Biochemical and Biophysical Research Communications 399 (2010) 593–599

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reduction of amyloid β-peptide accumulation in Tg2576 transgenic mice by oral vaccination

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ARTICLE INFO

Article history:
Received 26 July 2010
Available online 1 August 2010

Keywords:
Alzheimer's
Amyloid β-peptide
Oral vaccination
Transgenic mice
Green pepper

ABSTRACT

Alzheimer's disease (AD) is pathologically characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles. Amyloid β-peptide (Aβ) is the main component of senile plaques, and the pathological load of Aβ in the brain has been shown to be a marker of the severity of AD. Aβ is produced from the amyloid precursor protein by membrane proteases and is known to aggregate. Recently, immune-mediated cerebral clearance of Aβ has been studied extensively as potential therapeutic strategy. In previous studies that used a purified Aβ challenge in a mouse model of AD, symptomatic improvement was reported. However, a clinical Alzheimer's vaccine trial in the United States was stopped because of severe side effects. Immunization with the strong adjuvant used in these trials might have activated an inflammatory Th1 response.

In this study, to establish a novel, safer, lower-cost therapy for AD, we tested an oral vaccination in a wild-type and a transgenic mouse model of AD administered via green pepper leaves expressing GFP-Aβ. Anti-Aβ antibodies were effectively induced after oral immunization. We examined the immunological effects in detail and identified no inflammatory reactions. Furthermore, we demonstrated a reduction of Aβ in the immunized AD-model mice. These results suggest this edible vehicle for Aβ vaccination has a potential clinical application in the treatment of AD.

1. Introduction

Alzheimer's disease (AD) is one of the major causes of chronic and progressive age-associated cognitive decline, with characteristic pathological hallmarks including extracellular senile plaques, intracellular hyperphosphorylated tau in neurofibrillary tangles, and brain weight loss [1,2].

Amyloid β-peptide (Aβ), which is reported to aggregate spontaneously, is the major constituent of the senile plaques in patients with AD. Aβ has been reported to accumulate on the outside of nerve cells or in the cerebral vascular walls. According to the amyloid cascade hypothesis, the gradual cerebral accumulation of soluble and insoluble assemblies of Aβ in the limbic and association cortices triggers a cascade of biochemical and cellular alterations that produce the clinical phenotype of AD. Therefore, soluble or insoluble Aβ is regarded as a therapeutic target of the progression of AD.

Aβ is generated by the sequential proteolytic processing of the amyloid precursor protein (APP) by membrane proteases. In human brains, the longer Aβ peptide, Aβ42, has a greater propensity to aggregate than the shorter Aβ40.

In 1999, a vaccination therapy to Aβ was developed as a novel therapeutic method against AD. These experiments employed APP transgenic mouse model of AD (PDAPP) mice, which overexpress mutant human APP and progressively develop senile plaques or neurofibrillary tangles in an age- and brain region-dependent manner. In the study, transgenic mice were immunized with synthesized Aβ42 either before (at 6 weeks of age) or after (11 months) the onset of neuropathologies. The study reported that the antibody titer against Aβ42 was elevated, and immunization of the young animals essentially prevented the development of senile plaques, neurofibrillary tangles, and neuritic atrophy. Treatment of the older animals also markedly reduced the extent and progression of these AD-like neuropathologies [3]. This reduction in the number of senile plaques was observed not only with active immunization but also with passive immunization [4] and with the mucosal immunization delivered via the nose [5].

Transgenic mouse models of AD, such as PDAPP mice, begin to display a learning disability derived from the cognitive disorder

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.07.120

in a water maze test at around 15 months of age, but this learning disorder improves with the Aβ42 immunization [6–9]. In 2001, the Elan Corporation in the United States examined the clinical application of Aβ immunization. However, during the phase II trial, some patients presented with acute meningitis as a side effect after the second immunization, and this clinical test was suspended [10]. Adjuvants used in the muscular injection of the synthesized Aβ42 have high immunological activity and induce cell-mediated immune responses such as T-lymphocyte activation. In some cases, Aβ or APP-reactive killer T cells may have invaded the brain causing allergic meningitis. Despite the suspension of the clinical trial, some data suggest that the vaccination therapy may be effective for Aβ clearance [11]. Overall, these results indicate that safer and cheaper vaccination methods are needed, in particular those that do not require injection, strong adjuvants, and expensive synthesized peptides.

As for the safety of the vaccine, physiological immunoreactivity against the vaccine is of great importance. In the human body, immunoreactivity against "the self" is usually suppressed, a phenomenon called immunotolerance. "Self" immunotolerance is affected by the balance between Th1 and Th2 helper T cells. When the balance between Th1 and Th2 T cells changes and Th1 cells are activated, cellular reactions to "the self" increase, tending to give rise to autoimmune diseases such as meningitis. When Th2 cells are activated, antibody production is induced and anti-inflammatory cytokines are released to alleviate the inflammation induced by Th1 cells. Safer vaccine therapies that induce the Th2 reaction via mucosal immunization are now being tested in animal models. In mucosal immunization, immune activity tends to be feeble, immunotolerance can be induced, and adjuvants delivered with the target antigen proteins must be taken into consideration. In an experiment in which a DNA vaccine was administered via the nose, Kim et al. delivered Aβ and adenovirus vectors coding granulocyte colony-stimulating factor (G-CSF) as the adjuvant, and effective elevation of anti-antibody titer and continual induction of Th2 reaction was reported [14].

Many studies of vaccination therapy for AD have been conducted, such as subcutaneous or nasal mucosal vaccination using synthetic peptide or DNA [5,15,16], but oral vaccination using an edible, virally transduced plant as a vehicle has some benefits [17,18]. First, because only the target subunits or fragments are expressed, and not all the pathogenic virus or proteins, it is a safer vaccine. Furthermore, purification of the target protein and cold storage are not required, and the cost is low. Plant biotechnology provides methods for introducing animal genes into plants.

In this study, we tested a plant-based vaccination therapy in an animal model of AD. We expressed green fluorescent protein (GFP)-conjugated Aβ in pepper leaves using a plant virus and fed the leaves to wild-type or transgenic mice. We used the cholera toxin B subunit (CTB) as the oral adjuvant. CTB suppresses Th1 cell reactivity and is known to form a pentamer, binding to GM1 gangliosides on the cell surface. Then we examined the immunological reaction in mice and checked the effectiveness and safety of this plant-based vaccination.

We succeeded in stably expressing GFP-conjugated Aβ in green pepper leaves using a plant virus, TocJ. Oral vaccination of wild-type and Tg2576 transgenic mice resulted in the elevation of Aβ antibody titer without significant side effects.

2. Materials and methods

2.1. Mice

We used female Tg2576 transgenic mice (670th amino acid of APP mutated from Lys to Asn, and 671st from Met to Leu), a model of Swedish familial AD, and female wild-type B6 mice.

2.2. Plants

For the plant-based vaccine, we used leaves of *Capiscum annuum* var. angulosum virally transduced with GFP-conjugated Aβ42 using a plant virus, TocJ [18,21]. Viral transduction with GFP alone was used as a negative control.

