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Ji-Hoon Sohn[1], Jung On So[1], Hyun Joo Hong[1], Jong Won Kim[2], Duk Ryul Na[2], Manho Kim[3], Hee Kim[4], Eunjoo Nam[4], Hee Jin Ha[4], Young Ho Kim[4], Inhee Mook-Jung[1]

[1]Department of Biochemistry and Cancer Research Institute, Seoul National University College of Medicine, Seoul, 110-799, Korea, [2]Department of Neurology, Samsung Medical Center, Seoul, 135-710, Korea, [3]Department of Neurology, Seoul National University College of Medicine, Seoul, 110-799, Korea, [4]Digital Biotech, Inc., Gyeongi, 425-838, Korea

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1. ABSTRACT

Alzheimer's disease (AD) is characterized by two major neurological features: amyloid deposits and neurofibrillary tangles in the brain. According to the amyloid cascade hypothesis, accumulation of amyloid-beta peptide (A-beta) plays a central role in the pathogenesis of AD. Several lines of evidence suggest that antibodies against A-beta play a protective role in the neuropathology of AD. In this study, we describe the purification of an autoantibody against A-beta from human serum using affinity purification method. The purified autoantibody recognized A-beta deposits in the brain of aged Tg2676 mice, an animal model of AD. The serum levels of anti- A-beta autoantibody correlated inversely with age in both AD patients and control non-demented elderly subjects. Furthermore, the levels were significantly lower in AD patients compared with the age-matched control subjects. It is the first time to show the identification of endogenous anti-A-beta autoantibody in human serum and suggesting that serum levels of anti-A-beta autoantibody might be a good biomarker for AD patients.

2. INTRODUCTION

Alzheimer's disease (AD) is the most common demented disorder of the elderly that causes severe cognitive impairment (1). Epidemiological studies have confirmed that aging is the major risk factor for AD; the prevalence of AD doubles every 5 years after age 65 and approaches 50% by age 85 (2). Amyloid plaque, a dense core composed of sticky amyloid beta peptide (A-beta), is the central pathological feature in the brain of AD (3). According to the amyloid cascade hypothesis, accumulation of A-beta triggers a widespread neuronal dysfunction including synaptic injury, gliosis and formation of neurofibrillary tangles (4). Accordingly, much effort has been made to develop strategies to reduce the production and/or accumulation of A-beta. Immunization against A-beta has been one of the main approaches pursued to combat AD since the promising outcome observed in transgenic mice of AD (5). Both active and passive immunization results in prevention of amyloid deposition and even clearing of existing plaques in the brain, with associated improvement of behavioral/cognitive deficits (6-9). Although a clinical trial using aggregated A-beta $_{1-42}$ has been suspended because 6% of patients developed

meningoencephalitis, beneficial effects were also observed in those patients who generated anti-A-beta antibodies (10). In order to have a deeper insight considering putative roles of anti-A-beta antibodies in AD, a previous research characterized anti-A-beta antibodies derived from multiple B cell lines from a patient with AD (11). A circulating serum antibody against A-beta, however, has not identified yet.

In the present study, we describe the isolation of naturally-occurring antibody against A-beta from the human serum. The binding activity of purified anti-A-beta autoantibody to A-beta plaque in the brain was then determined in Tg2676 mice that overexpress the Swedish mutation of human amyloid precursor protein (APP). We next analyzed the levels of serum anti-A-beta autoantibody in a large number of subjects. The results indicated that serum levels of anti-A-beta autoantibody diminish with aging and are significantly lower in AD patients than in age-matched control subjects.

3. MATERIALS AND METHODS

3.1. PATIENTS AND CONTROLS

Blood samples were collected from patients with AD (n = 136, mean mini-mental state examination score±SD = 17.3±6.9) and non-demented elderly control subjects (n = 210) at Samsung Medical Center in Seoul, Korea, under the approval of the Institutional Review Board. All subjects consented to the study. Patients with AD met the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable AD (12). Supplementary table 1 summarizes the demographic characteristics of participating subjects.

