Comprehensive Proteomic Profiles of Mouse AApoAII Amyloid Fibrils Provide Insights into the Involvement of Lipoproteins in the Pathology of Amyloidosis

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Abstract

Amyloidosis is a disorder characterized by extracellular fibrillar deposits of misfolded proteins. The amyloid deposits commonly contain several non-fibrillar proteins as amyloid-associated proteins, but their roles in amyloidosis pathology are still unknown. In mouse senile amyloidosis, apolipoprotein A-II (ApoA-II) forms extracellular amyloid fibril (AApoAII) deposits with other proteins (AApoAII-associated proteins) in many organs. We previously reported that R1.P1-*Apoa2*^c mice provide a reproducible model of AApoAII amyloidosis. In order to investigate the sequential alterations of AApoAII-associated protein, we performed a proteomic analysis of amyloid fibrils extracted from mouse liver tissues that contained different levels of AApoAII deposition. We identified 6 AApoAII-associated proteins that constituted 20 of the top-ranked proteins in mice with severe AApoAII deposition. Although the amount of AApoAII-associated proteins increased with the progression of amyloidosis, the relative abundance of AApoAII-associated proteins changed little throughout the progression of amyloidosis. On the other hand, plasma levels of these proteins showed dramatic changes during the progression of amyloidosis. In addition, we confirmed that AApoAII-associated proteins were significantly associated with lipid metabolism based on functional enrichment analysis, and lipids were co-deposited with AApoAII fibrils from early stages of development of amyloidosis. Thus, these results demonstrate that lipoproteins are involved in AApoAII amyloidosis pathology.

1. Introduction

The amyloidoses constitute a group of disorders in which misfolded proteins lead to pathologic accumulation of extracellular amyloid fibrils in various tissues and organs. Currently, 36 amyloid proteins have been found in human and animal amyloidosis as represented by Alzheimer's disease (AD), familial amyloid polyneuropathy (FAP) and amyloid light chain (AL) amyloidosis.[1] Recent comprehensive studies of the molecular composition in amyloid deposits have shown that they contain a variety of proteins, glycosaminoglycans and lipids as amyloid-associated molecules.[2, 3] Regardless of the differences between precursor proteins of amyloid fibrils and the tissues in which they are deposited, several amyloid-associated proteins, such as apolipoprotein E (ApoE), serum amyloid P-component (SAP), clusterin and heparin sulfate proteoglycans are common among various amyloidosis.[2] ApoE is identified as a primary genetic risk factor for late-onset AD from meta-analysis of genome-wide association studies[4] as well as isoform-specific effects on protofibril to fibril conversion of amyloid beta (A β).[5] SAP and clusterin are known to act as extracellular chaperones that either increase solubility of extracellular insoluble aggregates and maintain protein homeostasis, or protect amyloid fibrils from degradation.[6] Many kinds of glycosaminoglycans and proteoglycans (including heparan sulfate) unfold amyloid proteins and catalyze their subsequent nucleus formation and stabilize the amyloid fibrils.[7] Thus, it is suggested that amyloid-associated proteins are involved in the pathogenesis of many kinds of amyloidoses. However, their role is still controversial.

Recent proteomic studies suggested that the proteins related to molecular transport, cell death or survival, extracellular matrix, lipid metabolism and mitochondrial functions may be involved in amyloidosis pathogenesis.[8, 9] These proteomic studies analyzed the tissues from patients with severe amyloid deposition. Thus, the observed biological pathways reflected both constituents of amyloid deposits and cellular responses in tissues around the amyloid deposits. For that reason, the progressive changes in proteomic profiles of amyloidosis are not well

1 understood.

2 In the present study, we analyzed sequential proteomic profiles using a reproducible mouse
3 model of senile amyloidosis, which is an age-associated systemic amyloidosis characterized by
4 apolipoprotein A-II (ApoA-II) amyloid fibril (AApoAII) deposition. ApoA-II is the second most
5 abundant apolipoprotein of plasma high-density lipoprotein (HDL) and C-terminal extension of
6 human ApoA-II derived from a mutation in the normal stop codon causes human AApoAII
7 amyloidosis.[10, 11] In mouse AApoAII amyloidosis, we previously reported that there are
8 seven alleles of the ApoA-II gene (*Apoa2*) among inbred laboratory strains,[12] and the mouse
9 strain with the type C allele of *Apoa2* (*Apoa2^c*) frequently exhibits accelerated spontaneous
10 AApoAII amyloidosis.[13, 14] Importantly, a congenic strain of mice with the amyloidogenic
11 *Apoa2^c* allele on the genetic background of the Senescence-Accelerated Resistant Mouse 1
12 (SAMR1), named R1.P1-*Apoa2^c* mice, rarely exhibits spontaneous AApoAII amyloidosis under
13 specific pathogen-free condition. However, intravenous injection of AApoAII amyloid fibrils to
14 R1.P1-*Apoa2^c* mice induces incremental AApoAII amyloidosis.[15] So, R1.P1-*Apoa2^c* mice are
15 useful for analyzing the development of amyloidosis pathology.

16 By using R1.P1-*Apoa2^c* mice, we identified proteomic profiles of amyloid fibril fractions
17 derived from livers with different levels of AApoAII deposition. Identification of
18 AApoAII-associated proteins and sequential alterations of their abundance were determined
19 through use of mass spectrometry, and the changes in plasma were also determined.

2. Materials and Methods

2.1. Animals

R1.P1-*Apoa2^c* mice, a congenic strain of mice with the amyloidogenic *Apoa2^c* allele of Senescence-Accelerated Mouse Prone 1 (SAMP1) strain on the genetic background of the SAMR1 strain,[16] were reared in the Division of Animal Research, Research Center for Support of Advanced Sciences, Shinshu University, under specific pathogen-free conditions at 24 ± 2 °C with a controlled light regimen (12 h light/dark cycle). A commercial diet (MF; Oriental Yeast, Tokyo, Japan) and tap water were available *ad libitum*. All experiments were performed with the approval of the Committee for Animal Experiments of Shinshu University. Female mice were used in this study in order to avoid serum amyloid A (SAA) amyloidosis (AA amyloidosis) or other adverse impacts caused by fighting among male mice reared in the same cage. Three through five biological replicates of each mouse sample were used for each experiment.

