から比較的容易に回収できるAβを含む数少ない材料である。これが脳内のAβ沈着量を反映していれば、特定の設備を持たなくとも、CSFの値から脳内Aβ沈着量を予測することができるだろう。ただし、少なくとも年齢とAβ沈着量には差がなかった。Aβ沈着量との関連は次年度への課題である。

E. 結論

食物回収試験は高齢のサルでも試行に取り組むことが可能であり、食物の位置を認知する機能を評価する第一段階はクリアできた。サルのCSFは年齢との関係はなかったが、今後、脳内Aβ沈着量との関連を調べていく予定である。

G. 研究発表

1．論文発表

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2．学会発表

1. Nakamura S, Okabayashi S, Ageyama N, Koie H, Sankai T, Ono F, Fujimoto K, Terao K. Transthyretin amyloidosis and two other aging-related amyloidoses in an aged vervet monkey. 1st meeting of Asian Society of Veterinary Pathologist. Taipei, Taiwan. Aug, 2007.

H. 知的財産権の出願・登録状況

該当なし。

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Tabira, T et al.	Current Topics in Neuroimmunology	Tabira, T., Yamamura, T. Kira, J.	Current Topics in Neuroimmunology.	Medimond	Bologna, Italy	2007	
Hara, H., Tabira, T.	Oral Aβ vaccine using adeno-associated virus vector for Alzheimer's disease.	Tabira, T., Yamamura, T. Kira	Current Topics in Neuroimmunology	Medimond	129-137	2007	129-137

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Lakshmana, M.K., Hara, H. Tabira, T.	Amyloid beta protein-related death-inducing protein induces G2/M arrest: Implications for neurodegeneration in Alzheimer's disease.	J. Neurosci. Res.	85	2262-71	2007
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Liu, H., Wang, J.,Sekiyama, A., Tabira T.	Juzen-taiho-to, an Herbal Medicine, Activates and Enhances Phagocytosis in Microglia/Macrophages.	Tohoku J. Exp. Med .			in press
Wang, J., Hara, H., Makifuchi, T. Tabira,T.	Development and characterization of a TAPIR-like mouse monoclonal antibody to Aβ.	J. Alzheim. Dis			in press

Oral Aβ Vaccine Using Adeno-associated Virus Vector for Alzheimer's Disease

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Summary

We developed Aβ vaccine using recombinant adeno-associated virus vector and Aβ1-43 or Aβ1-21 cDNA (rAAV/Aβ). When rAAV/Aβ was given once orally, antibodies to Aβ were elevated and high titers were maintained for more than 40 weeks. The antibody subtypes were IgG1 and IgG2b. This vaccine reduced amyloid burden significantly in APP transgenic mice (tg2576) without any complications such as meningoencephalitis. Among cytokines TGF-β1 was significantly reduced in the serum and brain of vaccinated mice. The vaccinated mice showed significant improvement in cognitive functions such as Y-maze test, two novel object recognition test and water-maze test. Here we discuss about advantage of this oral vaccine.

1. Invention of vaccination therapy and clinical trial

Alzheimer's disease (AD) is characterized by progressive loss of cognitive function associated with β amyloid deposits in the central nervous system (1). Immunization of amyloid precursor protein (APP)-transgenic mice with synthetic Aβ in complete and subsequently incomplete Freund's adjuvant showed a marked reduction of amyloid burden in the brain (2). Repetitive passive transfer of Aβ antibodies (3) was also effective for reducing the amyloid deposits. Although Aβ is not an infectious agent, this treatment is now widely accepted as "vaccination" from its analogy in the mechanism. Since vaccinated mice showed an improvement of memory loss in mice (4, 5), clinical trials were performed in humans in USA and Europe. The phase II trial of AN-1792 vaccine composed of synthetic Aβ1-42 and adjuvant QS21 was halted because of a serious complication, subacute meningoencephalitis which appeared in 6% of patients (6). However autopsy cases with or without the

complication suggested effective clearance of β amyloid by vaccination (7-9), and a series of patients at an institute, who produced tissue amyloid plaque immunoreactive (TAPIR) antibodies showed less cognitive decline than those who did not produce such antibodies (10). Although the cognitive function decline in a whole patients who attended the trial was not different between patients with high ELISA antibodies and those without the antibodies, treated patients who developed high antibody titers showed significantly better scores in some memory functions (11). Therefore, Ab vaccination seems to be a promising way to delay the onset or to slow down the progression of AD, if the complication is minimized.

2. Production of AAV/Aβ vaccine

We have developed an oral Aβ vaccine using the recombinant adeno-associated virus vector (AAV), which successfully reduced amyloid burden in tg2576 APP transgenic mice without any complications (12). The AAV vector (serotype 2) carrying human Aβ1-43 or Aβ1-21 was constructed using plasmid DNA pTRUF2 and the secreted form of Aβ was made by linking the APP signal sequence (SS) to the Aβ sequence. Human embryonic kidney (HEK) 293 cells were co-transfected with SS-Aβ pTRUF2 and plasmid pXX2 and pXX6 as described (13).

To confirm the secretion of Aβ, we transfected HEK293 cells with the SS-Aβ1-43pTRUF2 expression vector. An immunoprecipitation and Western blot method revealed Aβ monomers in the cell lysate and Aβ oligomers in the conditioned medium. When AAV/Aβ was given in mice, Aβ expression was observed immunohistochemically in the lamina propria of the upper part of the small intestine. There was no increase of Aβ1-43 or Aβ1-21 in the serum. Transduction of AAV was confirmed by PCR in intestinal cells, but not in the liver, spleen, heart, lung and kidney 4, 11 and 21 weeks after treatment, suggesting an absence of widespread infection of the virus vector.

3. Vaccination and tissue examinations

AAV/Aβ43 or AAV/Ab21 was diluted with PBS, and $5x10^{11}$ genome in a final volume of 0.1 ml was administered once to tg2576 mice (Taconic) using an orogastric tube in the treated group; control mice received 0.1 ml of PBS similarly. Since immunization with vector alone did not show any significant difference in our preliminary study, we used PBS as control. Mice were randomized to four groups; a group treated at age 15 weeks; a group treated at age 30 weeks; a group treated at age 45 weeks; and control groups treated with PBS at each age. Each group consisted of 4-6 mice. At the age of 56 weeks all mice were anesthetized with Nembutal, and their brains were fixed in 4% paraformaldehyde with 0.1 M phosphate buffer, pH 7.6 for immunohistochemical analysis.

Oral vaccination with AAV/Aβ43 or AAV/Aβ21 resulted in marked reduction of Aβ deposition in all treated groups compared to the control examined at age 56 weeks (Fig. 1). Quantitative image analyses in three differ-

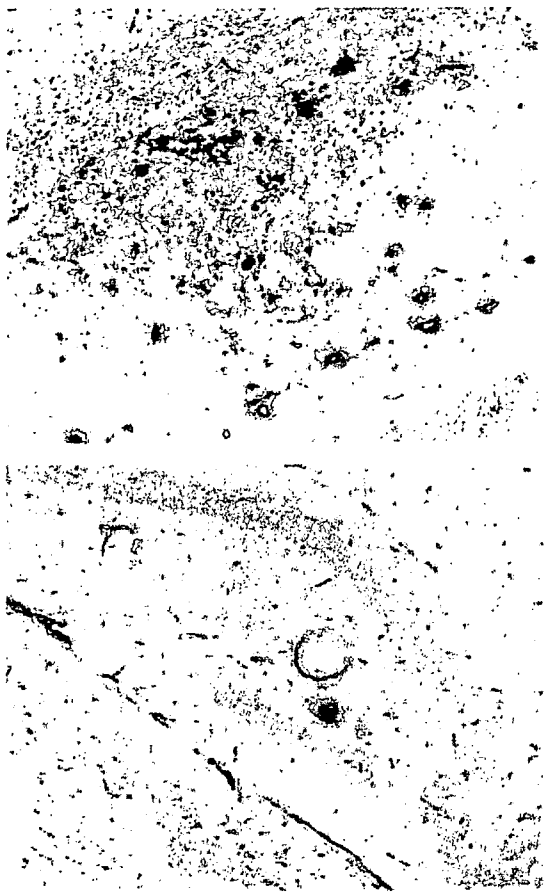

Fig. 1. Amyloid plaques in the brain of control and vaccinated mice. Immuno-stained senile plaques with an anti-Aβ antibody were significantly reduced in mice received AAV/Aβ vaccine at 15 weeks, 30 weeks and 45 weeks old than control. Shown is a brain specimen obtained from a control mouse (A) and a mouse vaccinated at 30 weeks of age (B).

ent regions of the brain showed a significant decrease of Aβ burden in all groups of treated mice compared to controls.

