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Knockout of plasminogen activator inhibitor 1 gene reduces amyloid beta peptide burden in a mouse model of Alzheimer's disease

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Abstract

Accumulation of amyloid beta peptide (A β) in the brain is a pathological hallmark of Alzheimer's disease (AD); the underlying mechanism, however, is not well understood. In this study, we show that expression of plasminogen activator inhibitor 1 (PAI-1), a physiological inhibitor of tissue type and urokinase type plasminogen activators (tPA and uPA), increases with age in the brain of wild type and A β precursor protein-presenilin 1 (APP/PS1) transgenic mice as well as in AD patients. Most importantly, we show that knocking out the PAI-1 gene dramatically reduces A β burden in the brain of APP/PS1 mice but has no effect on the levels of full-length APP, alpha or beta C-terminal fragments. Furthermore, we show that knocking out the PAI-1 gene leads to increases in the activities of tPA and plasmin, and the plasmin activity inversely correlates with the amounts of SDS insoluble A β 40 and A β 42. Together, these data suggest that increased PAI-1 expression/activity contributes importantly to A β accumulation during aging and in AD probably by inhibiting plasminogen activation and thus A β degradation.

Keywords

PAI-1; Amyloid beta peptide; plasmin; Alzheimer's; disease; aging; knockout

1. Introduction

Alzheimer's disease (AD), an age-related neurodegenerative disease, is a major cause of dementia in the elderly. One of the major pathological features of AD is the formation of senile plaques in the brain, which are composed mainly of amyloid beta peptide (A β)

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(Bramblett et al., 1993; Sturchler-Pierrat et al., 1997). Numerous studies have shown that A β burden in the brain correlates directly with the severity of the pathological changes and memory impairment in AD (Lambert et al., 1998; Walsh et al., 2002; Klyubin et al., 2005; Arendash et al., 2006; Ohno et al., 2007). Accumulation of both soluble and insoluble A β in the brain has therefore been suggested to be the central disease-causing and disease-promoting event (Selkoe, 2000). Except for rare genetic forms of AD, in which the production of A β or pathogenic isoform of A β , A β 42, is increased due to mutations in the genes coding for amyloid precursor protein (APP) or presenilin1/presenilin 2 (PS1/PS2), a transmembrane protein and the catalytic component of γ -secretase complex, the mechanism underlying A β accumulation in the majority of AD cases (i.e., the sporadic form), which occur after age 65, remains unclear. Importantly, A β accumulation in the brain also occurs during the normal aging process in different animal species including fish, dog, monkey, and humans (Vaucher et al., 2001; Head and Torp, 2002; Maldonado et al., 2002; Costantini et al., 2005; Inestrosa et al., 2005) and these A β deposits have the same characteristics as those found in Alzheimer's disease (Fukumoto et al., 1996). Moreover, even in transgenic mice overexpressing mutant forms of human APP or APP plus presenilin-1 genes, A β accumulation does not occur until a certain age and AD pathological features develop gradually with increasing age (Hsiao et al., 1996; Oyama et al., 1998; Chapman et al., 1999; Takeuchi et al., 2000). These lines of evidence suggest that age-related changes contribute importantly to A β accumulation in AD.

The levels of A β in the brain represent a dynamic equilibrium state as a result of their biosynthesis and degradation. Although the enzymes responsible for the degradation of A β have not been well defined, several proteinases including neprilysin (NEP), insulin-degrading enzyme (IDE), endothelin-converting enzymes (ECE), metalloproteases (MMPs), and plasmin have been found to be able to degrade A β (Ledesma et al., 2000; Eckman et al., 2001; Iwata et al., 2001; Selkoe, 2001; Farris et al., 2003; Yan et al., 2006). Plasmin, a serine protease, plays a critical role in A β degradation. It can degrade A β with physiologically relevant efficiency (Van Nostrand and Porter, 1999; Ledesma et al., 2000; Tucker et al., 2000a; Exley and Korchazhkina, 2001) and is the only enzyme that efficiently degrades aggregated A β among these proteases (Tucker et al., 2000b). It has also been reported that plasmin enhances APP α -cleavage (Ledesma et al., 2000), suggesting that plasmin may reduce the toxic build-up of A β by either diverting APP away from the β cleavage pathway or by directly degrading existing A β . Importantly, plasmin activity decreases with age and in AD patients (Aoyagi et al., 1994; Ledesma et al., 2000; Ledesma et al., 2003); the underlying mechanism, however, is unclear.

