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Tissue plasminogen activator arrests Alzheimer's disease pathogenesis

Shin Bi Oh [a], Catherine Jeonghae Byun [a], Jin-Ho Yun [c], Dong-Gyu Jo [c], Peter Carmeliet [d], Jae-Young Koh [b], Joo-Yong Lee [a,b,*]

[a] Asan Institute for Life Sciences, Asan Medical Center, Seoul, Republic of Korea
[b] Department of Neurology, University of Ulsan College of Medicine, Seoul, Republic of Korea
[c] The School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea
[d] Laboratory of Angiogenesis and Neurovascular Link, VIB, Vesalius Research Center, Department of Oncology, University of Leuven, Belgium

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ABSTRACT

The progressive deposition of amyloid-β (Aβ) in the brain is a pathologic feature of Alzheimer's disease (AD). This study was aimed to determine whether endogenous tissue plasminogen activator (tPA) modulates the pathogenic process of AD. tPA expression and activity developed around amyloid plaques in the brains of human amyloid precursor protein–overexpressing Tg2576 mice, which were weakened by the genetic ablation of tPA. Although the complete loss of tPA was developmentally fatal to Tg2576 mice, tPA-heterozygous Tg2576 mice expressed the more severe degenerative phenotypes than tPA wild-type Tg2576 mice, including abnormal and unhealthy growth, shorter life spans, significantly enhanced Aβ levels, and the deposition of more and larger amyloid plaques in the brain. In addition, the expression of synaptic function–associated proteins was significantly reduced, which in turn caused a more severe impairment in learning and memory performance in Tg2576 mice. Thus, endogenous tPA, preferentially its aggregate form, could degrade Aβ molecules and maintain low levels of brain Aβ, resulting in the delay of AD pathogenesis.

1. Introduction

Alzheimer's disease (AD) is a progressive and degenerative brain disease that results in the most common form of dementia. The most prominent pathologic feature of AD is the accumulation of amyloid-β peptides (Aβ), which consist of 39–43 amino acids that are cleaved from amyloid precursor protein (APP). Because Aβ is neurotoxic and synaptotoxic, the brain should normally degrade and remove it. The imbalance between Aβ generation and clearance in the brain is central to the progression of AD. Whereas the excessive production and deposition of Aβ causes early-onset familial AD, decline in Aβ clearance could be responsible for sporadic AD, which is more common than familial AD (Selkoe, 2000; Tanzi et al., 2004). Several Aβ-degrading proteases have been identified as contributors to Aβ clearance, including insulin-degrading enzyme, angiotensin-converting enzyme, endothelin-converting enzyme, neprilysin, and matrix metalloproteinases (Miners et al., 2008).

Tissue plasminogen activator (tPA) could participate in Aβ clearance because it converts plasminogen into active plasmin, which is capable of dissolving peptide fibrils. Earlier findings suggested the possibility that tPA-activated plasmin may suppress Aβ aggregation and attenuate neurotoxicity. The addition of tPA with plasminogen reduces Aβ levels and toxicity in human amyloid precursor protein (hAPP)-transfected cells and primary cultured neurons (Ledesma et al., 2000; Tucker et al., 2000a, 2000b). tPA-activated plasmin attacks Aβ fibrils and aggregates in aqueous environments or when added to the brain tissues of Tg2576 hAPP-transgenic mice (Lee et al., 2007; Tucker et al., 2000a, 2000b). In an animal study, when synthetic Aβ was injected into the hippocampus, a brain area where tPA is prevalent (Qian et al., 1993; Teesalu et al., 2004), it rapidly disappeared and inflicted less neuronal degeneration in wild-type mice, but the deposits persisted longer and resulted in extensive neuronal loss in mice lacking either tPA or plasminogen (Melchor et al., 2003).

However, it is still unknown whether the physiologic level of endogenous tPA indeed influences AD pathogenesis. This study is the first to show that loss of endogenous tPA could cause an abnormal expression of synaptic function–associated proteins and in turn affect the behavioral performance in Tg2576 mice, which is principally due to the drop in Aβ-degradation or clearance capacity.