HE staining of the brain sections of the treated mice showed no lymphocytic infiltration in either leptomeninges, cerebral cortex or white matter, and there was no hemorrhagic lesion in the brain. Immunohistochemical studies did not reveal any cellular infiltration positive for CD3, CD4, CD86, CD19, and CD11b in brain sections. Iba-1$^+$ activated microglia were numerous in vaccinated mice, and some microglia cells containing phagocytosed Aβ were observed. In contrast, GFAP$^+$ cells were less frequent in vaccinated mice.

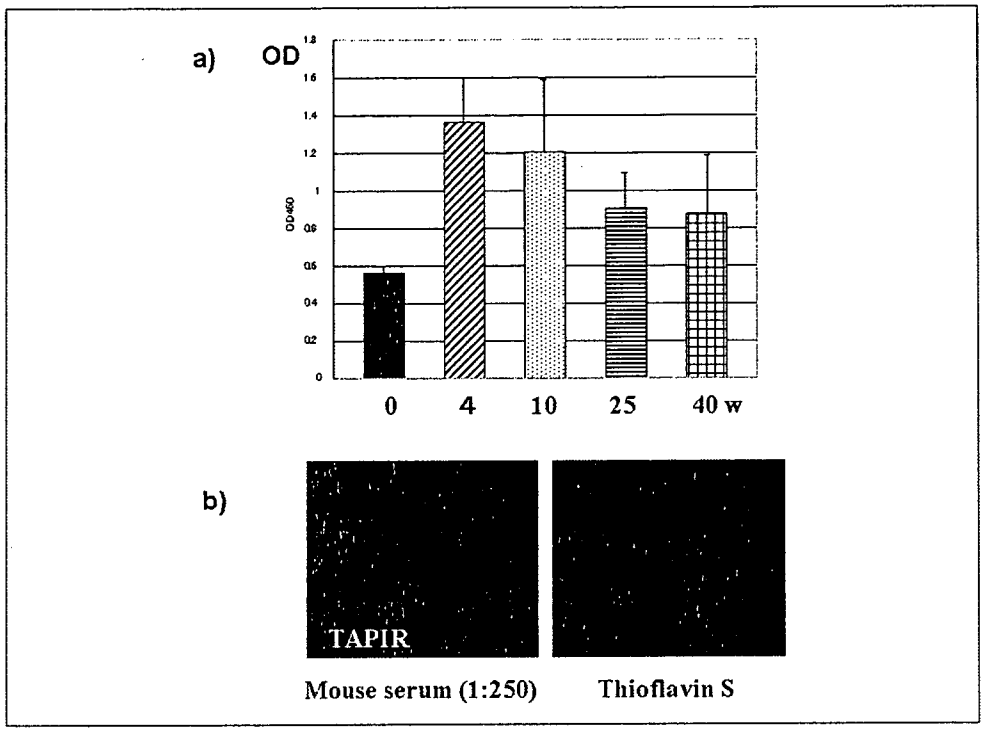

Fig. 2. Antibody responses in AAV/Aβ vaccine-treated mice. Oral AAV/Aβ vaccine was given once and serum antibodies were measured by ELISA. a. The antibody to Aβ was well elevated 1 month after vaccination and the levels were kept high at 40 weeks after vaccination. b. Sera (1:250 dilution) from mice vaccinated with AAV/Ab vaccine reacted with senile plaque amyloid in the brain of AD patients (red), which was stained with thioflavin S (green).

4. Immune responses to Aβ

In the treated tg2576 mice, IgG antibodies were detected in the serum at 4 weeks, and kept elevated for more than 6 months (Fig. 2a). The antibody isotypes were mainly IgG1 and to a lesser amount IgG2b, but IgG2a was not detected and IgA was low. The TAPIR antibody was positive, in other words, the immune sera from vaccinated mice stained the amyloid plaques of the AD brain (Fig. 2b). The proliferative response of spleen T cells against Aβ peptide was not detected in the vaccinated mice as well as in control mice.

Cytokines were measured in the serum using antibody-coated beads against various cytokines. Most of cytokines were not different in vaccinated mice except for TGF-β1 which was significantly lower in vaccinated mice (Fig. 3a). Immunohistochemical staining of the brain with antibodies to TGF-β1 confirmed the reduced expression of TGF-β1 mainly in the senile plaques (Fig. 3b).

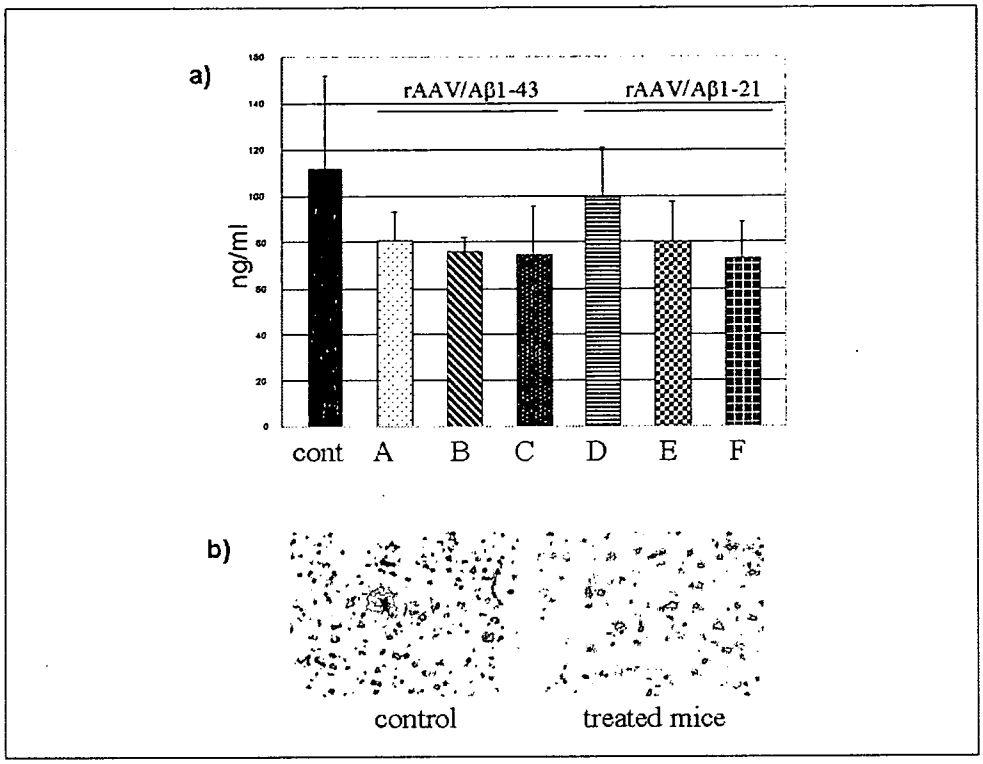

Fig. 3. TGF-β1 is significantly reduced in vaccinated mice. a. Serum cytokines were measured by antibody-coated microbeads. Most of cytokines were not significantly changed except for TGF-β1 which was significantly decreased in vaccinated mice. b. Immunohistochemical staining of tg-2576 mice brain showed reduced staining of TGF-β1 in senile plaques and vessels.

5. Vaccination improved cognitive functions

The treated mice showed significant improvement in several cognitive functions (14). The Y-maze test was done at 6 months of age and tg2576 mice showed significant abnormalities, although senile plaques were not observed at this age (Fig. 4a). AAV/Aβ43 vaccine was given similarly at 10 months of age and the Y maze test was done at 13 months of age. The vaccinated mice showed significant improvement in this test (Fig. 4b).

The novel object recognition test was done similarly. This function was not disturbed at 6 months of age, but it was significantly worse in transgenic mice at 10 months of age. When AAV/Aβ43 vaccine was given at 10 months of age and the test was done at 13 months of age, the vaccinated mice showed significant improved in this function. We also performed Morris water maze test similarly and vaccinated mice showed significant improvement.