3.2. PURIFICATION OF HUMAN ANTI-A-BETA ANTIBODY

A-beta $_{1-42}$ (US peptide Inc, Rancho Cucamonga, CA) was conjugated with BSA with glutaraldehyde (5), followed by coupling to CNBr-activaed Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden). Anti-A-beta antibody was purified from 100 μl of normal human serum according to the instructions provided by the manufacturer.

3.3. ANTI A-BETA ANTIBODY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Microtiter wells (Maxisorp, Nunc, Roskilde, Denmark) were coated with 1 μg/well monomeric human A-beta $_{1-42}$ (Bachem, Bubendorf, Switzerland) and blocked with 10% FBS/PBS buffer. The plates were then incubated with blood serum samples diluted at 1:100 in 10% FBS/PBS buffer. After washing, the wells were incubated with secondary antibody of sheep anti-human IgG antibody conjugated to horseradish peroxidase (1:2000 dilution, Amersham Pharmacia Biotech, Buckinghamshire, UK). Color reaction was performed with a substrate, 3,3′,5,5′-tetramethylbenzidine (Pierce, Rockford, IL) followed by reading on a plate reader (PowerWave XS, Biotek, VT) at 450 nm.

3.4. ANIMALS AND IMMUNOHISTOCHEMISTRY

Tg2576 mice carrying human APP_{695} with the Swedish mutation were generated as described previously (13). A-beta deposits were visualized immunohistochemically as described previously (14) except using mAb 4G8, which recognizes the A-beta 17-28 region (Senetek, St. Louis, MO) and purified human anti-A-beta antibody, at 1:100 and 1:2 dilution, respectively. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Seoul National University.

3.5. STATISTICAL ANALYSIS

Comparisons of the levels of anti- A-beta autoantibody were performed by unpaired *t*-test. Correlations between anti-A-beta autoantibody level and age were examined using Pearson correlation analysis. P value less than 0.05 denoted the presence of a statistically significant difference. All statistical analyses were performed using Graphpad Instat 3.0 (GraphPad Software Inc., San Diego, CA) statistical software package.

4. RESULTS AND DISCUSSION

We first purified anti-A-beta autoantibody from human serum using affinity purification to investigate its putative role in the neuropathology of AD and normal aging. Coomassie brilliant blue staining showed a purified human anti-A-beta autoantibody (Figure 1 A) after SDS-PAGE. Immunohistochemical staining of coronal brain sections of 24-month-old Tg2576 mice showed that the purified human anti-A-beta autoantibody recognized the A-beta deposit in hippocampal regions of the brains (Figure 1 B), confirming that the purified antibody is a naturally occurring serum anti-A-beta autoantibody. Although purified anti-A-beta autoantibody was not as sensitive as mAb 4G8, specific bindings were validated by comparing the locations of stained A-beta deposits by 4G8 and by purified anti- A-beta autoantibody on the serial sections (Figure 1 C and D). We next measured the serum levels of anti-A-beta autoantibody in AD patients (n = 136, age: 70.0±10.0 years, mean±SD) and normal nondemented elderly individuals (n = 210, age: 70.0±9.8 years). The mean level of anti-A-beta autoantibody in the serum was significantly lower in patients with AD compared with the control (Figure 2 A, $P < 0.0001$). Serum anti-A-beta autoantibody levels of 71% of AD patients were lower than the mean level (0.32±0.01 optical density, mean±SEM) of the control (Figure 2 B). Consistent with previous reports (15, 16), no significant difference were observed in serum IgG concentrations between control and AD (data not shown) suggesting that the low levels of anti-A-beta autoantibody in the sera of AD patients was not simply due to age-associated nonspecific immune deficiency. It is

