Biochimica et Biophysica Acta 1854 (2015) 1010–1018

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Monocytic elastase-mediated apolipoprotein-E degradation: Potential involvement of microglial elastase-like proteases in apolipoprotein-E proteolysis in brains with Alzheimers disease

Midori Suenaga [a], Akiko Furuta [b], Koichi Wakabayashi [c], Toshiji Saibara [d], Yoichi Matsunaga [a,*]

[a] Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima 770-8514, Japan
[b] Department of Cellular and Molecular Neuropathology, Juntendo University, School of Medicine, Hongo 2-1-1, Bunkyou-ku, Tokyo 113-8421, Japan
[c] Department of Neuropathology, Institute of Brain Science, Hirosaki University, Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
[d] Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 9 April 2015
Accepted 28 April 2015
Available online 6 May 2015

Keywords:
Alzheimer's disease
Elastase
Protease
Microglia
Apolipoprotein-E

ABSTRACT

Impaired clearance of soluble Aβ (amyloid-β) promotes Aβ aggregation in brains with Alzheimer's disease (AD), while apolipoprotein-E (ApoE) in microglia mediates Aβ clearance. We studied the protease responsible for $ApoE_4$ degradation in human peripheral monocyte extracts, which are from the same lineage as microglia. We detected the hydrolytic activity for $ApoE_4$ in high-salt extracts with 2 M NaCl and found that the activity was inhibited by a serine protease inhibitor and an elastase-specific inhibitor, but not by other protease inhibitors. The extracts exhibited higher activity for the elastase substrate, and we followed the activity with ion-exchange and gel-filtration chromatography. Through silver staining, we partially purified a protein of 28 kDa, which was clarified as elastase by liquid chromatography–tandem mass spectrometry. These observations suggest that elastase is the key protease for $ApoE_4$ degradation. We also detected $ApoE_4$ hydrolytic activity in high-salt extracts in mouse microglial (BV-2) cell lysates, and showed that the $ApoE_4$ fragments by the BV-2 extracts differed from the fragments by the monocyte extracts. Though the $ApoE_4$ degradation by the extracts was not inhibited with elastase-specific inhibitors, it was inhibited by an elastase-specific monoclonal antibody, suggesting that elastase-like proteases in microglia differ from those of monocytes. Immunohistochemistry revealed that both elastase and ApoE were expressed in the senile plaques of brains with AD. *In vitro* studies also disclosed the localization of elastase in the microglial cell line, BV-2. Our results suggest that elastase-like proteases in the microglial cells surrounding Aβ plaques are responsible for ApoE degradation in the brain.

1. Introduction

The senile plaque, associated with activated microglia and astrocytes, is the pathological hallmark in the brains of patients with Alzheimer's disease (AD). Extracellular accumulation of insoluble amyloid-β protein (Aβ) is the main component of the plaque that induces synaptic dysfunction and neuronal loss resulting in progressive dementia [1].

Quantitative analyses have shown that, on average, 60% of all plaques contain Aβ42 and 31% contain Aβ40, and that the newly deposited Aβ was only partially co-localized with pre-existing Aβ and apolipoprotein-E (ApoE) [2]. Neurons produce Aβs by the proteolytic cleavage of amyloid precursor protein (APP), and are normally cleared though efflux into the peripheral circulation [3]. Aβs are then degraded by insulin-degrading enzymes secreted from astrocytes [4]. Impaired clearance of soluble Aβ rather than its overproduction is implicated in Aβ accumulation. The burden of extracellular Aβ induces an increase in intraneuronal Aβ accumulation [5] that is derived from internalized Aβ by neurons from the extracellular space. This occurs through ApoE-dependent and ApoE-independent pathways [6] and from intrinsic Aβ that escapes exocytosis after APP processing in the endocytic compartment [7].

ApoE is secreted from astrocytes/glia into the interstitial fluid and is important for metabolizing Aβ because of its propensity for binding Aβ as a cholesterol and phospholipid acceptor in reverse cholesterol transport [8]. Aβ oligomers show a direct toxicity to neurons, and ApoE inhibits oligomer formation of Aβ peptides in solution because of its ability to bind Aβ [9].