2.2. Induction of AApoAII Amyloidosis

Two-month-old female R1.P1-*Apoa2^c* mice were treated with intravenous injection of the AApoAII amyloid fibril fraction (isolation methods are described below) to induce AApoAII amyloidosis. The mice were sampled at 1, 2, 4, 7 and 10 months after injection and termed the induced group (IJ- groups); 2-, 6- and 12-month-old mice constituted the non-treated control group (NT- groups) (Fig. 1A). A portion of the tissues were fixed in 10% (v/v) neutral buffered formalin followed by embedding in paraffin for histochemical examinations, prefixed in 2.5% (w/v) glutaraldehyde in PBS (pH 7.4) and postfixed in 1% osmium tetroxide followed by embedding in epoxy resin for transmission electron microscopy (TEM). The remaining tissues were frozen at -80°C until isolation of amyloid fibril fractions and biochemical analysis.

2.3. Isolation of Amyloid Fibril Fractions

AApoAII amyloid fibril fractions were isolated from the livers of R1.P1-*Apoa2*^c mice by the
Pras method with some modification.[17, 18] All procedures were carried out at 4°C. Initially,
300 mg of liver tissue and 3 mL of 0.15 M NaCl were homogenized with a Polytron
homogenizer (T25 basic; IKA, Baden-Württemberg, Germany) for 90 sec, and the mixture was
centrifuged at $40,000 \times g$ for 30 min using a high-speed centrifuge (Model 6900; KUBOTA,
Osaka, Japan). The supernatant was discarded, and the precipitate was resuspended in 300 μ L of
0.15 M NaCl. The mixture was homogenized and centrifuged as before. These operations were
repeated 7 times. At this point, most of the soluble proteins have been removed from the
samples. The last precipitate was then resuspended in 300 μ L distilled deionized water (DW) to
remove salts. The mixture was homogenized and centrifuged at $30,000 \times g$ for 30 min. The
supernatant containing only small quantities of protein was discarded, and the precipitate was
resuspended in 300 μ L of DW. The mixture was homogenized and centrifuged as before. The
supernatant containing micellar proteins was collected, and the precipitate was suspended in 300
 μ L of DW. Collection of the supernatants was repeated 3 times. Finally, the collected solution
was ultracentrifuged at $100,000 \times g$ for 60 min with an ultracentrifuge (CS100FNX; Hitachi
Koki Co., Ltd, Tokyo, Japan). The supernatant was discarded, and the precipitate was
resuspended in DW and sonicated with a homogenizer (VP-5S; TIETECH, Nagoya, Japan) for
30 sec on ice. This solution contained amyloid fibrils of varying lengths (Supplementary Fig. 1).

As we previously reported [19], protein concentrations were estimated using a BCA protein
quantification kit (Pierce BCA Protein Assay Kit; Thermo Scientific, Tokyo, Japan) according to
the manufacturer's instructions. Amyloid fibril fractions from three individuals showing severe
AApoAII-deposition (10 months after injection of AApoAII amyloid fibrils) were pooled for the
induction of AApoAII amyloidosis.

2.4. Preparation of Trypsin-Digested Peptides

To improve protein solubility prior to tryptic digestion, a total of 40 μ L containing 1.5 μ g of

an amyloid fibril fraction was mixed with 0.05% (w/v) RapiGest (Waters Corporation, Milford, MA, USA) that had been reconstituted in 50 mM ammonium bicarbonate. The mixture was then heated at 99°C for 5 min. For reduction and alkylation, samples were incubated in 5 mM dithiothreitol for 30 min on a 60°C heating block, followed by incubation in 15 mM iodoacetamide for 30 min at room temperature in the dark. Sequencing grade trypsin, reconstituted in 50 mM ammonium bicarbonate, was added at an enzyme/amyloid fibril fraction ratio of 1:1 (w/w), and incubated overnight at 37°C. The surfactant (RapiGest) was degraded by incubating in 0.5% (v/v) trifluoroacetic acid (TFA) at 37°C for 30 min. The samples were then centrifuged for 15 min at 14,500 rpm. Digested peptides in the supernatant were dried in a vacuum concentrator (VC-15SP; TIETECH, Nagoya, Japan) and resuspended in 0.1% TFA. The samples were purified by Zip Tip C18 (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions. After drying in a vacuum concentrator, the samples were resuspended in 0.1% TFA and used for liquid chromatography coupled online to a tandem mass spectrometer (LC-MS/MS) analysis.

2.5. Nanoflow LC-MS/MS

A 3- μ L aliquot of trypsin-digested peptides was loaded on a nanoACQUITY UPLC Xevo Q-TOF (Waters Corporation). Peptides were trapped on a 5 μ m nanoACQUITY SymmentryC18 Trap Column (180 μ m $\times$ 20 mm) and washed with 99% solvent A (0.1% formic acid in water) and 1% solvent B (0.1% formic acid in acetonitrile) for 3 min at a flow rate of 10 μ L/min. Peptides were then eluted with a 1-40% B linear gradient solvent over 90 min on a 1.7 μ m nanoACQUITY BEH130 C18 column (75 μ m $\times$ 150 mm) at a flow rate of 300 nL/min. The column was washed with 95% B until 95 min, followed by a 25 min post run with 99% A and 1% B solvent for re-equilibration at initial condition.

Mass spectrometric analysis was performed using the nanoLC coupled by a nanoESI emitter to a QTOF mass spectrometer (Waters Corporation). Data were collected using MassLynx

version 4.1 (Waters Corporation) in data independent analysis (DIA or MS^E) positive ion mode,[20] in which alternating 1 sec scans of low (6 V) or high (20-45 V) collision energies were used to obtain both precursor ion information and full-scan accurate mass fragment information. The mass range was set to 50-1990 Da with a scan time of 1 sec. Glu-1-fibrinopeptide B was infused as lock mass for the external mass calibrant at a flow rate of 500 nL/min. Three technical replicates were performed for each biological sample.

