Accumulation of pro-apolipoprotein A-II in mouse senile amyloid fibrils

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Apolipoprotein A-II (apoA-II), the major apoprotein of serum high-density lipoprotein, is deposited as amyloid fibrils (AApoAII) in murine senile amyloidosis. We have identified and purified a more basic amyloid protein from old-mouse liver. N-terminal sequencing of the protein revealed that the pro-segment of five amino acid residues (Ala-Leu-Val-Lys-Arg) extended from the N-terminal glutamine residue of mature apoA-II protein. MS analysis revealed the deposit of intact pro-apoA-II protein (molecular mass 9319 Da). Antiserum was prepared for staining of the AApoAII amyloid deposition. The relative abundance of pro-apoA-II to mature apoA-II in the amyloid-

fibril fraction isolated from livers of mice with severe amyloidosis was 14.1 %. The similar abundance of pro-apoA-II in the amyloid fibril fraction from the spleen (16.3 %) suggested that deposited pro-apoA-II originated from the blood. The concentration of pro-apoA-II was much lower in the serum (1.5 % of mature apoA-II) than in the amyloid-fibril fraction. There was no difference in the content of pro-apoA-II between the amyloidogenic R1.P1-*Apoa2^c* and amyloid-resistant SAMR1 strains at the age of 3 months. The abundance of pro-apoA-II in the amyloid-fibril fraction compared with the serum suggested that it plays a key role in the initialization of mouse senile amyloidosis.

INTRODUCTION

Amyloidosis refers to a group of biochemically diverse conditions characterized by the extracellular accumulation of fine amyloid fibrils to which normally innocuous soluble proteins polymerize [1]. Amyloidosis associated with aging is common in humans, mice and other animal species [2].

ApoA-II, an apolipoprotein in serum high-density lipoproteins (HDL) and a precursor of murine senile amyloid fibrils (AApoAII) first isolated from SAMP1 mice [3,4], was later found to be present universally in mice [5–7]. Three variants of apoA-II protein (types A, B and C) with different amino acid substitutions at four positions are present in inbred strains of mice [8]. The strains with a high incidence of senile amyloidosis have type C apoA-II (*Apoa2^c*) with glutamine at position 5, whereas the strains with a low incidence of amyloidosis have type A (*Apoa2^a*) or type B (*Apoa2^b*) apoA-II with proline at position 5. Examination of the genotype of apoA-II gene and amyloid deposition in the F1, F2 and back-crossed mice made by the crossing of strains with *Apoa2^c* and strains with *Apoa2^a* or *Apoa2^b* showed that early deposition of AApoAII is linked to *Apoa2^c* [8,9]. A congenic strain (R1.P1-*Apoa2^c*) of mice which has the amyloidogenic apoA-II gene (*Apoa2^c*) of SAMP1 on the genetic background of SAMR1 was established, and these mice showed age-associated severe amyloid deposition [10,11].

Despite recent biochemical and molecular-genetic advances pertinent to various amyloid proteins, the pathogenesis of amyloidosis is not fully known. Kinetic analysis of amyloid-fibril polymerization *in vitro* using the amyloid-fibril-specific fluorescence of thioflavine T revealed that type C apoA-II monomers attach to the ends of existing fibrils and produce amyloid fibrils [12]. Nucleation-dependent polymerization described above is postulated to be a model which explains well the kinetics of fibrilization of amyloid proteins in Alzheimer's disease (A β

protein), scrapie (Prion protein), secondary amyloidosis (AA protein) and familial amyloid polyneuropathy (TTR protein) [13–17]. Several substances associated with amyloid deposits are considered to influence the nucleation and/or the elongation phase of amyloid-fibril formation. These include the serum amyloid P component [18,19], heparan sulphate proteoglycan [20,21], apolipoprotein E [22,23] and advanced glycation end products [24]. In addition, two C-terminal variants of A β protein, A β 40 and A β 42(43) have been identified in amyloid deposits in Alzheimer's-disease brain. Amyloid formation by the kinetically soluble A β 40 may be initiated by nucleation of A β 42(43) which has a greater propensity to aggregate than A β 40 [25,26].

In the present study we isolated and analysed proteins from the murine senile amyloid deposits (AApoAII) to clarify the minor substances which might affect the fibrilization of amyloid protein. Sequence analysis revealed the presence of pro-apoA-II protein, which has five extra amino acid residues from the N-terminus of mature apoA-II. The relative abundance of pro-apoA-II to mature apoA-II in the amyloid fibril fraction was compared with that in the blood to evaluate the role of pro-apoA-II in amyloidogenesis.