Plasmin is converted from the zymogen plasminogen by tissue type and urokinase type plasminogen activators (tPA and uPA). The activities of tPA and uPA, in turn, are controlled by plasminogen activator inhibitors, mainly plasminogen activator inhibitor 1 (PAI-1), under physiological conditions. It has been reported that PAI-1 expression is increased in senescent cells (Comi et al., 1995; Mu and Higgins, 1995; West et al., 1996; Park et al., 2004) and in the murine aging model, *klotho* mutant (*kl/kl*) mice (Takeshita et al., 2002). It has also been shown that PAI-1 protein levels increase with age in plasma (Hashimoto et al., 1987; Sundell et al., 1989; Aoyagi et al., 1994; Tofler et al., 2005; Yamamoto et al., 2005) and in the cerebrospinal fluid of AD patients (Sutton et al., 1994). A recent study further shows that PAI-1 mRNA level is increased in APP transgenic mice (Cacquevel et al., 2007). Whether increased expression/activity of PAI-1 is responsible for the increase in A β deposition/accumulation observed in the elderly and in AD patients, however, is unknown.

In this study, we show that PAI-1 expression increases with age in the brain of wild type and APP/PS1 transgenic mice, and in AD patients. Most importantly, we show that knockout of the PAI-1 gene, which leads to increases in the activities of tPA and plasmin, significantly

reduces the amounts of SDS-soluble and insoluble A β 42 and A β 40 as well as A β plaques in the brain of APP/PS1 mice. These data suggest that increased PAI-1 expression/activity may underlie the decline in the plasmin activity and the increase in A β accumulation during aging and in AD.

2. Materials and Methods

2.1. Generation of triple transgenic-knockout mouse model

The homozygous PAI-1 deficient mice (PAI-1^{-/-}), purchased from JAXMICE, were generated and maintained on a C57BL6 background (Carmeliet et al., 1993). APP/PS1 double transgenic mice, purchased from JAXMICE, were generated by co-injection of human APP and PS1 transgene constructs containing AD mutations [a mutant human presenilin1 (DeltaE9) and a chimeric mouse/human Amyloid Precursor Protein] and were maintained on a mixed C57BL/6 x C3H genetic background (Jankowsky et al., 2001). Since the APP and PS1 transgenes are co-integrated at the same locus and transmit as non-segregating units, the APP/PS1 mice breed like a “single” transgenic line (Jankowsky et al., 2001). To generate APP/PS1 transgenic-PAI-1 knockout mice (APP/PS1/PAI-1^{-/-}), a two-step breeding strategy was used. For the first step, APP/PS1 mice were bred with PAI-1^{-/-} mice to produce APP/PS1/PAI-1^{+/-} and non-APP/PS1 transgenic PAI-1^{+/-} mice. The second-step mating of APP/PS1/PAI-1^{+/-} mice with PAI-1^{+/-} mice produced progenies with six genotypes: APP/PS1 transgenic mice (APP/PS1/PAI-1^{+/+}, APP/PS1/PAI-1^{+/-}, and APP/PS1/PAI-1^{-/-}) and non-APP/PS1 transgenic mice (PAI-1^{+/+}, PAI-1^{+/-}, and PAI-1^{-/-}). For unknown reasons, the birth and survival rates of APP/PS1/PAI-1^{+/+} mice were very low. Therefore, only APP/PS1/PAI-1^{+/-} and APP/PS1/PAI-1^{-/-} mice as well as the corresponding non-APP/PS1 transgenic PAI-1^{+/-} and PAI-1^{-/-} littermates were used in this study. The genotypes of these mice were determined by PCR of tail genomic DNA using gene construct specific primers. All the mice were maintained on a 12-h light/dark cycle at 22°C with free access to water and food.