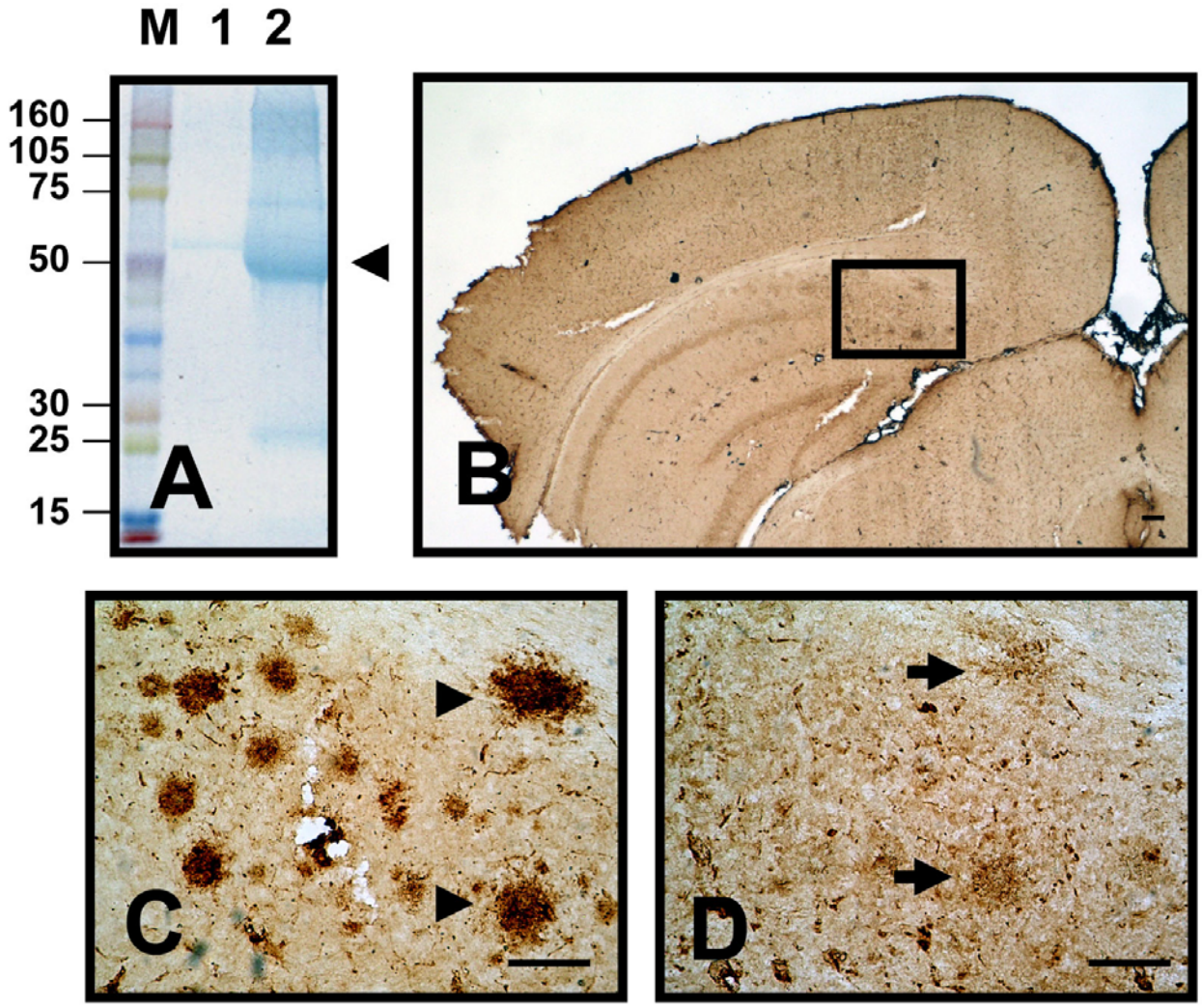

Figure 1. Purified human anti-A-beta autoantibody recognized A-beta deposits in the brain of 24-month-old Tg2576 mouse. (A) Purified anti-A-beta antibody was visualized by Coomassie brilliant blue staining after SDS-PAGE. The two major bands are 50 kDa (arrowhead) IgG heavy and 25 kDa IgG light chain, respectively. M: prestained size marker; lanes 1 and 2: 0.6 and 51 μg of purified anti-A-beta autoantibody, respectively. (B - D) Immunohistochemical staining using purified anti-A-beta antibody showed A-beta deposits in hippocampal regions of coronal brain sections of 24-month-old Tg2576 mice. The two relatively large A-beta deposits stained with purified anti-A-beta antibody (D, arrows) were also stained with mAb 4G8 (C, arrowheads) at the same locations on the neighbor sections. Scale bar = 200 μm.

Table 1. Demographic characteristics of AD patients and nondemented elderly control subjects

	Number of participating subjects	Age (years)											
		30 - 39		40 - 49		50 - 59		60 - 69		70 - 79		80 - 90	
		F	M	F	M	F	M	F	M	F	M	F	M
Control	210			5	2	5	20	25	28	32	59	15	19
AD	136	2		2	1	6	7	25	12	50	12	15	4

F: female, M: male

possible that the low autoantibody level is due to T cell tolerance of A-beta. Cultured T cells from patients with AD or from APP transgenic mice showed impaired proliferative responses against A-beta (17, 18). In addition, the levels of anti-A-beta autoantibody were correlated inversely with age in both AD patients ($P < 0.005$) and control subjects ($P < 0.0001$). In the entire group of 346 subjects, anti-A-beta autoantibody levels were inversely and significantly correlated with age (Pearson's $r = -0.27$, $P < 0.0001$, 95% confidence interval = -0.37 to -0.17, Figure 2 C). The reason for the more significant correlation in control than in AD is probably due to the larger proportion of elderly individuals among the control group than AD group (Table 1). Lastly, we analyzed the differences in anti-A-beta