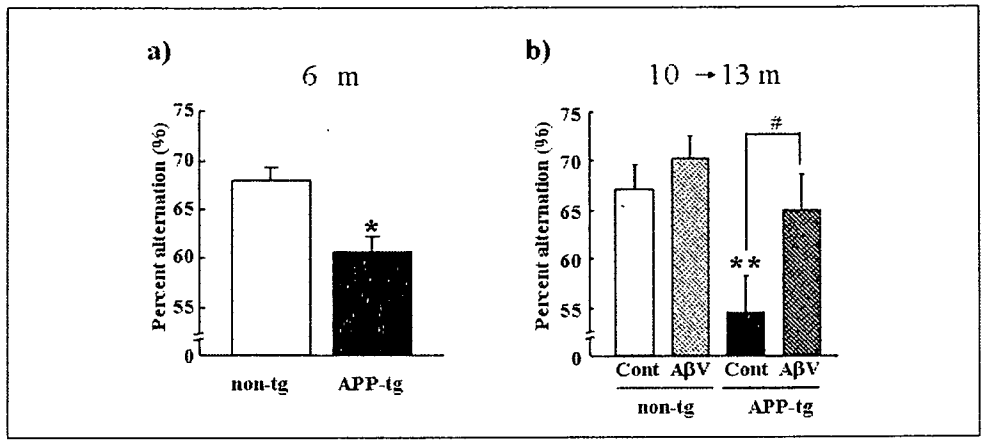

Fig. 4. Vaccinated mice showed improvement in Y-maze test. a. Y-maze test was abnormal at 6 months of age in tg-2576 mice. b. Oral Aβ vaccine was given at 10 months of age and Y-maze test was examined at 13 months of age. The result shows significant improvement in vaccinated mice. App-tg, APP transgenic mice; AbV, Aβ vaccine.

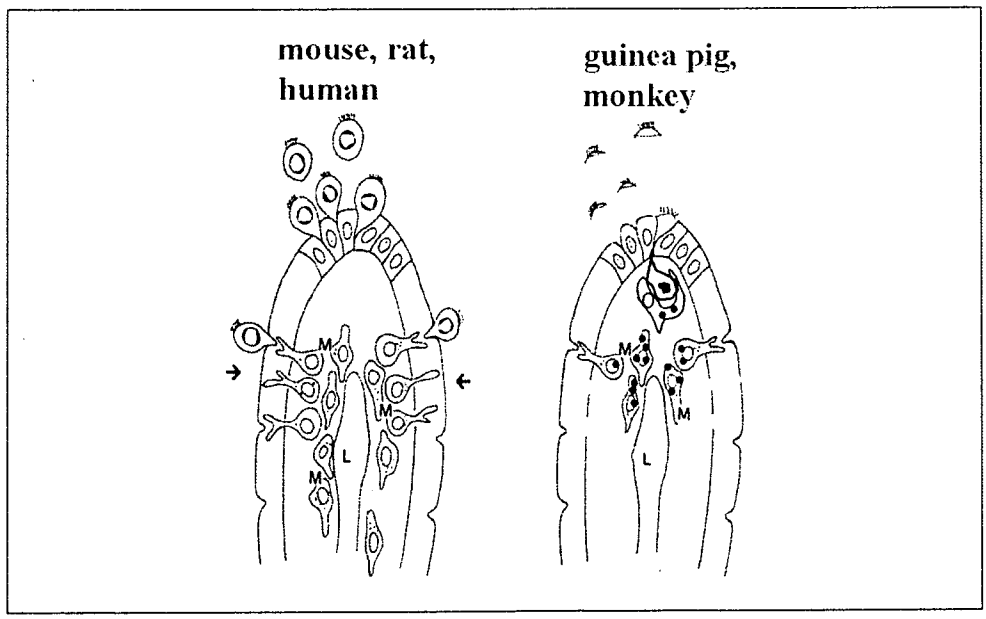

Fig. 5. Renewal of gut epithelium. Gut epithelial cells are renewed in a few days by exfoliations of apoptotic cells in mice, rats and probably in humans, while they are engulfed by macrophages in guinea pigs and monkeys[18].

6. Aβ vaccine-mediated meningoencephalitis

The exact mechanism of meningoencephalitis in patients who received AN-1792 vaccine is not known yet. The autopsied brain showed cellular infiltrates composed mainly of $CD4^+$ T cells and $CD8^+$ T cells. The MRI findings were similar to post-vaccinal encephalomyelitis, although gray matter lesions were more pronounced in AN-1792-related meningoencephalitis. It has been reported that there exist Aβ-reactive T cells in the human peripheral blood, and the frequencies are higher in the elderly (15). Thus, it is highly probable that the AN-1792-related meningoencephalitis is autoimmune encephalitis, probably due to Th1 immune responses to Aβ. If this is the case, it may be possible to induce similar conditions experimentally in animals. Although there is a report showing experimental meningoencephalitis in B6 mice immunized with Aβ (16), we and others could not confirm their observation. However, Monsonego et al. could induce encephalitis in APP mice crossed with interferon-γ transgenic mice which have augmented Th1 immune responses (17). Thus, it is reasonable to think that the meningoencephalitis is mediated by autoimmune Th1 T cells reactive to Aβ.

7. Gut immune system and advantage of AAV/Aβ vaccine

It is well known that the gut immune system is strongly shifted to Th2. There are two types of T helper cells, Th1 and Th2. Th1 T cells mainly help cellular immune responses and suppress Th2 cells. It is known that effector T cells for autoimmune encephalomyelitis are Th1 type. On the other hand, Th2 T cells mainly help humoral immune responses and suppress Th1 cells. Thus, the use of the gut immune system has a big advantage to induce antibodies and suppress adverse T cell immune responses. Since anti-Aβ antibodies were continuously elevated for more than 40 weeks in mice which received our oral Aβ vaccine, it might be sufficient for patients to take the oral vaccine once or twice a year. In addition, AAV was detected only in the gut without spreading over in other organs. Adeno-associated virus DNA normally does not integrate into the cellular genome, instead it remains in the episomal region. Moreover, since the turnover of epithelial cells of the GI tract is relatively quick, the recombinant AAV is eliminated along with the course of renewal of the epithelial cells, suggesting a lower risk in case of unexpected events. It is interesting to know that the majority of apoptotic epithelial cells of the murine and probably human gut is exfoliated into the gut lumen, while those of the guinea pig and monkey gut are engulfed by macrophages in the lamina propria (18) (Fig. 5). Thus, majority of Aβ cDNA in the epithelial cells seems to be deleted along with exfoliation of gut epithelial cells in humans. However, we could detect AAV DNA by PCR amplification in the gut for 6 months. Thus, a small amount of AAV may be retained in certain cells including the stem cell of the gut epithelium. In the case of stem cells, viral DNA will be rapidly diluted along with mytosis.

8. The mechanism of Aβ vaccine

The mechanism by which Aβ vaccine clears b amyloid from the brain tissue is still unknown. There are several hypotheses. First, Fc receptor-mediated uptake of Aβ-antibody complexes by activated microglia (3). Second, antibody-mediated disaggregation of amyloid fibrils (19). Several reports indicated therapeutically active antibodies mainly recognize the residue 4-10 of Aβ peptide and these antibodies inhibit Ab fibrillogenesis and cytotoxicity. Third, DeMattos et al. (20) hypothesized that injected antibodies sequestrate Aβ from the peripheral blood and eventually pull Aβ out of the brain. In our vaccinated mice, some activated microglia contained Aβ, and sera from vaccinated mice showed inhibition of Aβ aggregation. Thus, all three mechanisms seem to be likely involved.

Recently, Rivest and his colleagues demonstrated that microglia migrated in senile plaques are bone marrow origin during younger ages, which eat amyloid more efficiently. However, in aged mice those are resident microglia and do not eat amyloid efficiently. To show this they used chimeric mice of APP transgenic mice transplanted with bone marrow cells from GFP transgenic mice (21). They found green cells in senile plaques in the chimeric mice up to 9 months old. If this is the case, it is more important to activate peripheral macrophages to eliminated amyloid plaque in Alzheimer's disease. Activation of peripheral macrophages may be an additional mechanism in Aβ vaccination therapy.