EXPERIMENTAL

Animals

R1.P1-*Apoa2^c*, a congenic strain of mice which has amyloidogenic type C apoA-II (*Apoa2^c*) of the SAMP1 strain on the genetic background of the amyloid-resistant SAMR1 strain [10], and the SAMR1 strain of mice were used. These mice were raised in our laboratory under conventional conditions at 24 $\pm$ 2 °C with a light-controlled regimen (12 h light/12 h dark cycle). A commercial diet (CE-2; Nihon CLEA, Tokyo, Japan) and tap water

Abbreviations used: apoA-II, apolipoprotein A-II; AApoAII, murine senile amyloid fibrils; HDL, high-density lipoprotein(s); GDAM, guanidinium chloride-denatured amyloid protein; AAP-1, amyloid-associated protein-1; CBB, Coomassie Brilliant Blue; ABC, avidin-biotinylated horseradish peroxidase complex; MALDI, matrix-assisted laser-desorption ionization.

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were available *ad lib*. The livers and spleens were obtained from R1.P1-*Apoa2^c* mice over 18 months of age and stored frozen until amyloid-fibril isolation. Female mice 3 months of age were deprived of food overnight and bled by cardiac puncture under diethyl ether anaesthesia. Blood was left to clot at room temperature and the serum was separated by centrifugation at 4 °C.

Isolation of amyloid proteins

The amyloid-fibril fraction was isolated as a water suspension from the livers and spleens of 16–19-month-old R1.P1-*Apoa2^c* mice as described by Pras et al. [27]. Isolated amyloid-fibril fraction was further purified by ultracentrifugation as described elsewhere [28]. Pellets after ultracentrifugation were resuspended in distilled water by mixing thoroughly with an ultradisperser (Ultra-Turrax T25; Janke and Kunkel G.m.b.h., KG, Staufen, Germany) and freeze-dried. The freeze-dried sample was denatured in 6 M guanidinium chloride containing 1.0 % 2-mercaptoethanol, left at room temperature for 24 h, and then was dialysed against 10 mM NH_4HCO_3 and freeze-dried again. PAGE in the presence of 6 M urea was performed by the method of Davis [29] on a 15 cm $\times$ 10 cm $\times$ 0.3 cm slab gel with 7.5 % acrylamide. The freeze-dried-denatured sample (2.5 mg of protein) was dissolved in 1.0 ml of sample buffer containing 6 M urea and incubated for 6 h at room temperature and applied on to a gel and electrophoresed overnight at 6 mA at 4 °C. Amyloid proteins contained in the bands located by stained reference gel were excised, diced into small pieces and suspended in 10 mM NH_4HCO_3 . Each amyloid-associated protein (AAP) was extracted three times with stirring for 12 h at 4 °C.

Gel-electrophoretic analysis

The method of Swank and Munkres [30] was used for 8M urea/SDS PAGE. Analytical alkaline-urea/PAGE was also performed by the method of Davis [29]. Gels were stained in 25 % methanol/10 % acetic acid/5 % Coomassie Brilliant Blue (CBB) R-250. Mouse serum (10 μl) was delipidated with 1.5 ml ethanol/diethyl ether (3:1, v/v) for 24 h at -10 °C and dried under nitrogen. De-lipidated sera (300 nl of the original serum) were subjected to analytical alkaline-urea/PAGE and electro-blotted on to PVDF membranes (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Protein bands reacting with antiserum against guanidinium chloride-denatured amyloid protein (GDAM) (1:4000), mature apoA-II (diluted 1:2000) and pro-apoA-II (diluted 1:1000) were detected using enhanced chemiluminescence induced by oxidation of luminol by the horseradish peroxidase-labelled anti-rabbit IgG (ECL System; Amersham International).

The intensity of bands stained with CBB and on the autoradiography films (X-OMAT; Eastman Kodak, New York, NY, U.S.A.) were determined with a video densitometric image analyser (Luzex 3U, Nikon, Tokyo, Japan).

Antisera and immunohistochemistry

Anti-GDAM and mature apoA-II antisera were obtained in rabbits as described in [31]. Antiserum to pro-apoA-II was prepared in rabbits by serial injection of 50 μg of the protein solubilized in 10 mM NH_4HCO_3 and emulsified in adjuvant (Titer Max; Vaxcel, Northwood, GA, U.S.A.). Immunohistochemical examination using the avidin-biotinylated horseradish peroxidase complex (ABC) methods were performed as described [10]. Paraffin-embedded sections of 4 μm thickness were allowed to react with GDAM, apoA-II and pro-apoA-II antiserum

and detected with 3,3'-diaminobenzidine tetrahydrochloride as substrate.

