calnexin were obtained from BD Biosciences (San Jose, CA). Polyclonal antibodies against integrins β1, α1, α2, α4, α5, α6, α7, αL, and the N-terminus of PS1 (H-70) were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against the loop of PS1 (MAB5232), which recognizes the C-terminal fragment of PS1, was purchased from Chemicon (Temecula, CA). A polyclonal antinicastrin antibody raised against the C-terminus of nicastrin (amino acids 693–709) was purchased from Sigma (Saint Louis, MO).

Electrophoresis, Immunoblotting, Deglycosylation, and Immunoprecipitation. Total protein (50 μg) from cell lysates was dissolved in SDS sample buffer, separated on 4–20% gradient gels, and transferred to nitrocellulose membranes (equal loading was confirmed by Western blotting for Bip/GRP78 or α-tubulin). The target proteins were visualized using SuperSignal (Pierce, Rockford, IL) with antibodies to integrins, nicastrin, Bip/GRP78, calnexin, syntaxin 6 and PS1. To assess integrin β1 maturation and nicastrin glycosylation, lysates from the wt and PS-ko cells were treated with PNGase F, *O*-glycanase, or sialidase A using an enzymatic deglycosylation kit according to the manufacturer's instructions (PROzyme, San Leandro, CA). For immunoprecipitation, the cells were homogenized in a solution of 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) containing a protease inhibitor cocktail, and the homogenate was centrifuged at 10,000*g* and 4 °C for 10 min. The supernatant was immunoprecipitated with a polyclonal antibody to integrin β1 or PS1 and protein G sepharose (Amersham Biosciences, Uppsala, Sweden). Coimmunoprecipitated PS1, nicastrin, integrin β1, and integrin α subunits were detected by Western blotting.

Subcellular Fractionation on Iodixanol Gradient. The wt and PS-ko cells were grown in eight 10-cm tissue culture dishes, and subcellular fractionation was performed as previously described (*23*). They were homogenized in an ice-cold homogenization buffer [10 mM HEPES (pH 7.4), 1 mM EDTA, and 0.25 M sucrose containing a protease inhibitor cocktail]. The postnuclear supernatant was centrifuged for 1 h at 4 °C and 65,000*g*. The resultant vesicle pellets were rehomogenized in 0.8 mL of the homogenization buffer and layered on a step gradient consisting of 1 mL of 2.5%, 2 mL of 5%, 2 mL of 7.5%, 2 mL of 10%, 0.5 mL of 12.5%, 2 mL of 15%, 0.5 mL of 17.5%, 0.5 mL of 20%, and 0.3 mL of 30% (v/v) iodixanol (GIBCO). After centrifugation at 90,000*g* (SW41 rotor, Beckman) for 2.5 h at 4 °C, 11 fractions were collected from the top of the gradient.

Transfection, γ-Secretase Inhibitors Treatment and Aβ ELISA. The retrovirus-mediated gene expression of human APP695, PS1, PS2, PS1D257A, PS1D385A, PS1ΔE 9, PS1I143F, PS1R278K, and PS1L392V was carried out as previously described (*24*). The fibroblasts were transfected at 10% confluence and maintained in DMEM containing 10% fetal calf serum. The transfection efficiency was nearly 100% in this study, as estimated by the control transfection of the pMX-green fluorescent protein (pMX-GFP). γ-secretase inhibitors, namely, DAPT and L-685,458, were added to the wt cells stably expressing hAPP695 immediately after passage. The culture medium was collected two days after confluence, and the level of Aβ1-40 secreted was measured using an Aβ ELISA kit (Wako Pure Chemical, Osaka, Japan).

Cell Surface Biotinylation and Cell Surface Uptake of Integrin β1. Cell surface biotinylation was carried out using a Pinpoint cell surface protein isolation kit (Pierce). The wt and PS-ko cells were grown in four 10-cm tissue culture dishes, and washed twice with ice-cold PBS (GIBCO). The cells were incubated in 10 mL of ice-cold 0.25 mg/mL sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) (Pierce) in ice-cold PBS for 30 min at 4 °C. Then, 500 μL of the quenching solution was added to each dish to quench the reaction. The cells were scraped and washed twice with Tris-buffered saline (TBS) [10 mM Tris/HCl (pH 7.5) and 150 mM NaCl] and lysed in the lysis buffer containing protease inhibitors. Each lysate was incubated with streptavidin-agarose beads (Pierce) at 4 °C for 60 min, and captured proteins were eluted with 50 mM DTT in Laemmli's SDS sample buffer. To assess cell surface integrin β1 internalization, immunostaining was performed as previously reported (*14*). The cells plated on a fibronectin-coated culture slide were washed in ice-cold PBS, and incubated on ice with a monoclonal antibody against integrin β1 at 1:200 dilution in PBS containing 0.1% BSA. After 20 min, the cells were washed with ice-cold PBS, and then incubated in prewarmed culture medium for various durations at 37 °C. After the indicated durations, the cells in the culture slides were fixed with 4% paraformadehyde in PBS for 20 min. After rinsing three times in PBS, permeabilization was achieved in 0.1% Triton X-100/PBS for 5 min, and the slides were incubated with rhodamine-coupled goat antimouse IgG