2. Methods and Materials

2.1. Animals

This animal study used tPA-knockout mice, which are genetically deficient in the tPA protein (Carmeliet et al., 1994), and Tg2576

* Corresponding author at: Asan Institute for Life Sciences, Asan Medical Center, Seoul 138-736, Republic of Korea. Tel.:+82 2 3010 4143; fax: +82 2 3010 4680.
E-mail address: jlee@amc.seoul.kr (J.-Y. Lee).

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hAPP transgenic mice, which overexpress the Swedish double-mutant form of human amyloid precursor protein (K670N/M671L) (Hsiao et al., 1996). We first collected Tg2576/tPA-heterozygous (F1 Tg2576/$tPA^{+/-}$) mice by crossbreeding Tg2576 ($hAPP^{+/-}$) mice with tPA-knockout ($tP^{-/-}$) mice, which were again mated to generate F2 Tg2576 mice that carried either wild-type (Tg2576/$tPA^{+/+}$), heterozygous (Tg2576/$tPA^{+/-}$), or knockout tPA (Tg2576/$tPA^{-/-}$). All animals were housed with free access to food and water. All animal experiments complied with the guidelines of the Asan Institute for Life Sciences for laboratory animal care and use.

2.2. Tissue preparations

Immediately after the mice were sacrificed by decapitation under deep anesthesia, their brains were dissected into the right and left hemispheres for subsequent biochemical and histochemical analyses, respectively. Shortly thereafter, these tissues were snap frozen in liquid nitrogen. Sagittal slices of the left hemisphere (12 μm thick) were collected onto 1% poly-L-lysine-coated glass slides using a cryostat (HM550; Microm, Walldorf, Germany).

2.3. In situ zymography

To examine tPA/plasmin activity in frozen sections, we conducted in situ caseinolytic zymography using the EnzChek protease green fluorescence assay kit (Invitrogen, Camarillo, CA, USA) (Lee et al., 2007). Briefly, unfixed brain sections (12 μm thick) were incubated in phosphate-buffered saline (PBS) that contained BODIPY FL-labeled casein (0.1 mg/mL) and human plasminogen (20 μg/mL; Millipore, Billerica, MA, USA) for 3 hours at 37 °C. The fluorescence of the BODIPY FL-labeled peptides that were released in the brain by plasmin-dependent caseinolysis was examined and photographed using a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan) fitted with a FITC filter (dichroic, 505 nm; excitation, 465–495 nm; barrier, 515–555 nm).

2.4. Immunohistochemistry

To evaluate Aβ deposition in the brain, we immunologically stained tissue sections with the primary antibody, anti-APP/Aβ (4G8, 1:1000; Covance, Emeryville, CA, USA) or anti-Aβ42 antibody (1:500; Abcam, Cambridge, UK). After fixation with 4% paraformaldehyde, the endogenous peroxidase activity of the tissue was depleted by treatment with 3% hydrogen peroxide. The sections were blocked with 3% normal serum and 0.3% Triton X-100 in PBS then reacted with the primary antibody and a biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA, USA). Next, the sections were treated with avidin-horseradish peroxidase (Vector Laboratories), developed using 0.015% diaminobenzidine and 0.001% hydrogen peroxide (Vector Laboratories) and then examined and photographed using a light microscope.

2.5. Histologic evaluation of the amyloid plaques

Compact core amyloid plaques emit blue fluorescence in situ under ultraviolet (UV) illumination (Lee et al., 2012). Thus, we evaluated the formation of blue fluorescent amyloid deposits in the brain under a fluorescence microscope (Eclipse 800i) with a UV-2A filter (dichroic, 400 nm; excitation, 330–380 nm; barrier, 420 nm).