Amino acid sequence analysis

The purified amyloid associated protein, AAP-1 (50 μg) was subjected to 8 M urea/SDS/PAGE and transferred to PVDF membrane for 12 h in 60 mM boric acid, pH 8.0, with 30 % methanol at a constant current of 100 mA and at 4 °C. The protein band stained with CBB R-250 was cut out and used for N-terminal amino acid determination. N-terminal amino acid analysis was completed with automated Edman degradation and determination of amino acid derivatives with an on-line phenylthiohydantoin-amino acid analyser using an automatic sequence analyser (PPSQ-10; Shimadzu, Kyoto, Japan).

Matrix-assisted laser-desorption ionization (MALDI) MS

The MALDI mass spectra of AAP-1 (10 μg) were recorded on a Kompact MALDI III time-of-flight mass spectrometer (Kratos-Shimadzu, Kyoto, Japan). Sinapinic acid was employed as a UV-absorbing matrix. The total flight path of the instrument was 0.7 m and ionization and desorption were effected with a 337 nm nitrogen laser (3 ns pulse). The analyser was operated in the positive-ion linear mode at an accelerating voltage of 20 kV. The laser power setting was adjusted using a computer-controlled neutral-density filter and gradually increased until a threshold level was attained at which the MALDI process was activated. Satisfactory mass spectra were obtained when the laser power was set at a value slightly in excess of the MALDI threshold level. Under these conditions the resolving power represented by the 50 % valley definition of adjacent symmetrical peaks ($m/\Delta m$) was 250–350. For optimal laser power values the instrument dial setting in arbitrary units (maximum is 180) was 63. The mass spectrum was the average of 100 shots from regular scanning across the sample spot. Mass calibration was achieved externally using bovine insulin (molecular mass 5735 Da). The mass numbers (m/z) refer to the average masses of the protonated molecular ions ($M+H$)⁺.

Statistical analysis

All data are presented as the means $\pm$ S.E.M. Student's *t* test was used for comparison of the content of pro-apoA-II in the amyloid-fibril fraction and in the serum.

RESULTS

Isolation and purification of AAP-1

Amyloid-fibril fraction was isolated from the liver of an 18-month-old female R1.P1-*Apoa2^c* mouse. After denaturation with 6 M guanidinium chloride, amyloid proteins were analysed by 8 M urea/SDS/PAGE and alkaline-urea/PAGE (Figure 1). On SDS/PAGE, AAP-1 was a major band with a molecular mass of 8.7 kDa. A minor protein band (AAP-1) with a slower migration rate than AAP-1 was detected by alkaline-urea/PAGE without SDS. AAP-1 was extracted from the alkaline-urea/PAGE gel. AAP-1 (98.8–134.8 μg) was purified from 2.5 mg of amyloid proteins depending on the extraction trials. Simultaneously, 805.8–1284.0 μg of AAP-1 were purified. Purified AAP-1 migrated as a single protein band with a molecular mass slightly higher than that of AAP-1. On alkaline-urea/PAGE, AAP-1 migrated as a single principal band with the same migration rate as the minor band of amyloid proteins.

Figure 1 Purification of AApoAll and AAP-1

Urea (8 M)/SDS/PAGE (12.5% gel) electrophoresis of amyloid proteins in the amyloid-fibril fraction (lane A). To obtain purified A β poAlI (lane B) and AAP-1 (lane C), the bands from alkaline-urea/PAGE of amyloid proteins were cut out. Molecular-mass standards (kDa) were prestained aprotinin (7400), lysozyme (19400), soybean trypsin inhibitor (27800), carbonic anhydrase (33600), and ovalbumin (50700). Alkaline (pH 9.2) urea/PAGE (7.5%): lane A, amyloid proteins; lane B, purified A β poAlI; lane C, purified AAP-1.

Figure 2 Amino acid sequence of AAP-1 and apoA-II

The primary translation product of mouse type C apoA-II protein was predicted from the cDNA sequence [32]. The signal sequence and propeptide are underlined with double and single lines respectively. Mature apoA-II protein, which starts with the glutamine residue (pyrrolidine-carboxylic acid in the mature protein) at position 1 consists of 78 amino acids. The lower line in *italics* is the sequence of AAP-1 determined with an automated protein sequencer. Asterisks are the positions at which amino acid residues could not be identified clearly.