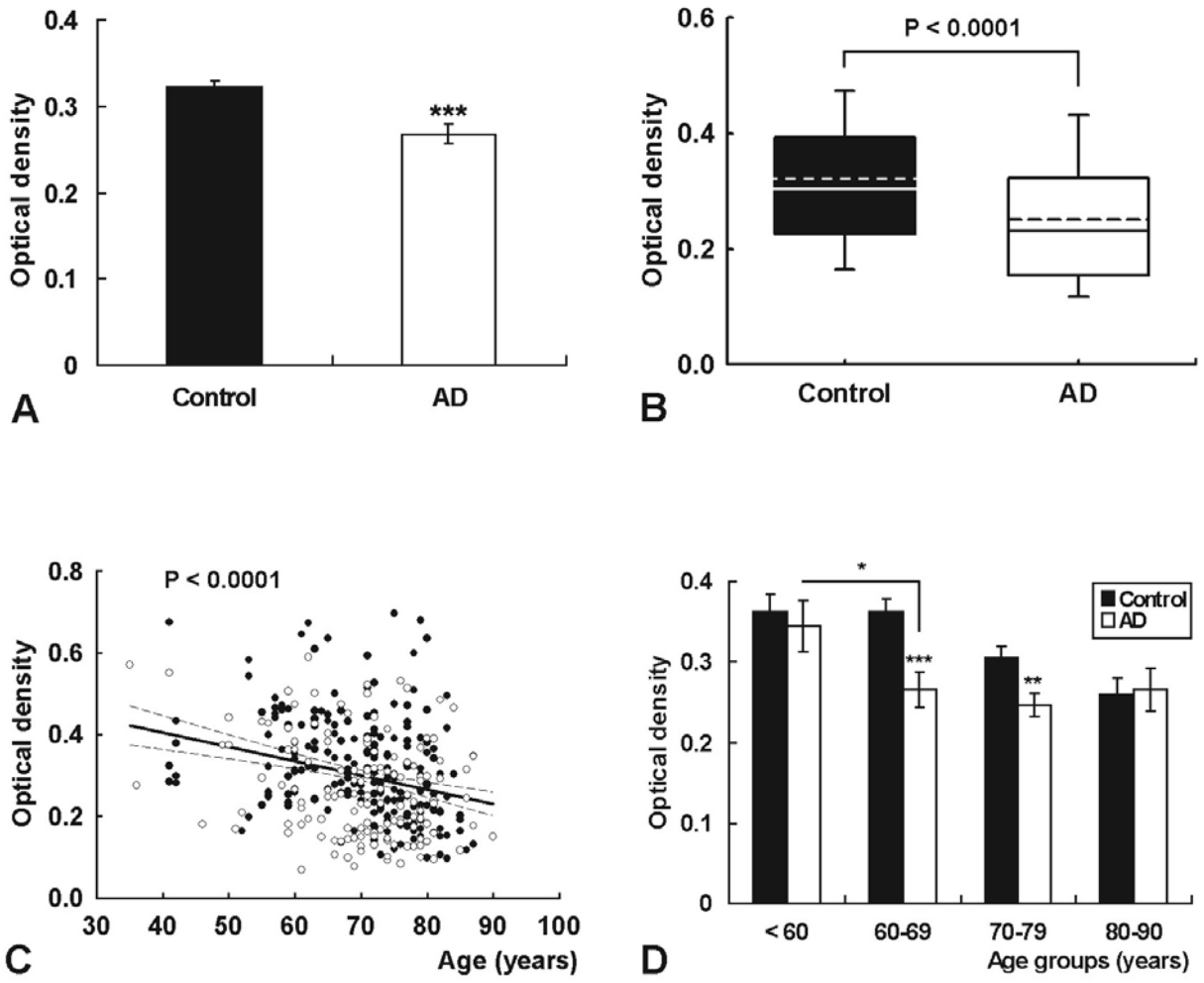

Figure 2. Effects of age and AD on serum levels of anti-A-beta autoantibody. (A) Serum levels of anti-A-beta autoantibody in patients with AD (n = 136) compared with control subjects (n = 210). Data are mean±SEM. ***P < 0.0001, by unpaired *t*-test. (B) Box plots denote 10th, 25th, 75th and 90th percentiles for each group. Solid and dashed lines within the box represent median and mean values, respectively. (C) Correlation between serum levels of anti-A-beta autoantibody of control subjects and AD patients with age. Closed circles: control subjects, open circles: patients with AD. Dashed line indicates 95% confidence interval (-0.37 to -0.17). Pearson's r = -0.27, P < 0.0001. (D) Serum levels of anti-A-beta autoantibody were significantly lower in 60-69 year old AD age group. Differences between control subjects and AD subjects were the most significant for the 60-69 year old group. Note also that the level of the autoantibody diminished progressively with age in both groups. Data are mean±SEM. ***P = 0.0003, **P = 0.003, *P < 0.05, by unpaired *t*-test.

autoantibody levels between control and AD stratified according to age. The most significant reduction was noted in the 60-69 years old AD patients (P = 0.0003), relative to other age groups and the control. The differences between control and AD tended to diminish in older groups (70-79 group, P = 0.003; 80-90 group, P = not significant). Furthermore, the mean levels of anti-A-beta autoantibody were similar between control subjects and AD patients aged less than 60 years (Figure 2 D). Considered together, the above results indicate that serum levels of anti-A-beta autoantibody are significantly lower in the 60-69 year old AD patients, which coincides with the sharp rise in the incidence of AD during that age period.

In conclusion, our study suggests that serum levels of anti-A-beta autoantibody decrease with age, thus increasing the risk of AD in the elderly, and that insufficient production of anti-A-beta autoantibody in patients with AD, which persists after the age of 60, could contribute to the progression of the disease.

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Send correspondence to: Inhee Mook-Jung, Department of Biochemistry & Cancer Research Institute, Seoul National University College of Medicine, 28 Yongon-dong, Jongno-gu, Seoul 110-799, Korea. Tel: 82 2 740 8245; Fax: 82-2-744-4534. E-mail: inhee@snu.ac.kr

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