Deposition of Alpha2-Macroglobulin in Fibrillar Type of Senile Plaques in the Brain of PS/APP-Transgenic Mice

Takayuki Kondo^{1,2} and Ikuo Tooyama¹

¹Molecular Neurobiology Research Center, Shiga University of Medical Science, Otsu 520–2192, Japan and ²Center for Dementia Research, Nathan Kline Institute/New York University School of Medicine, Orangeburg, NY 10962, U.S.A.

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Alpha2-macroglobulin (A2M), a pan-proteinase inhibitor, accumulates in senile plaques of Alzheimer disease. In this study, we investigated the immunohistochemical localization of A2M in the brain of PS/APP mice. In control mice, A2M immunoreactivity was restricted to the vessels of brain parenchyma. In PS/APP mice, A2M deposition occurred in the brain parenchyma. A2M-positive deposits appeared after three months of

age when senile plaque formation began to occur. The number of A2M-positive deposits increased with age. Double-staining for A2M and thioflavin S revealed that A2M accumulated in a subpopulation of thioflavin S-positive fibrillar type of amyloid beta deposits. These results indicate that A2M is implicated in the formation of fibrillar type senile plaques in PS/APP mice.

Key words: alpha2-macroglobulin, Alzheimer disease, senile plaque, transgenic mouse

I. Introduction

Human alpha2-macroglobulin (A2M) is a high molecular protein (720 kDa) and has been identified as a pan-protease inhibitor distributed in various body fluids [5, 6, 22]. Proteases cleave an internal thiol ester bond in the “bait” region, causing a conformational change of A2M leading to its activation. Several studies indicate that A2M plays an important role in pathogenesis of Alzheimer disease (AD). For example, A2M gene polymorphism is likely to influence the development of AD and AD-type neuropathological changes [4, 18]. Also A2M is detected in senile plaques [3, 25] and likely to be associated with clearance or prevention of β -amyloid protein (A β) accumulation [8, 15, 24]. In the brain, A2M is synthesized mainly by astrocytes [10, 12]. A2M and its activated form can make a complex with A β *in vitro* [8, 15]. This complex can be cleared by a low-density lipoprotein receptor-related protein (LRP) mediated pathway in cultured cells [20]. These studies suggest the possibility that A2M plays a role in the clearance of A β from the brain. However, the direct significance of A2M in the pathogenesis of AD has been unresolved. In order to clarify the issues, *in vivo* studies using animal models of AD is of great importance.

Mutations in the amyloid precursor protein (APP) and presenilin-1 (PS-1) and presenilin-2 (PS-2) genes have been known to be associated with AD. Transgenic mice carrying the mutant APP (APP_{k670N, M671L}) gene show a marked elevation in A β protein levels and A β deposition in the cerebral cortex and hippocampus from approximately one year of age [11]. Mutant PS1 transgenic mice do not show abnormal changes, but mice carrying both mutant genes (PS/APP) develop AD-like deposits composed of A β from three months of age [9]. Moreover, the development of senile plaques has been well investigated in the PS/APP model [17]. Using the model animals, we set forth to ascertain: 1) whether A2M deposition is reproduced in the animal model of AD (PS/APP mice) or not, if A2M deposition is detected, 2) when A2M deposition occurs, and 3) in what type of senile plaques is A2M detected.

II. Materials and Methods

Animals

This experiment was conducted with strict adherence to the principles of laboratory animal care (NIH, revised 1985). A total of fifteen PS/APP transgenic mice (one at 5 weeks, four at 3 months, five at 7 months, and two at 12 months) were used in this study. A total of seven littermate PS-1 mice (three at 3 months, two at 7 months, and two at 12 months) were used as a control. All animals were deeply anesthetized, and were then perfused transcardially with 10 mM

Correspondence to: Ikuo Tooyama, M.D., Ph.D., Molecular Neuroscience Research Center, Shiga University of Medical Science, Seta, Otsu, Shiga 520–2192, Japan.

phosphate buffered saline (PBS) to wash out the blood. Subsequently, the animals were perfused with a chilled fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was quickly removed, and was post-fixed for 18 hr with 4% paraformaldehyde in 0.1 M PB at 4°C. After cyroprotection by incubating the block in 0.1 M PB containing 15% sucrose and 0.1% sodium azide at 4°C at least for 2 days, coronal sections were cut at 30 µm in a freezing microtome (Physitemp, Clifton, NJ, USA and Leica, Heiderberg, Germany) and collected in 0.01 M PBS (pH 7.4).

Immunohistochemical procedures

The sections in a free-floating state were incubated with 0.5% H₂O₂ in PBS containing 0.05% Tween 20 (PBST) for 20 min to suppress endogenous peroxidase activity, washed 3 times for 10 min each with PBST, and then incubated overnight at 4°C with a rabbit polyclonal anti-A2M antibody (Dako Japan, Osaka, Japan, 1:5,000). After washing 3 times for 10 min each with PBST, the free-floating sections were incubated for 1 hr at room temperature with a peroxidase-labeled anti-rabbit IgG Fab' antibody (HISTOFINE, Nichirei Co., Tokyo, Japan; diluted 1:10) in PBST. The immunoreactivity for A2M was visualized by reacting with a mixture containing 0.02% 3,3'-diaminobenzidine (DAB), 0.6% nickel ammonium sulfate and 0.005% H₂O₂ in 50 mM Tris-HCl buffer (pH 7.6) for 5 min.