(Chemicon) for 20 min. Confocal images were taken with a Zeiss LSM 510 confocal system (Carl Zeiss, Jena, Germany).

Cell Attachment Assay. Ninety-six- and 6-well plates (Corning Inc., Corning, NY) were coated with 10 μg/mL fibronectin or laminin (Sigma) for 8 h at 4 °C. After aspirating the coating reagent, 0.2 or 1.5 mL of 10 mg/mL filtered, heat-denatured bovine serum albumin (BSA) (Sigma) was dispensed into the wells, and the plates were incubated at 4 °C for 16 h. The cell attachment assay using single resuspended cells was carried out in 96-well plates as previously reported (*25*). Subconfluent cells were washed with HEPES-buffered saline [HBS, 150 mM NaCl, and 25 mM HEPES (pH 7.5)] and resuspended at a density of 0.2 to 1 $\times$ 10^6 cells/mL. A 50-μL aliquot of the cell suspension was then added to each well. The plates were incubated for 30 min at 37 °C in 5% (v/v) CO_2. Unbound or loosely bound cells were removed by aspiration and gentle washing with HBS. To assess the total number of cells added, 100%, 75%, 50%, 25%, and 0% cells were added to the wells and fixed by adding 1/10 vol of 50% (v/v) glutaraldehyde. To assess the attachment strength of the wt and PS-ko cells after the cells reached confluence, the cell attachment assay was performed in 6-well plates. After aspirating the conditioning medium, the cells were incubated in 1 mM EDTA for 30 min at room temperature. Detached cells were washed out with HBS. The cells were fixed in the wells by adding 5% (v/v) glutaraldehyde in HBS and stained with 0.1% (w/v) crystal violet in 200 mM MES (pH 6.0) for 60 min. The solution in the wells was then aspirated, and the wells were washed with water. Acetic acid [10% (v/v)] was dispensed into the wells of the 96-well plates, and the absorbance at 570 nm of each well was measured with a multiscan plate reader. Images of the 6-well plates were taken when the wells were dried, and the area of the wells occupied by adherent cells was measured using Image J 1.36b software (NIH, Bethesda, MD).

RESULTS

Integrin β1 is synthesized as an 87-kDa polypeptide that undergoes glycosylation in ER and the Golgi apparatus. In ER, the most prevalent, incompletely glycosylated immature integrin β1 has a mass of 105 kDa. The mature form of this 105 kDa integrin β1 has a mass of 125 kD (*26, 27*). The immature form is not found on the cell surface and has no role in cell adhesion or cell signaling (*26, 28*). These features of integrin β1 in terms of maturation are similar to those of nicastrin, a member of the γ-secretase complex. To determine how the absence of PS affects integrin β1 maturation, we performed a Western blotting of cell lysates prepared from wt, PS-ko, PS1(−/−), and PS2(−/−) fibroblasts. In the wt cells, integrin β1 was detected as a nonglycosylated core protein (87 kDa), a poorly glycosylated immature protein (105 kDa), and a highly glycosylated mature protein (125 kDa). Most of these integrin β1 isoforms are of the immature form. Interestingly, a marked increase in the expression level of mature integrin β1 and a decrease in that of ER-localized immature integrin β1 were observed in the PS-ko cells, suggesting that PS proteins exert an inhibitory effect on the post-translational maturation of integrin β1 (Figure 1A). The PS1(−/−) or PS2(−/−) cells exhibited an intermediate increase in the expression level of mature integrin β1 and a