Sequence and MS analysis of AAP-1

Automated Edman degradation revealed the N-terminal sequence of the first 47 amino acid residues of AAP-1 (Figure 2). The sequence from the sixth to the 47th amino acid residue (glutamine to phenylalanine) was completely identical with the type C apoA-II (AApoAII) [4]. The first five amino acid residues, Ala-Leu-Val-Lys-Arg were identical with the propeptide residues of mouse apoA-II protein predicted from the cDNA sequence [32].

The MALDI mass spectra of purified AAP-1 yielded high-quality spectra (Figure 3). The primary structure of each peptide was deduced by comparing the observed mass with the calculated mass for peptides derived from the apoA-II sequence. The most intense and major signal (peak B) with an observed mass of 9319 Da, corresponded to full-length pro-apoA-II (from -5 to 78 residues) with a mass calculated to be 9321.9 Da. These results indicated that AAP-1 is the pro-apoA-II protein. The minor signal (peak A) with an observed mass of 8733 Da seemed to be an apoA-II variant with an open-circle glutamine residue as an N-terminus, with a mass calculated to be 8738.8 Da.

Figure 3 MALDI mass spectrum of AAP-1

AAP-1 purified by alkaline-urea/PAGE from amyloid proteins of old mouse liver was analysed with an MALDI mass spectrometer using sinapinic acid as the UV-absorbing matrix. Inset: molecular masses and predicted sequence of AAP-1 peaks A and B.

Immunochemical characterization of pro-apoA-II

Anti-pro-apoA-II antisera were produced by serial immunization of purified pro-apoA-II in two rabbits. The specificity of the antiserum was analysed by Western-blot analysis (Figure 4). The antiserum produced in one of two rabbits detected only the pro-apoA-II band separated by alkaline-urea/PAGE from amyloid proteins, but the anti-(mature apoA-II) antiserum detected both mature and pro-apoA-II bands. Antiserum against GDAM detected also both pro-apoA-II and mature apoA-II (results not

Figure 4 Immunological reactivity of antiserum against amyloid proteins

Partially purified amyloid proteins (250 ng) were separated by alkaline-urea/PAGE and transferred to PVDF membrane. Antibody labelling antiserum against purified mature apoA-II (1:2000) (lane B) and antiserum against purified pro-apoA-II (1:1000) (lane D) was performed using the ECL system. Lanes A, C and E are the references for immunoblotting 2.5 μ g of purified AApoAII (mature apoA-II), 5.0 μ g of amyloid proteins and 2.5 μ g of purified pro-apoA-II respectively separated by alkaline-urea/PAGE and stained with CBB. Lane F, 2.5 μ g of AApoAII as the reference. Lane E, labelling of 250 ng of AApoAII electroblotted with the anti-pro-apoA-II antiserum (1:1000).

Figure 5 Immunological staining of amyloid deposits with antiserum against purified pro-apoA-II

(A) Green birefringence in specimens stained with Congo Red under polarized microscope. (B), (D) and (E) ABC immunostaining of amyloid depositions using antiserum (1:1000 dilution) against purified pro-apoA-II. (C) ABC staining of amyloid depositions using antiserum (1:4000) against GDAM [31]. (A), (B) and (C) are sections of the heart, (D) the liver and (E) of the spleen of an 18-month-old R1.P1-*Apoa2*^o mouse. The scale bar represents 25 μ m.

shown). This pro-apoA-II-specific antiserum stained amyloid deposition in the liver, kidneys, spleen, lungs, heart, testis, tongue, intestine and vessels of R1.P1-*Apoa2*^o (1:1000) (Figure 5). Staining was evident in the extracellular spaces of these organs, and the distribution was the same as that of deposits stained using anti-(mature apoA-II), GDAM antiserum and by the green birefringence in Congo Red-stained sections by polarizing microscopy. Positive staining with anti-pro-apoA-II was not seen after absorption with purified pro-apoA-II.

Relative abundance of pro-apoA-II in the amyloid-fibril fraction

Amyloid-fibril fraction was isolated from the liver and spleen of three female R1.P1-*Apoa2*^o mice, 18, 19 and 25 months old. Marked AApoAII deposition was graded 4 [31] in immunohistochemically stained sections of the liver and spleen of all these mice. Amyloid proteins in the amyloid-fibril fraction isolated from each mouse were subjected to alkaline-urea/PAGE and the ratio of pro-apoA-II to mature apoA-II was calculated (Figure 6). The average ratio of pro-apoA-II to mature apoA-II

in the liver was $14.1 \pm 4.1\%$ (mean $\pm$ S.E.M.), and not significantly different from that in the spleen ($16.1 \pm 0.6\%$) (Table 1).