2.3. Quantification of Aβ expression in leaves of Capiscum annuum var. angulosum

The leaves were suspended in phosphate-buffered saline (PBS), homogenized, and centrifuged at 10,000*g* for 10 min (4 °C), then the supernatant was separated from the pellet. Protein samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels, transferred to polyvinylidene fluoride (PVDF) membranes (2 mA/cm^2 for 90 min), blocked with 5% skim milk for 60 min, and incubated in the mouse monoclonal antibody 6E10 at 4 °C overnight. Blots were then incubated with a horseradish peroxide (HRP)-conjugated secondary antibodies at room temperature for 10 min and then at 37 °C for 45 min. For detection and analysis of the luminescence, we used a lumino-image analyzer (Fujifilm, Tokyo, Japan).

2.4. Preparation of the leaves for immunization

The quantity of green pepper leaves administered to each mouse was adjusted to deliver 60 μg of Aβ. Samples for subcutaneous injection were prepared as follows: the green pepper leaves were suspended in PBS, centrifuged at 10,000*g* for 10 min (4 °C), and the supernatant was added to an equal volume of Freund's adjuvant and stirred to form an emulsion. Samples for oral administration were prepared as follows: crushed green pepper powder stored in liquid nitrogen was mixed with CTB (10 μg per mouse) in a 5% sucrose solution.

2.5. Mouse immunization

For subcutaneous immunization, each mouse was anesthetized with diethyl ether and injected with 100–200 μL of extract at several points using a 21G needle. For oral immunization, the mixture was orally delivered by gastric intubation.

We examined three groups of wild-type B6 mice. Five wild-type mice vaccinated orally with GFP-only served as negative controls. The rest of the wild-type mice were immunized with GFP-Aβ42 either subcutaneously ($n = 5$) or orally ($n = 6$). All wild-type mice received doses of vaccine biweekly from 3 to 11 months of age. Two weeks after a booster given at 11 months, whole blood was collected from the heart.

We examined three groups of Tg2576 mice. Ten Tg2576 mice vaccinated orally with GFP-only served as negative controls. The rest of the transgenic mice were immunized with GFP-Aβ42 either subcutaneously ($n = 8$) or orally ($n = 10$). All transgenic mice received doses of vaccine biweekly from 5.5 to 18 months of age. Two weeks after a booster at 18 months, whole blood was collected from the heart. Other than during vaccine dosing, animals were fed regular mouse chow during the entire experiment. All animals were housed 3–5 to a cage and maintained on ad libitum food and water with a 12 h light/dark cycle.

2.6. Quantification of the anti-Aβ antibody titer with ELISA

Well plates were coated with Aβ42 dissolved in 0.15 M ammonium solution and then washed five times with wash buffer (0.45% NaCl and 0.05% Tween 20). Next, the plates were blocked with 3% skim milk for 30–60 min at room temperature and washed four times.

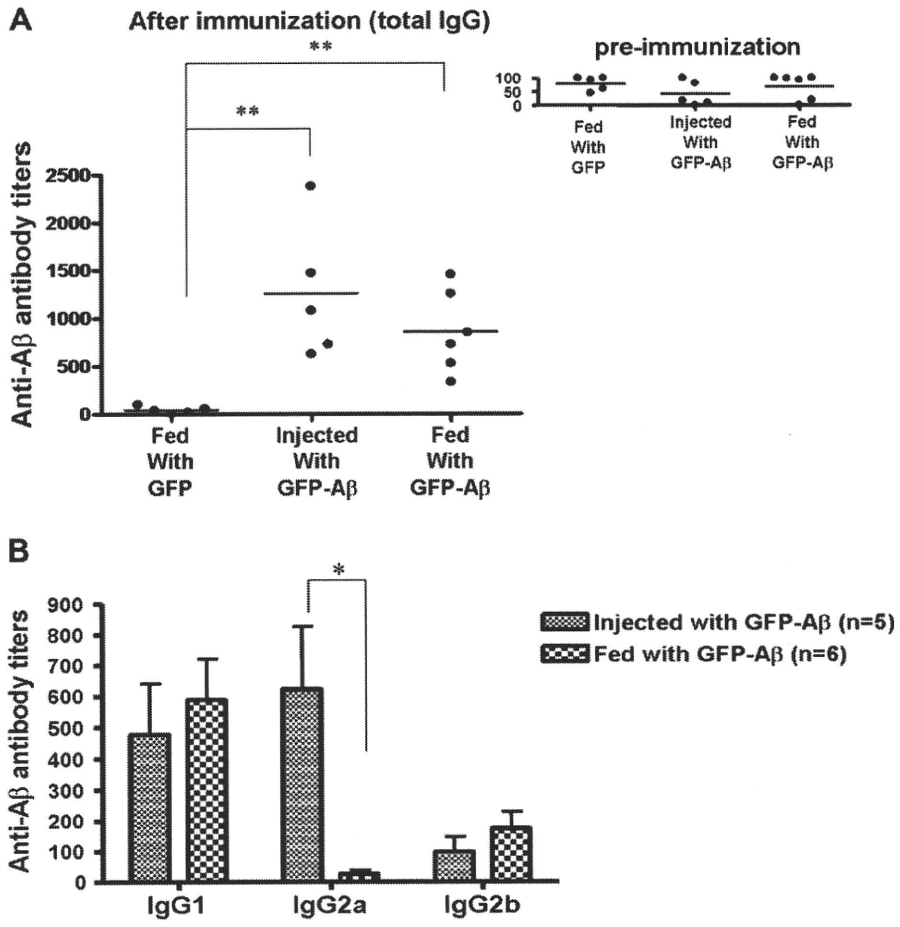

Fig. 1. (A) Elevation of the anti-Aβ titer in wild-type B6 mice. The antibody titer of each wild-type B6 mouse is shown in this figure ($^{**}P < 0.01$). Mice fed GFP-expressing leaves served as a control. (B) IgG isotyping of anti-Aβ antibodies in wild-type B6 immunized mice. Serum collected from GFP-Aβ-treated mice was used for IgG isotyping of anti-Aβ antibodies. Anti-Aβ antibody titers of each IgG isotype are shown for each condition. Mean ± SE. $^{*}P < 0.05$.

Blood plasma samples were diluted 10–10^5 times. Monoclonal anti-Aβ antibody 6E10 was diluted 10,000–160,000 times as a positive control. Each sample as applied to a well and incubated at 37 °C for 1 h. After washing six times, each well was blocked at room temperature for 30 min with 3% skim milk and again washed five times. Wells were incubated with an HRP-conjugated secondary antibody at 37 °C for 1 h and washed 10 times. To complete the enzyme-linked immunosorbent assay (ELISA), wells were incubated with tetramethylbenzidine (TMB) substrate at room temperature in the dark. After sufficient color reaction occurred, 2 M phosphate buffer stopped the reaction. We measured the absorbance at 450 nm with a spectrophotometer and calculated antibody titers using the statistics software, PRISM version 4.