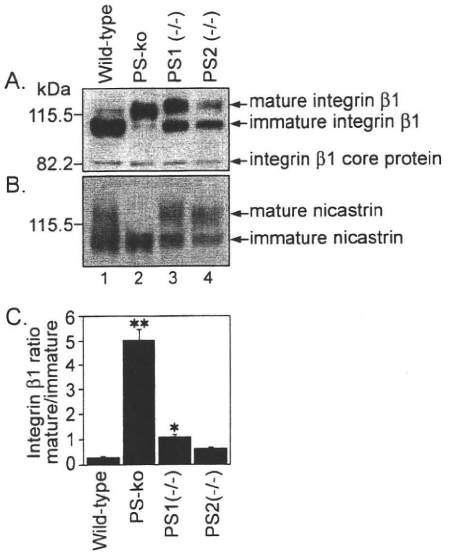

FIGURE 1: Presenilin deficiency promotes integrin β1 maturation and inhibits nicastrin maturation. The wt, PS-ko, PS1(−/−), and PS2(−/−) fibroblasts were lysed in RIPA buffer 2 days after reaching confluence. Western blots of 50 μg of total protein from the cells were probed with an anti-integrin β1 monoclonal antibody (A) or antinicastrin polyclonal antibody (B). Three isoforms of integrin β1 were observed in the cells: the ~87-kDa core protein, ~105-kDa immature glycosylated form, and ~125-kDa mature glycosylated form (indicated by arrows). Note that the predominant isoform of integrin β1 in the wt fibroblasts was the immature form and that in the PS-ko fibroblasts was the mature form. For nicastrin, the immature form of ~105 kDa and the mature form of ~125 kDa were observed in the wt fibroblasts. The mature form of nicastrin was absent in PS-ko fibroblasts. The expression level ratio of mature integrin β1 to immature integrin β1 was determined by densitometry (C). Data represent the means ± SEM; $n = 3$, $*p < 0.05$, $**p < 0.001$, PS-ko or PS1(−/−) vs wt, Bonferroni/Dunn test.

decrease in that of immature integrin β1, suggesting that the regulation of the post-translational maturation of integrin β1 is PS-dependent, not cell-line-dependent (Figure 1A). The PS-ko cells showed a level ratio of mature integrin β1 to immature integrin β1 18-fold higher than that of the wt cells. The PS1(−/−) cells showed a 4-fold increase in the level ratio of mature integrin β1 to immature integrin β1 (Figure 1C). In contrast to integrin β1, the 125-kDa mature nicastrin species showed a significant decrease in expression level (Figure 1B), in agreement with previous reports (*10–12*). These results suggest that PS regulates the maturation of membrane proteins in opposite directions.

To determine whether the mature integrin β1 with an increased expression level is glycosylated in PS-ko cells as it is in wt cells, we experimentally examined the glycosylation of mature integrin β1. Similar to nicastrin, both immature and mature integrin β1s were sensitive to PNGase F. Digestion with PNGase F decreased the apparent size of mature and immature integrin β1s to 95 kDa as a major species in the wt cells (Figure 2A, lane 5). Integrin β1 in the PS-ko cells was partially resistant to PNGase F. In addition to the 95-kDa species, a 105-kDa species was detected after digestion with PNGase F. This PNGase F-resistant 105-kDa integrin β1 was likely generated from the 125-kDa mature integrin β1 because of its abundance in the PS-ko cells and scarcity in the wt cells (Figure 2A, lane 6). However, the wt cells showed a 105-kDa nicastrin PNGase F-resistant species in addition to the 70-kDa