2.2. Animal sacrifice and tissue collection

One- or twelve-month old female APP/PS1 mice or five-month old female APP/PS1/PAI-1^{+/-}, APP/PS1/PAI-1^{-/-} PAI-1^{-/-}, and PAI-1^{-/-} mice were euthanized with 100 mg/kg sodium pentobarbital and transcardial perfusion performed with cold phosphate buffered saline (PBS). Only female mice were used in this study as they develop A β plaques at an earlier age than male mice. Brains were cut sagittally into right and left hemispheres with the right hemisphere being fixed in 10% PBS buffered formalin for immunohistochemical staining of A β or PAI-1 while the left hemispheres were used for dissection of cerebral cortex and hippocampus. The dissected tissues were frozen in liquid nitrogen immediately for biochemistry analyses of mRNA, protein, and enzyme activity. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

2.3. Human brain samples

Frontal cortex samples were obtained from University of Alabama at Birmingham Alzheimer's Disease Research Center Brain Resource Program for each of the following two groups: 1) 65 years or older female healthy individuals; 2) 65 years or older female sporadic AD patients. All the brain samples were collected within 6 hours of death. The AD patients had undergone testing, including cranial imaging, thyroid panel, B12 level, syphilis testing, blood counts and blood chemistries, to eliminate treatable causes of dementia and met NIA-Reagan Institute criteria for the diagnosis of AD (McKhann et al., 1984). Both patients and controls were excluded if they suffered from cancer, liver disease, temporal arteritis, rheumatoid arthritis, diabetes mellitus, Parkinson's disease, or myocardial

infarction within the last 6 months. This study was approved by the University of Alabama at Birmingham Institutional Review Board prior to its start.

2.4. Determination of mRNA content by Northern hybridization

RNAs were isolated using TRIzol reagent following the manufacturer's protocol. Northern hybridizations were carried out as described before (Liu and Choi, 2000). Briefly, 20 μ g total RNA were subjected to electrophoresis and then transferred onto nylon membranes, which were hybridized with cDNA probes specific for PAI-1 or 18S (used as RNA loading control) for 2 hours sequentially after stripping. After hybridization, the membranes were washed and radioactivities were quantified using an Instantimager (Packard Instrument Company, Meriden, CT).

2.5. ELISA analyses of PAI-1 and A β proteins in the brain tissue

To measure PAI-1 protein in the brain, tissues were homogenized in Tris-HCl buffer containing 0.1% Triton-X100, pH 8.5. After ultracentrifugation at 100,000 *g* for 1 hour, the supernatants were used for the measurement of total PAI-1 protein levels using an ELISA kit as we have described before (Vayalil et al., 2005). The results were calculated based on the protein concentrations.

For the measurement of soluble and insoluble A β , mouse brain tissues were prepared according to the method described before (Kawarabayashi et al., 2001). Briefly, the brain tissues were homogenized in a A β extraction buffer containing 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 1% Triton X-100, 2% SDS, and protease inhibitors (complete protease inhibitor cocktail, Boehringer Mannheim, Mannheim, Germany), and centrifuged at 100,000 *g* for 1 hour. Supernatant was collected (SDS soluble) and stored at -80°C until analysis. Pellets were dissolved in 70% formic acid (FA), incubated at room temperature with gentle shaking for 2 hours, and then centrifuged at 100,000 *g* for 1 hour. The supernatants were collected (SDS insoluble/FA soluble). ELISA was performed to quantify SDS soluble and SDS-insoluble/formic acid soluble A β using the A β 40 or A β 42 ELISA kits from Covance (Emeryville, CA), which have been used by many investigators and shown to be specific for their specific isoforms (Zhou et al., 2005; Gunstad et al., 2008). The FA extraction solution was neutralized and diluted 20 times with a neutralizing buffer containing 1 M Tris, 0.5 M Na₂HPO₄, and 0.05% NaN₃ before analysis by ELISA.

2.6. Immunohistochemical staining of A β deposits in mouse brain

Immunohistochemical staining of A β deposits in the brain was conducted according to a previously described protocol (Li et al., 2003). Briefly, formalin-fixed and paraffin-embedded tissue sections were subjected to the avidin-biotin immunoperoxidase method to detect A β using a Vectastain ABC kit (Vector, Burlingame, CA) and monoclonal anti human A β antibody 6E10 (Covance, Emeryville, CA). The amyloid burden in the cerebral cortex and hippocampus was quantified using a histomorphometry system consisting of a Leica DMR research microscope equipped for fluorescence, polarizer/analyzer, and brightfield microscopy, a SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI), and Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, MD) capable of color segmentation and automation via programmable macros. The A β peptide stained areas were expressed as a percentage of total area of brain tissue.