2.6. Data Processing and Protein Identification

LC-MS^E data were processed with Proteins-Lynx Global Server (PLGS) version 2.5 (Waters Corporation). Each processed file was searched against a mouse protein database from Uniprot (www.uniprot.org). The mouse *Apoa2*^c sequence was added,[12] and the database consisted of 28,108 entries. Each search parameter used a default setting as follows: a minimum of 3 consecutive product ions for matching a peptide, a minimum of 7 total product ions for matching a protein, a minimum of 1 peptide for matching a protein and a maximum 4% false positive rate against the randomized database. Oxidation of methionine and a fixed carbamidomethyl-cysteine were allowed for variable modifications. A maximum of 1 missed tryptic cleavage site was also allowed.

In excel data summaries of the processed MS spectra, each protein was quantified in the column “protein.top3MatchedPeptideIntenSum” representing the total spectral intensities of the top 3 matched peptides. In order to perform semi-quantitative analysis, each identified protein was ranked in ascending order according to this parameter. The content ratio of each protein in the sample was calculated as the ratio of the spectral intensities of each protein to the total of all identified proteins. The proteins identified in severe AApoAII deposits were detected by comparing IJ-12 to NT-12 groups using Expression mode of PLGS2.5 (Table 1). As the data set included suspicious proteins (represented only 1 sample), only the proteins represented more than 4 times and the 20 top-ranked in IJ-12 group (Table 2) were assigned to

AApoAII-associated protein (Fig. 1A).

2.7. Histology and immunohistochemistry

Sections (4- μ m thickness) from paraffin-embedded tissues were used for immunohistochemistry and Congo red staining. Each section was deparaffinized in Hemo-De (FALMA, Tokyo, Japan) and rehydrated with decreasing concentrations of ethanol (final concentration of 70% ethanol). AApoAII fibrils and AApoAII-associated proteins were detected immunohistochemically using the horseradish peroxidase-labeled streptavidin-biotin method (Agilent Technologies, Santa Clara, CA, USA) with rabbit antiserum against mouse ApoA-II prepared in our laboratory,[21] and antibodies against ApoE (1:50; Santa Cruz Biotechnology #sc-6384, Santa Cruz, CA, USA), clusterin (1:50; Santa Cruz Biotechnology #sc-8354), vitronectin (1:50; Santa Cruz Biotechnology #sc-15332) and CD81 (1:50; Santa Cruz Biotechnology #sc-9158). Analysis was performed by light microscopy (BX43; Olympus Corporation, Tokyo, Japan) and Olympus cellSens software (Olympus Corporation). For quantitative analysis, 5 liver sections were randomly captured with $\times 200$ magnification and areas stained by anti-ApoA-II antiserum were calculated by ImageJ (NIH). Amyloid deposition was also detected in sections stained with 1% (w/v) Congo red with polarized-light microscopy (Axioskop 2; Carl Zeiss, Jena, Germany).[13]

For lipid staining, formalin-fixed tissues were cryoprotected in increasing concentrations of sucrose solution (from 5%, to 10% to 20% (w/v)), prior to sectioning with a freezing microtome (CM1950; Leica Biosystems, Wetzlar, Germany). Frozen sections of 7- μ m thickness were incubated in a saturated solution of Oil red O prepared in 60% (v/v) 2-propanol for 15 min at 37°C followed by differentiation in 60% (v/v) 2-propanol. The sections were counterstained with Mayer's hematoxylin and then differentiated in 0.05% (w/v) lithium carbonate.

2.8. Transmission electron microscopy

For TEM experiments, sections of 1- μ m thickness from epoxy resin-embedded tissues were stained with 1% (w/v) toluidine blue to detect the sites of AApoAII deposition under light microscopy (BX43). Ultrathin sections of 100-nm thickness around the AApoAII deposits were stained with 1% (w/v) uranyl acetate for 10 min followed by incubation with lead citrate for 5 min at room temperature. The stained sections were viewed with a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan). The amyloid fibrillar structures were detected at $\times 10,000$ magnifications.

2.9. Western blot analysis

For Western blot analysis, 3-10 μ g of total proteins from amyloid fibril fractions or 0.5-1.0 μ L of plasma samples were separated by electrophoresis on a Tris/Tricine 16.5% or 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene difluoride (PVDF) membranes using a semi-dry Western blot apparatus (Nihon Eido, Tokyo, Japan). The AApoAII-associated proteins were probed with the same antibodies used for immunohistochemistry. The relative level of each protein was measured by ImageJ as the fold-change relative to the 2-month-old NT- group (NT-2) samples.

2.10. Functional Enrichment Analysis

Functional analyses were examined by DAVID Bioinformatics Resources (National Institute of Allergy and Infectious Diseases (NIAID), NIH, david.ncifcrf.gov). The specific proteins from the IJ-12 group showing severe AApoAII deposition were compared to the NT-12 group lacking deposition. Biological functions were evaluated using Uniprot knowledgebase (UniprotKB) keywords. The modified Fisher Exact p-value of each keyword was adjusted by the Benjamini-Hochberg procedure for multiple testing and significant keywords that had a false discovery rate (FDR) of < 0.05 and a p-value < 0.05 are listed.

3. Results

3.1. Collection of R1.P1-*Apoa2^c* Mouse Liver Samples with Different levels of AApoAII Deposits

We injected AApoAII amyloid fibrils into 2-month-old R1.P1-*Apoa2^c* mice and sampled 1, 2, 4, 7 and 10 months after induction of amyloidosis (Fig. 1A). Initially, we assessed AApoAII deposition with Congo red-stained sections under polarized light (Fig. 1B). In the liver, R1.P1-*Apoa2^c* mice exhibited progressive AApoAII deposition after induction. Neither the non-treated (NT-) nor the three-month-old induced group (IJ-3) (1 month post-treatment) had amyloid deposits. In contrast, deposition was first noted in the four-month-old induced group (IJ-4) (2 months post-treatment). AApoAII amyloid fibrils were initially deposited around blood vessel, especially the portal vein, and they then spread around hepatocytes. The levels of AApoAII deposition were also determined by immunohistochemistry with anti-ApoA-II antiserum (Fig. 1C). Consistent with Congo Red staining, AApoAII deposition was observed in the IJ-4 group and developed with time. As we previously reported, AApoAII deposition was also detected in other organs such as the small intestine, heart, and tongue (Supplementary Fig. 2).