In conclusion, this oral Aβ vaccine seems to be safe and beneficial for AD and clinical trial should be considered after confirmation of its efficacy and safety in old monkeys.

Acknowledgements

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primary antibodies diluted in 3% BSA-PBST. The slides were washed in PBST and incubated in Alexa Fluor®594-conjugated donkey anti-rat IgG and Alexa Fluor®488-conjugated donkey anti-goat IgG or Alexa Fluor®488-conjugated goat anti-rabbit IgG secondary antibodies (Invitrogen) in 3% BSA-PBST. After washing, fluorescent microscopy was performed with an LSM510 confocal microscope (Carl Zeiss).

We investigated whether particular experimental conditions modify the expression of BACE, and found a novel alternative splicing variant. cDNA from SH-SY5Y cells treated with protein synthesis inhibitors was subjected to PCR amplification using the human BACE cDNA-specific primers A and B. Treatment with protein synthesis inhibitors generated a 180 bp band in addition to the 368 bp (derived from wild-type BACE), 293 bp (I-476), 236 bp (I-457) and 161 bp (I-432) bands (Fig. 1B). Sequence analysis showed that the 180 bp transcript was produced by utilizing an internal splicing donor site in exon 3 (a deletion of BACE cDNA nucleotides 380–567). This splicing event led to generation of an internal stop codon, resulting in a coding sequence of 127 amino acids (GenBank accession no. AB089958). To confirm that I-127 contains exon 1 to 4, cDNA from SH-SY5Y cells treated with anisomycin was amplified by PCR using several primer pairs targeting sequences in exon 1 and exon 4. Sequence analysis of the PCR products showed that the 247 bp, 187 bp, and 163 bp fragments contained identical sequences to the expected sequence of I-127 (Fig. 1C). The novel isoform, designated I-127, lacked 375 amino acids that contained a conserved aspartic protease active site domain, four all *N*-glycosylation sites, a transmembrane domain, and a C-terminal cytoplasmic tail. We also found that low levels of I-127 mRNA were expressed in brains of human AD patients and non-AD controls (Fig. 1D). Since postmortem brain tissue displays varying degree of gross RNA degradation, we could not quantify differences in I-127 expression levels between AD patients and non-AD controls.

To characterize the intracellular localization of I-127, HEK293 cells were transfected with an empty vector or an expression vector with an HA epitope tag fused to the carboxy-terminus of BACE or I-127. Cytosol and membrane fractions as well as the culture medium were subjected to SDS-PAGE, followed by Western blot analysis using an anti-HA antibody, a propeptide-specific antibody (BACE 26–45), and an antibody (BACE 46–67) raised against the amino terminus of mature BACE. Fig. 2 shows that transfected I-127-HA migrated at about 15 kDa and that this band was also recognized by BACE 26–45 and BACE 46–67. These data suggest that the propeptide of I-127-HA was not cleaved. BACE-HA and I-127-HA were exclusively found in the membrane fraction and not in the cytosol fraction or culture medium. To examine the subcellular distribution of I-127-HA, HEK293 cells doubly expressing BACE-HA and I-127-HA were homogenized, and the post-nuclear supernatant was subjected to an iodixanol gradient. Proteins present

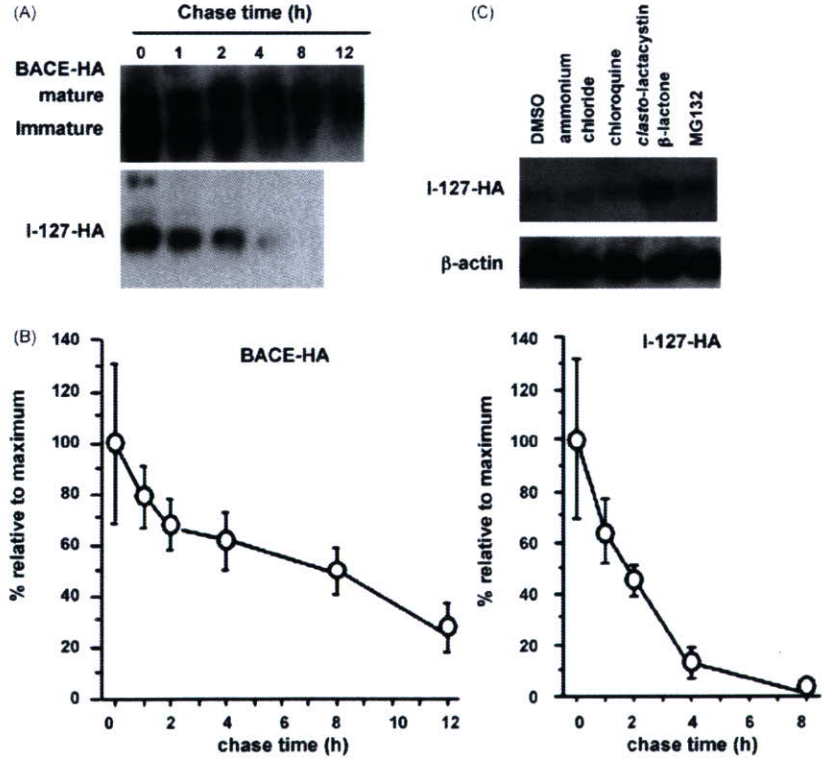

Fig. 4. Turnover and proteasome-dependent degradation of I-127. (A) Representative autoradiograms. HEK293 cells transiently expressing BACE-HA or I-127-HA were metabolically labeled for 1 h and chased for the indicated time points. Cells were then lysed and immunoprecipitated with 3F10. (B) The radioactivity of total BACE-HA (immature and mature) and I-127-HA. The results are expressed as percentages (±S.D., $n=2$) of total BACE-HA or I-127-HA radioactivity at the 0-h time point. (C) I-127-HA cDNA-transfected HEK293 cells were treated with vehicle DMSO (lane 1) or inhibitors of lysosomal or proteasome degradation (lanes 2–5). I-127 HA and β-actin were detected by 3F10 and anti-β-actin antibody (AC-15, Sigma).

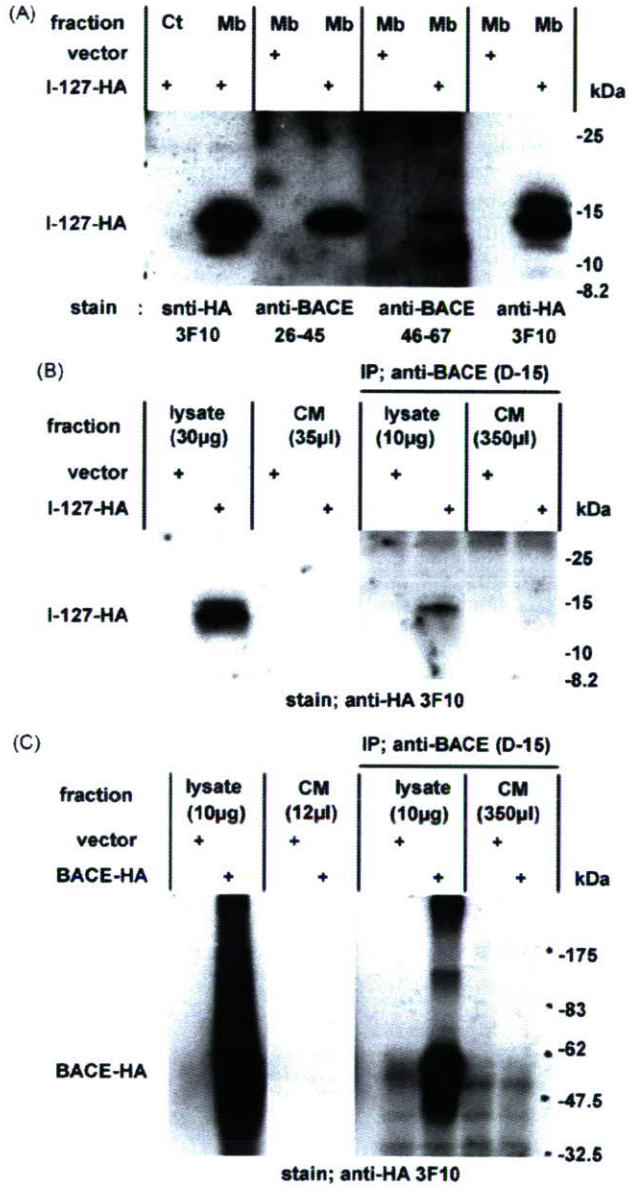

Fig. 2. Localization of I-127 and lack of propeptide cleavage. (A) Membrane fractions (Mb) and cytosol fractions (Ct) of HEK293 cells transfected with either pcDNAZeo or pcDNAZeo/I-127-HA were subjected to Western blot analysis using 3F10, BACE 26–45, and BACE 46–67. (B) The total cell lysate (30 μg) and 35 μl of the culture medium (CM) were subjected to Western blot analysis using 3F10. The total cell lysate (10 μg) and 350 μl of the CM were also immunoprecipitated with D-16 and subjected to Western blot analysis. (C) The total cell lysate (10 μg) and 12 μl of the CM were subjected to Western blot analysis using 3F10. The total cell lysate (10 μg) and 350 μl of the CM were also immunoprecipitated with D-16 and subjected to Western blot analysis.

