Elevated amyloid-β plaque deposition in dietary selenium-deficient *Tg2576* transgenic mice

Mamoru Haratake,* Sakura Yoshida, Megumi Mandai, Takeshi Fuchigami and Morio Nakayama*

Graduate School of Biomedical Sciences, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan. E-mail: haratake@nagasaki-u.ac.jp (M. Haratake); morio@nagasaki-u.ac.jp (M. Nakayama)

Selenium-containing proteins (e.g., glutathione peroxidases) are important antioxidants in neuronal defense against oxidative stress. In this study, the production of amyloid-β (Aβ) plaques in the brain of the *Tg2576* transgenic mice was investigated under a dietary selenium-deficient condition. The 16-week-old mice were fed a selenium-deficient diet (0.004 μg-selenium g^{-1}-diet) or a selenium-adequate diet (0.386 μg-selenium g^{-1}-diet) for 76-week. The selenium concentrations of the organs/tissues in the selenium-deficient diet-fed mice were significantly decreased in comparison to those in the selenium-adequate diet-fed mice; 1.7% of that in the selenium-adequate diet-fed mice in the liver and 43% of that in the selenium-adequate diet-fed mice in the brain. The Aβ plaques formed in the brain were fluorescently stained with thioflavin T, and then the obtained images of the brain slices were qualitatively analyzed. The feeding of the selenium-deficient diet to the *Tg2576* transgenic mice resulted in more than a two-fold increase in the total area of the Aβ plaques in comparison to that of the selenium-adequate diet. The elevated Aβ plaque deposition in the selenium-deficient mice can be explained as a consequence of decreases in the selenium concentration, which suggests that the selenium status is associated with the production

and/or the clearance of the Aβ peptide. The selenium-deficiency could possibly promote the onset and/or progression of Alzheimer's disease (AD) dementia, if the Aβ peptides initiate a sequence of events that lead to AD dementia. Consequently, the results shown here suggest that AD has an important relation with the selenium status *in vivo*.

Introduction

The deposition of insoluble plaques containing amyloid-β (Aβ) peptides in the brain represents a pathological hallmark feature of Alzheimer's disease (AD) dementia. The amyloid cascade hypothesis, which posits that the deposition of the Aβ peptide is a central event in AD pathology, has dominated research for the past three decades.[1] The human brain, which claims ~2% of total body mass, is responsible for ~20% of total body oxygen consumption.[2–4] In consequence of the high oxygen demand, the brain inevitably induces the generation of large amounts of reactive oxygen species (ROS), which are thought to be associated with the onset and/or progression of AD dementia due to the ROS-mediated injury to certain brain regions from the early stages of the illness.[5–7] Thus, oxidative stress is a key feature in the AD patient brain and manifests as lipid peroxidation.

Selenium is an essential antioxidant nutrient for humans and other higher animal species and is incorporated into selenoproteins in the form of selenocysteine that is known as the 21st amino acid.[8] The brain is the organ that remains selenium replete the longest under conditions of selenium deficiency, suggesting that selenium plays a critical role in brain functions. Accumulating papers have pointed out that decreases in selenium-dependent glutathione peroxidase (GPx) activity in the brain are associated with the onset of neurodegenerative diseases including AD dementia.[9–12] In particular, the role of GPx-4 appears more important than other antioxidant enzymes due to the high lipid content in the brain.[13,14] In addition, selenoprotein P (SelP),[15] which is the brain-specific selenium transport

protein in the plasma, is known to be essential for neuronal survival and function[16] and is also associated with the AD pathology.[17] Recently, AD dementia patients were reported to have significantly lower selenium concentrations in the plasma, red cells and nails (32.59, 43.74 and 0.302 μg g^{-1}) compared with the healthy control subjects (50.99, 79.16 and 0.400 μg g^{-1}).[18]

The purpose of this study was to investigate whether the dietary selenium status affects the production of Aβ plaques in the brain. Experiments were conducted to study the effect of dietary selenium deficiency on the Aβ plaque deposition using the *Tg2576* transgenic mice. So far, scant information on the relevance of selenium status to the production of the Aβ plaques *in vivo* has been reported.[19,20] To our best knowledge, this is the first paper showing that the dietary selenium-deficiency increases the Aβ plaques formed in the brain of the *Tg2576* transgenic mice.