Serum was isolated from 3-month-old female R1.P1-*Apoa2*^o and SAMR1 mice after 16 h of fasting. Serum from six mice per strain was de-lipidated and subjected to alkaline-urea/PAGE and transferred to a PVDF membrane. Pro-apoA-II and mature apoA-II were detected immunochemically using the anti-GDAM antiserum and the ECL system (Figure 7). We usually use 30 nl of serum for measuring the apoA-II concentration using the ECL system [10]. Since we could not detect the pro-apoA-II band from 30 nl of delipidated serum, we applied 300 nl of delipidated serum and increased the time of exposure to the X-ray film. The average ratio of pro-apoA-II to mature apoA-II was $1.6 \pm 1.0\%$ in the R1.P1-*Apoa2*^o strain and $1.4 \pm 1.1\%$ in the SAMR1 strain (Table 1). The ratio in the serum of each strain was significantly lower than that in the amyloid-fibril fraction isolated from the liver and spleen ($P < 0.01$). The concentrations of pro-apoA-II and mature apoA-II in each strain were determined using mature apoA-II in the pooled control serum of 2-month-old female SAMR1 mice as the standard. The serum concentration of

Figure 6 Pro-apoA-II in the amyloid-fibril fraction isolated from the liver and spleen

Amyloid-fibril fraction was isolated from old R1.P1-*Apoa2^c* mice and further purified by ultracentrifugation. Amyloid proteins (2.5 µg) from the liver of an 18-month-old mouse (lane A), the liver of an 19-month-old mouse (lane B), the spleen of an 18-month-old mouse (lane C) and the spleen of an 19-month-old mouse (lane D) were separated by alkaline-urea/PAGE and stained with CBB.

Table 1 Relative abundance of pro-apoA-II in the amyloid-fibril fraction isolated from the liver and spleen and in the serum

Amyloid-fibril fraction was isolated from amyloid deposits in 18-, 19- and 25-month-old R1.P1-*Apoa2^c* mice. Relative abundance of pro-apoA-II to mature apoA-II in the amyloid-fibril fraction was determined by alkaline-urea/PAGE with CBB staining. Serum concentrations of mature apoA-II, pro-apoA-II and relative abundance of pro-apoA-II in the de-lipidated serum of 3-month-old female R1.P1-*Apoa2^c* and SAMR1 mice were determined by Western blotting with anti-GDAM antiserum (1:4000). All values are means ± S.E.M. Values with different letters are significantly different by Student's *t* test ($P < 0.01$).

Tissue	No. of mice	Mature apoA-II (mg/dl)	Pro-apoA-II (mg/dl)	Mature apoA-II (%)
Amyloid-fibril fraction				
Liver	3			14.1 ± 4.1 ^a
Spleen	3			16.1 ± 0.6 ^a
Serum				
R1.P1- <i>Apoa2^c</i>	6	31.8 ± 4.3 ^a	0.5 ± 0.5	1.6 ± 1.0 ^b
SAMR1	6	52.4 ± 6.4 ^b	0.7 ± 0.6	1.4 ± 1.1 ^b

mature apoA-II in the amyloidogenic R1.P1-*Apoa2^c* was significantly lower than that in SAMR1. However, the concentrations and the relative abundance of pro-apoA-II were not significantly different between R1.P1-*Apoa2^c* and SAMR1. In the assay condition used for estimation of the pro-apoA-II levels, mature apoA-II bands might be overexposed. Thus there was no linear relationship between the amount of mature apoA-II and pro-apoA-II, and the amount of pro-apoA-II might have been overestimated.