3.2. Identification of AApoAII-associated Proteins by LC-MS/MS Analysis and Semi-Quantitative Changes in the Amyloid Fibril Fractions

AApoAII amyloid fibril fractions were extracted from each mouse liver through the use of a modified Pras method.[18] To determine the suspected sequential alterations in the abundance of individual AApoAII-associated proteins, we conducted comprehensive analyses of protein composition by LC-MS/MS. First, we detected 89 proteins that were specific for the IJ-12 group with severe AApoAII deposition compared to the control NT-12 group (Table 1). Second, we identified their overlap with the 20 top-ranked major proteins in IJ-12, termed AApoAII-associated proteins (Table 2). These overlapping proteins were ApoA-II, ApoE,

clusterin, vitronectin, CD81 and 17 beta hydroxysteroid dehydrogenase 13 (Fig. 2A). To compare the relative abundance of these proteins between groups, we ranked each identified protein from the data set of semi-quantitative spectral intensity. As the most common liver protein detected in the amyloid fibril fraction, glutamate dehydrogenase 1 mitochondrial was identified in most samples and ranked in the top 10 to 30 (Fig. 2B). As expected, the amyloid fibril protein ApoA-II was identified from IJ-4 and was most abundant from IJ-6 onwards (Fig. 2B). Although the rank of ApoA-II in the IJ-9 and IJ-12 group was varied, the raw data of spectral counts showed that interpretation was incorrect due to the excess of ApoA-II content and ApoA-II rank was underestimated (data not shown). While ApoE showed changes similar to ApoA-II and was the second most abundant protein, other AApoAII-associated proteins (clusterin, vitronectin, CD81 and 17 beta hydroxysteroid dehydrogenase 13), were identified in IJ-6 and later samples (Fig. 2B). Co-deposition of AApoAII-associated proteins with AApoAII amyloid fibrils was confirmed by immunohistochemistry (Supplementary Fig. 3). We further evaluated the relative abundance of AApoAII-associated proteins and their changes. Our semi-quantitative analysis revealed that the ApoE content was 7- to 10-fold higher than other AApoAII-associated proteins from IJ-6 onwards and this tendency did not change with the progress of AApoAII deposition (Fig. 2C). Therefore, these data suggest that the AApoAII-associated proteins increased as components of AApoAII deposits with the development of AApoAII amyloidosis pathology rather than specifically accumulate in response to deposited AApoAII amyloid fibrils.

3.3. Qualitative Changes of AApoAII-associated Protein in the Amyloid Fibril Fractions

Previous studies demonstrated that extracted amyloid fibril proteins and ApoE had different molecular weights compared to their full-length monomers. In addition to semi-quantitative changes, we also determined the state of AApoAII-associated proteins by SDS-PAGE (Fig. 3). As we previously reported, the amount of full length ApoA-II monomer (MW ~7,800 Da) and

polymerized forms (dimer, trimer, tetramer and so on) in the amyloid fibril fractions were clearly increased in IJ-6 onwards. In contrast, the increase of ApoE was mostly derived from intact monomer (MW ~ 36 kDa) and various fragmented ApoE peptides ranging from 10 to 36 kDa. The ~ 34 kDa secreted clusterin consisted of disulfide linked α - and β -cleaved clusterin chains (MWs of both chains were ~34 kDa). These were detected from IJ-9 onwards. The fragmented band (MW ~ 37 kDa) of vitronectin (MW ~ 75 kDa) was detected from IJ-9 onwards. In addition, the 15 kDa fragmented band of vitronectin was detected in IJ-12. Finally, CD81 decreased rather than increased with AApoAII deposition. This result might be due to lower reliability of each mass spectrum compared to other AApoAII-associated proteins. Age-dependent increase was not detected in NT- groups in any determined protein.

3.4. Enrichment of Proteins Associated with Lipid Metabolism and Lipid Co-deposition with AApoAII Amyloid Fibrils

In order to assess biological functions, we further analyzed the 89 specific proteins in the IJ-12 group compared to the NT-12 group with DAVID Bioinformatics Resources and identified UniprotKB keywords associated with AApoAII amyloidosis. Our enrichment analysis showed that the AApoAII amyloidosis-specific proteins were enriched in regard to HDL-associated lipid metabolism or secreted proteins (Table 3). The terms that related to keratin family proteins (“intermediate filament”, “keratin” and “coiled coil”) were not pursued in further experiments because their spectra might have originated from contaminating proteins.

We initially focused on the term “secreted” that was linked to most AApoAII-associated proteins, including ApoA-II, ApoE, clusterin, and vitronectin. Thus, we determined plasma levels of these secreted proteins by SDS-PAGE (Fig. 4). We confirmed that both plasma ApoA-II and ApoE were dramatically decreased with intensifying AApoAII amyloidosis pathology. Secreted clusterin in plasma was present from IJ-9 onwards as well as in the liver amyloid fibril fraction. In contrast, it was likely that plasma vitronectin was not significantly

1 changed with AApoAII amyloidosis pathology.

2 Next, we focused on HDL-associated lipid metabolism terms (“hdl”, “lipid binding” and
3 “lipid transport”). To determine the relationship between AApoAII amyloidosis and lipids, we
4 confirmed the existence of lipid in the AApoAII amyloid deposits. Oil red O staining revealed
5 that liver AApoAII deposits consisted of apolipoproteins as well as some neutral lipids and fatty
6 acids (Fig. 5A). Moreover, the lipid deposits were present in the IJ-4 group, the early stage of
7 AApoAII deposition. Lipid deposits with AApoAII amyloid fibrils were also found in the lung
8 (Supplementary Fig. 4). Electron microscopy also showed the existence of electron-lucent
9 structures, suggestive of lipid materials depositing along with amyloid fibrils (Fig. 5B). Thus,
10 these data suggest that several molecules contained in both apolipoproteins, lipids and
11 lipoproteins, may have a functional role in the development of AApoAII amyloid deposits.