Experimental

Animals and diets

Female 16-week-old *Tg2576* transgenic mice were obtained from Taconic Farms, Inc. (Germantown, NY, U.S.A.) and cared for in accordance with the guidelines of Nagasaki University on Animal Care. The mice were randomly divided into two groups (five in each) and housed one per cage on a 12 h light/12 h dark schedule at 23 ± 2 °C and 60% relative humidity. The mice were freely fed a selenium-deficient diet [Oriental Yeast Co. Ltd., Tokyo, Japan, Nutrient contents (% by weight): torula yeast, 30; sucrose, 55.7; lard, 5; liver oil, 3; minerals, 5; vitamins, 0.9; methionine, 0.3; choline chloride, 0.2][21] or a selenium-adequate regular breeding diet CE-2 (Clea Japan, Inc., Tokyo, Japan) for 76 weeks. Selenium concentrations of the selenium-deficient and selenium-adequate diets used in this

study were 0.004 and 0.386 μg-selenium g^{-1}-diet, respectively. During the feeding of these diets, the body weights of the selenium-deficient diet-fed *Tg2576* transgenic mice were not significantly different from those of the selenium-adequate diet-fed mice. *Ad libitum* purified water (> 18 MΩ · cm) generated by a Milli-Q Biocel system (Millipore Corp., Billerica, MA, U.S.A.) was supplied as the drinking water to the mice throughout the feeding experiment.

Determination of selenium concentration

The selected organs/tissues were removed from the mice [92-week-old (≈ 21-month-old)] under deep ether anesthesia and thoroughly rinsed with saline. After the removal of remaining saline drops with paper towels, an appropriate amount of organs/tissues in the wet state was placed in a glass tube, and digested with a one to four mixture by volume of perchloric acid and nitric acid. Selenium concentrations of the digested samples were fluorometrically determined using 2,3-diaminonaphtharene (DAN) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (excitation wavelength, 375 nm; emission wavelength, 525 nm) subsequent to acid digestion using a one to four mixture by volume of perchloric acid and nitric acid.[22,23] The selenium standard solution [1000 ppm as selenium (IV) dioxide in 0.5 M nitric acid] for the fluorometry was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). For the quality control of the determination of the selenium concentrations, organ/tissue samples from the conventional mice were spiked with a given volume of the selenium standard solution and then subjected to the wet digestion and the subsequent fluorometry. Three samples from each diet used in this study were randomly collected, and their selenium concentrations were also determined by a procedure similar to that described above. All other chemicals were of commercial reagent grade and used as received.

Preparation of brain slice and its staining with thioflavin T

The average brain weight of the selenium-deficient diet-fed and the selenium-adequate diet-fed mice were 0.356 and 0.371 g in the wet state, respectively. Serial 10-μm-thick brain vertical slices (50 pieces for each mouse) were prepared from the middle part of the mice brains in a frozen state on a microtome CM1900 (Leica Microsystems, Wetzlar, Germany) and then mounted on non-fluorescent slide glasses. To detect the Aβ plaques, the brain slice-immobilized slide glasses were soaked in 10 μM thioflavin T (ThT) (Nacalai Tesque, Inc., Kyoto, Japan) 50% ethanol-Milli-Q water solution for 5 min and then sequentially washed in three Milli-Q water-filled glass chambers with moving the slides up and down gently. After the removal of remaining water drops, the brain slice-mounted slides were left in a lightproof box for ≈ 12 h at room temperature and then subjected to fluorescence microscopic evaluation.