The culture media and total cell lysates (amounts indicated in Fig. 2) were immunoprecipitated with the goat anti-BACE antibody D-16 (Santa Cruz) raised against a peptide mapped to the internal region of human BACE and UltraLink™ immobilized protein A/G overnight at 4 °C. The beads were washed three times with wash buffer (PBS containing 0.2% NP-40 and protease inhibitors), boiled for 5 min in SDS sample buffer, and the elutions were collected. Total cell lysates, the cytosol and membrane fractions, and the immunoprecipitates were fractionated by 15 or 8% SDS-PAGE and then subjected to Western blot analysis using the rat anti-HA antibody 3F10 (Roche), D-16, the rabbit anti-BACE antibody BACE 26–45 (Chemicon) specific for amino acids 26–45 of human BACE, or the rabbit anti-BACE antibody BACE 46–67 (Chemicon) specific for amino acids 46–67 of human BACE. Discontinuous iodixanol gradient fractionation was performed according to Majoul et al. [12]. Ten 1 ml fractions were collected from the top of the gradient. For immunoblotting, fractions were diluted to 2 ml with PBS, and membranes were pelleted by centrifugation at 125,000 *g* for 1 h. Pellets dissolved in SDS sample buffer were subjected to Western blot analysis. Marker proteins for specific organelles were detected by the appropriate antibodies for the ER (Grp78 N-20, Santa Cruz; ribophorin I C-15, Santa Cruz), ER-Golgi intermediate compartment (ERGIC) (rab6 C-19, Santa Cruz), and Golgi (γ1-adaptin M-300, Santa Cruz).

Twenty-four hours after transfection, cells were seeded on Lab-Tek II chamber slides (Nalge Nunc), fixed in 4% paraformaldehyde in PBS at room temperature (15 min), and permeabilized with 0.2% Triton X-100 in PBS. After blocking by incubation in 4% BSA in PBS containing 0.05% Tween 20 (PBST) (1 h), the slides were incubated at 4 °C (16 h) in

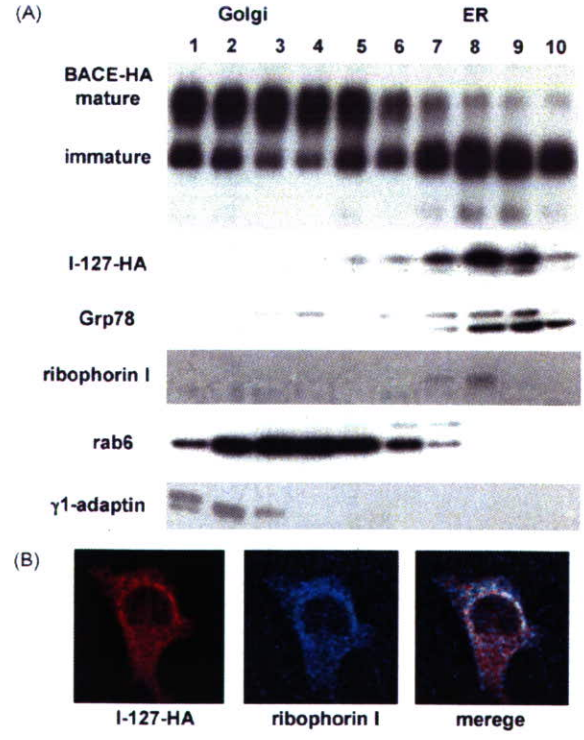

Fig. 3. Iodixanol gradient fractionation and immunocytochemical analysis. (A) Iodixanol gradient fractionation. HEK293 cells doubly expressing BACE-HA and I-127-HA were homogenized. The post-nuclear supernatant was subjected to iodixanol fractionation and Western blot analysis using the appropriate antibodies. (B) Immunocytochemical analysis. I-127-HA-transfected cells were double-stained with 3F10 and anti-ribophorin I, and the positive staining showed a partially overlapping pattern.

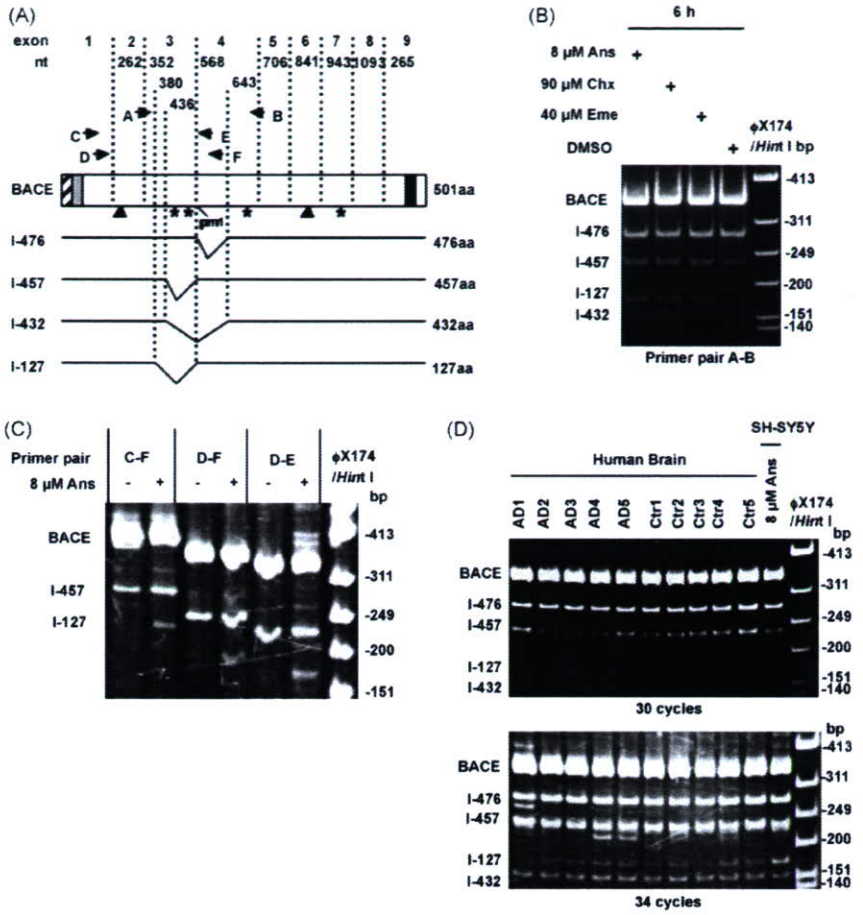

Fig. 1. Detection of alternatively spliced transcripts by PCR. (A) Schematic representation of the coding exons and alternatively spliced transcripts of BACE. The translation initiation site, 5′ nucleotide sequence of each exon, end of the coding sequence, and the internal splice site in exons 3 and 4 are shown. The signal peptide, propeptide, and transmembrane domains are indicated by a hatched rectangle, a grey box, and a black box, respectively. Triangles and asterisks denote protease aspartic active sites and *N*-glycosylation sites, respectively. pmt; premature termination codon. The positions of primers A (nt 344–363), B (antisense of nt 711–692), C (nt 180–199), D (nt 220–239), E (antisense of nt 590–570) and F (antisense of nt 614–595) are indicated by arrows. (B) cDNA from SH-SY5Y cells treated with vehicle (DMSO), 8 μM anisomycin (Ans), 90 μM cycloheximide (Chx), or 40 μM emetine hydrochloride (Eme) for 6 h was subjected to PCR amplification (30 cycles) using primers A and B [16]. (C) Presence of exons 1–4 in I-127. cDNA from SH-SY5Y cells treated with 8 μM Ans was amplified by PCR (30 cycles) using the indicated primer pairs. The arrowhead, square, and circle denote the PCR products derived from BACE, I-457, and I-127, respectively. (D) Detection of I-127 mRNA in the human brain. cDNA from SH-SY5Y cells treated with 8 μM Ans and from cortical tissues were subjected to PCR amplification using primers A and B under the indicated cycling conditions. AD1 to AD5 were AD samples. Ctr1 to 5 were non-AD control samples.

female, age 68, Ctr4; male, age 82, Ctr5; male, age 58). All brain samples were approved to be used for research by informed consent from the family of each patient.