4. Discussion

Several proteomic studies of amyloidosis have revealed that proteins related to molecular transport, cell death / survival, extracellular matrix, lipid metabolism and mitochondrial functions may be involved in the pathogenesis of amyloidosis.[8, 9] In order to gain a better understanding of the mechanism, we analyzed sequential alteration of amyloid-associated proteins in the amyloid fibril fractions extracted from mice at different stages of progression of AApoAII amyloidosis. In our mouse model of AApoAII amyloidosis, disease is induced by injection of amyloid fibrils.[15] The mice exhibited reproducible progression of amyloid fibril deposition in the liver (Fig. 1)[15] Although the relative abundance of these AApoAII-associated proteins increased in the fibril fraction with AApoAII amyloidosis pathology (Fig. 2 and Fig. 3), these proteins showed variable changes in plasma, and these changes were independent among the proteins (Fig. 4). Furthermore, enrichment analysis revealed that major AApoAII-associated proteins were enriched in lipid metabolism-related or secreted proteins (Table 3) as well as co-deposition of lipids with AApoAII fibrils (Fig. 5). These results suggest that lipoproteins contribute to the pathology of amyloidosis. It is possible that amyloid-associated proteins may be derived from lipoproteins.

Initially, we focused on the major proteins observed in mice experiencing severe deposition of AApoAII in comparison to a control group (Table 1 and Table 2). Here, they were defined as “AApoAII-associated proteins”. The list contained well-known amyloid-associated proteins reported in various types of amyloidosis,[8, 9, 22, 23] including ApoE, clusterin, and vitronectin. ApoE has isoform-specific effects on the conversion of protofibrils to fibrils of A β [5]. It is known that clusterin inhibits the formation of amyloid fibrils.[24] Recent studies have reported that ApoE and clusterin facilitate cellular uptake of A β -complexes by binding to several endocytic receptors, including low-density lipoprotein receptor-related protein 1 (LRP1)[25] and triggering receptor expressed on myeloid cells 2 (TREM2)[26] in vascular smooth muscle

1 cells and microglia. Since the internalized amyloid fibrils are known to be degraded by the
2 autophagy-lysosomal pathway,[27, 28] the increased levels of these proteins may contribute to
3 the clearance of amyloid deposits.

4 In the present study, we employed amyloid fibril fractions as analytical samples extracted by
5 the modified Pras method[18]. The amyloid fibril fraction is supposed to contain proteins that
6 interact with amyloid fibrils or have a low solubility similar to that of amyloid fibrils. Although
7 we identified AApoAII-associated proteins, several well-known co-deposited proteins, such as
8 SAP and apolipoprotein A-I (ApoA-I)[2] were not identified in liver samples of
9 AApoAII-positive mice. We confirmed immunohistochemically that the AApoAII deposits
10 contained SAP and ApoA-I (Supplementary Fig. 4). It is possible that SAP requires calcium ions
11 to interact with other molecules such as amyloid fibrils[29, 30] and that soluble ApoA-I was
12 removed from amyloid fibril fractions.

13 Consistent with previous proteomic analyses of human amyloidosis tissues, our study of
14 amyloid fibril fractions implicated the involvement of lipid metabolism in amyloidosis
15 pathology. However, other previously reported biological pathways such as cellular responses
16 and disrupted functions were not found in our enrichment keyword analysis. We suggest that the
17 amyloid fibril fractions mainly contain constituents of amyloid deposition and thus reflect the
18 involvement of lipid metabolism.

19
20 Among the identified AApoAII-associated proteins, ApoE was the most abundant, and
21 several ApoE fragments were observed (Fig. 3). Previous studies have reported that ApoE
22 fragments truncated at both carboxyl-[31] and amino-termini,[32] and neuron-specific human
23 ApoE- expressing mice accumulated carboxyl terminus-truncated ApoE fragments in an
24 age-dependent manner.[31] However, this was not seen in astrocyte-specific human
25 ApoE-expressing mice.[31] It was reported that the carboxyl terminus-truncated ApoE
26 fragments induced intracellular neurofibrillary tangle-like inclusions in neurons.[33] Our

Western blot analysis might have detected artificially fragmented ApoE bands generated by repeated homogenization during the isolation process. However, the molecular weights of various ApoE fragments were similar to those in previous studies.[33, 34] The plasma levels of ApoA-II and ApoE decreased dramatically with the progression of AApoAII amyloidosis. The rapid decrease of circulating SAA amyloid protein was associated with the induction of amyloid deposition.[35] Therefore, we suggest that plasma levels of ApoA-II and ApoE decreased due to their removal from plasma into amyloid deposits in tissues.

Clusterin is also known as a major genetic risk factor of AD [4] (AlzGene: www.alzgene.org). The risk allele is associated with reduced expression levels of clusterin whereas the protective allele is associated with increased expression levels.[26, 36, 37] Previous studies have reported that clusterin has inhibitory effects on A β , transthyretin (TTR) and apolipoprotein C-II (ApoC-II) amyloid fibril formation via extracellular chaperone activity.[38-40] Plasma levels of clusterin were dramatically increased with AApoAII amyloidosis progression (Fig. 4). Clusterin expression is induced by several cellular stress agents such as oxidants, heat shock, ionizing radiation and chemotherapeutic drugs.[41, 42] It is also upregulated in a wide variety of pathologies such as AD, prion disease and atherosclerosis.[43-45] These diseases induce cellular stresses via injury, reactive oxygen and dysregulation of signal transduction pathways.[41] These findings suggest that the elevated level of circulating clusterin may be a response to tissue damage by extracellular AApoAII deposits.[15]

Vitronectin is a multifunctional glycoprotein known to be present within amyloid deposits in patients with AA, AL, A β , ATTR, amyloid β 2-microglobulin (β 2M), amyloid insulin (Ins) and AApoAI amyloidosis.[23] Although fragmented vitronectin (~36 and ~15 kDa) increased in amyloid fractions, the plasma levels of vitronectin (full length) did not change with the progression of AApoAII amyloidosis (Fig. 3 and Fig. 4). It is apparent that further studies are needed to elucidate the role of vitronectin in AApoAII amyloidosis pathogenesis.