ApoE expression is transcriptionally induced through the action of the nuclear receptor, peroxisome proliferator-activated receptor-γ, and liver X receptors (LXRs) in coordination with retinoid x receptors [10]. Oral administration of retinoid x receptor agonists promotes the

* Corresponding author at: Department of Medical Pharmacology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan. Tel.: +81 88 602 68420; fax: +81 88 655 3051.
E-mail address: matsunaga@ph.bunri-u.ac.jp (Y. Matsunaga).

http://dx.doi.org/10.1016/j.bbapap.2015.04.028
1570-9639/

clearance of soluble Aβ and improves a variety of symptoms in mouse models of AD [11].

The lipidation of ApoE by the lipid transporter ATP-binding cassette transporter A1 (ABCA1), a major regulator of high-density lipoprotein metabolism, helps ApoE bind Aβ, while LXRs regulate the expression of both ABCA1 and ApoE and their activation increases the levels of lipidated ApoE [12]. The lack of ABCA1 causes a significant decrease in ApoE levels and a significant increase in amyloid deposition in the brains of ABCA1 knock-out mice, indicating that lipidated ApoE is essential for clearing amyloid deposition in the brain [13]. The intracellular degradation of Aβ by microglia is principally carried out by neprilysin and related proteases [14] whose activity can be enhanced with ApoE. Thus, ApoE is essential for Aβ internalization into the microglia, the promotion of Aβ proteolytic degradation [15–18], and the inhibition of Aβ toxicity in the brain.

However, a recent report suggests that ApoE affects Aβ metabolism not by directly binding to Aβ in physiological fluids such as cerebrospinal fluid or interstitial fluid, but by creating competition for the same clearance pathways as Aβ, resulting in Aβ accumulation in the brain [19]. The beneficial effects of decreased ApoE expression that occur following AD treatment are supported by the fact that decreases in ApoE under haploinsufficiency of human ApoE results in less Aβ deposition in amyloid mouse models, which is independent of ApoE isoforms [20,21]. Given that ApoE does appear to be involved in Aβ metabolism, ApoE metabolism may serve as a potential therapeutic target in the AD brain, although little is known about the fate of ApoE in the brain.

In the present study, we identified the proteases responsible for ApoE degradation in human peripheral monocytes, mouse microglial (BV-2) cells, and microglia from brain tissue with AD.

2. Materials and methods

2.1. Materials

Lymphoprep was purchased from Axis-Shield PoC (Oslo, Norway), and the Monocyte isolation kit II and FITC conjugated anti-CD4 for the flow cytometric analyses were from Miltenyi Biotec Inc. (CA, USA). The $ApoE_4$ (monomer) was from Peprotech (Rocky Hill, NJ, USA) and the $ApoE_4$ (dimer) was from Merck (Darmstadt, Germany). The anti-human $ApoE_4$ monoclonal antibody (moAb) was from IBL Co., Ltd. (Tokyo, Japan). The synthetic peptides that were used as substrates for protease activity were purchased from Peptide Institute, Inc. (Minoh, Osaka, Japan) including Suc(OMe)-Ala-Ala-Pro-Val-MCA of elastase, Boc-Phe-Ser-Arg-MCA of trypsin, Suc-Ala-Ala-Pro-Phe-MCA of chymotrypsin, and Zn-Phe-Arg-MCA of cysteine proteases. Protease inhibitors were purchased from Roche (Mannheim, Germany) including antipain for trypsin, bestatin for aminopeptidases, chymostatin for chymotrypsin, E-64 for cysteine proteases, leupeptin for serine and cysteine proteases, phosphoramidon for metalloendopeptidases, Pefabloc-SC for serine proteases, ethylenediaminetetraacetic acid (EDTA)-Na_2 for metalloproteases, pepstatin for aspartic proteases, and aprotinin for serine proteases. The elastase-specific inhibitor, Elasase Inhibitor IV, and the anti-ApoE moAb (E6D7), common to ApoE isoforms, were from Calbiochem (Darmstadt, Germany). Elastinal was from Enzo Life Science (Farmingdale, NY, USA), neutrophil elastase was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany), and the anti-elastase moAb for Western blots was from Abcam (Cambridge, MA, USA). The second antibody, horse radish peroxidase-conjugated anti-mouse immunoglobulin G antibody, for Western blots was from GM healthcare (Fairfield, CT, USA).