The entire coding sequence of BACE or I-127 cDNA, tagged with a hemagglutinin (HA)-epitope sequence at the C-terminus, was amplified and cloned into the pcDNAZeo vector (Invitrogen). HEK293 cells were transfected with pcDNAZeo, pcDNA/BACE-HA or pcDNA/I-127-HA using LipofectAMINE^{TM} 2000 transfection reagent (Invitrogen). At 5 h after transfection, the medium was changed and at 48 h post-transfection, cells and culture media were harvested for subcellular fractionation. Metabolic labeling and pulse-chase experiments were performed as described in [16] and the cells were chased in MEM supplemented with 10% FBS for the times indicated in the figure. For treatment with proteasome or lysosomal degradation inhibitors, 50 μM MG132 (Merck), 20 μM *clasto*-lactacystin β-lactone (Merck), 20 mM ammonium chloride (Sigma) or 100 μM chloroquine (Sigma) were added at 6 h before cell harvesting at 30 h post-transfection.

Cells were washed with ice-cold PBS and removed in the presence of removing medium [12]. For the preparation of total cell lysate, the cells were sonicated in RIPA buffer [16]. For subcellular fractionation, the cells were homogenized in homogenization medium [12] by repeated aspiration: 10 times each through 22- and 25-gauge needles, respectively. The homogenates were spun at $1000 \times g$ for 10 min and the supernatants were spun at $3000 \times g$ for 10 min. The obtained supernatants were further centrifuged at $100{,}000 \times g$ for 30 min, and the resulting supernatants and pellets dissolved in SDS sample buffer were used as cytosol and membrane fractions, respectively. The collected culture media were precleared with UltraLink^{TM} immobilized protein A/G (Pierce).

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A novel beta-site amyloid precursor protein cleaving enzyme (BACE) isoform regulated by nonsense-mediated mRNA decay and proteasome-dependent degradation

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Abstract

Proteolytic cleavage of amyloid beta-peptide (Aβ) from amyloid precursor protein (APP) is a key event in the pathogenesis of Alzheimer's disease. Beta-site amyloid precursor protein cleaving enzyme (BACE) cleaves the APP at the N-terminus of Aβ. We investigated whether particular stress conditions modify the expression and activity of BACE, and found that treatment of human neuroblastoma cells with protein synthesis inhibitors induced expression of a novel splice variant of BACE. This unusual transcript, I-127, is produced by usage of an internal splicing donor site in exon 3. The splicing event leads to a premature termination codon, as well as elimination of one of two conserved aspartic protease active sites, a transmembrane domain, and a C-terminal cytoplasmic tail from BACE. Low levels of this mRNA were found in the human brain. When expressed in cells, I-127 had no effect on Aβ secretion and was retained in the endoplasmic reticulum without propeptide removal. It was also unstable with a turnover $t_{1/2}$ of ~2 h; normal BACE had a turnover $t_{1/2}$ of ~8 h. Finally, I-127 was degraded in a proteasome-dependent manner. Thus, I-127 is regulated by both nonsense-mediated mRNA decay (NMD) and proteasome-dependent degradation.

Keywords: Alternative splicing; Alzheimer's disease; BACE; mRNA surveillance; Nonsense-mediated mRNA decay; Proteasome-dependent degradation

Alzheimer's disease (AD) is the most common type of senile dementia and characterized by the progressive formation in the brain of insoluble amyloid plaques consisting of the 4-kD Aβ protein. Aβ is sequentially cleaved from APP by two proteolytic enzymes, the β- and γ-secretases. BACE has been shown to have all the properties of β-secretase [18]. The BACE open reading frame encodes a protein of 501 amino acids containing an N-terminal 21-amino acid signal peptide, a 24-amino acid propeptide, two conserved aspartic protease active site domains, a transmembrane domain (residues 461–477), and a 24-amino acid C-terminal cytoplasmic tail (Fig. 1A). Immunostaining has demonstrated predominant localization of BACE to the Golgi and endosomes [18]. BACE is expressed initially as a preproprotein and then cotranslationally *N*-glycosylated. This process produces a ~60-kDa immature BACE in the endoplasmic reticulum (ER), when then undergoes propeptide cleavage and further complex glycosylation, producing a final ~70-kDa mature BACE in the Golgi [3,5,7]. Three alternatively spliced variants of the BACE gene have been described [2,16]. These variants are the result of the alternative splicing of parts of exon 3 and/or exon 4, which produces in-frame deletions of 75 (I-476), 132 (I-457), and 207 (I-432) nucleotides, and results in protein isoforms with different enzymatic activities [2,16]. In contrast to BACE, I-476 and I-457 are retained in the ER in an immature state [2,4,16]. We have investigated whether particular stress conditions modify the expression and activity of BACE. Here we report a novel splicing variant I-127 induced by treatment of human neuroblastoma cells with protein synthesis inhibitors.

Tissue samples were taken from the frozen frontal lobes of patients (AD AD1; male, age 74, onset 69, AD2; female, age 82, onset unknown, AD3; female, age 76, onset unknown, AD4; male, age 82, onset 80; AD5, female, age 65, onset 50; Non-AD control, Ctr1; male, age 65, Ctr2; female, age 78, Ctr3;

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in each subcellular fraction were analyzed by Western blot analysis (Fig. 3A). The majority of mature BACE-HA was found in the Golgi-enriched fractions, whereas immature BACE-HA was mainly located in the ER-enriched fractions. However, I-127-HA was exclusively localized to the ER-enriched fractions. To determine the subcellular localization of I-127, we also performed double immunofluorescence staining using antibodies against ribophorin I (a marker for the ER), γ1-adaptin (a marker for the Golgi), and EEA1 (a marker for endosomes). The specificities of these antibodies were confirmed by Western blotting of cell lysates (data not shown). When I-127-HA transfected cells were double-stained with anti-HA and anti-ribophorin I, the positive staining patterns partially overlapped (Fig. 3B). Anti-HA immunoreactivity was not clearly colocalized with that of anti-γ1-adaptin and anti-EEA1 (data not shown). These data suggest that I-127 is localized to the ER.

To compare the fates of I-127 and BACE, I-127-HA or BACE-HA cDNA-transfected HEK293 cells were metabolically labeled and chased for various time periods, and cell extracts were analyzed after immunoprecipitation with anti-HA antibody. Fig. 4 shows that I-127-HA was unstable with a turnover $t_{1/2}$ of ~2 h, whereas BACE had a turnover $t_{1/2}$ of ~8 h. This result suggests that I-127 is regulated by post-translationally. Treatment with the proteasome inhibitors MG132 and *clasto*-lactacystin β-lactone prevented I-127 degradation (Fig. 4C). However, treatment with inhibitors of lysosomal degradation, ammonium chloride and chloroquine, did not protect I-127 from degradation. These data suggest that when I-127 was expressed in cells, it was unstable, retained in the ER without propeptide removal, and degraded in a proteasome-dependent manner.

We examined whether I-127 affects the secretion of Aβ or BACE activity. Fig. 5A shows that overexpression of BACE-HA increased the secretion of Aβ40 and Aβ42 by about 71% and 87%, respectively, when compared to pcDNAZeo-transfected cells. However, overexpression of I-214-HA did not significantly affect the secretion of Aβ40 and Aβ42. Furthermore, Fig. 5B shows that overexpression of BACE-HA increased the secretion of Aβ40 and Aβ42 by about 50% and 48%, respectively, compared with pcDNAZeo-transfected cells, and that co-transfection with I-127-HA did not influence BACE-HA-mediated effects on Aβ secretion. Thus, I-127 affected neither the secretion of Aβ nor the activity of BACE.