2.7. Western analysis of A β monomer/oligomers, full-length APP, and alpha/beta C-terminal fragments in the mouse brain tissue as well as PAI-1 protein in human brain tissues

Mouse brain tissues were homogenized in a tissue extraction buffer containing 2% SDS and different protease inhibitors. Western analyses of full-length APP, A β monomer/oligomers, and alpha/beta C-terminal fragments (α -CTF/ β -CTF) in mouse brain homogenates were performed using two different concentrations of sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE gel): 10% SDS-PAGE gel for 40- to 140-kDa proteins and 16.5% Tris-Tricine SDS-PAGE for 3- to 40-kDa proteins. A β monomer/oligomers and full-length APP proteins were detected with 6E10 antibody (Covance, Emeryville, CA) while α - and β -CTFs were determined using rabbit polyclonal antibody CT695 against the C terminus of APP (Invitrogen). Western analyses of PAI-1 protein in human brain tissues were performed according to a procedure described before (Vayalil et al., 2005) using anti human PAI-1 antibody. Semi-quantitation of the bands was performed by densitometric scanning (a Bio-Rad Model GS-670 densitometer and Molecular Analyst™/PC software.) and normalized by β -actin.

2.8. Zymographic analysis of tPA and uPA activities

The activities of t-PA and u-PA in the brain tissue were determined by zymographic analysis according to our previously described protocol (Vayalil et al., 2005). Briefly, equal amounts of proteins were loaded onto 12% SDS-polyacrylamide gel containing 2 mg/ml casein and 5 (μ g/ml plasminogen. After electrophoresis, the enzyme reactions were initiated by incubating the gel in 0.1 M glycine-NaOH (pH 8.3) at 37°C for 16 hours. The lytic bands (tPA and uPA activity) were revealed by Coomassie blue staining and quantified using Bio-Rad Fluor-s MultiImaging system. Gels without plasminogen were also run simultaneously to ensure that the lytic bands are due to plasminogen activators. The tPA and uPA bands were identified based on both molecular weight and the loss of lytic activity upon inclusion of the plasmin inhibitor aprotinin (2 μ g/ml).

2.9. Analysis of plasmin activity

Plasmin activities were measured using a specific chromogenic substrate Tosyl-glycyl-prolyl-lysine-4-nitranilide-acetate (Chromzyme PL, from Roche Applied Sciences) as described before (Vayalil et al., 2005). The reaction mixture contained 33 mM Tris (pH 8.2), 6.4 mM NaCl and 0.5mM Chromozym PL. The time-dependent production of 4-nitraniline was followed by monitoring the absorbance at 405 nm. Plasmin activity was calculated using the 4-nitraniline extinction coefficient ($\epsilon_{405\text{nm}} = 1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and expressed based on the protein concentration.

2.10. Statistics

Data were expressed as mean $\pm$ SEM and evaluated by one-way ANOVA. Statistical significance was determined by Fisher LSD test. $P < 0.05$ was considered significant. Correlation studies were conducted using Pearson correlation analysis.

3. Results

3.1. PAI-1 mRNA and protein levels increase with age in the brain of APP/PS1 transgenic and non-transgenic mice

To determine whether PAI-1 gene expression is increased with age in the brain, PAI-1 mRNA and protein content in one- and twelve-month old APP/PS1 transgenic and non-transgenic mice were determined by Northern blot analysis and ELISA, respectively. The results show that the PAI-1 mRNA levels in the brain of twelve-month old APP/PS1 mice

are significantly increased compared to the levels in one-month old APP/PS1 mice (Fig 1A). ELISA data further show that PAI-1 protein levels increase with age in both APP/PS1 transgenic and non-transgenic mice and that 12-month old APP/PS1 mice have significant higher levels of PAI-1 compared to age-matched APP/PS1 non transgenic littermates (control, Fig 1B). Furthermore, we show that PAI-1 mRNA content is also significantly increased with age in the brain of wild type C57BL/6 mice (Fig 1C). The results suggest that PAI-1 expression in the brain is increased with age, which may be further stimulated by overexpression of APP/PS1.