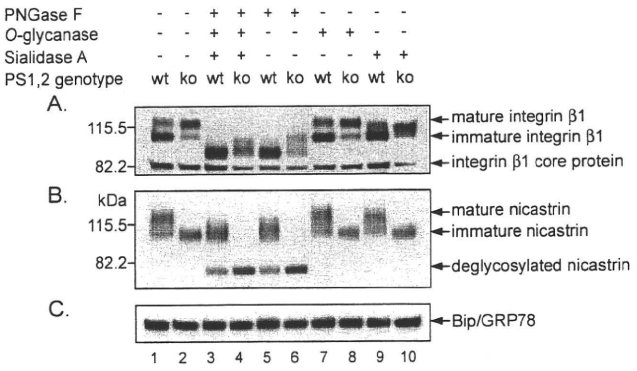

FIGURE 2: Deglycosylation of integrin β1 and nicastrin. Total protein (50 μg) from the wt or PS-ko fibroblast lysate was digested with PNGase F (0.1 U/mL), *O*-glycanase (0.025 U/mL), or sialidase A (0.1 U/mL) and analyzed by SDS−PAGE and immunoblotting. Western blots were probed with an anti-integrin β1 monoclonal (A), antinicastrin polyclonal (B), or anti-Bip/GRP78 (C) antibody. Lanes 1 and 2, no treatment; lanes 3 and 4, PNGase F, *O*-glycanase, and sialidase A treatment; lanes 5 and 6, PNGase F treatment; lanes 7 and 8, *O*-glycanase treatment; and lanes 9 and 10, sialidase A treatment. The mature form of integrin β1 in the PS-ko cells and the mature form of nicastrin in the wt cells are partially PNGase F-resistant. Equal amounts of protein loaded are shown by the Western blot of Bip/GRP78.

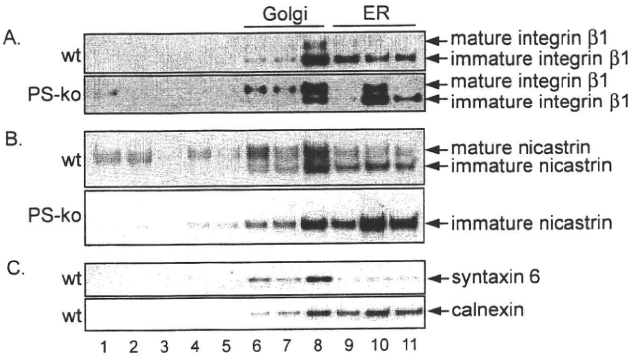

FIGURE 3: Absence of PS1 and PS2 enhances integrin β1 maturation in the Golgi apparatus and has no effect on the intracellular distribution of integrin β1. The wt and PS-ko fibroblasts were harvested and fractionated on iodixanol gradients. Fractions rich in ER (lanes 9–11) are at the bottom, and 11 fractions were collected from top to bottom. Golgi-apparatus-rich fractions are shown in lanes 6–8. The fractions were analyzed by immublotting with an anti-integrin β1 antibody (A), an antinicastrin antibody (B), and antibodies to the Golgi apparatus marker protein syntaxin-6 and ER marker protein calnexin (C). Note that nicastrin was retained in the ER fractions of the PS-ko cells, but not integrin β1. The maturation of integrin β1 in PS-ko fibroblasts in the Golgi apparatus was markedly accelerated compared with that in wt fibroblasts.

deglycosylated species; the PS-ko cells showed that the apparent size of the 105-kDa immature nicastrin as a single species decreased to 70 kDa (Figure 2B, lanes 1, 2, 5, and 6). Treatment with *O*-glycanase had no effect on the SDS−PAGE mobility of either integrin β1 or nicastrin, indicating the absence of an *O*-linked glycosylation of these two proteins (Figure 2A and B, lanes 7 and 8). The mature forms, not the immature forms of integrin β1 and nicastrin were sensitive to sialidase A digestion, indicating the sialylation of the mature forms of both proteins (Figure 2A and B, lanes 9 and 10). The ER protein Bip/GRP78 served as the internal control protein, which indicated the same amount of protein loaded in each lane (Figure 2C). These results show that (i) in contrast to that of nicastrin, whose mature form is absent in PS-ko cells, the maturation of integrin β1 in PS-ko cells is enhanced compared with that in wt cells and that (ii) mature integrin β1 in PS-ko cells is normally glycosylated by *N*-glycans, the characteristics of which are similar to those of *N*-glycans in mature nicastrin in wt cells.

The results described above demonstrate that the absence of PS1 and PS2 promotes integrin β1 maturation. We also confirmed that the absence of PS1 and PS2 inhibits nicastrin maturation, as shown by previous studies. To investigate how the absence of PS1 and PS2 disrupts the processing and intracellular distribution of integrin β1 and nicastrin, we carried out iodixanol gradient fractionation to separate the Golgi apparatus and ER-derived membranes (*23*). Syntaxin 6 and calnexin in the wt cells served as the Golgi apparatus and ER markers, respectively (Figure 3C). The distributions of syntaxin 6 and calnexin in PS-ko cells did not differ from those in wt cells (data not shown). In the wt cells, integrin β1 and nicastrin underwent normal maturation and ER-to-Golgi apparatus trafficking, with most of their immature forms localizing in the ER, and most of their mature forms localizing in the Golgi apparatus. The mature and immature forms of nicastrin showed patterns similar to those of the mature and immature forms of integrin β1. In contrast, in the PS-ko cells, mature forms of nicastrin were absent, and most of the immature forms of nicastrin were restricted in the ER fractions, suggesting the disrupted exit of nicastrin from ER. Interestingly, in the PS-ko cells, the expression level of mature integrin β1 increased predominantly in the Golgi apparatus, whereas that of immature integrin β1 significantly decreased in the Golgi apparatus, as compared with those in the wt cells, indicating that the maturation of integrin β1 in the Golgi apparatus is enhanced. In addition, there was no selective retention of integrin β1 in the ER in the PS-ko cells. These results suggest that the trafficking of immature integrin β1 from ER to the Golgi apparatus is accelerated in PS-ko cells (Figure 3A and B).