2.7. Aβ quantification in Tg2576 mouse brains using sandwich ELISA

At the end of the vaccination protocol, we removed the brains of Tg2576 mice and homogenized one hemisphere in with Tris-buffered saline (TS) and centrifuged this sample at 4 °C and 200,000*g* for 20 min. The supernatant was used as the soluble fraction. After the pellet was washed with TS, we homogenized the pellet with 6 M guanidine–Tris buffer. After sonification at room temperature for 30 s, the sample was incubated for 1 h at room temperature and then centrifuged at 4 °C and 200,000*g* for 20 min. We made a 1:12 dilution of the sample in sandwich ELISA dilution buffer. The precipitate was regarded as the insoluble fraction.

We measured the amount of Aβ42 in each brain using a sandwich ELISA kit (Wako, Osaka, Japan). First, we incubated Aβ42 peptide standards and brain extract samples at 4 °C overnight on microplates coated with a monoclonal antibody raised against the human Aβ42 N-terminus. The plate was washed with wash buffer five times, and then incubated at 4 °C overnight with BC05, a HRP-conjugated antibody raised against the Aβ42 C-terminus. After five washes, a color reaction was carried out at room temperature in the dark and then stopped after 30 min. We measured the absorbance at 450 nm and calculated the concentration of Aβ42 from the standard curve.

2.8. Immunostaining of Tg2576 mouse brain slices

We fixed the remaining brain hemisphere with 4% paraformaldehyde overnight. Then, the brains were embedded in paraffin, sliced at 4 μm thickness, and immunostained using the 6E10 primary antibody and a diaminobenzidine (DAB) color reaction.

3. Results

3.1. Quantification of Aβ expression in plant leaves

We performed SDS–PAGE and Western blotting for green pepper leaf extracts in PBS. We used the mouse monoclonal antibody 6E10 as the primary antibody to detect Aβ. Then we quantified the expression of Aβ using synthesized mouse IgG as the protein standard. The amount of Aβ was 100–600 μg for 1 g of plant leaves, depending on the preparation.

3.2. Production of anti-Aβ antibodies in wild-type B6 mice

To study the course of antibody production after vaccination and the precise immunological response, subcutaneous or oral immunization of wild-type B6 mice was performed starting at 3 months of age and continued for 8 months. The vaccine was administered once every 2 weeks. We quantified and calculated the serum anti-Aβ antibody titer (Fig. 1A). Compared to the GFP controls, serum anti-Aβ titer was significantly elevated in the groups receiving GFP-Aβ.

3.3. IgG isotyping in wild-type mice

To check the safety of the food vaccination, we determined the isotype of anti-Aβ antibodies produced in the serum and checked for inflammatory reactions in GFP-Aβ vaccinated mice (Fig. 1B). IgG1 and IgG2b are known to be the non-inflammatory Th2 isotype IgGs and IgG2a is the inflammatory Th1 isotype IgG. This latter isotype is used as a marker of inflammation. We found that the amount of IgG2a was significantly reduced in mice administered the vaccine orally compared to those receiving the subcutaneous injection.

3.4. Anti-Aβ antibody production in a transgenic mouse model of AD

To examine whether the food Aβ vaccination was effective in a mouse model of AD in which Aβ accumulates in an age-dependent manner, we immunized Tg2576 mice subcutaneously or orally. The mice received the vaccine biweekly from 5 .5 to 16.5 months of age. Samples were collected at the age of 18 months. We first scored the anti-Aβ antibodies and calculated the antibody titers (Fig. 2A). Compared to control mice treated with GFP-only orally, serum anti-Aβ antibody titer was significantly elevated in GFP-Aβ-treated mice. The increase in titer was the same for those injected with vaccine.

3.5. IgG isotyping in Tg2576 mice

To examine the safety of food vaccination in Tg2576 mice, we examined anti-Aβ antibody isotypes and inflammatory reaction in the GFP-Aβ-treated mice (Fig. 2B). Compared to the subcutaneous injection group, serum IgG2a was significantly reduced in the oral administration group.

3.6. Aβ burden in the mouse brains

We homogenized one hemisphere of the brain in Tris-buffered saline, centrifuged the sample, homogenized the pellet in 6 M guanidine–Tris buffer, and quantified the amount of Aβ with sandwich ELISA (Fig. 3A). In the GFP-Aβ-treated mice, the amount of intracerebral Aβ was significantly reduced compared to control mice. However, the amount in the insoluble Aβ samples of subcutaneously injected mice was not significantly different from that of control mice.

The other hemisphere of the brain was used for immunological staining with anti-Aβ antibody 6E10 (Fig. 3B). Fewer senile plaques were observed in GFP-Aβ-treated mice than in control mice.

4. Discussion

The hypothesis that Aβ vaccination should prevent senile plaque formation was raised in an experiment by Schenk et al. The

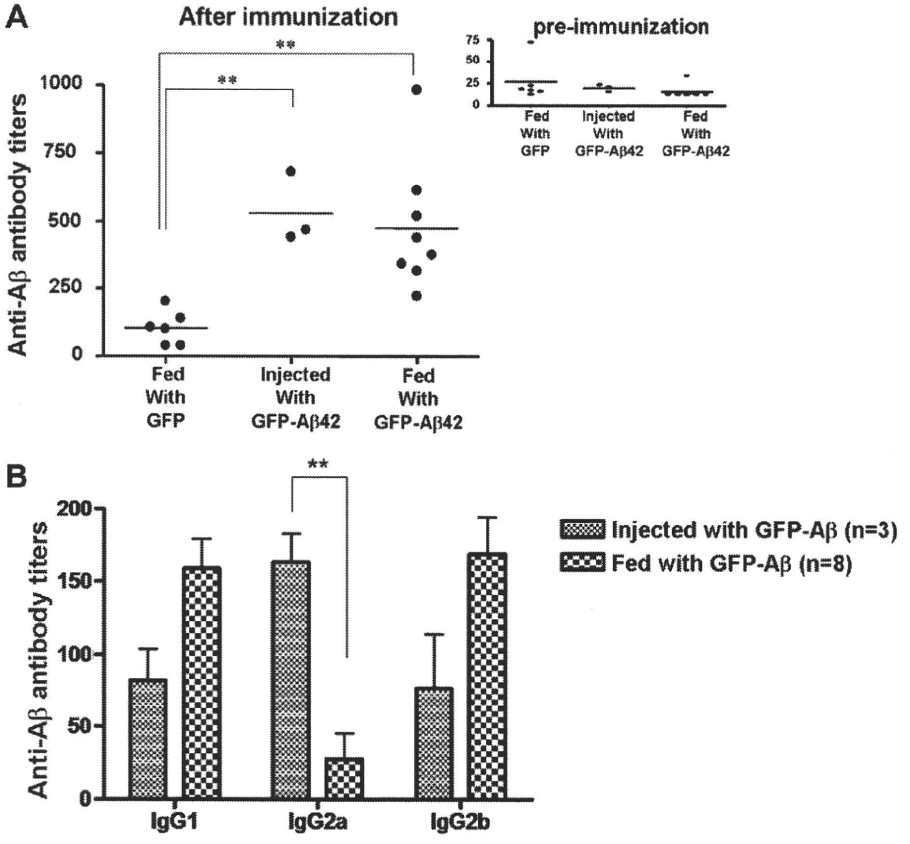

Fig. 2. (A) The anti-Aβ antibody titer is elevated in Tg2576 mice. The titer of antibodies against the Aβ protein increased after immunization. $^{**}P < 0.01$. (B) IgG isotyping of anti-Aβ antibodies in immunized Tg2576 mice. Serum collected from GFP-Aβ-treated mice was used for IgG isotyping of anti-Aβ antibodies. Anti-Aβ antibody titers of each IgG isotype are shown for each condition. Mean ± SE. $^{**}P < 0.01$.