For immunohistochemical control, an immunoabsorption test was employed using the A2M antiserum preincubated overnight with 20 µg/ml of human A2M at 4°C.

Western blotting

Mouse A2M in serum and human A2M purified from plasma (Calbiochem, La Jolla, CA) were analyzed by Western blot analysis as described below. Briefly, 8 µl of PS/APP mouse serum as well as 8 µg of human A2M was denatured by heat shock (98°C, 10 min). They were electrophoresed on a 5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under reducing conditions, and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Nippon Millipore Ltd., Tokyo, Japan). Molecular weight markers were supplied from BioRad (Nippon BioRad Laboratories, Japan).

After incubation for 1 hr at room temperature with 5% skim milk in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS, pH 7.4), the membrane was reacted overnight at 4°C with the A2M antibody at the working dilution in TBS containing 1% skim milk. After washing in TBS containing 0.05% Tween 20 (pH 7.5), the membrane was incubated at room temperature with peroxidase-labeled Fab' part of anti-rabbit IgG (HISTOFINE, Nichirei Co., Tokyo, Japan; diluted 1:10) in TBS containing 1% skim milk for 1.5 hr. We used the Precision marker (BioRad, Japan), which was incubated for 30 min with Precision streptation-HRP (BioRad diluted 1:10,000). The labeling was visualized by the chemiluminescence method with ECL Western blotting detection reagents (Amersham Pharmacia Biotech., Buck-

inghamshire, England).

Double immunostaining for A2M and CD34

Double immunostaining was performed according to the previous reports [1, 7] with slight modification. Sections were incubated overnight with a mouse monoclonal anti-CD34 antibody (6 µg/ml, IgG2a, κ, BO Bioscience Pharmingen, San Diego, CA, USA) at 4°C. After washing with PBST, the sections were incubated for 2 hr with a peroxidase-labeled anti-mouse IgG Fab' antibody (HISTOFINE, Nichirei Co., Tokyo, Japan; diluted 1:10). The immunoreactivity for CD34 was colorized as a purple reaction product by reacting with a mixture containing 0.02% DAB, 0.6% nickel ammonium sulfate and 0.005% H₂O₂ in 50 mM Tris-HCl buffer (pH 7.6).

After the first immunohistochemical staining for CD34, the sections were washed for 10 min with Tris-HCl buffer, and then incubated for 20 min with 0.5% H₂O₂ in PBS-T to kill peroxidase activity. After washing with PBST, sections were incubated overnight with a rabbit polyclonal antibody to A2M (1:5,000) at 4°C. After washing with PBST, sections were incubated for 2 hr with a peroxidase-labeled anti-rabbit IgG Fab' antibody (HISTOFINE, Nichirei Co., Tokyo, Japan; diluted 1:10). The immunoreactivity for A2M was visualized as a brown reaction product by reacting with the DAB solution in the absence of nickel ammonium sulfate.

Double staining with A2M immunohistochemistry and thioflavin S histochemistry

The coronal sections of PS/APP mice at three and seven months of age were used. Double staining with A2M immunohistochemistry and thioflavin S histochemistry were employed as described before [23]. In brief, after the immunohistochemical staining for A2M, the sections were counterstained with aqueous 1% thioflavin S for 10 min at room temperature. Sections were then briefly washed with distilled water, followed by dehydration in 70–100% ethanol and xylene. Finally, sections were embedded with Entellan.

III. Results

Figure 1 shows the result obtained by Western blot analysis. The A2M antibody detected a band of 180 kDa, which corresponded to the molecular weight of the human A2M (Lane 1). In the mouse serum (Lane 2), the A2M antibody recognized a weak band of about 180 kDa (arrowhead) and an intense band of about 165 kDa (arrow).

For immunohistochemical control, an immunoabsorption test was employed using the A2M antiserum preincubated human A2M. No positive staining was seen (data not shown).

In control mice A2M immunoreactivity was detected in some capillaries, but the distribution pattern of A2M-positive capillaries was not uniform in the cerebral cortex (Fig. 2A, B). No A2M-positive deposits were seen. Figure 2C showed that CD34 immunostaining (purple) detected the entire part of capillaries, but on the contrary A2M

immunostaining (brown) detected only some part of CD 34 positive capillaries.

In the PS/APP transgenic mice, no positive deposits were seen in the brain of parenchyma at five weeks of age. At 3 months of age, a few A2M deposits were initially visible in the neuropil intermingled with A2M-immunoreactive vessels in the frontal cortex (Fig. 3A, B). The number of A2M-positive plaques increased with age (Fig. 3C, D).