Acquisition and analysis of fluorescence image

An inverted microscope BZ-8100 equipped with a GFP-BP filter set (excitation filter, 450–470 nm; dichroic mirror, 495 nm; emission filter, 515–565 nm) and a 4-fold objective lens (numerical aperture, 0.2; working distance, 20 mm) (KEYENCE Corp., Osaka, Japan) was used for the fluorescence microscopic observation. All fluorescence images of the ThT-treated brain slices (1360 × 1024 pixels) were acquired for the same exposure time (0.05 sec) and saved as super fine-grade JPEG files. The digitized fluorescence images of the brain slices were qualitatively analyzed by using MetaMorph (Premier) software (Molecular Devices, LLC, Sunnyvale, CA, USA). The fluorescence intensity above a threshold value from the images of the selenium-adequate diet-fed mice was considered as signals.

Statistical analysis

All data were presented as the mean ± standard error. Statistical analyses were performed using a program PRISM 4 (GraphPad Software, Inc., San Diego, CA, U.S.A.). Multiple mean values were compared by a two-way ANOVA with a Bonferroni post-hoc test with treatment and selenium concentration in the diets as factors. Comparisons were considered statistically significant at $p < 0.05$.

Results

To examine the selenium status in the *Tg2576* transgenic mice after the feeding experiment, the selenium concentrations of the selected organs/tissues were determined by the fluorescence method using DAN. The selenium concentrations of the organs/tissues in the selenium-deficient diet-fed mice were significantly decreased in comparison to those in the selenium-adequate diet-fed mice (Fig. 1). Typically, the selenium level in the selenium-deficient diet-fed mice liver represented 1.7% of that in the selenium-adequate diet-fed mice. The selenium concentration of the brain in the selenium-deficient diet-fed mice was also decreased to less than half of that in the selenium-adequate diet-fed mice (43%), although the brain retains a relatively high selenium concentration even under conditions of persistent dietary selenium deficiency.[24]

The Aβ plaques formed in the brain slices were detected using the most widely used amyloid staining fluorescent dye ThT whose fluorescence originates only from the dye bound to amyloid fibrils.[25,26] Typical fluorescence images of the brain slices in the two mice groups are represented in Fig. 2. The fluorescent signals arising from the ThT-treated brain slice of the selenium-deficient diet-fed mice (Fig. 2 A) were seemingly more intense in the calvarial and temporal regions than those of the selenium-adequate diet-fed mice (Fig. 2 B).

Subsequently, the obtained fluorescence images of the brain slices were qualitatively analyzed using MetaMorph software (Fig. 3). The feeding of the selenium-deficient diet to the *Tg2576* transgenic mice resulted in more than a two-fold increase in the total area of the ThT-stained Aβ plaques in the brain slices in comparison to that of the selenium-adequate diet (Fig. 3 A). Similar trends were also observed for the total fluorescence intensity (Fig. 3 B) and the number of the ThT-stained Aβ plaques (Fig. 3 C), while no significant difference in the average area of the Aβ plaque stained was found between the selenium-deficient and selenium-adequate diet-fed mice (Fig. 3 D). These results demonstrated that the dietary selenium-deficiency influences the number, not the size, of the Aβ plaque formed in the brain. Consequently, the dietary selenium-deficiency resulted in the significant increase in the number of the Aβ plaques formed in the brain of the *Tg2576* transgenic mice.

Discussion

The *Tg2576* transgenic mice overexpress a mutant variant of human amyloid precursor protein (APP) (Lys670Asn, Met671Leu) and are well characterized models of AD amyloidosis because the mice develop extensive extracellular Aβ plaque deposits.[27] Increased lipid peroxidation was reported to precede the Aβ plaque formation in the brain of the *Tg2576* transgenic mice[7]; Homogenates from the cerebral cortex and hippocampus of the *Tg2576* transgenic mice had significantly higher levels of a marker (8,12- *iso*-iPF2a-VI) of lipid peroxidation than those from the wild-type mice starting at 8 months old. In contrast, a surge in Aβ peptide levels as well as Aβ deposits in the *Tg2576* transgenic mice brains occurs later, at 12 months old. The decreased selenium concentrations in the organs/tissues including the brain (Fig. 1) implicated decreases in the concentration of selenoproteins, such as GPx-4 and SelP, in the early evolution of Aβ amyloidosis. GPx-4 heterozygous knockout (*GPx-4*+/–) mice have higher levels of β-secretase activity than age-matched wild-type