The primary antibodies used in the immunohistochemical and immunocytochemical studies were obtained as follows: elastase (rabbit polyclonal, 1:100, LS Bio, Seattle, WA, USA), ApoE (mouse moAb, 1:200, Millipore, Billerica, MA, USA), APP (mouse moAb, 1:200, Cell Signaling Technology, Beverly, MA, USA), PHT-tau (AT8, mouse moAb, 1:2000, Innogenetics, Ghent, Belgium), glial fibrillary acidic protein (GFAP, mouse moAb, GA5, 1:400, Sigma, Gillingham, UK), CD68 (mouse moAb, 1:200, Sigma, Gillingham, UK), and Mac2 (rat moAb, 1: 500, Cedarlane, Ontario, Canada).

For the immunohistochemical study, the hippocampal tissue from formalin-fixed autopsied brains with AD (4 cases, 2 males and 2 females, aged 76–78 years, each Braak stage III-C, III-C, IV-C, V-C) and non-demented individuals (4 cases, 2 males and 2 females, aged 37–85 years, each Braak stage 0-0, I-0, II-0, II-B) (Table 1) was examined in accordance with the Ethics Committee guidelines of Hirosaki University, Aomori, Japan. The mouse microglial (BV-2) cells were kindly provided by Dr. Donato [22,23], and the mouse neuroblastoma (Neuro-2a) cells and human neuroblastoma (IMR-32) cells were from the JCRB Cell Bank (Osaka, Japan).

2.2. Cell cultures

BV-2 cells were maintained at 37 °C in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2, while Neuro-2a cells and IMR-32 cells were maintained at 37 °C in Minimum Essential Media containing 10% fetal bovine serum under 5% CO_2.

2.3. Preparation of monocytes from human peripheral blood

Heparinized venous blood from healthy volunteers was obtained in accordance with the Ethics Committee Guidelines of Tokushima Bunri University, Tokushima, Japan. Peripheral blood mononuclear cells were isolated by density gradient centrifugation at 500 ×*g* for 30 min at 20 °C using Lymphoprep. The buffy coat layer was collected and washed twice with saline at 200 ×*g* for 15 min at 4 °C and the supernatant was removed. Monocytes were isolated according to the manufacturer's instructions (MACS, Monocyte isolation kit II), *i.e.*, the cell pellets were resuspended in 30 μL of MACS buffer (phosphate buffered saline [PBS] containing 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2) per 1×10^7 cells and mixed well with 10 μL FcR blocking regent (human immunoglobulin) while 10 μL of biotin-conjugated moAb cocktail was added containing anti-CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A and incubated for 10 min at 4 °C to react with non-monocytes. Then, 30 μL of MACS buffer was added with 20 μL of anti-biotin microbeads and incubated for an additional 15 min at 4 °C. After washing the cells with MACS buffer, the cells were resuspended to 1×10^8 cells in 500 μL of MACS buffer, applied to the MACS separator column in the magnetic field, and rinsed with MACS buffer to obtain the passed through populations as monocyte fractions. We confirmed the purity of the monocyte fractions as CD14-positive cell populations by flow cytometry.

2.4. Preparation of crude cell extracts

The purified monocytes (1.16×10^6 cells), BV-2 cells (8.96×10^5 cells), Neuro-2a cells (6.58×10^5 cells), and IMR-32 cells (5.99×10^6 cells) were suspended in 500 μL of the indicated concentrations of NaCl solutions and were sonicated for 20 s on ice and centrifuged at 15,000 ×*g* for 30 min at 4 °C with some modifications from our previous report [24]. The supernatants were collected as the starting samples and

Table 1
Characteristics of the autopsied brain cases.

	Age	Gender	Braak stage
AD 1	76	Male	IV-C
AD 2	77	Male	III-C
AD 3	77	Female	III-C
AD 4	78	Female	V-C
Control 1	37	Male	0-0
Control 2	71	Female	II-0
Control 3	84	Male	II-B
Control 4	85	Female	I-0

AD: Alzheimer' disease.