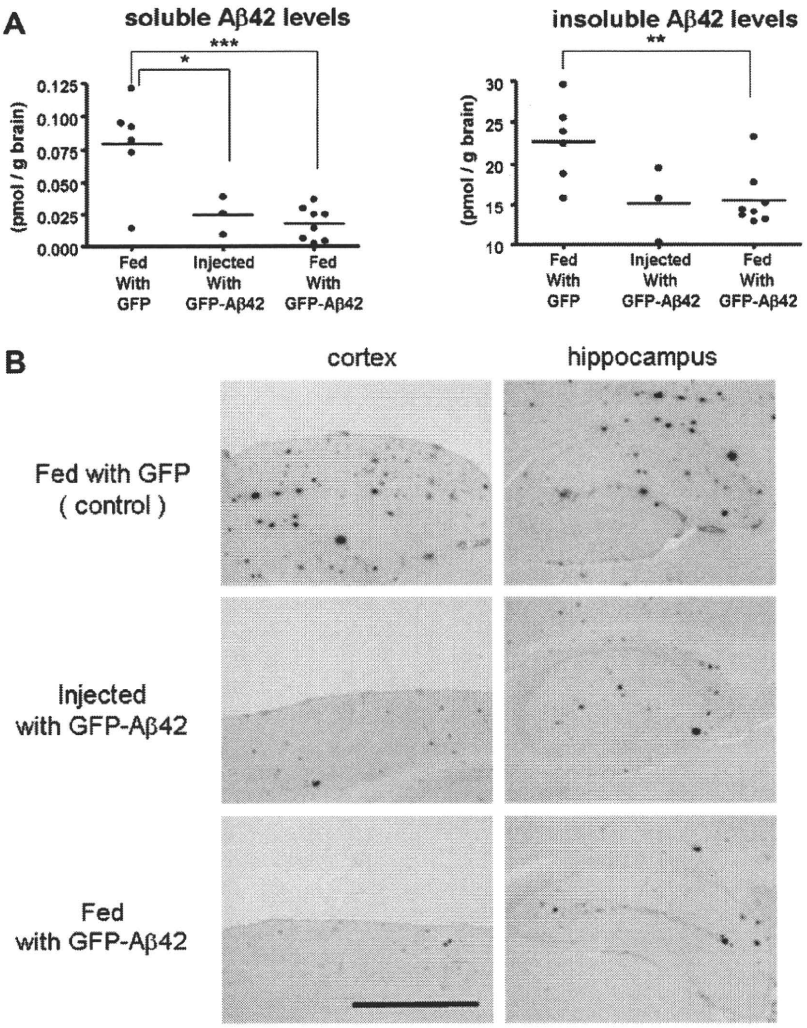

Fig. 3. (A) Intracerebral Aβ42 content in Tg2576 mice. A plot of the concentration of Aβ42 per 1 g of brain for each mouse. (a) The level of soluble Aβ42. (b) The level of insoluble Aβ42. (B) Immunostaining of Tg2576 mouse brains (left: cortex; right: hippocampus). Bar = 1 mm.

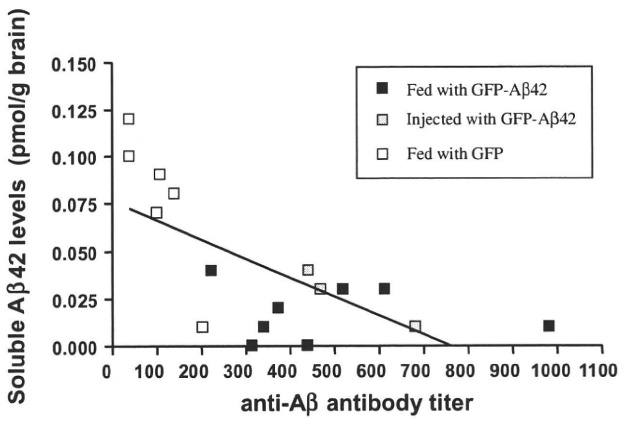

Fig. 4. The anti-Aβ antibody titer correlates with the level of soluble intracerebral A 42 in Tg2576 mice. These data were recalculated from those of Figs. 2A and 3A. $r = -0.686$, $p = 0.0023$.

authors put several kinds of antibodies against Aβ on frozen brain slices with senile plaques either from transgenic mice or from human patients with AD. Then the brain slices were incubated with a reagent containing microglia and phagocytes. If the antibodies were specific to Aβ, the microglia phagocytized Aβ on the brain section. This did not happen with antibodies to other proteins. These results supported the amyloid phagocytizing hypothesis that anti-Aβ antibodies bind to Aβ and then Aβ is phagocytized by microglia via Fc receptors [4]. When a monoclonal antibody against Aβ, m266, was peritoneally injected into transgenic mice, the amount of senile plaques was reduced in the brain and monomers and dimers of Aβ in the blood were elevated 1000 times over control 24 h after the injection. These findings also support the sink hypothesis that anti-Aβ antibodies mainly bind to and promote the clearance of peripheral Aβ, causing the Aβ to be drawn out of the central nervous system [12,13].

Most vaccines are administered by hypodermic injection. The antigens are either inactivated proteins, attenuated viruses, or bacteria. The protein antigen itself does not invite infection and is safe. These vaccines must be stored at cold temperatures and can be costly. Other vaccines are administered by mucosal application. Mucosal immunization utilizes the alimentary tract-associated lymphoid system. This mucosal epidermis is the first place from where some external antigens enter the body, and many lymphocytes are in the mucosal lymphoid tissue. In the mucosal immunity strategy, these lymphoid tissues are needed to accept only the target antigen specifically and effectively. The vaccination method used in the present study utilized the nasal mucous membrane-associated lymphoid system.

In this study, to make a plant-based vaccine, we used a plant virus that is the relative of the *Tobacco mosaic virus* classified in the genus *Tobamovirus*. We can easily synthesize viral particles in plants, and can preserve them at a cold temperature for a long time. Therefore, using a plant to develop an oral vaccination is only practicable when viral particles can be used to infect the plant. Hori et al. established a way of stably expressing an external gene without also expressing viral coat proteins using the *Tobamovirus* [19,20].

From preliminary data, we found that 50–150 μg of antigen was enough for one vaccination dose. In this study, we immunized mice by using 60 μg of antigen for each dose. Over the course of vaccination, the total intake of green pepper leaves was 0.3 g per mouse.

