Partial Identification of Amyloid-β Degrading Activity in Human Serum

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ABSTRACT

The major hallmarks of Alzheimer's disease (AD) are the extracellular accumulation of pathological amyloid beta (Aβ) in the brain parenchyma and Aβ deposition in cerebral blood walls (cerebral amyloid angiopathy; CAA). Although CAA occurs in more than 80% of AD patients, the mechanisms of Aβ deposition and clearance around the vessel walls are unknown. We found Aβ-degrading activity in human serum during analysis of the regulatory mechanism of Aβ production in human endothelial cells. To elucidate the metabolic dynamics of Aβ surrounding the brain microvessels, we identified Aβ-degrading activity in human serum (blood Aβ-degrading activity: BADA) by column chromatography and LC/MS. BADA exhibited characteristics of an acidic protein, pI 4.3, which had two different protein surface charges (low and high affinity cations). Both BADA fractions had a relative molecular mass of greater than 400 kDa. Furthermore, BADA in the low affinity cation fraction was inhibited by the serine protease inhibitor 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). We clarified alpha-2-macroglobulin (a2M) and several serine proteases from this BADA by LC-MS. Moreover, we demonstrated that BADA is increased by approximately 5000-fold in human serum by column chromatography. Therefore, BADA may play an important role in the circulation and metabolism of Aβ in human brain microvessels.

Keywords: Alzheimer's disease, amyloid-beta-degrading activity, human serum, brain microvessel

INTRODUCTION

The major hallmarks of Alzheimer’s disease (AD) are amyloid plaques, which are formed by the accumulation of amyloid beta (Aβ) peptides, and neurofibrillary tangles caused by aggregation of hyper-phosphorylated tau, leading to cognitive dysfunction with aging.1,2 The two basic types of AD are familial and sporadic. Familial AD (FAD), known as early-onset, is associated with muta-
tions of several genes and is a very rare form of the disease affecting 10% of those diagnosed with AD.\textsuperscript{3} In contrast, sporadic AD, known as late-onset, is the most common form affecting approximately 90% of patients.\textsuperscript{3} However, most research has been performed using transgenic mouse models harboring the FAD mutation.\textsuperscript{4} Although there has been marked progress in our understanding of the pathogenesis of AD over the past several decades, the exact mechanism of AD remains unknown.

Based on an autopsy series, the prevalence of cerebral amyloid angiopathy (CAA) is significantly higher (approximately 80\%) in AD brains, and ranges from 10\% to 57\% in the general population.\textsuperscript{5} CAA is defined as the deposition of A\textsubscript{\beta} in the meningeal and intracerebral vessels. Although the source of A\textsubscript{\beta} in AD and CAA is considered to mainly originate from neurons in vessel walls, the detailed mechanism is unclear. Recently, it was reported that A\textsubscript{\beta} is generated from component cells in brain microvessels such as endothelial cells, astrocytes and pericytes.\textsuperscript{6,7} Regarding pericytes, amyloid precursor protein was confirmed to be the precursor molecule whose proteolysis generates A\textsubscript{\beta}.\textsuperscript{8} These cells, including neurons, have the potential to cause accumulation of A\textsubscript{\beta} in blood vessel walls. Thus, AD and CAA may be caused by the disruption of A\textsubscript{\beta} metabolism in cerebral vessels.

We observed A\textsubscript{\beta}-degrading activity in pooled normal human serum during our analysis on the regulatory mechanism for the production of A\textsubscript{\beta} in endothelial cells. The activity, which we termed BADA (blood A\textsubscript{\beta}-degrading activity), was characterized by the combination of ammonium sulfate precipitation, ion-exchange chromatography, isoelectric focusing, gel filtration and hydroxyapatite chromatography. The results demonstrated that the partially purified fraction of BADA was significantly activated by hydroxyapatite chromatography. This fraction of BADA was eluted as a high molecular weight protein (greater than 400 kDa) by gel filtration, and its isoelectric point was an acidic protein of pH 4.3 by IEF. We found that BADA exhibited different affinities to cations by hydroxyapatite and ion-exchange chromatography. Furthermore, it was inactivated by heat treatment and suppressed by serine protease inhibitors. We identified one of the major BADA proteins as alpha-2-macroglobulin (a2M) and several proteases using LC/MS analysis. A2M forms a complex with several proteases to rapidly degrade A\textsubscript{\beta}.\textsuperscript{9} A2M tightly binds to A\textsubscript{\beta}, attenuates fibrillogenesis and the neurotoxicity of A\textsubscript{\beta}, and mediates A\textsubscript{\beta} degradation.\textsuperscript{9,11} Moreover, a2M is genetically associated with AD.\textsuperscript{12} Therefore, the a2M-serine protease complex may play an important role for the circulating A\textsubscript{\beta} and metabolic microenvironment in the neurovasculature. The A\textsubscript{\beta}-degrading activity of the a2M-serine protease complex in blood should be investigated as a valuable biomarker and possible target for AD in the future.