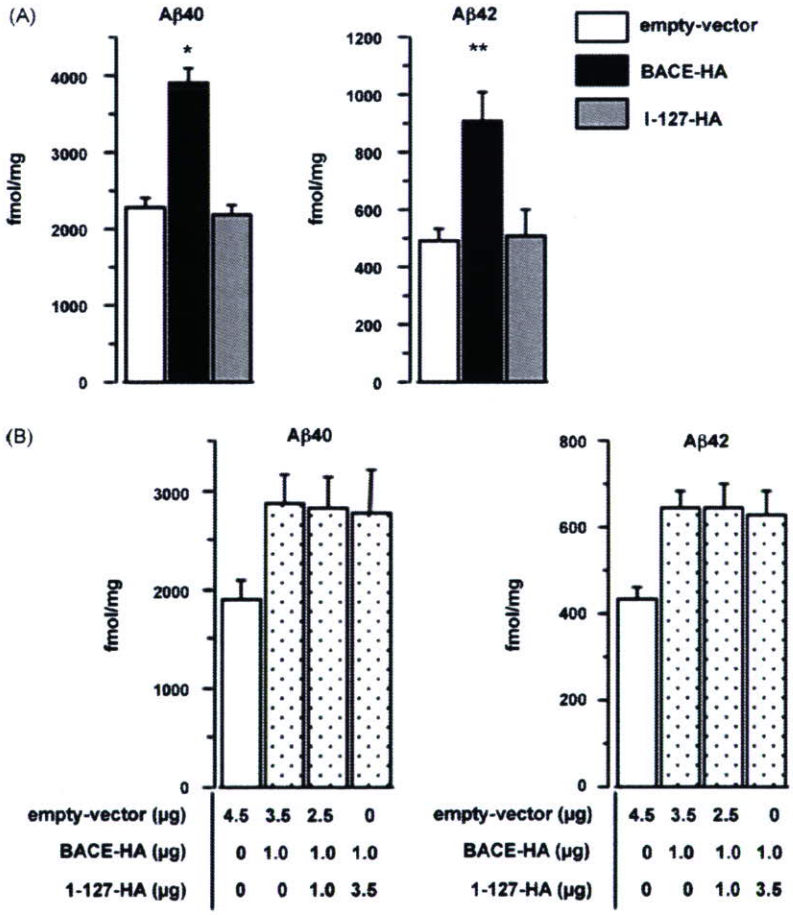

Fig. 5. Effect of I-127 on Aβ production in transiently transfected cells. HEK293 cells that stably overexpressed APP695 [16] in 6-well plates (4×10^5 cells per well) were transfected with the expression vectors indicated in the figure. Forty-three hours after the final change of medium, the media were collected. Aβ sandwich ELISA was performed as previously described [15]. The quantity of Aβ40 and Aβ42 was normalized to the protein content of the cells. (A) Effect of I-127 on Aβ secretion. Results are the mean ± S.D. of four samples. $^{*}P < 0.0001$, $^{**}P < 0.0005$ compared with pcDNAZeo-transfected cells (unpaired *t*-test). (B) Effect of co-transfection with I-127 on Aβ secretion from BACE-HA cDNA-transfected cells. Results are the mean ± S.D. of five samples.

Alternative splicing has been shown to affect more than one-third of human genes, and one-third of the alternative transcripts have been found to contain premature termination codons [11]. mRNA surveillance facilitates the detection and destruction of mRNA that contains premature termination codons through a process called nonsense-mediated mRNA decay (NMD), presumably to prevent the production of potentially deleterious proteins [10]. We found that treatment of SH-SY5Y cells with protein synthesis inhibitors induced a novel BACE splice variant retaining a premature termination codon, suggesting that I-127 is regulated by NMD. In agreement with the NMD mechanism, which operates in a translation-dependent manner, inhibition of protein synthesis by the inhibitors resulted in the enrichment of I-127 transcript. When expressed in cells, I-127 was retained in the ER and degraded in a proteasome-dependent manner to prevent the production of non-functional I-127. It has been reported that I-457 and its soluble variant were degraded in the ER by proteasome-dependent ER-associated degradation (ERAD) [13], and that I-476 [4,16] and BACE containing the single mutations P54H, A183T, Y184H, A333T, L346P or L455F were efficiently retained in the ER [4]. These findings suggest that BACE is very sensitive to small structural changes and may receive tight quality control. A presenilin 1 splicing variant with a spliced-out transmembrane domain has only been found in an AD patient [1]. Moreover, unusual alternative splicing of presenilin 2 and out-of-frame splice variants of GFAP have been reported to be expressed in the sporadic AD brain [6,14]. Thus, some alternative splicing variants have been associated with AD. The expression level of I-127 mRNA in the brain is low. However, frameshift mutants of APP and ubiquitin-B have been described to be associated with sporadic AD, even though only about one in 10,000 cells carry a mutation [17]. Recent reports show that proteasome activity is decreased in AD [8,9]. The functional importance of the alternatively spliced I-127 and its relation to AD pathology may therefore be a subject for further study.

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A molecular chaperone inducer protects neurons from ER stress

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The endoplasmic reticulum (ER) stress response is a defense system for dealing with the accumulation of unfolded proteins in the ER lumen. Recent reports have shown that ER stress is involved in the pathology of some neurodegenerative diseases and cerebral ischemia. In a screen for compounds that induce the ER-mediated chaperone BiP (immunoglobulin heavy-chain binding protein)/GRP78 (78 kDa glucose-regulated protein), we identified BiP inducer X (BIX). BIX preferentially induced BiP with slight inductions of GRP94 (94 kDa glucose-regulated protein), calreticulin, and C/EBP homologous protein. The induction of BiP mRNA by BIX was mediated by activation of ER stress response elements upstream of the BiP gene, through the ATF6 (activating transcription factor 6) pathway. Pretreatment of neuroblastoma cells with BIX reduced cell death induced by ER stress. Intracerebroventricular pretreatment with BIX reduced the area of infarction due to focal cerebral ischemia in mice. In the penumbra of BIX-treated mice, ER stress-induced apoptosis was suppressed, leading to a reduction in the number of apoptotic cells. Considering these results together, it appears that BIX induces BiP to prevent neuronal death by ER stress, suggesting that it may be a potential therapeutic agent for cerebral diseases caused by ER stress.
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The endoplasmic reticulum (ER) is an 'assembly plant' for the manufacture of secretory and membrane proteins. However, from time to time 'inferior goods', that is, unfolded/misfolded proteins in the ER are inevitable. Under normal physiological conditions, unfolded proteins are degraded; under conditions of ER stress, however, unfolded proteins can accumulate in the ER lumen. Eukaryotes utilize the unfolded protein response (UPR) to overcome the critical status induced by ER stress.[1] The UPR consists of the following three pathways: (1) suppression of protein translation to prevent the generation of more unfolded proteins; (2) facilitation of refolding of unfolded proteins by the induction of ER chaperones; and (3) activation of ER-associated degradation (ERAD) to degrade the unfolded proteins accumulated in the ER, by the ubiquitin–proteasome pathway. If these strategies are unsuccessful, cells go into ER stress-induced apoptosis.

Recent reports show that dysregulation of the UPR is implicated in much important pathology, including some neurodegenerative diseases and cerebral ischemia. Previously, the ER transducers inositol-requiring kinase 1 (IRE1), PKR(protein kinase regulated by RNA)-like ER-associated kinase (PERK), and activating transcription factor 6 (ATF6) were reported to be downregulated in presenilin-1 mutant neurons, causing the vulnerability to ER stress seen in cases of familial Alzheimer disease (AD).[2–4] Caspase 4, the human homologue of murine caspase 12, was also reported to play a critical role in ER stress-induced neuronal cell death in AD.[5] One inherited form of Parkinson's disease is associated with the accumulation of the protein Parkin-associated endothelin receptor-like receptor in the ER of dopaminergic neurons as a result of defective ERAD by mutant Parkin, a ubiquitin protein ligase (E3).[6,7] Analysis of the polyglutamine tract associated with the spinocerebrocellular atrophy protein (spinocerebellar ataxia type 3) in Machado–Joseph disease suggests that cytoplasmic accumulation of this protein can inhibit proteasome function, thereby interfering with ERAD and eliciting ER stress-induced apoptosis.[8,9] It was also reported that cerebral ischemia activates the UPR.[10,11] These findings show that many cerebral disorders are related to an impaired UPR and ER stress-induced apoptosis.