CD81 is a member of the tetraspanin membrane protein superfamily, and it mediates the

1 influx of lipoprotein-virus complexes into hepatocytes.[46] Regarding CD81, our results were
2 confusing because LC-MS/MS analysis exhibited an increase of CD81 content with AApoAII
3 amyloidosis pathology (Fig. 2), whereas Western blot analysis did not show similar profiles (Fig.
4 3). We determined that CD81 did not co-deposit with AApoAII (Supplementary Fig. 3).
5 Therefore, we suggest that LC-MS/MS profiles were false positives for unknown reasons.
6 Further investigations are needed to reveal which peptides were assigned to the sequences of
7 CD81.

8
9 Interestingly, several AApoAII-associated proteins (ApoE, clusterin and vitronectin) were
10 commonly found as constituents of extracellular deposits associated with amyloidosis as well as
11 with other degenerative diseases with extracellular aggregates, such as atherosclerosis[47] and
12 age-related macular degeneration (AMD).[48, 49] For that reason, it has been suggested that
13 some common mechanisms underlie these disease.[50] Subendothelial retention of lipoprotein
14 might be an initial key event in the development of lesions of both atherosclerosis and AMD,
15 followed by macrophage-mediated inflammatory response.[49, 51, 52] Herein, we observed that
16 lipids were also constituents of AApoAII amyloid deposits in the early stage of AApoAII
17 amyloidosis pathology (Fig. 5A). ApoA-II is a component of HDL particles. Thus, it would be
18 logical to assume that AApoAII amyloid deposits contained lipids. Indeed, other amyloidosis
19 studies have reported that amyloid deposits in AD, FAP, dialysis-related amyloidosis (DRA) and
20 hereditary ApoA-I amyloidosis were enriched in lipids.[53-55] Recent proteomic studies of both
21 human and mouse HDL-associated proteins identified ApoE, clusterin and vitronectin.[56, 57]
22 In addition, the HDL proteomic profiles are significantly enriched in proteins associated with
23 “amyloid” or “amyloidosis” keywords in UniProtKB, such as TTR, β 2M, SAA and gelsolin.
24 Furthermore, it is well known that many apolipoproteins form amyloid fibrils, including
25 ApoA-I,[58] ApoA-II,[13] ApoA-IV,[59] ApoC-II,[60] ApoC-III[61] and SAA.[62]
26 Sequence-based computational approaches predicted that amyloidogenic segments of

apolipoproteins could be located in the lipid-binding amphipathic α -helices in the native state of apolipoproteins,[63] and these predictions have been verified experimentally for ApoA-I,[64] ApoA-II,[14] ApoC-II[65] and SAA.[66] Importantly, the involvement of apolipoproteins in amyloidosis correlates with the strength of protein-lipid association rather than the amino acid sequence propensity to form amyloid.[63] These data suggest that lipoproteins may contribute to amyloidosis pathology. The common protein components among degenerative diseases with lipid-rich deposits could be accounted for by the subendothelial lipoprotein retention model, termed “lipoprotein-derived debris”.[49]

In future studies, we will focus on the lipidomic profiles of amyloid deposits. Whereas both atherosclerotic and AMD plaques are derived from apolipoprotein B- (ApoB) containing lipoproteins,[51] lipidomic profiles of AApoAII amyloid deposit would make it possible to determine the types of lipoproteins that are involved in AApoAII amyloidosis pathology. Then, nutritional strategies aimed at changing plasma lipid profiles could be performed. Caloric restriction alleviates amyloid deposition in a mouse model of AD and AApoAII amyloidosis,[67, 68] and high fat diets induce accelerated amyloid deposition in AD and AA amyloidosis.[69, 70] We have also reported that unsaturated fatty acids in the diet effect amyloid deposition in AApoAII amyloidosis.[71, 72] These strategies may also effect apolipoprotein amyloidosis from the aspect of protein stability because the increased levels of lipid content in lipoprotein particles lead to shifts in lipoprotein-bound stable apolipoproteins to free amyloidogenic apolipoproteins.[73] These studies should help to determine the involvement of lipoproteins in the development of non-apolipoprotein amyloidosis.

Study Limitations

Our study has several limitations in its experimental design and the interpretation of findings. First, we used 5 different time points for the IJ group but only 3 time points for the NT group. The comparison between NT and IJ groups at different sequential points was an unconventional

strategy to determine the changes attributable to progression of amyloidosis vs. ageing. Second, our MS experiments did not ensure that the same amount of total peptide was loaded in each case. Therefore, we semi-quantified AApoAII-associated proteins by rank order or the content ratio. Third, we did not pursue experiments for the terms related to the keratin family of proteins, including intermediate filament, keratin and coiled coil. Keratins are common contaminants in proteomic studies, although intermediate filaments have several important biological functions in AD pathology.[74] Finally, in this study, we determined the proteomic profiles of only mature amyloid fibril-associated proteins. Since soluble oligomers and amorphous aggregates of amyloid proteins have more inherent toxicity than mature amyloid fibrils,[75] we need to establish a way to differentiate between physiological soluble oligomers and pre-fibrillar oligomers.

5. Conclusions

We identified 6 amyloid-associated proteins in proteomic analysis of amyloid fibril fractions in mouse liver tissues with different levels of AApoAII deposits and characterized ApoA-II, ApoE, clusterin and vitronectin in the pathology of amyloidosis. We found that the relative abundance of these associated proteins in the amyloid fibril fractions did not change throughout the development of AApoAII amyloidosis pathology, although their concentrations in plasma changed dramatically with progression of the disease. Further analysis of amyloid-associated proteins showed that they were enriched in terms of lipid metabolism and lipids were co-deposited with AApoAII fibrils. Thus, our proteomic analysis indicated a likely relationship between lipoproteins and amyloidosis pathogenesis.

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Figure Legends

Figure 1. Experimental design and AApoAII deposition in R1.P1-*Apoa2*^c mice after AApoAII fibril injection. (A) The induction of AApoAII amyloidosis was performed by intravenous injection of AApoAII fibrils into 2-month-old female R1.P1-*Apoa2*^c mice. (B) Amyloid deposition was confirmed by Congo red staining. Bright field photos represent enlarged views of polarized-light field (white squares). Scale bars represent 100 μ m. (C) AApoAII deposition was evaluated by immunostaining of each mouse liver with ApoA-II antibody. Scale bars in photomicrographs represent 100 μ m, and each bar in the graphic field represents the mean and $\pm$ S.D. (N = 3 (NT-12) and 5 (others)).