controls, as a result of up-regulation of β-secretase expression.[28] APP transgenic mice with reduced brain GPx-4 (*APP GPx-4*+/–) mice significantly increased amyloid plaques compared with *APP GPx-4*+/+ mice.[28] In addition, in our previous studies using normal mice, a similar selenium-deficient state occurred due to the 8-week feeding of the selenium-deficient diet used in this study.[23,29] Thus, the selenium-deficient diet-fed *Tg2576* transgenic mice were also thought to already reach a selenium status as low as that shown in Fig. 1, at around 2 months after the feeding of the diets started before the Aβ plaque production. The brain of *Tg2576* mice is thought to ubiquitously exhibit an increased level of oxidative stress than the wild-type mice, because the sites of amyloid plaque deposition do not reflect the regional expression of the *Tg2576* transgenes, which are widely expressed in neurons throughout the brain.[30] Overall, the elevated Aβ plaques deposition in the brain of the selenium-deficient diet-fed mice can be explained as a consequence of decreases in the selenium concentration.

The present study demonstrates the elevated Aβ plaque deposition in the brain of the selenium-deficient diet-fed *Tg2576* transgenic mice, which suggests that the selenium status in the brain is associated with the processes of the production and/or the clearance of the Aβ peptide from APP. The selenium-deficiency (or low selenium status) could possibly promote the onset and/or progression of AD dementia, if the oligomeric aggregates and/or deposits of the Aβ peptide formed in the brain initiate a sequence of events that lead to AD dementia. Thus, the results presented in this study allowed us to suggest that AD has an important relation with the selenium status *in vivo*.

At the present, the number of AD dementia patients is increasing all over the world, currently estimated at 36 million, and will triple by 2050.[31] Although many potent Aβ peptide-targeted therapeutics (e.g., crenezumab by Genentech, Inc., a humanized anti-Aβ peptide-antibody) are now in various stages of development, several candidates (e.g., semagacestat by Eli Lilly & Co.) that were purported to reduce its production or aggregation

are not yet successful in a Phase III clinical trial. Preventing cognitive impairment and dementia in the elderly is a major public health challenge for this century and all hypotheses should be explored. In addition to the development of effective therapeutics, based on the protective roles of selenium from the ROS-mediated injury to the brain, a large prevention trial involving 6,600 elderly males in the United States (the Prevention of Alzheimer's Disease by Vitamin E and Selenium, PREADVISE) was conducted,[32,33] but was unfortunately discontinued due to the ironic fact that long-term selenium supplementation with selenized yeast could cause type 2 diabetes.[34] Antioxidative selenoproteins and/or selenium-containing species are one of the factors that may affect the risk of cognitive decline. Early mechanistic roles of antioxidant selenium in the production and/or clearance of the Aβ peptide will be the next issues to be investigated, which can provide not only useful information for the prevention of the onset of AD dementia but also a potential target for the development of Aβ-targeted therapeutics.