**MATERIALS AND METHODS**

*Production of A\textsubscript{\beta} from HUVECs*

Human umbilical vein endothelial cells (HUVECs) from Cell Applications, Inc. were cultured in endothelial cell basal medium (ECBM) with endothelial cell growth supplement containing fetal bovine serum in 35-mm dishes (3 x 10\textsuperscript{5} cells/dish). To measure A\textsubscript{\beta}40 in the medium after seeding for 24 h, the culture medium was changed to ECBM containing 3\% (v/v) heat-inactivated pooled human serum purchased from Innovative Research, Inc., 5 ng/ml of rhFGF-basic, 5 ng/ml of rhEGF, 0.75 U/ml of heparin and 0.1 \mu g/ml of hydrocortisone. Cells were incubated at 37\degree C in 5\% CO2. Culture supernatant from HUVECs was harvested at 24, 48 and 72 h after changing the medium containing pooled normal human serum. The production of A\textsubscript{\beta}40 from HUVECs was measured using the Human/Rat beta-Amyloid (40) ELISA kit (Wako pure chemicals, co, ltd.) according to the manufacturer’s protocol.
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**Assay of Aβ-degrading activity in human serum**

Aβ-degrading activity was based on the remaining Aβ40 measured by ELISA after incubation with 100 pM synthetic human Aβ40 (Peptide Institute, Inc.) in human serum or 10–100 μl of purified proteins from the human serum fraction at 37°C. To precipitate the protein, trichloroacetic acid (TCA) at a final concentration of 5% (w/v) was added and placed on ice for 1 h. The supernatant was removed after centrifugation at 18,000 x g for 10 min at 4°C. The pellet was washed with cold acetone and centrifuged at 18,000 x g for 10 min at 4°C. The wash step was repeated to remove all of the TCA and acetone, and the pellet was then dried at room temperature. Next, the pellet was mixed with 70% formic acid. It was completely dissolved and neutralized with a 20-fold volume of 1 M Tris-base. The solution was immediately dispensed into an ELISA well. One unit was defined as the amount of enzyme required to digest 50% of 100 pM Aβ40 in 72 h at 37°C.

**Autoradiography and Western blotting**

The purified protein fraction from human serum (Innovative Research Inc.) by DEAE chromatography with 2 nM Aβ40, including 30,000 cpm (3.5 pM) of 125I-Aβ40 (PerkinElmer, Inc.), was incubated at 37°C for 72 h. The protein was precipitated by the TCA/acetone method described above. Sample buffer was added to the fraction, which was then boiled at 95°C for 5 min. The fraction was separated on a 4–20% gradient tricine gel (Cosmo Bio Co., Ltd.). Proteins were then transferred onto a PVDF membrane. Phosphate buffer saline at pH 7.4 (PBS) was added to the membrane in a hybrid bag, and assessed by phosphor imaging to detect 125I-Aβ40 by Typhoon FLA 9500 (GE Healthcare Life Sciences Inc.). The membrane with PBS in the hybrid bag was boiled for 5 min and left at room temperature. The membrane was blocked for 1 h at room temperature using Blocking One solution (Nacalai Tesque, Inc.). The membrane was washed three times with TBST for 10 min. The membrane was incubated with the primary antibody, 6E10 (1/10,000; Covance, Inc.), in Can Get Signal Solution 1 (Toyobo Co.) at 4°C overnight. The membrane was then washed three times with TBST for 10 min. Next, it was incubated with the diluted secondary antibody, Mouse IgG-HRP (1/10,000; Jackson ImmunoResearch Laboratories, Inc.), in Can Get Signal Solution 2 (Toyobo) for 1 h at room temperature. The membrane was similarly washed, and proteins were detected using ECL Prime (GE Healthcare Life Sciences, Inc.), followed by exposure to Kodak BioMax MR film (Sigma-Aldrich, Inc.).