3.2. PAI-1 protein content is increased in the cerebral cortex of AD patients

To determine whether PAI-1 gene expression is increased in the brain of AD patients, we compared the amounts of PAI-1 protein in the frontal cortex and cerebellum of sporadic AD patients and the age-gender matched healthy controls by Western blot analysis. The results show that PAI-1 protein level is significantly increased in the frontal cortex, but not in the cerebellum (data not shown), of AD patients compared to the age-gender matched healthy controls (Fig 2).

3.3. Knockout of the PAI-1 gene reduces A β burden in the brain of APP/PS1 transgenic mice

To delineate the role of PAI-1 in A β deposition/accumulation, APP/PS1/PAI-1^{+/-} and APP/PS1/PAI-1^{-/-} mice were generated by crossing APP/PS1 mice with PAI-1^{-/-} mice (for unknown reasons, the birth and survival rates of APP/PS1/PAI-1^{+/+} mice were very low and insufficient mice with this genotype were generated for these experiments). Five-month old female APP/PS1/PAI-1^{+/-} and APP/PS1/PAI-1^{-/-} mice as well as non-APP/PS1 transgenic PAI-1^{+/-} and PAI-1^{-/-} littermates were sacrificed. A β deposition in the brain was assessed by immunohistochemical staining while the amounts of A β 40 and A β 42, both soluble and insoluble, in the brain were determined by ELISA after extraction with 2% sodium dodecyl sulfate (SDS-soluble) and 70% formic acid (SDS-insoluble/FA-soluble), respectively. Immunohistochemical staining shows that the amounts of A β deposits are decreased by half in the brain of APP/PS1/PAI-1^{-/-} mice compared to APP/PS1/PAI-1^{+/-} mice (Fig 3). ELISA analyses further show that the amounts of SDS insoluble (FA-soluble) A β 42 and A β 40 are decreased by 41% and 42%, respectively, while SDS soluble A β 42 and A β 40 are decreased by 28% and 27%, respectively, in the brain of APP/PS1/PAI-1^{-/-} mice as compared with APP/PS1/PAI-1^{+/-} mice (Fig 4). It is notable that most of A β is present in the SDS-insoluble fraction (10 times the level of A β in the SDS-soluble fraction) and that PAI-1 homozygous deletion leads to a bigger reduction in SDS-insoluble A β than SDS-soluble A β . No A β deposits or proteins were detected by immunohistochemical staining or ELISA in APP/PS1 non-transgenic mice with or without PAI-1 gene (data not shown).

3.4. Knockout of the PAI-1 gene has no effect on the steady state level of APP, α -CTF, or β -CTF

To elucidate the mechanism whereby knocking out the PAI-1 gene reduces A β burden in APP/PS1 mice, the amounts of A β monomer/oligomers, full-length APP (FL-APP), α -CTF, and β -CTF were determined by western analyses. The results show that there are no significant differences in the amounts of FL-APP ($\approx$ 100kD), α -CTF, or β -CTF between APP/PS1 mice bearing PAI-1 homozygous or heterozygous deletion (Fig 5A&5B). However, the amounts of A β monomer (4 kD) and oligomers (16–20 kD) were dramatically reduced in the brain of APP/PS1/PAI-1^{-/-} mice as compared with APP/PS1/PAI-1^{+/-} mice (Fig 5A). The results suggest that knocking out the PAI-1 gene reduces A β burden in the brain of APP/PS1 mice probably not by decreasing APP synthesis or β -cleavage of APP or increasing α -cleavage of APP but by increasing A β degradation.

3.5. Knockout of the PAI-1 gene leads to increases in the activities of tPA and plasmin while expression of APP/PS1 transgenes is associated with decreases in tPA and plasmin activities