Mice that were immunized to GFP-Aβ had a significantly elevated antibody titer to GFP-Aβ as measured in an ELISA compared to mice that were immunized to GFP only. The antibody titer measured for mice receiving the vaccine orally was elevated to a similar level as in those receiving the vaccine through subcutaneous injection.

In general, the immunological effect tends to be weak for vaccines relying upon oral–intestinal mucosal immunization, and this method induces immunological tolerance. However, oral immunological tolerance can be suppressed by the use of specific adjuvants. In this study, we used CTB as the oral adjuvant and found that it was an effective adjuvant that effectively induced an immunological reaction against Aβ.

When we immunized Tg2576 mice between 8 and 12 months of age, a drastic decrease in senile plaques was observed at 16.5 months of age. Also, the serum anti-Aβ antibody titer was significantly elevated in mice administered the vaccine orally compared to controls. We demonstrated that our immunization methods successfully decreased the burden of intracerebral Aβ42 and senile plaques as measured via immunological staining. Together, these results indicate that food vaccination of Aβ is not only effective at inducing novel anti-Aβ antibody production, but also reduces intracerebral Aβ. Furthermore, we found a significant correlation between the anti-Aβ antibody titer and the level of soluble, intracerebral Aβ42 (Fig. 4). Individual differences in the level of insoluble intracerebral Aβ42 were too large to evaluate the effect of immunization on this measure.

To determine the safety of food vaccination, we investigated the IgG isotypes, markers of inflammation, of the anti-Aβ antibodies found in the serum of vaccinated mice. IgG2a, an inflammatory Th1 immunological globulin, was significantly reduced in both GFP-Aβ-treated wild-type B6 and Tg2576 mice compared to GFP-treated controls. These results suggest that oral Aβ immunization is less likely to induce inflammatory reactions than subcutaneous Aβ injection. In most clinical cases, vaccinations are usually delivered by subcutaneous injection. Although this method results in a high level of immunization, patients must endure injection, and such vaccinations are expensive due to the need for refrigerating the antigen. In contrast, food vaccination is easy to administer, without a need for refrigeration. Our findings may help to develop a safe immunotherapy protocol.

A 6-year follow-up of patients in the first phase I clinical trial of active immunization for treating AD was recently completed [22]. The results of this study indicated that cortical Aβ loads were lower in patients who were immunized compared to the control group. Patients with higher antibody responses had more extensive Aβ removal. This demonstrates that anti-Aβ immunization therapy can influence cerebral Aβ deposition. However, this study did not find statistically different cognitive impairment or survival for immunized patients. The results raised concerns that anti-Aβ therapy may be an ineffective treatment for AD. However, whether vaccination in this clinical trial decreased the concentration of neurotoxic Aβ oligomers has not yet been determined. Therefore, we should not prematurely abandon Aβ vaccination. Further animal experiments and human clinical trials are necessary to determine the effectiveness of immunization to Aβ. Also, such therapies should start long before the onset of symptoms in people at risk of AD when neurons are still intact.

In this study, we confirmed the safety and immunological effectiveness of an Aβ food-based vaccination in mice, the first step toward the development of an effective AD vaccination therapy. Plant-based vaccines would be safer than those produced in animal tissues because the chance of unidentified human pathogen hitching a ride would be small. In addition, an Aβ vaccination that preferentially induces a Th2 response would be highly desirable to prevent side effects. However, the individual's predisposition toward specific T-cell response is not fully understood and it is not possible to identify who is at risk of autoimmune reaction before vaccination. Selection of adjuvant and coadministration of IL-12 and IL-4 which shifts the subtype of T-cell toward Th2 would reduce the risk of Th1 response. Future clinical trial is necessary to elucidate these problems.

Acknowledgments

We thank Miss Hisaka Sato for valuable discussions. This work was supported in part by the Human Frontier Science Program and a research grant from the Ministry of Health, Labor and Welfare, Japan to S.I.

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Biosci. Biotechnol. Biochem., **75** (2), 396–400, 2011

Communication

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

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Received December 2, 2010; Accepted December 27, 2010; Online Publication, February 7, 2011
[doi:10.1271/bbb.100861]

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

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

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

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

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

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

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

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

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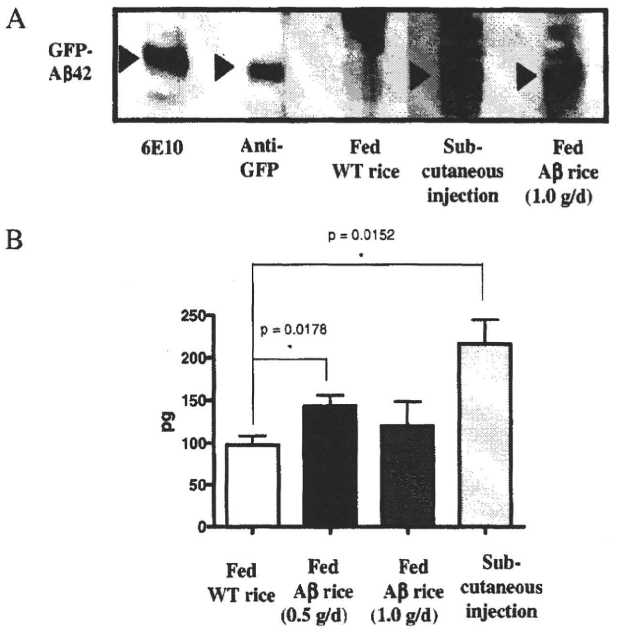

Fig. 1. Detection of Anti-Aβ Antibody in Mice.