**Purified fractions of BADA**

Ammonium sulfate (AS) was slowly added to 10 ml of pooled human serum (Innovative Research Inc.) until 30% saturation, and allowed to precipitate for 30 min at 4°C with stirring. After centrifuging at 10,000 x g for 15 min at 4°C, ammonium sulfate was slowly added to the supernatant until 40% saturation for 30 min at 4°C with stirring. The pellet was then dissolved in PBS (0–30% fraction). After centrifuging at 10,000 x g for 15 min at 4°C, the supernatant was collected (30–40% fraction), and the pellet was again dissolved in PBS (40–100% fraction). Each fraction was dialyzed against PBS at 4°C. The protein concentration of each fraction after dialysis was measured using the BCA assay. Aβ40 was added to each fraction. After incubation for 72 h at 37°C, the amount of remaining Aβ40 was measured by ELISA. The isoelectric points of the 30–40% AS fraction were determined using a pH gradient electrophoresis system (Nihoneido Co.). A linear sucrose gradient from 50 to 25% was loaded onto a 90-ml electrophoresis column in the presence of 1% pharmalyte (GE Healthcare Life Sciences, Inc.). After the protein sample containing 25% sucrose and 1% pharmalyte was loaded into the top of the column, a linear sucrose gradient from 25 to 0% was loaded into the same column. Electrophoresis was performed at a constant voltage of 400 V for 48 h at 6°C. After electrophoresis, samples were fractionated
every 1.7 ml. Each fraction was neutralized by 1 M potassium phosphate buffer (KPB, pH 7.5) immediately after the pH value of each fraction was measured on ice with a pH meter (Horiba, Co. Ltd). The protein concentration of each fraction was measured by its absorbance at 280 nm. Purification was performed on an FPLC system (GE Healthcare Life Sciences, Inc.) using each column. Pooled fractions at pH 4.3 were exchanged into 5 mM potassium phosphate buffer (KPB) at pH 7.5 using the Amicon Ultra-15 centrifuge device (10,000 NMWL; Merck Millipore Inc.). After concentrating, BADA fractions were applied to a hydroxyapatite column (Bio-Rad Inc.) equilibrated with 5 mM KPB (pH 7.5). BADA was eluted with a linear gradient from 5 mM to 250 mM KPB (pH 7.5), and subsequently eluted with 500 mM KPB (pH 7.5). The absorbance at 280 nm was measured with 0.5 ml of each collected fraction. Ten microliters of each fraction was mixed with 100 pM Aβ40, and after incubation for 3 h at 37°C, the amount of remaining Aβ40 was measured by ELISA. Eluted BADA with 150 mM KPB (pH 7.5) as a low affinity cation (LAC-BADA) was exchanged into PBS and concentrated. Similarly, eluted BADA with 500 mM KPB (pH 7.5) as a high affinity cation (HAC-BADA) was similarly exchanged. Each sample was applied onto a gel filtration column (Superose12; GE healthcare Life Sciences, Inc.) equilibrated with PBS at pH 7.4. The concentration of each eluted fraction with PBS was measured by its absorbance at 280 nm. Twenty microliters of each fraction was mixed with 100 pM Aβ40, and after incubation for 3 h at 37°C, the amount of remaining Aβ40 was measured by ELISA. The molecular weight of the proteins was calculated using a gel filtration calibration kit HMW (GE healthcare Life Sciences, Inc.). Each BADA fraction purified by gel filtration was exchanged into 20 mM Tris-HCl (pH 8.0) and concentrated. Each sample was applied to an equilibrated DEAE column (GE Healthcare Life Sciences, Inc.) with 20 mM Tris-HCl (pH 8.0). BADA was eluted with a linear gradient from 0 mM to 250 mM NaCl in 20 mM Tris-HCl (pH 8.0), and subsequently eluted with 500 mM NaCl in 20 mM Tris-HCl (pH 8.0). Then, 10 μl of each fraction was mixed with 100 pM Aβ40, and after incubation for 12 h at 37°C, the amount of remaining Aβ40 was measured by ELISA. The protein concentration of all purified samples was measured by the BCA assay.

Identification of BADA by LC-MS

Purified LAC-BADA by DEAE chromatography was separated by 12.5% SDS-PAGE, and the bands were excised from gels with Oriole Fluorescent Gel Stain (Bio-Rad, Inc.). The gel slices were digested with trypsin (Promega, Inc.), and subjected to peptide-mass fingerprinting analysis using LC-MS (Thermo Fisher Scientific, Inc.) and the MASCOT search program (Matrix Science Inc.).

Inhibitor of BADA

Aliquots of 15 μl of LAC-BADA purified by DEAE chromatography were mixed with 100 pM Aβ40 with 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) or 1 mM ethylenediaminetetraacetic acid (EDTA), a serine protease and metalloprotease protease inhibitor, respectively. To examine the effects of heat treatment, LAC-BADA was incubated for 5 min at 95°C and Aβ40 was added to the solution. After incubation for 16 h at 37°C, the amount of remaining Aβ40 was measured by ELISA.