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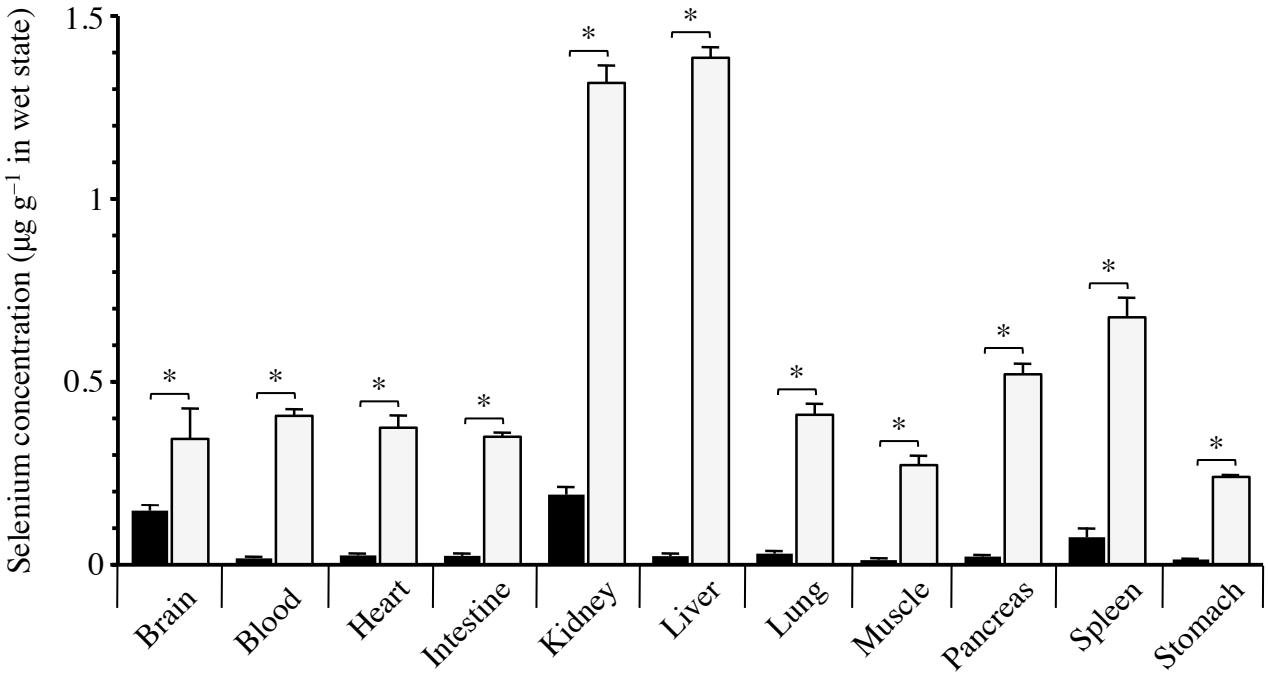

Fig. 1 Selenium concentrations of organs/tissues of *Tg2576* transgenic mice at 92-week-old after the diet feeding. Black column, selenium-deficient diet-fed mice; gray column, selenium-adequate diet-fed mice. All selenium concentrations in the selenium-deficient diet-fed mice were significantly different from those in the selenium adequate diet-fed mice. Data express mean ± standard error (n = 5). Asterisks indicate the organs/tissues that were significantly different ($P < 0.05$) between the diets.

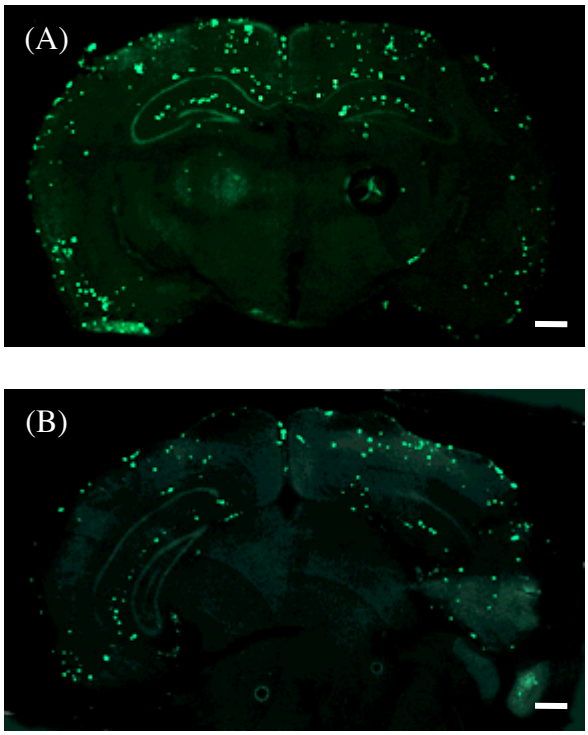

Fig. 2 Typical thioflavin T fluorescence images of the brain slices of the *Tg2576* transgenic mice. (A), selenium-deficient diet-fed mice; (B), selenium-adequate diet-fed mice. White scale bars of the lower right of the pictures represent 0.5 mm.