PAI-1 is a physiological inhibitor of tPA and uPA, which activate plasminogen. To examine whether knocking out the PAI-1 gene reduces A β burden in the brain of APP/PS1 mice by increasing the activity of the plasminogen cascade, we measured tPA and uPA activities in mouse brain tissue by zymography techniques and plasmin activity by a chromogenic substrate. The results show that the activity of tPA, but not uPA, is increased by 30% in PAI-1 homozygous deletion (PAI-1^{-/-}) mice as compared with PAI-1 heterozygous deletion (PAI-1^{+/-}) mice bearing no APP/PS1 transgenes (Fig 6A). This increased tPA activity is associated with an increase in plasmin activity (Fig 6B). Importantly, the expression of APP/PS1 transgenes leads to inhibition of tPA and plasmin activities not only in APP/PS1 mice bearing one single copy of PAI-1 gene (APP/PS1/PAI-1^{+/-}) but also in APP/PS1 mice bearing no PAI-1 gene (APP/PS1/PAI-1^{-/-}) (Fig 6A&6B). Correlation study results show that the amounts of SDS-insoluble (formic acid soluble) A β 40 and A β 42 in mouse brain are inversely correlated with the plasmin activity (Fig 7). These results suggest that knockout of the PAI-1 gene reduces A β burden in APP/PS1 mice probably by increasing plasmin activity and therefore A β degradation. The results also suggest that overexpression of APP may have a direct inhibition on tPA/plasmin expression/activity.

4. Discussion

PAI-1 has pleiotropic functions and plays an important role in homeostasis and wound healing. Increased PAI-1 expression has also been implicated in various pathological conditions including fibrosis, atherosclerosis, obesity, asthma, and tumor angiogenesis. In this study, we show that the expression of PAI-1 increases in the brain of old mice with or without APP/PS1 transgenes and in AD patients. Most importantly, we show that knocking out the PAI-1 gene, which leads to increases in the activities of tPA and plasmin, dramatically reduces A β burden in the brain of APP/PS1 transgenic mice. These data indicate that PAI-1 plays a critical role in A β accumulation and therefore in AD pathology. The data also suggest that age-associated increase in PAI-1 expression may underlie A β accumulation during aging and in AD. This notion is further supported by a recent study which shows that PAI-1 inhibitors significantly lower the plasma and brain A β levels and restore the memory functions in APP/PS1 transgenic mice (Jacobsen et al., 2008).

The mechanism underlying the reduction of A β in APP/PS1 mice bearing no PAI-1 gene is unclear. PAI-1 exerts its biological functions mainly by inhibiting the activities of tPA and uPA and therefore the activation of plasminogen (plasmin formation). As emerging evidence shows that plasmin plays a critical role in the degradation of A β (Ledesma et al., 2000; Tucker et al., 2000b; Melchor et al., 2003), it is speculated that the reduction of A β deposition/accumulation in the brain of APP/PS1/PAI-1^{-/-} mice results from increased degradation of A β secondary to loss of the inhibitory effect of PAI-1 on tPA and subsequent plasminogen activation. Indeed, we show in this study that the activities of tPA and plasmin are increased in the brain of PAI-1 homozygous knockout with or without APP/PS1 transgenes (Fig 6) and that the amounts of SDS insoluble A β 40 and A β 42 are inversely correlated with the plasmin activity (Fig 7). We also show that knocking out the PAI-1 gene dramatically reduces the amounts of A β monomer and oligomers but has no effect on the amount of full-length APP, α -CTF, or β -CTF (Fig 5A&5B). These data suggest that knockout of the PAI-1 gene, which leads to increased plasmin activity, reduces A β burden in the brain of APP/PS1 mice probably by increasing A β degradation rather than by suppressing APP synthesis or β -cleavage of APP (Fig 8). Ledesma et al. reported that incubation of human APP695 infected hippocampal neurons with plasmin increased α -cleavage of APP (Ledesma et al., 2000). In this study, however, we did not detect significant

increases in the amounts of α -CTF in PAI-1 knockout APP/PS1 mice as compared with PAI-1 heterozygous APP/PS1 mice (Fig 5B). The reason for such a discrepancy is unclear, probably due to the difference in the amounts of plasmin used in Ledesma's study and present in the mouse brain in our animal model. Nonetheless, these data further suggest that knockout of the PAI-1 gene decreases A β burden by increasing A β degradation rather than by decreasing A β 42 synthesis through increasing α -cleavage.