RESULTS

Amyloid β peptide-degrading activity in human serum

To investigate the time-dependent production of Aβ in endothelial cells, we measured
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endogenous Aβ40 in the medium using HUVECs by ELISA at 24, 48 and 72 h. Although the production of Aβ40 increased for 24 and plateaued at 48 h, Aβ40 decreased at 72 h in the medium (Fig. 1a). To examine whether the decreased Aβ was due to Aβ-degrading activity, we added synthesized Aβ40 to the conditioned medium. Aβ40 was significantly decreased by 50 and 80% in the conditioned medium at 24 and 48 h, respectively (data not shown). We next evaluated Aβ-degrading activity in human serum because Aβ40 was also significantly decreased by 20% to 40% in the 3% human serum (Fig. 1b). We first purified human serum by DEAE chromatography, and measured the Aβ-degrading activity by western blotting, autoradiography and ELISA (Fig. 1c-e). Proteins with Aβ-degrading activity in human serum were eluted with a linear gradient containing 0 to 1 M NaCl. We selected ELISA to explore purified fractions of the Aβ-degrading activity, termed blood amyloid-β-degrading activity (BADA), in human serum as it is the simplest method.

Fig. 1 Aβ40-degrading activity was observed in human serum
a Aβ40-degrading activity in HUVEC-conditioned medium. b Aβ40-degrading activity in human serum. c The remaining Aβ40 was measured by western blotting and d autoradiography in purified HS using ion exchange column chromatography. e The remaining Aβ40 was compared among measurement methods using ELISA.
Purification of BADA fractions

A protocol was developed for the high-yield purification of BADA. Using four chromatographic steps, highly pure fractions of BADA were obtained (Fig. 2a). We first purified by ammonium sulfate precipitation to eliminate contaminating proteins from the human serum. Saturated 30–40% ammonium sulfate separated the BADA (Fig. 2b). We were able to remove contaminating proteins while maintaining the Aβ-degrading activity in human serum. Next, following dialyzed saturated 30–40% ammonium sulfate precipitation in PBS. The fractions of BADA were purified by column isoelectric focusing (IEF) in immobilized pH gradients. pH gradients ranging from 2.5 to 5.0 were formed using carrier ampholytes in sucrose solutions. The BADA was present at pH 4.3 among the three elution peaks (Fig. 2c). The separated proteins by IEF gained a positive charge in low pH environments. Subsequently, BADA at pH 4.3 was applied to hydroxyapatite (HA) chromatography after exchanging the potassium phosphate buffer using membrane ultrafiltration. The fraction of BADA was eluted by a potassium phosphate buffer gradient. The BADA was found to have two different affinities to the HA column in a low affinity cation fraction (LAC) and high affinity cation fraction (HAC) (Fig. 2d). The BADA was increased by 11.3- and 45.9-fold by HA chromatography relative to IEF in the LAC and HAC fractions, respectively (Table 1).

Both LAC- and HAC-BADA fractions were purified separately by gel filtration and size-exclusion chromatography. The BADA fractions were eluted by PBS, and the molecular weight was measured using a calibration curve. Both BADA fractions had a relative molecular mass of greater than 400 kDa (Fig. 2e). LAC-BADA was completely separated to an albumin peak (Fig. 2e, LAC). Although the BADA was increased more by gel filtration than HA chromatography, several gel bands were detected by SDS-PAGE (data not shown). The protein peaks were also not consistent with the BADA peak. We then additionally purified the BADA proteins by ion exchange chromatography.

The fractions of BADA were eluted using the weak anion exchanger DEAE (diethyaminoethyl). The fractions of LAC- and HAC-BADA were eluted with low- or high-salt buffer, respectively (Fig. 2f). The different salt concentrations were consistent with HA chromatography (Fig. 2d). The BADA of acidic proteins may have different surface charges. The LAC-BADA was finally