These accumulated data concerning the involvement of ER stress in cerebral disorders encouraged us to investigate this

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Keywords: endoplasmic reticulum stress; chaperone; apoptosis; cerebral ischemia; neurodegeneration

Abbreviations: AD, Alzheimer disease; ATF6, activating transcription factor 6; BiP, immunoglobulin heavy-chain binding protein; CHOP, C/EBP homologous protein; EDEM, ER degradation-enhancing α-mannosidase-like protein; eIF2α, eukaryotic translation initiation factor 2 subunit α; ERAD, ER-associated degradation; ERdj4/MDG1, ER-localized DnaJ 4/microvascular differentiation gene 1; ERSE, ER stress response element; GRP78, 78 kDa glucose-regulated protein; GRP94, 94 kDa glucose-regulated protein; HSP70, 70 kDa heat-shock protein; IRE1, inositol-requiring kinase 1; MCA, middle cerebral artery; MEF, mouse embryonic fibroblast; PERK, PKR(protein kinase regulated by RNA)-like ER-associated kinase; $p58^{IPK}$, protein kinase inhibitor of 58 kDa; Tg, thapsigargin; Tm, tunicamycin; TTC, 2,3,5-triphenyltetrazolium chloride; UPR, unfolded protein response; XBP1, X-box binding protein 1

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field in an effort to identify therapeutic targets for the treatment of these disorders. We speculate that a therapeutic strategy that induces the UPR might prevent neuronal death induced by ER stress. According to the UPR pathway, we could try to (1) induce the expression of ER molecular chaperones; (2) block translation of proteins; or (3) artificially stimulate the degradation of misfolded proteins by the proteasome, as therapeutic approaches. Indeed, some chemical compounds that induce particular UPR pathways have been developed. For example, Boyce *et al.*[12] identified salubrinal, a selective inhibitor of cellular complexes that dephosphorylates eukaryotic translation initiation factor 2 subunit α (eIF2α), and thereby blocks translation. They concluded that salubrinal might be useful in the treatment of diseases involving ER stress.[12] Kim *et al.*[13] reported that valproate, a widely prescribed drug for epilepsy and bipolar disorder, increases the expression levels of the ER chaperones immunoglobulin heavy-chain binding protein (BiP), GRP94 (94 kDa glucose-regulated protein), protein disulfide isomerase, and calreticulin as well as the cytosolic chaperone HSP70 (70 kDa heat-shock protein). They also showed that valproate induces these chaperones without evoking the UPR, and speculated that inhibition of glycogen synthase kinase-3 by valproate might lead to the induction of chaperones.[13]

Previous reports have shown that induction of BiP, an ER molecular chaperone, prevents neuronal death induced by ER stress.[2,14–16] By contrast, inhibition of GRP78 (78 kDa glucose-regulated protein) mRNA induction increases cell death in response to calcium release from the ER, oxidative stress, hypoxia, and T-cell-mediated cytotoxicity.[17–19] Therefore, in this paper, we searched for a chemical compound that induces BiP for possible use as a neuroprotective agent against ER stress. We have identified such a chemical compound, BiP inducer X (BIX), and shown its protective effect against ER stress-induced apoptosis *in vivo* and *in vitro*, suggesting that this compound might be useful in the treatment of cerebral disorders associated with ER stress, such as cerebral ischemia.

Results

BIX preferentially induces BiP. To identify chemical compounds that induce BiP mRNA, we utilized high-throughput screening (HTS) with a BiP reporter assay system. Of the screened compounds, 1-(3,4-dihydroxy-phenyl)-2-thiocyanate-ethanone (Figure 1a) showed the highest level of induction of BiP mRNA; thus, we named this compound BiP inducer X (BIX).

First, we examined whether BIX does indeed induce the expression of BiP mRNA. Northern blot analysis and real-time PCR of SK-N-SH neuroblastoma cells treated with BIX showed that BIX induces BiP mRNA in a dose-dependent manner; however, the level of BiP mRNA induced by BIX is less than that induced by thapsigargin (Tg) (Figure 1b). Additionally, treatment of cells with 50 μM BIX caused the highest induction of BiP and little toxicity to cells. Because BIX generated cytotoxicity at higher dosages, we did not use it at concentrations greater than 50 μM in further analyses. Semiquantitative RT-PCR and real-time PCR showed that the BiP signal peaked at 4 h after the addition of 5 μM BIX and remained at that level until 6 h after treatment, with a subsequent reduction in level after this point (Figure 1c). To determine whether the induction of BiP mRNA by BIX results in an increase in the level of BiP protein, we carried out immunoblot analysis. As shown in Figure 1d, the level of BiP protein was increased by 5 μM BIX in a time-dependent manner, consistent with the changes in mRNA levels.

Next, we performed semiquantitative RT-PCR analysis to investigate whether BIX affects the expression of any other ER stress response-related genes, such as GRP94, calreticulin, X-box binding protein 1 (XBP1), ER-localized DnaJ 4/microvascular differentiation gene 1 (ERdj4/MDG1), ER degradation-enhancing α-mannosidase-like protein (EDEM), protein kinase inhibitor of 58 kDa (p58^{IPK}), C/EBP homologous protein (CHOP), and asparagine synthetase (ASNS) (Figure 2a). According to the time-course study of BiP induction by BIX (Figure 1c), a 6 h treatment of cells with BIX was adopted into this study. XBP1 mRNA, which is spliced under ER stress induced by 1 μM Tg or 1 μg/ml tunicamycin (Tm), was not processed in cells treated with 5 μM BIX. Compared with a control sample, ERdj4/MDG1, EDEM, p58^{IPK}, and ASNS were scarcely induced by BIX. On the other hand, GRP94, calreticulin, and CHOP were induced by BIX, but their inductions were not as prominent as that of BiP. We also performed time-course analyses on the expressions of ER stress response-related genes by real-time PCR in SK-N-SH cells treated with 50 μM BIX as well as 5 μM BIX. A 5 μM portion of BIX induced GRP94, calreticulin, and CHOP mRNA as well as BiP. However, BiP was definitely induced from 2 to 6 h (Figure 2b). The time courses for EDEM, p58^{IPK}, and ASNS were not changed by treatment with 5 μM BIX (Figure 2b). A 50 μM portion of BIX also induced BiP from 4 to 6 h and transiently induced calreticulin and CHOP. Even 50 μM BIX did not induce EDEM, p58^{IPK}, or ASNS (Figure 2b).

Moreover, we performed immunoblot analysis of GRP94 and phosphorylated eIF2α to examine whether BIX affects other signaling pathways that participate in the ER stress response. Treatment of SK-N-SH cells with BIX caused very slight induction of GRP94 protein compared with its induction by 1 μM Tg (Figure 2c). This result was consistent with that of semiquantitative RT-PCR analysis of the GRP94 mRNA level. Treatment of cells with 1 μM Tg increased the level of phosphorylated eIF2α, but BIX did not cause its phosphorylation (Figure 2c). These results indicate that BIX invokes little ER stress, but almost preferentially induces BiP.

The induction of BiP by BIX is mediated by activation of ERSEs through the ATF6 pathway. To investigate the mechanism by which BiP is induced by BIX, we performed reporter assays using 132 bp BiP-pGL3 reporter plasmids as described in the Materials and Methods section. A BiP (132)-pGL3 plasmid (Figure 3a) was transfected into SK-N-SH cells and the cells were treated with 5 μM BIX, 300 nM Tg, or 0.5 μg/ml Tm for either 6 or 16 h. The reporter activities in transfectants were elevated in response to stimulation with Tg or Tm, and maintained at a long-lasting high level (Figure 3b). By contrast, reporter activities in cells treated with BIX were transiently induced at 6 h after stimulation and then downregulated to basal levels by 16 h (Figure 3b). This