Because PS1 and PS2 can regulate cell signaling pathways via γ-secretase activity (*29*), we determined whether the blockade of γ-cleavage leads to an enhanced maturation of integrin β1. We treated hAPP-transfected wt cells with two major γ-secretase-specific inhibitors, namely, DAPT and L-685,458, to inhibit γ-secretase activity and monitored the level of Aβ1-40 secreted in the culture medium to evaluate γ-secretase activity (Figure 4). γ-secretase inhibitors at a concentration higher than 2.5 μM completely inhibited γ-secretase activity but did not facilitate integrin β1 maturation, indicating that the inhibition of γ-secretase activity is not sufficient to facilitate integrin β1 maturation (Figure 4 A and B). In Figure 1, we show that the expression level of mature integrin β1 was enhanced in three independent PS-deficient cell lines, namely, PS-ko, PS1(−/−), and PS2(−/−). The expression level of mature integrin β1 inversely correlated with the expression level of PS in these three independent cell lines. The transfection of PS-ko cells with human PS1 and PS2 restored the normal expression of mature nicastrin and inhibited the maturation of integrin β1, suggesting that PS is essential for post-translational maturation of nicastrin and for inhibiting the maturation of integrin β1 (Figure 4C). The transfection with PS1 aspartate mutants lacking γ-secretase activity, namely, PS1 D257A and PS1 D385A, did not restore the expression of mature integrin β1, whereas it restored the normal expression of mature

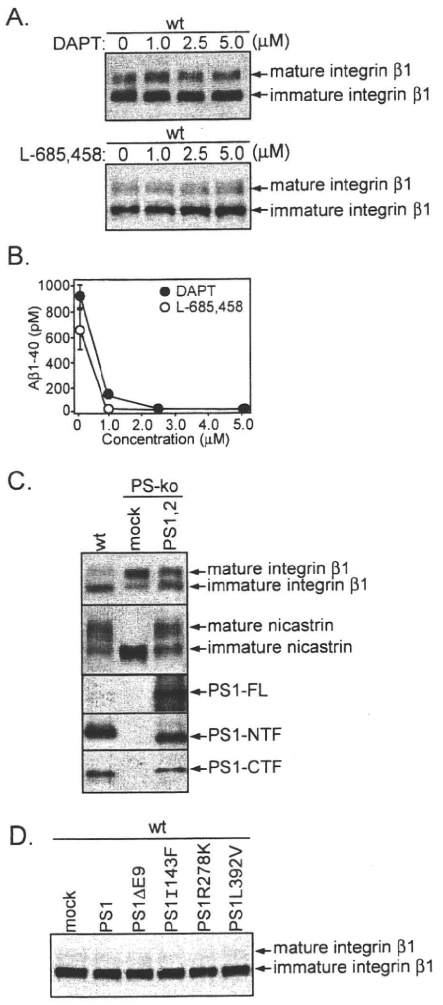

FIGURE 4: Effects of γ-secretase inhibitors and FAD PS mutants on integrin β1 maturation and restoration of integrin β1 and nicastrin maturation by transfection with PS1 and PS2. The wt fibroblasts stably expressing human APP695 were treated with or without DAPT or L-685,458 immediately after passage. The cells were lysed after reaching confluence, and the lysate was analyzed by immunoblotting with an anti-integrin β1 antibody (A). The level of Aβ1-40 secreted to the culture medium was measured using an Aβ1-40 ELISA kit (Wako) (B). γ-secretase inhibitors at a concentration greater than 2.5 μM completely inhibited γ-secretase activity, which was monitored by analyzing Aβ1-40 secretion; however, integrin β1 maturation remained unchanged. The PS-ko fibroblasts were transfected with human PS1 and PS2. Western blots of 50 μg of total protein from the transfected PS-ko fibroblasts were probed with an anti-integrin β1 antibody, an antinicastrin antibody, and anti-PS1 antibodies (C). Double transfection of PS-ko fibroblasts with human PS1 and PS2 restored the maturation of integrin β1 and nicastrin. The wt fibroblasts were transfected with human PS1 and FAD PS1 mutants, and integrin β1 maturation remained unchanged (D). FL, full-length; NTF, N-terminal fragment; CTF, C-terminal fragment.