| Step                        | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Fold |
|-----------------------------|--------------------|--------------------|--------------------------|-----------|------|
| Pooled human (10 ml)        | 1335               | 66647              | 2                        | 100       | 1    |
| Ammonium sulfate saturation (30–40%) | 1224               | 13556              | 9                        | 92        | 5    |
| Isoelectric focusing (pI 4.3) | 170                | 1177               | 14                       | 13        | 7    |
| Low-affinity cation (LAC)   |                    |                    |                          |           |      |
| Hydroxyapatite              | 456                | 2.90               | 157                      | 34        | 79   |
| Gel filtration              | 852                | 0.28               | 3048                     | 64        | 1522 |
| DEAE (fraction no.41)       | 330                | 0.03               | 10776                    | 25        | 5380 |
| High-affinity cation (HAC)  |                    |                    |                          |           |      |
| Hydroxyapatite              | 422                | 0.66               | 643                      | 32        | 321  |
| Gel filtration              | 552                | 0.66               | 840                      | 41        | 419  |
| DEAE (fraction no.61)       | 72                 | 0.01               | 9360                     | 5         | 4673 |
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Fig. 2 Purification of BADA fractions

a) Schematic representation of the purification protocol. b) Fractionation of BADA with ammonium sulfate saturation from human serum. c) Isoelectric focusing of BADA. d) BADA from hydroxyapatite chromatography. BADA was separated into low- and high-affinity cation fractions (LAC and HAC). e) BADA from gel-filtration chromatography. Each LAC and HAC fraction of BADA (upper, lower) was exchanged to PBS at pH 7.5, and then eluted into high molecular weight (400 kDa) fractions by gel filtration with PBS at pH 7.5. The molecular weight of the protein was measured using gel filtration markers. f) LAC- and HAC-BADA were purified using a linear gradient of 250 mM sodium chloride in 20 mM Tris-HCl buffer (pH 7.5) by diethylaminoethyl (DEAE) anion exchange chromatography. LAC-BADA was eluted by DEAE chromatography with low levels of sodium chloride (upper). HAC-BADA was eluted by DEAE chromatography with high levels of sodium chloride (lower).
purified from normal human serum at 5400-fold, and it exhibited an increased specific activity of approximately 10,000 U/mg protein (Table 1). The fraction of LAC-BADA was eluted as a single peak, which matched the activity peak, but several bands were observed on SDS-PAGE (Fig. 3a). Thus, we identified the LAC-BADA protein by LC-MS because HAC was eluted as a broad peak that was not consistent with the activity peak.
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Identification of BADA by LC-MS and Suppression of BADA by Serine Protease Inhibitor

To identify LAC-BADA among the several bands detected by SDS-PAGE, we identified proteins by LC/MS from several bands and found that the fraction correlated with Aβ-degrading activity (Fig. 3a. frac. no.41). The numerous bands were identified as alpha-2 macroglobulin. The others were characterized as serine proteases such as trypsin and plasma kallikrein (Fig. 3b). To examine whether LAC-BADA is suppressed by serine protease inhibitors, we added 1 mM AEBSF with Aβ40 to the LAC-BADA fraction and measured residual Aβ40 by ELISA. The LAC-BADA was suppressed by AEBSF but not EDTA, a metalloproteinase inhibitor (Fig. 3c). These results suggest that the LAC-BADA includes a serine protease.

DISCUSSION

The hallmark of AD is Aβ accumulation with aging in the brain. Aβ production and clearance are important to balance Aβ deposition. There have been many studies on Aβ deposition, including APP overexpression and excessive quantities of Aβ exerting toxic effects other than Aβ clearance. Although the roles of neprilysin (NEP) and insulin-degrading enzyme (IDE), which are major Aβ-degrading enzymes in brain parenchyma, were demonstrated in transgenic AD model mice, data supporting an association between Aβ-degrading enzymes and AD are limited. We reported Aβ-degrading activity in human serum (BADA). BADA was characterized by several chromatographic columns as large acidic proteins. The acidic protein having BADA was separated by different charges on the protein surface. Alpha-2 macroglobulin (a2M) containing some serine proteases was identified by LC-MS analysis in the LAC-BADA fraction. Previously reported major Aβ-degrading enzymes in the brain (NEP and insulin-degrading enzyme) and blood (plasmin) were not found in LAC-BADA.

A2M is a well-known protease inhibitor present in the blood that forms a tetramer including a protease (monomer: 180 kDa). However, a2M itself is not a protease, and several studies have reported that the a2M-protease complex plays a key role in Aβ clearance. A2M, including trypsin, has degradation activity in vitro. Furthermore, a2M binds Aβ with high affinity and the a2M-protease complex inhibits amyloid formation. The a2M-protease complex can degrade Aβ deposited around cerebral vessel walls because a2M has a binding site on LRP1 (low density lipoprotein receptor-related protein 1), which transports Aβ from the brain to the blood across the blood-brain barrier. Although a2M-protease is assumed to play an essential role in Aβ clearance, the relationship between AD and a2M-protease activity in human serum remains unknown. In the future, elucidation of its activity will be important for early diagnosis and new treatment strategies for AD.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.
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