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

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

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

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

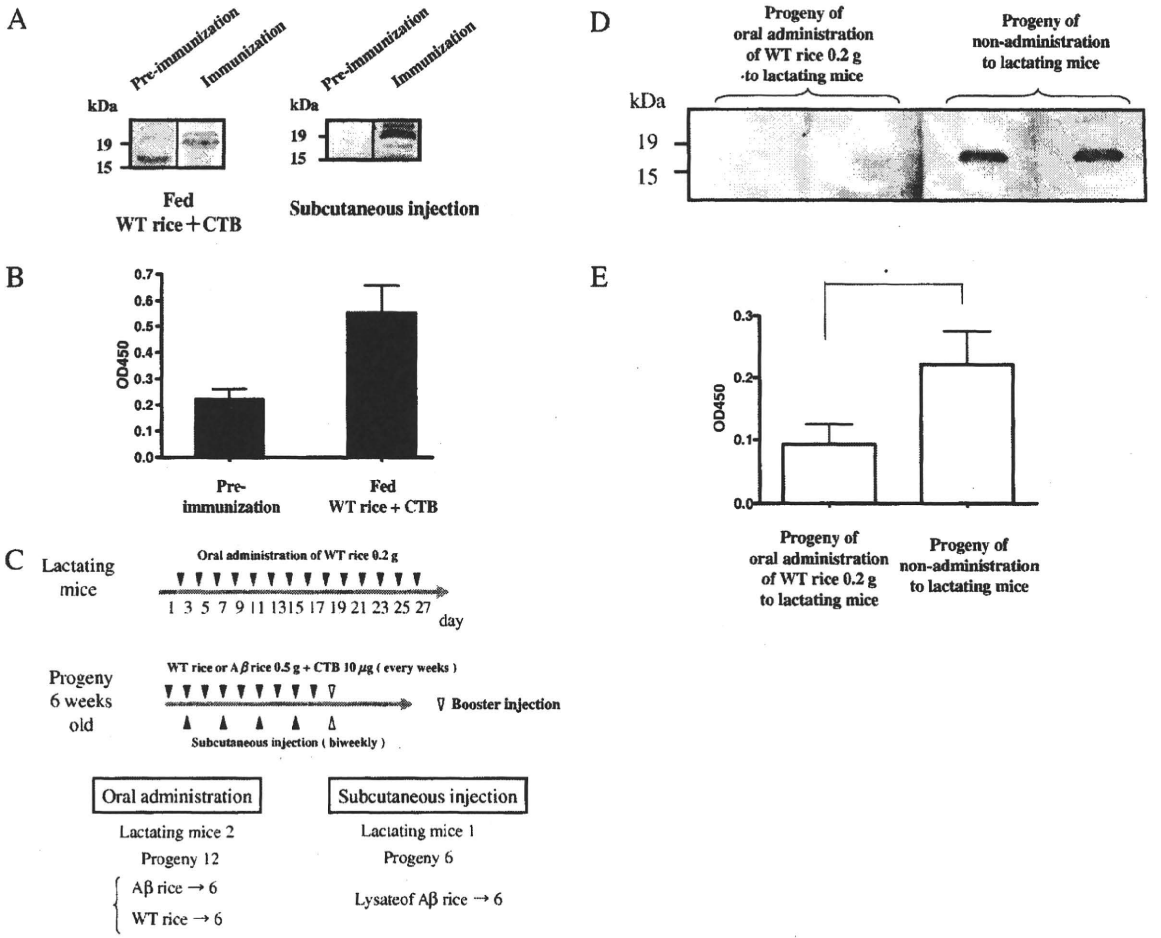

Fig. 2. Production of Anti-Rice Storage Protein Antibodies in Mice.

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

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

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

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

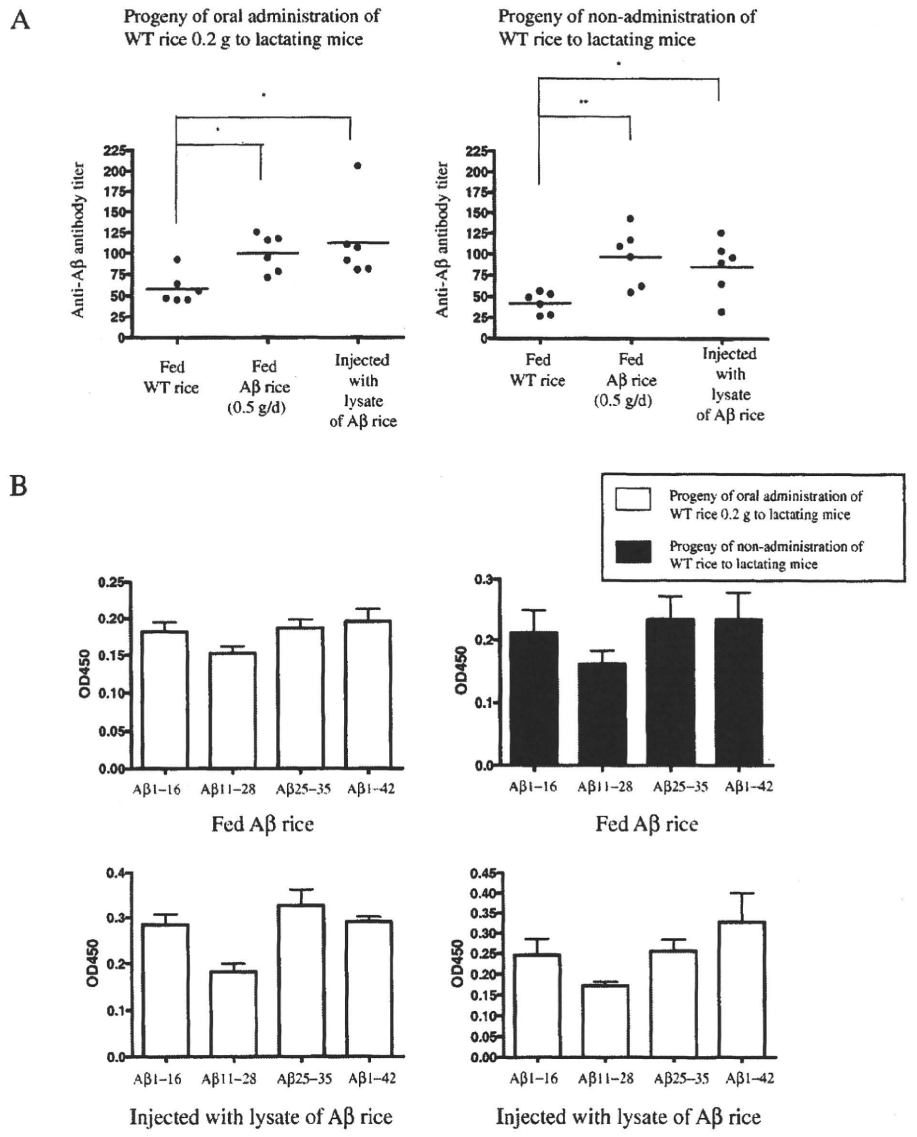

Fig. 3. Detection of Anti-Aβ Antibody in Mice and Epitope Mapping.
A, The Aβ-antibody titers of the mice are shown ($^*p < 0.05$, $^{**}p < 0.01$). B, The epitope mapping of Aβ. The peptides of Aβ1-16, Aβ11-28, and Aβ25-35 were used as antigens. Sera from mice fed Aβ rice or injected with the lysate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

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

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

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

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

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

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International Journal of Biological Sciences
2011; 7(3):301-307

Short Research Communication

Transgenic Rice Expressing Amyloid β-peptide for Oral Immunization

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Received: 2010.12.11; Accepted: 2011.03.22; Published: 2011.03.25