To examine congophilic amyloid deposits, brain sections were stained with hematoxylin (Gill type III; Merck, Darmstadt, Germany) and Accustain Congo red amyloid staining solution (Sigma, St. Louis, MO) according to the manufacturer's instructions. After washing in absolute ethanol, the stained tissue sections were examined using a light or polarized microscope (Eclipse 80i).

2.6. Measurement of Aβ40/42 using a sandwich enzyme-linked immunosorbent assay

Whole brain hemispheres were weighed (wet weight) and homogenized in PBS (pH 7.4; 1:10, w/v) containing protease inhibitors (Complete protease inhibitor cocktail; 1 tablet/50 mL; Roche Diagnostics, Mannheim, Germany). After the homogenate was centrifuged at 100,000 *g*, the supernatant was collected (PBS-soluble fraction) and the PBS-insoluble pellets were sonicated in 2% sodium dodecyl sulfate (SDS, in water). The resulting homogenate was centrifuged again at 100,000 *g* (SDS-soluble fraction). To analyze insoluble Aβ, the SDS-insoluble pellets were solubilized in 70% formic acid (FA) and the FA-soluble fraction was collected by centrifugation at 100,000 *g*. Immediately before performing the sandwich enzyme-linked immunosorbent assay using the human Aβ40/42 ELISA Kit (Invitrogen), the SDS- and PBS-soluble fractions were diluted in EC buffer. FA-soluble fractions were neutralized with 1 M Tris (adjusted to pH 11 using 5 M NaOH), then diluted in the EC buffer. Measurements were taken at 450 nm using the Synergy H1 Hybrid microplate reader (BioTek, Winooski, VT, USA). Finally, the Aβ40/42 content of each brain was calculated as moles per gram of wet tissue.

2.7. Western blot analysis

The following primary antibodies were used: anti-APP/Aβ (6E10; 1:500; Covance), anti-zinc transporter-3 (1:1000; provided by Dr Robert D. Palmiter, University of Washington, Seattle, WA, USA), anti-PSD (1:2000; Sigma), anti-synaptobrevin/vesicle-associated membrane protein 1 and 2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti–brain-derived neurotrophic factor (1:500; Santa Cruz Biotechnology), anti-NR2A (1:1000; Rockland, Gilbertsville, PA, USA), anti-NR2B (1:1000; Rockland), anti-PSD95 (1:2000; Applied Biological Materials, Richmond, Canada), anti-ankyrin G (1:1000; Santa Cruz Biotechnology), and anti-β-actin antibody (1:5000; Santa Cruz).

Whole brain hemispheres were homogenized in PRO-PREP protein extraction solution containing protease inhibitors (1.0 mmol/L phenylmethylsulfonyl fluoride, 1.0 mmol/L ethylenediaminetetraacetic acid, 1 μmol/L pepstatin A, 1 μmol/L leupeptin, and 1 μmol/L aprotinin; iNtRON, Seongnam, Korea). Proteins were boiled with the sample buffer that contained 62.5 mmol/L Tris (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% mercaptoethanol, and 50 mmol/L dithiothreitol, and then separated using SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore) using semidry blotters (TE70 PWR; Amersham Biosciences, Uppsala Sweden). For Western blot analysis of the Aβ peptides, proteins were electrophoresed on 16.5% Mini-PROTEAN Tris-Tricine Precast Gel (Bio-Rad, Hercules, CA, USA). After blocking with 5% skim milk and 1% bovine serum albumin in TBS-T buffer (25 mM Tris, 150 mM NaCl, 0.1% Tween 20; pH 7.4), the blots were reacted with a primary antibodies and subsequently with horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). Immunoreactive proteins were developed using an enhanced chemiluminescence detection reagent (SuperSignal West Pico Luminol/Enhancer solution; Thermo, Rockford, IL, USA) and examined and photographed using the Davinch-Chemi Chemiluminescence Imaging System (CAS-400SM; CoreBio, Seoul, Korea). The protein densities of the blots were quantitatively analyzed by the ImageJ program (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin, which was used as the loading control.