tPA is a major type of plasminogen activator expressed in the brain and plays an important role in hippocampal long-term potentiation (Mizutani et al., 1996; Nakagami et al., 2000; Pang et al., 2004). In this study, we show that the mouse brain has much higher activity of tPA than uPA (Fig 6), further suggesting an important role of tPA in the central nervous system. Interestingly, we show that A β deposition/accumulation is associated with a decrease in the tPA activity not only in APP/PS1/PAI-1^{+/-} mice but also in APP/PS1/PAI-1^{-/-} mice, suggesting that overexpression of APP may have a direct inhibitory effect on tPA expression/activity (independent of PAI-1 effect). It has been reported that neuroserpin mRNA expression is increased with age in the brain of the transgenic mice expressing human APP751 isoform (Cacquevel et al., 2007). Therefore, it is also possible that neuroserpin expression is increased in APP/PS1 mice, which leads to an inhibition of the tPA activity. It should be mentioned that although several studies including ours have shown that tPA activity was decreased in the brain of APP transgenic mice, associated with an increase in PAI-1 expression/activity (Melchor et al., 2003; Cacquevel et al., 2007; Jacobsen et al., 2008), other studies have reported that tPA and uPA mRNA content or tPA activity was increased in A β treated neurons or in APP transgenic mice (Tucker et al., 2000b; Lee et al., 2007). The reason for such a discrepancy is unclear, and probably reflects differences in the animal models used (Tg2576 vs. APP/PS1 mice), the age of the mice, and the techniques used to measure the activities of these enzymes. For example, we used zymography techniques to determine the tPA activity and a chromogen to measure plasmin activity in the brain homogenates while Lee et al used in situ zymography techniques to reveal the activities of tPA and plasmin in the areas surrounding the plaques (Lee et al., 2007).

PAI-1 expression is increased in senescent cells (Comi et al., 1995; Mu and Higgins, 1995; West et al., 1996; Park et al., 2004), in murine model of aging (Takeshita et al., 2002), and in the plasma of the elderly (Aillaud et al., 1986; Tofler et al., 2005). In this study, we show that PAI-1 expression is increased with age in the brain of wild type C56BL/6 mice and APP/PS1 transgenic or non-transgenic littermates. The mechanism underlying the increase in PAI-1 expression during aging is unclear. Many types of cells including endothelial cells, adipocytes, fibroblasts, hepatocytes, smooth muscle cells, and epithelial cells can synthesize PAI-1. The production of PAI-1 by these and other cells can be induced by various growth factors such as transforming growth factor beta and basic fibroblast growth factor, insulin-like growth factor, cytokines such as interleukin-1 and interleukin-6, and hormones such as corticosteroids (Loskutoff et al., 1993; Loskutoff and Samad, 1998; Dellas and Loskutoff, 2005; Dimova et al., 2005; Dong et al., 2007). Whether age-associated increase in PAI-1 expression results from increased production of any of these growth factors/cytokines/hormones remains to be determined. Our data and others (Melchor et al., 2003; Cacquevel et al., 2007; Jacobsen et al., 2008) also show that expression of APP/PS1 transgenes or accumulation of A β is associated with an increase in PAI-1 expression. Most interestingly, our data show that PAI-1 protein is increased in the cerebral cortex, but not in the cerebellum, a non-A β deposition prone area, of AD patients. The mechanism underlying the induction of PAI-1 expression in these APP/PS1 transgenic mice and in the cortex of AD patients is also unknown and needs to be further explored.

Although the cause is not clear, it has been reported that mice carrying the human APP transgene died early (about 2–3 month old) (Hsiao et al., 1995). Interestingly, we found, in

this study, that the mortality of APP/PS1/PAI-1^{+/+} mice was dramatically increased (around 43%) as compared with APP/PS1/PAI-1^{-/-} or APP/PS1/PAI-1^{+/-} mice (21% and 33%, respectively) (data not shown). The mechanism underlying such a dramatic increase in the mortality of APP/PS1/PAI-1^{+/+} mice is unknown. As there is no casualty among non-APP/PS1 transgenic PAI-1^{+/+} littermates, it is suggested that increased mortality in APP/PS1/PAI-1^{+/+} mice is related to APP/PS1 transgenes. Interestingly, homozygous deletion of PAI-1 increases the survival rate of APP/PS1 transgenic mice. Nonetheless, the mechanism underlying such effect of PAI-1 on the survival rate of APP/PS1 mice is beyond the scope of this study but warrants further investigation.

Taken together, the data presented in this manuscript suggest that PAI-1 plays an essential role in A β metabolism and that increased expression of PAI-1 may contribute importantly to the increased A β accumulation in the brain during aging and in AD. These results provide a strong rationale for developing PAI-1 inhibitors for the treatment of AD.