Figure 4 shows double staining for A2M and thioflavin S. It is evident that A2M is deposited in a subpopulation of thioflavin S-positive fibrillar type of senile plaques.

IV. Discussion

Specificity of the anti-A2M antibody

The absorption test and Western blotting analysis characterized the specificity of the antibody used in this study, which is a rabbit polyclonal antibody against human A2M. In the mouse serum, the antibody recognized a weak band of about 180 kDa (arrowhead) and an intense band of

Fig. 1. Western blot analysis of the human A2M (Lane 1) and mouse serum (Lane 2) probed with the A2M antibody. In mouse serum (Lane 2), a weak band of about 180 kDa (arrowhead) and an intense band of about 165 kDa (arrow) are seen.

Fig. 2. Immunostaining for A2M (A and B), and double immunostaining for A2M and CD34 (C) in the cerebral cortex of a control mouse at 7 months of age. **A:** A2M-positive capillaries widely distribute to brain parenchyma, but the density of positive capillaries varies among region to region. A black arrowhead indicates an area showing a relatively high density of positive capillaries. White arrowheads indicate areas displaying a low density of positive capillaries. **B:** High magnification of the area indicated by the black arrowhead in A. **C:** Double immunostaining for A2M and CD34. A2M-positive capillaries (brown) were only part of CD34-positive capillaries (purple). Bars=100 μ m (A) and 25 μ m (B, C).

about 165 kDa (arrow). The results are well in accordance with previous reports showing that mouse plasma A2M consists of several subunits such as 185 kDa and 165 kDa subunits [21, 26]. Our results indicate that this antibody recognized not only human A2M but also mouse A2M.

Immunohistochemistry

Previous studies demonstrated that A2M was deposited

in senile plaques of AD patients [3, 25]. We report here for the first time a deposition of A2M in senile plaques of transgenic animal models of Alzheimer's disease. Our findings indicate that the deposition of A2M in senile plaques of AD patients does not occur by postmortem processes, but rather by certain pathological processes common to both AD patients and PS/APP mice. Since PS/APP mice show a marked deposition of A β , but no deposition of tau [13,

Fig. 3. Immunostaining for A2M in the cerebral cortex of PS/APP transgenic mice at the age of 3 months (A, B) and 7 months (C, D). A2M-positive plaques were seen in the parenchyma of the frontal cortex. Bars=100 μ m (A, C) and 25 μ m (B, D).

Fig. 4. Microphotographs of double staining for A2M (A) and thioflavin S (B) in the cerebral cortex of the PS/APP mice at the age of 7 months. Overexposure (C) clearly shows that A2M is localized to a subpopulation of thioflavin S-positive plaques (black arrows). The white arrow indicates a thioflavin S-positive plaque without A2M deposition. Bar=250 μ m.

17], our results suggest that A2M accumulation is closely relevant to A β deposition, but not to tau deposition.

The previous *in situ* hybridization study showed that A2M mRNA was detected in astrocytes [12]. However, our finding of the lack of A2M immunoreactivity in the astrocytes of PS/APP mice points to the possibility that A2M in senile plaques of the mice may originate in the serum. In order to clarify the origin of A2M in senile plaques, further studies, including *in situ* hybridization, will be needed.

Based on evidence that senile plaques were first visible in the frontal cortex of PS/APP transgenic mice at three months of age [17], our findings of the simultaneous appearance of A2M deposits in the same area implicate plaque formation from the early period of AD.

In this study, double staining for A2M and thioflavin S showed localization of A2M in a subset of thioflavin S-positive fibrillar type plaques. Our results suggest that A2M is not related to the first process, but instead to a developmental stage of senile plaque formation.

A β peptides *in vivo* include two different species, A β 1-42(43) and A β 1-40 [19]. A β 1-42(43) has two additional hydrophobic residues (Ile and Ala) and displays higher potential to polymerize than A β 1-40. A recent study showed that A β 1-42(43), but not A β 1-40, induced pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in cultured microglia [16]. These cytokines have been shown to induce the expression of A2M [12, 14]. Thus, it is hypothesized that deposition of A2M in senile plaques is one of the inflammatory responses induced by A β 1-42(43). This hypothesis is supported by the previous studies showing the localization of variable immune/inflammatory markers in fibrillary type senile plaques of PS/APP mice [18] and AD patients [2].

In both control and PS/APP mice, we observed A2M-immunoreactivity in some parts of CD34-positive capillaries. A2M-positive capillaries widely distribute to brain parenchyma, but the density of positive capillaries varies among region to region. The reason for this remains unclear. Further studies are needed to clarify this issue.

V. Conclusion

The present study clearly demonstrated that A2M accumulated in a subpopulation of thioflavin S-positive fibrillar type of senile plaques. A2M-positive plaques were first seen at three months of age when plaque formation occurred, and the number of A2M-positive plaques increased with age. The present results suggest that PS/APP mice will provide a useful animal model to analyze roles of A2M in AD, *in vivo*.

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VII. References

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