Figure 2. Changes of AApoAII-associated proteins with AApoAII amyloidosis pathology. (A) Venn diagram illustrating the overlap between AApoAII amyloidosis-specific proteins (Table 2, left circle) and the 20 top-ranked proteins (Table 1, right circle) in the IJ-12 group. Their overlap was termed “AApoAII-associated proteins”. (B) Rank order of contents for common protein (*Glud1*) or AApoAII-associated proteins in the liver amyloid fibril fraction. (C) Percentage of each AApoAII-associated protein contents except ApoA2 in amyloid fibril fraction. Each bar represents the mean $\pm$ S.D. (N = 3 (NT-12) or 5 (others)).

Figure 3. Comparison of AApoAII-associated proteins in the amyloid fibril fractions. Each group of liver amyloid fibril fractions were subjected to Western blot analysis with antibodies against ApoA-II, ApoE, clusterin, vitronectin and CD81.

Figure 4. Plasma levels of AApoAII-associated proteins. Each group of plasma samples was subjected to Western blot analysis with antibodies against ApoA-II, ApoE, clusterin and vitronectin.

Figure 5. Lipid co-deposition with AApoAII amyloid fibrils. (A) Staining of the liver sections with Oil red O confirmed the presence of neutral lipids and fatty acids in AApoAII deposits. (B) Electron microscopic photos show electron-lucent suggestive lipid materials (arrows) along with amyloid fibrils (arrowheads). Each scale bar represent 500 nm (left) and 200 nm (right).

Supplemental Figure 1: Electron microscopic photo of extracted AApoAII amyloid fibrils from the IJ-12 group. Scale bar represents 50 nm.

Supplemental Figure 2: Histological analysis of AApoAII deposits in other organs. Each scale bar represents 100 μ m.

Supplemental Figure 3: Immunohistochemical analysis of (A) selected AApoAII-associated proteins (ApoE, clusterin, vitronectin and CD81) and (B) not identified common amyloid-associated proteins in amyloid fibril fractions (SAP and ApoA-I). Each scale bar represents 20 μ m.

Supplemental Figure 4: Histological analysis of lipid deposit in lung. Each scale bar represents 50 μ m.

Supplemental Table 1: List of 20 top-ranked major proteins in each experimental group.

Supplemental Table 2: List of proteins associated with enrichment UniprotKB keyword.

Table 1. List of Specific Proteins in IJ-12 Group Compared to NT-12 Group.

protein description ^a	gene symbol
1300001I01Rik protein Fragment	<i>1300001I01Rik</i>
Kidney specific expressed protein 2	<i>1810022C23Rik</i>
UPF0690 protein C1orf52 homolog	<i>2410004B18Rik</i>
RIKEN cDNA A630073D07 gene	<i>A630073D07Rik</i>
Ankyrin repeat domain containing protein 16	<i>Ankrd16</i>
Apolipoprotein A I	<i>Apoa1</i>
Apolipoprotein A II	<i>Apoa2</i>
Apolipoprotein E	<i>ApoE</i>
Beta 2 glycoprotein 1	<i>ApoH</i>
Probable methyltransferase BCDIN3D	<i>Bcdin3d</i>
D beta hydroxybutyrate dehydrogenase mitochondrial	<i>Bdh1</i>
BPI fold containing family A member 2E Fragment	<i>Bpifa2e</i>
Protein BUD31 homolog	<i>Bud31</i>
CD81 antigen	<i>Cd81</i>
Centrosomal protein of 57 kDa	<i>Cep57</i>
Clusterin	<i>Clu</i>
Cytochrome P450 3A11	<i>Cyp3a11</i>
Cytochrome P450 3A41	<i>Cyp3a41a</i>
DNA segment, Chr 11, Wayne State University 99, expressed, isoform CRA_c	<i>D11Wsu99e</i>
DnaJ homolog subfamily B member 8	<i>Dnajb8</i>
Elongation factor 1 alpha 1	<i>Eef1a1</i>
Fatty acid binding protein liver	<i>Fabp1</i>
Fatty acid binding protein intestinal	<i>Fabp2</i>
Fibrinogen alpha chain	<i>Fga</i>
Fibrinogen beta chain	<i>Fgb</i>
Fibrinogen gamma chain	<i>Fgg</i>
Gamma glutamylcyclotransferase	<i>Ggct</i>
Predicted gene, 10674	<i>Gm10674</i>
Predicted gene, 17026	<i>Gm17026</i>
Predicted gene, 17027	<i>Gm17027</i>
Predicted gene, 17093	<i>Gm17093</i>
Predicted gene, 17124	<i>Gm17124</i>
Predicted gene, 17654	<i>Gm17654</i>
Predicted gene, 3072	<i>Gm3072</i>
Predicted gene, 3633	<i>Gm3633</i>
Predicted gene, 7970	<i>Gm7970</i>
Predicted gene, 7980	<i>Gm7980</i>
Predicted gene, 8005	<i>Gm8005</i>
Predicted gene, 8082	<i>Gm8082</i>
Predicted gene, 8138	<i>Gm8138</i>