nicastrin (data not shown). Because PS1 aspartate mutants do not form the mature, high molecular weight PS complexes (*30*), these results suggest that the formation of the high molecular weight PS complex may be required to inhibit integrin β1 maturation. The expression of transfected PS1 was confirmed by Western blotting. The exogenous human PS1 in PS-ko cells was maintained in larger amounts of the full-length form and smaller amounts of the N-terminal fragment (NTF) and C-terminal fragment (CTF) than those of endogenous mouse PS1 in wt cells. Interestingly, the human PS1-NTF in the PS-ko cells showed a lower molecular weight than the mouse PS1-NTF in the wt cells, whereas the full-length human and mouse PS1s showed the same molecular weight. In agreement with this result, the human PS1-CTF in the PS-ko cells showed a higher molecular weight than the mouse PS1-CTF in the wt cells (Figure 4C, bottom three panels). These results suggest that mouse and human PS1s may undergo principal endoproteolytic cleavage at different sites or that presenilinase cleaves PS1 at different sites in wt and PS-ko cells. We also examined whether the overexpression of the PS1 mutants of familial Alzheimer's disease (FAD) alters integrin β1 maturation in wt cells. The overexpression of human wt PS1 and FAD PS1 mutants, namely, PS1ΔE 9, PS1I143F, PS1R278K, and PS1L392V, did not affect integrin β1 maturation in the wt cells, suggesting that the loss of PS function, probably the loss of both the γ-secretase and the chaperone protein functions of PS, may facilitate integrin β1 maturation (Figure 4D).

Integrin β1 associates with multiple integrin α-subunits to form transmembrane receptors of extracellular matrix proteins, including fibronectin, collagen, and laminin (*31–33*). To determine whether mature integrin β1 with an increased expression level in PS-ko cells is delivered to the cell surface, we determined the expression level of integrin β1 on the cell surface by surface biotinylation. Neither immature integrin β1 nor immature nicastrin was biotinylated in the wt or PS-ko cells, indicating that no immature forms of the two proteins localize on the cell surface (Figure 5A and B). The expression level of surface-biotinylated mature integrin β1 in the PS-ko cells significantly increased compared with that in the wt cells, indicating that the cell-surface delivery of integrin β1 is enhanced in PS-ko cells (Figure 5A, lanes 3 and 6). The expression level of integrin β1 on the surface of the PS-ko cells was 2.5-fold that on the surface of the wt cells (Figure 5C). In contrast to integrin β1, surface-biotinylated nicastrin was detected in the wt cells; however, no apparent signal of this protein was detected in the PS-ko cells, indicating that the cell-surface delivery of nicastrin is impaired in PS-ko cells (Figure 5B, lanes 3 and 6). Because the increased surface expression level of mature integrin β1 can be induced by delayed internalization and accelerated trafficking to the cell surface, we investigated the internalization of mature integrin β1 in living wt and PS-ko cells. The cells were labeled on ice with an antibody that recognizes integrin β1, washed, and incubated at 37 °C to initiate internalization. At the indicated time points, the cells were fixed, permeabilized and processed for immunofluorescence staining. The PS-ko cells had a large amount of surface-labeled integrin β1 in the absence of incubation at 37 °C (Figure 5D), which was consistent with the results of biotinylation. After 10 min of incubation at 37 °C, surface integrin β1 was partially internalized, and after 30 min, surface integrin β1 disappeared and was completely internalized in both the wt and PS-ko cells, indicating that PS deficiency has no effect on integrin β1 internalization. Combined with the results of the iodixanol gradient fractionation, these results suggest that the increased cell-surface expression level of mature integrin β1 is induced by the accelerated trafficking of integrin β1 from ER to the Golgi apparatus and then to the cell surface.

To determine whether an increased cell-surface expression level of mature integrin β1 has the same effect in PS-ko cells as in wt cells, we performed a cell attachment assay to