Abstract

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

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

Introduction

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

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

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

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

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

Materials and Methods

Plant materials

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

Plasmid construction

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

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

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

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

Transformation

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

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

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

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

Southern blot analysis

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

Quantification of expression levels in seeds

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

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

Mouse immunization

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

Quantification of the anti-Aβ antibody titer with ELISA

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

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

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

Results and Discussion

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

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

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

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

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

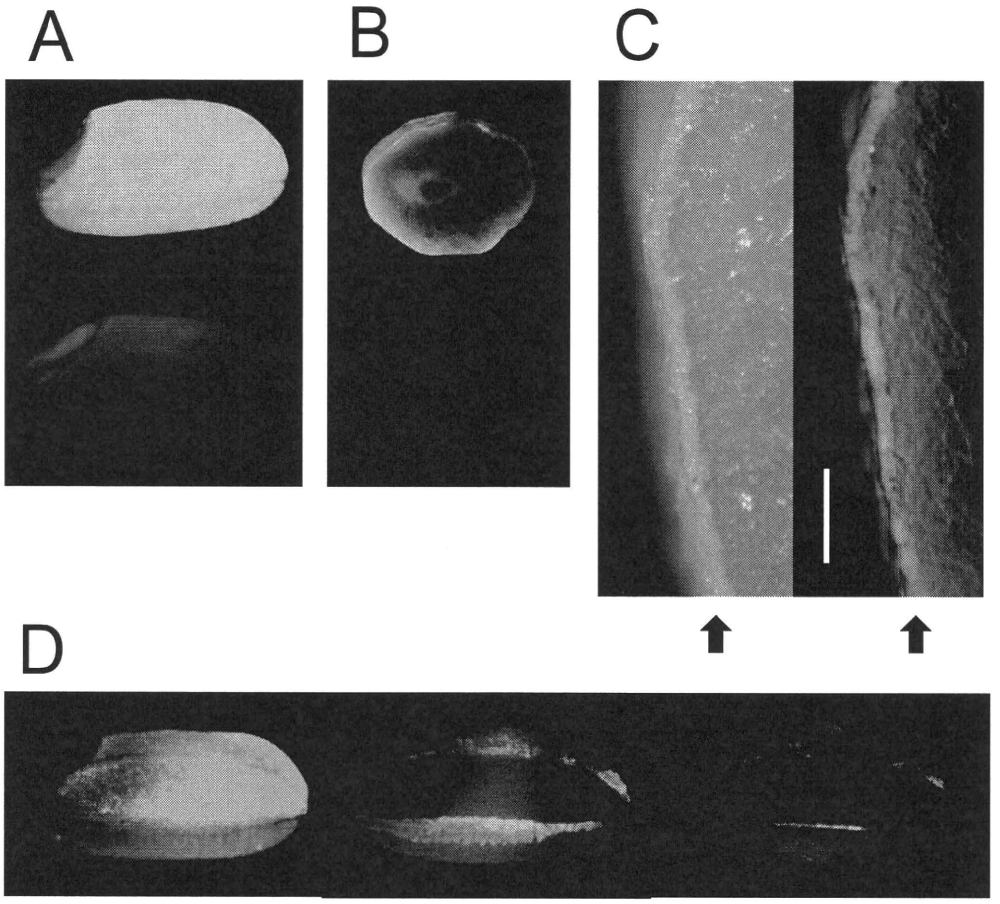

Fig. 1. Detection of GFP fluorescence in seeds (brown rice). (A) Transgenic brown rice (upper seed) and non-transgenic brown rice (lower seed). (B) Transverse sections of transgenic brown rice (upper seed) and non-transgenic brown rice (lower seed). (C) Aleurone layer in transverse section of transgenic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

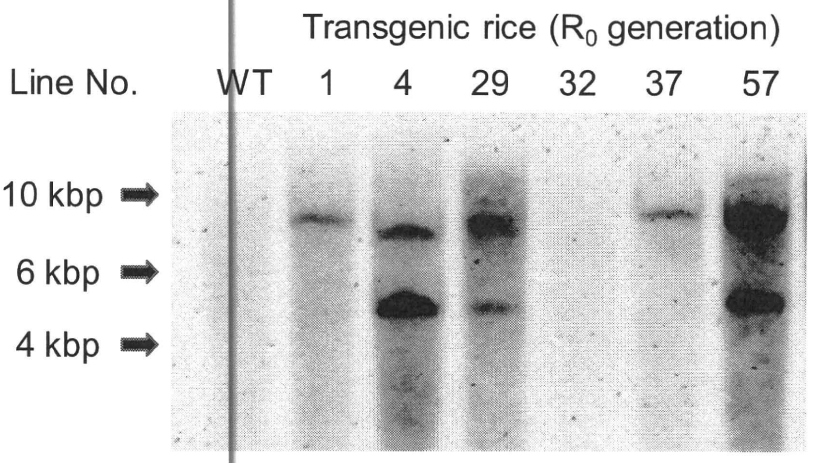

Fig. 2. Southern blot analysis of Xba I-digested total DNA probed for GFP-specific genes. WT, non-transgenic rice plant: R_0, primary transgenic rice plant.

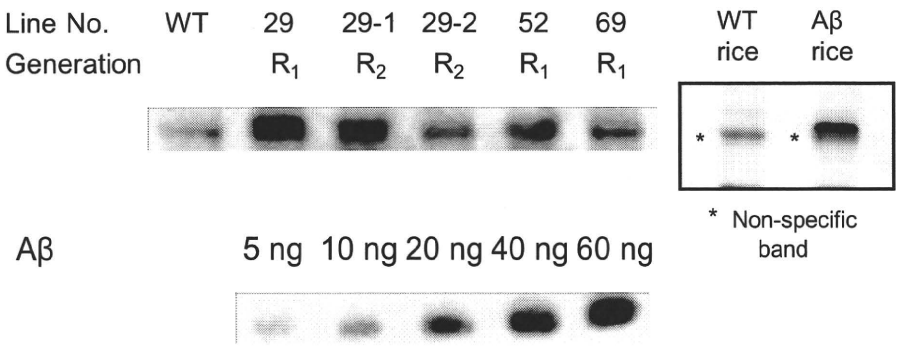

Fig. 3. Determination of Aβ42 expression levels. Brown rice samples were subjected to SDS-PAGE with Aβ42 at increasing concentration (5, 10, 20, 40 and 60 ng). Approximately 0.15 mg of crushed seeds was applied to each lane. R_0 seeds (R_1 generation) and R_1 seeds (R_2 generation) were used. WT; non-transgenic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