Predicted gene, 8165	<i>Gm8165</i>
Predicted gene, 8894	<i>Gm8894</i>
Predicted gene, 9493	<i>Gm9493</i>
Hemoglobin subunit alpha	<i>Hba</i>
Alpha globin	<i>Hba a1</i>
Hemoglobin alpha, adult chain 2	<i>Hba a2</i>
Homeodomain only protein	<i>Hopx</i>
17 beta hydroxysteroid dehydrogenase 13	<i>Hsd17b13</i>
78 kDa glucose regulated protein	<i>Hspa5</i>
Ig mu chain C region membrane bound form	<i>Igh 6</i>
Protein KIAA0664	<i>Kiaa0664</i>
Keratin type II cytoskeletal 1	<i>Krt1</i>
Keratin type I cuticular Ha1	<i>Krt31</i>
Keratin type I cuticular Ha3 I	<i>Krt33a</i>
Keratin type I cuticular Ha3 II	<i>Krt33b</i>
Keratin type I cuticular Ha4	<i>Krt34</i>
Keratin type II cytoskeletal 4	<i>Krt4</i>
Keratin type II cytoskeletal 71	<i>Krt71</i>
Keratin type II cytoskeletal 73	<i>Krt73</i>
Keratin type II cytoskeletal 1b	<i>Krt77</i>
Keratin type II cytoskeletal 79	<i>Krt79</i>
Keratin type II cuticular Hb2	<i>Krt82</i>
Lipopolysaccharide binding protein	<i>Lbp</i>
Leucine, glutamate and lysine-rich 1	<i>Lekr1</i>
MAA 76 protein	<i>maa 76</i>
Melanocortin-2 receptor accessory protein 2	<i>Mrap2</i>
Myosin light polypeptide 6	<i>Myl6</i>
NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit 4	<i>Ndufa4</i>
COUP transcription factor 1	<i>Nr2f1</i>
Protein disulfide isomerase	<i>P4hb</i>
Phospholipase A1 member A	<i>Pla1a</i>
Proteoglycan 4	<i>Prg4</i>
Protease, serine 1	<i>Prss1</i>
Parotid secretory protein	<i>Psp</i>
Syndecan 1	<i>Sdc1</i>
Sperm associated antigen 7	<i>Spag7</i>
Cornifin A	<i>Sprr1a</i>
Signal recognition particle 19 kDa protein	<i>Srp19</i>
Synaptotagmin like protein 5	<i>Sytl5</i>
Serotransferrin	<i>Tf</i>
Transmembrane protein 205	<i>Tmem205</i>
Tumor necrosis factor receptor superfamily member 10B	<i>Tnfrsf10b</i>
Trypsin 10	<i>Try10</i>

UDP-glucuronosyltransferase 1-7C	<i>Ugt1a10</i>
UDP glucuronosyltransferase 1 9	<i>Ugt1a9</i>
UDP glucuronosyltransferase 2 family polypeptide B36	<i>Ugt2b36</i>
Vimentin	<i>Vim</i>
Vitronectin	<i>Vtn</i>
DNA repair protein complementing XP C cells homolog	<i>Xpc</i>

^aprotein information and mouse gene symbols from www.uniprot.org database. Proteins shown in bold represent the AApoAII-associated protein.

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Table 2. List of the 20 Top-Ranked Proteins in the IJ-12 group

rank average ^a	protein description ^b	gene symbol	hit ^c
2.4	Apolipoprotein E	<i>Apoe</i>	5
4.0	Histone H4	<i>Gm11275</i>	5
7.3	Hist1h3e protein Fragment	<i>Hist1h3e</i>	4
9.2	Polyubiquitin C	<i>Ubc</i>	5
9.6	Keratin type II cytoskeletal 8	<i>Krt8</i>	5
11.4	Keratin type I cytoskeletal 18	<i>Krt18</i>	5
11.4	Clusterin	<i>Clu</i>	5
13.4	Uncharacterized protein	<i>Mgst1</i>	5
13.8	Apolipoprotein A II	<i>Apoa2</i>	5
15.0	Cytochrome b 5 isoform CRA d	<i>Cyb5</i>	4
16.8	Vitronectin	<i>Vtn</i>	5
17.6	ATP synthase subunit alpha mitochondrial	<i>Atp5a1</i>	5
19.0	CD81 antigen	<i>Cd81</i>	5
22.6	Histone H2A x	<i>H2afx</i>	5
23.4	17 beta hydroxysteroid dehydrogenase 13	<i>Hsd17b13</i>	5
25.5	Beta 2 globin Fragment	<i>Hbb</i>	4
29.3	Membrane associated progesterone receptor component 1	<i>Pgrmc1</i>	4
37.8	Very long chain acyl CoA synthetase	<i>Slc27a2</i>	4
38.6	Histone H2A V	<i>H2afv</i>	5
39.0	Trifunctional enzyme subunit alpha mitochondrial	<i>Hadha</i>	4

^arank average of content in the amyloid fibril fraction. ^bprotein information and mouse gene symbols from www.uniprot.org database. Proteins shown in bold represent the AApoAII-associated protein. ^cNumber of identifications of each protein among the 5 mice in the IJ-12 group. The 20 top-ranked proteins in other groups are listed in Supplementary Table 1.

Table 3. Functional enrichment Analysis of UniprotKB Keywords in the Mouse AApoAII Amyloid Fibril Fraction

Term ^a	# proteins ^b	p value ^c
intermediate filament	12	6.83E-14
keratin	11	1.19E-10
hdl	4	8.78E-04
lipid binding	3	8.78E-03
lipid transport	4	1.48E-02
coiled coil	15	2.13E-02
secreted	13	3.04E-02

Functional enrichment analysis was performed by DAVID Bioinformatics Resources (david.ncifcrf.gov). ^aThe 89 specific proteins in IJ-12 group compared to NT-12 group were subjected to enrichment analysis and significantly enriched terms were determined. ^bNumber of proteins associated with each term. ^cModified Fisher Exact p-values were adjusted by Benjamini-Hochberg procedure. Proteins associated with each term are listed in Supplementary Table 2.

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Figure 1.

Figure 2.

A

Specific proteins in IJ-12 group compared to control NT-12 group

Twenty top-ranked proteins in IJ-12 group

- AApoAll-associated proteins (Gene symbol)**
- ApoA-II (*Apoa2*)
 - ApoE (*ApoE*)
 - Clusterin (*Clu*)
 - Vitronectin (*Vtn*)
 - CD81 (*Cd81*)
 - 17 beta hydroxysteroid dehydrogenase 13 (*Hsd17b13*)

B

C

Figure 3.

Figure 4.

Figure 5.

Supplemental Figure 1.

Supplemental Figure 2.

Supplemental Figure 3.

A

ApoE

Clusterin

Vitronectin

CD81

B

SAP

ApoA-I

Supplemental Figure 4.

IJ-6

IJ-12