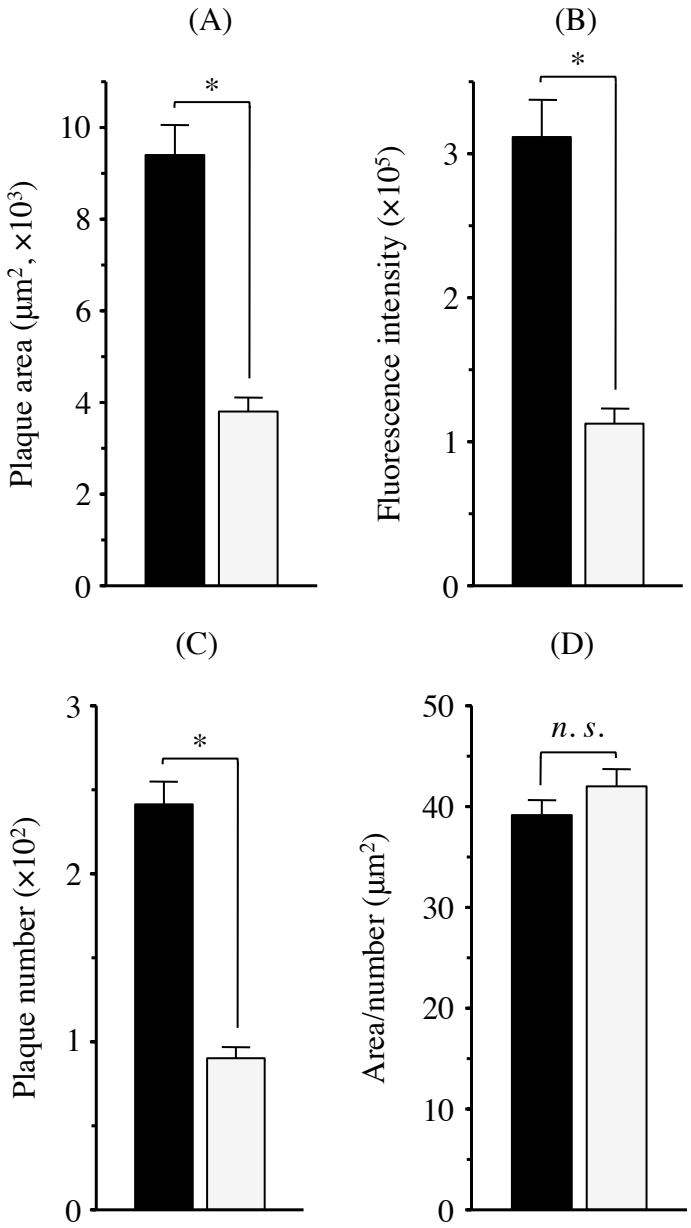

Fig. 3 Image analysis of thioflavin T fluorescence in the brain slices of the *Tg2576* transgenic mice. (A), total area stained with thioflavin T per brain slice; (B), total fluorescence intensity of stained area; (C), the plaque number; (D), ratio of the plaque area to the plaque number. Black column, selenium-deficient diet-fed mice; gray column, selenium-adequate diet-fed mice. Data express mean ± standard error (n = 250 specimens for each group). Asterisks indicate the parameters that were significantly different ($P < 0.05$) between the diets. *n. s.*, not significant.

Graphical abstract for the contents entry page

Dietary selenium-deficiency causes elevation of amyloid-β plaque deposition in the brain of *Tg2576* transgenic mice.