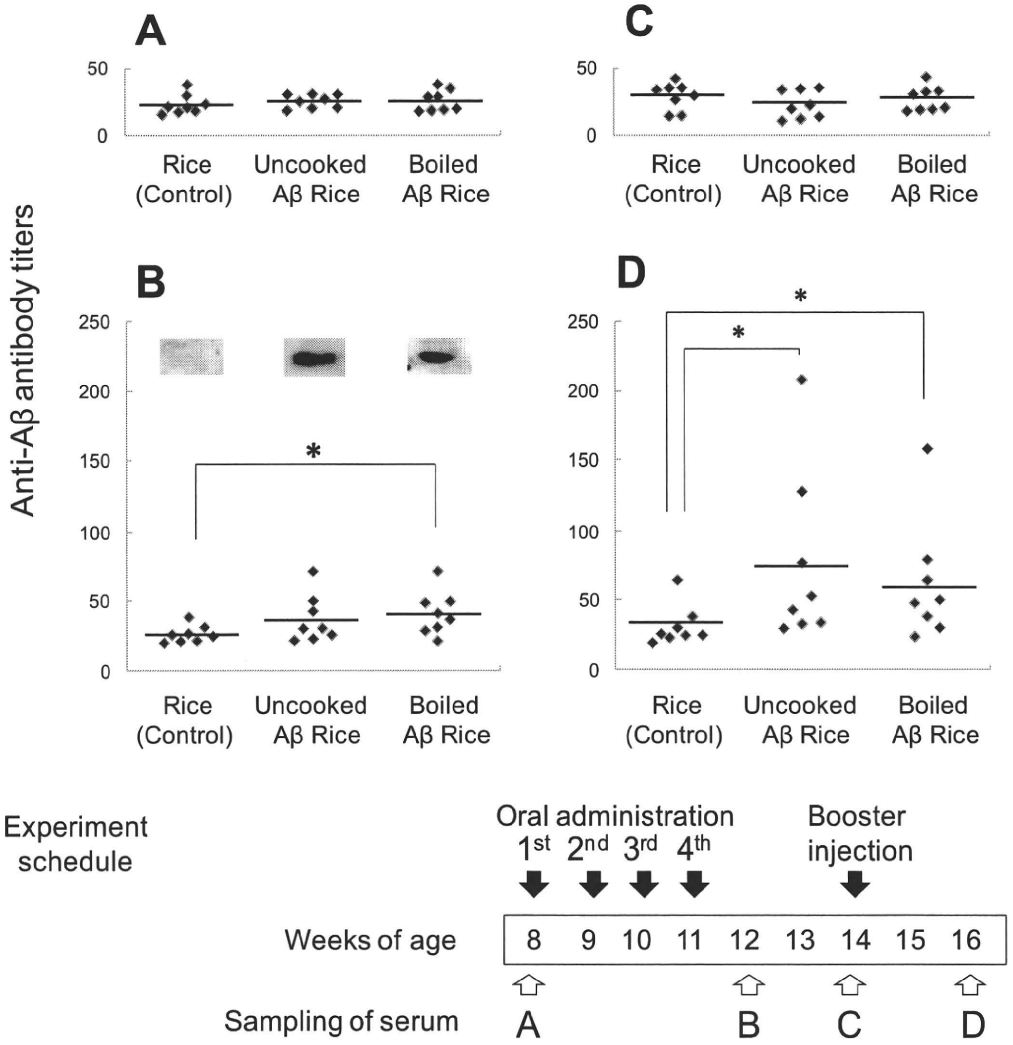

Fig. 4. Titers of antibodies against Aβ in serum from 8-week-old mice before immunization (A), 12-week-old and 14-week-old mice after immunization (B, C, each), and 16-week-old mice after booster injection (D). Anti-Aβ antibody titers for each mouse are shown. Horizontal lines show average.* $P < 0.05$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

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

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

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

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

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

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

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

Acknowledgements

We would like to thank Dr. S. Hidaka and Dr. N. Yamagishi for valuable advice. This work was supported in part by funding for Research for Promoting Technological Seeds from the Japan Science and Technology Agency.

Conflict of interests

The authors have declared that no conflict of interest exists.

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Biochemical and Biophysical Research Communications 396 (2010) 927–932

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ADAM19 autolysis is activated by LPS and promotes non-classical secretion of cysteine-rich protein 2

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ARTICLE INFO

Article history:
Received 17 April 2010
Available online 9 May 2010

Keywords:
ADAM19
CRIP2
α-Secretase
Alzheimer's disease
Non-classical secretion

ABSTRACT

ADAM family proteins are type I transmembrane, zinc-dependent metalloproteases. This family has multiple conserved domains, including a signal peptide, a pro-domain, a metalloprotease domain, a disintegrin (DI) domain, a cysteine-rich (Cys) domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic domain. The Cys and DI domains may play active roles in regulating proteolytic activity or substrate specificity. ADAM19 has an autolytic processing activity within its Cys domain, and the processing is necessary for its proteolytic activity. To identify a new physiological function of ADAM19, we screened for associating proteins by using the extracellular domain of ADAM19 in a yeast two-hybrid system. Cysteine-rich protein 2 (CRIP2) showed an association with ADAM19 through its DI and Cys domains. Sequence analysis revealed that CRIP2 is a secretable protein without a classical signal. CRIP2 secretion was increased by overexpression of ADAM19 and decreased by suppression of ADAM19 expression. Moreover, CRIP2 secretion increased in parallel with the autolytic processing of ADAM19 stimulated by lipopolysaccharide. These findings suggest that ADAM19 autolysis is activated by lipopolysaccharide and that ADAM19 promotes the secretion of CRIP2.

1. Introduction

ADAM family proteins are type I transmembrane, zinc-dependent metalloproteases. With multiple conserved domains, including a signal peptide (SP), a pro-domain (Pro), a metalloprotease (MP) domain, a disintegrin (DI) domain, a cysteine-rich (Cys) domain, an EGF-like (EGF) domain, a transmembrane domain, and a cytoplasmic domain, as shown in Fig. 1A. A pro-protein converting enzyme such as furin removes the pro-domain [1], and the activated ADAM mediates the ectodomain shedding of various transmembrane proteins, including growth factors, cytokines, growth factor receptors, cytokine receptors, and adhesion molecules [2]. Thus, growth factors and cytokines residing inactively in the membrane are cleaved by ADAM and secreted in their active forms. The shedding of extracellular matrix or adhesion proteins is important for cell–matrix and cell–cell interactions, and ADAM-mediated proteolysis on the cell surface plays a significant role in development, homeostatic control, and tissue repair. Furthermore, aberrant proteolysis is related to many diseases such as cancer, rheumatoid arthritis, and Alzheimer's disease (AD) [3].

One of the most important substrates of ADAM is amyloid precursor protein (APP). The cleavage of APP by β- or γ-secretase produces the neurotoxic amyloid β-peptide (Aβ), which produces the plaque characteristic of AD [4]. However, APP is normally cleaved in an alternative, non-amyloidogenic pathway, in which the Aβ sequence is cleaved by α-secretase, an ADAM, to release the neuroprotective ectodomain fragment referred to as soluble APP α (sAPP α) from the cell surface. The transmembrane stub is then cleaved by γ-secretase to generate a soluble p3 fragment, instead of Aβ. We have previously reported that ADAM19 has constitutive α-secretase activity [5].

Intriguingly, autolytic processing within the cysteine-rich domain of ADAM19 is necessary for its proteolytic activity [6] (Fig. 1A), although the regulatory mechanism is unknown. To clarify the regulatory mechanism and identify a new physiological role of ADAM19, we screened for associating proteins by using the extracellular domain of ADAM19 in a yeast two-hybrid system.

Here, we show that cysteine-rich protein2 (CRIP2) associates with ADAM19 through its DI and Cys domains, that CRIP2 is a secretable protein without a classical signal sequence, and that the secretion of CRIP2 is upregulated by ADAM19. The secretion of

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-peptide; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; sAPP α, soluble APP α; 3AT, s-aminotriazole; CRIP2, cysteine-rich protein-2; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; NRG, neuregulin; NSAID, non-steroidal anti-inflammatory drug; TRANCE, TNF-related activation-induced cytokine.

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.05.025