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Fig 1. PAI-1 mRNA and protein levels are increased with age in the brain of APP/PS1 transgenic and non transgenic mice

One and twelve month old APP/PS1 transgenic mice and non-transgenic littermates (control) as well as different aged wild type C57BL/6 mice were sacrificed and the brain collected as described in the Materials and Methods section. **A&C**, PAI-1 mRNA content was determined by Northern blot (the top panel) and the radioactivity was quantified using InstantImager (the bottom panel). **B**, PAI-1 protein content was determined by ELISA. * Significantly different from one- or three-month old group ($p < 0.05$, $n = 4-5$); †, significantly different from 12-month old control mice.

Fig 2. PAI-1 protein level is increased in the brain of AD patients

A, PAI-1 proteins were determined by western analysis using an antibody specific to human PAI-1. B, densitometry analysis of the intensity of the bands (normalized to β -actin). *

Significantly different from age- and gender-matched controls ($p < 0.05$, $n = 4$).

APP/PS1/PAI-1^{+/-}APP/PS1/PAI-1^{-/-}

Fig 3. Knocking out PAI-1 gene reduces A β deposition in the brain of APP/PS1 transgenic mice
 A β deposits in the brain of five month-old female mice were revealed by immunohistochemical staining using antibody specific to human A β (6E10). The amyloid burden in the cerebral cortex and hippocampus was quantified by the histomorphometry system and expressed as percentage of total area of brain. *, Significantly different from APP/PS1/PAI-1^{+/-} mice ($p < 0.05$, $n = 6-9$).

Fig 4. Knocking out PAI-1 gene leads to reduction of Aβ40 and Aβ42 in the brain of APP/PS1 mice
 APP/PS1 or non transgenic mice bearing heterozygous or null PAI-1 gene were sacrificed at 5 months of age. The amounts of Aβ40 and Aβ42, both soluble (right panel) and insoluble (left panel), in the brain were determined by ELISA and calculated based on the total protein content. * Significantly different from the corresponding APP/PS1/Pai-1^{+/−} mice (p<0.05, n=6–9).

Fig 5B

Fig 5. Knockout PAI-1 gene decreases the abundance of A β monomer and oligomers but has no effect on the level of full-length APP, α -CTF, or β -CTF in the brain of APP/PS1 transgenic mice Mouse brain tissues were homogenized in a tissue extraction buffer containing 2% SDS and different protease inhibitors. (A) Full-length APP (FL-APP) and A β monomer/oligomers in the brains of 5 month old APP/PS1 transgenic mice were determined by western analyses using 10% SDS-PAGE gel and 16.5% Tris-Tricine SDS-PAGE, respectively, and anti human A β antibody (6E10). (B) α -CTF and β -CTF were detected with an antibody against the C terminus of human APP (CT695). The intensities of the bands were semi-quantified by densitometric scanning and normalized by β -actin. * Significantly different from APP/PS1/PAI-1^{+/+} mice ($p < 0.05$, $n = 5$).

Fig 6. Knockout of PAI-1 gene leads to increases in the activities of tPA and plasmin while overexpression of APP/PS1 is associated with an inhibition of tPA and plasmin activities in the brain of APP/PS1 mice

tPA activity in the brain was determined by zymography while plasmin activity measured using a chromogenic substrate as described in the Materials and Methods section. The intensities of the bands were semi-quantified by densitometric scanning. ζ , Significantly different from the corresponding non APP/PS1 transgenic (control) mice; ϕ , significantly different from corresponding APP/PS1 transgenic or non-transgenic PAI-1^{+/-} mice ($p < 0.05$, $n = 6-9$).

Fig 7. The amounts of SDS insoluble A β 40 and A β 42 inversely correlate with the plasmin activity
Correlation studies of the amounts of formic acid (FA) soluble (SDS soluble) A β 40/A β 42 and the plasmin activity were performed by Pearson correlation analysis.

Fig 8. Potential mechanisms whereby knocking out the PAI-1 gene reduces Aβ burden in the brain of APP/PS1 mice

Knockout of the PAI-1 gene leads to an increase in tPA activity, which converts plasminogen into plasmin, a serin protease. Plasmin reduces Aβ accumulation in the brain by increasing Aβ degradation.