DISCUSSION

We isolated the pro-apoA-II protein as an amyloid-associated protein from the heavy amyloid deposits in the old R1.P1-*Apoa2^c*, which is a model mouse for severe senile amyloidosis. Interestingly pro-apoA-II was concentrated almost 10-fold in amyloid protein fractions compared with the contents in the

Figure 7 Relative abundance of pro-apoA-II in mouse serum

Sera (30 nL) from 3-month-old female R1.P1-*Apoa2^c* (lane A) and SAMR1 (lane B) mice were delipidated and separated with alkaline-urea/PAGE transferred to PVDF membrane and labelled with anti-GDAM antiserum (1:4000) using the ECL system. Exposure time to X-ray film was 20 s. Lane C, 300 nL of the serum from R1.P1-*Apoa2^c* mouse labelled with antiserum and exposed to X-ray film three times longer (60 s). Delipidated sera (300 nL) from the 3-month-old female R1.P1-*Apoa2^c* mice (lanes D, E and F) and SAMR1 mice (lanes G, H and I) were applied to alkaline-urea/PAGE. Pro-apoA-II and mature apoA-II were detected with anti-GDAM antiserum after transfer to PVDF membranes.

serum. Thus pro-apoA-II may play an important role in the pathogenesis, especially initiation of the fibrilization of apoA-II protein in mouse senile amyloidosis.

Previously we purified a minor protein which had more basic pI than the major mature apoA-II protein in the mouse senile amyloid fibril proteins by gel chromatography after water extraction. The calculated pI (5.76) of pro-apoA-II (type C) was similar to that of the minor amyloid protein reported previously [3]. A few minor bands of mouse plasma apoA-II with charge heterogeneity have been reported [33]. However, the lower pI of these proteins (4.9–5.2) suggested that they were not pro-apoA-II, and supported our finding that the pro-apoA-II level in the serum was very low.

MS analysis using MALDI revealed that intact (full-length) pro-apoA-II containing 83 amino acid residues deposited without degradation. The minor protein peak with a smaller molecular mass of 8733 Da was detected by MALDI spectrometry (Figure 3, peak A). From the calculated mass (8738 Da), this protein was considered to be an isoform of mature apoA-II which has free glutamine residues at the N-terminal and +1 pI from mature apoA-II. The molecular mass of mature type C apoA-II, which has the circularized pyroglutamic acid residue, was calculated to be 8721 Da. The presence of glutamine, alanine, aspartic acid, glycine, glutamic acid and aspartic acid, identified as minor amino acid residues at the first to sixth cycle of sequencing, respectively, supported this speculation (results not shown).

ApoA-II is the second major protein of HDL synthesized mainly by the liver in mice [32]. Analysis of the protein and DNA sequences of the mouse apoA-II gene [4] have revealed that the primary translational product of apoA-II mRNA consists of 102 amino acids including an 18-residue-long signal peptide, a 5-residue-long propeptide (Ala-Leu-Val-Lys-Arg), and a C-terminal lysine residue [32]. Several isoforms of apoA-II including proisoform of apoA-II have been detected in the human plasma, lymph and HepG2 cell media [34,35], but pro-apoA-II could be identified by its markedly different pI from that of the major isoform of mature apoA-II. This difference was produced by the lysine and arginine residues (in mice) and two arginine residues (in humans) at the cleavage site of propeptides. ApoA-II synthesized by HepG2 cells undergoes various intra- and extra-cellular modification [36]. The pro-segment is cleaved intra- and extra-

cellularly [36,37] by a thiol protease exhibiting cathepsin B-like activity in humans [38]. The propeptide might play a role in the translocation of newly synthesized protein in the cell [39]. However, the function of the propeptide of apoA-II remains unknown. Pro-apoA-II has been detected in very small quantities in human plasma [34] and was first demonstrated in mice in this study.

The nucleation-dependent polymerization model of amyloid proteins suggests that amyloid-fibril deposition in almost all types of amyloidosis occurs only in the later stage of life because of the long lag time for nucleation of amyloid proteins [13]. Several substances associated with amyloid deposits are considered to influence the nucleation of amyloid-fibril formation. Elucidation of the mechanism involved in the formation of the nucleus that induces polymerization of apoA-II would be useful for understanding the pathogenesis of mouse senile amyloidosis. The lipid-binding domain composed of four amphipathic α -helices has been localized at the C-terminal region of apoA-II and lipid binding in the HDL particle increases the α -helices [40]. However, in the amyloid-containing tissues of old mice, apoA-II protein is released from HDL particles and polymerized to amyloid fibrils with a β -pleated structure. Amyloidogenic type C apoA-II has glutamine instead of proline at the fifth position. The finding that an amino acid substitution in the N-terminal region which has no specific structure [41] accelerates amyloid-fibril formation suggested the importance of the N-terminal region of peptides for amyloidogenesis. Analysis of the secondary structure [42] of pro-apoA-II revealed that addition of the five-amino-acid-residue propeptide extends the N-terminal non-structural region. These findings suggest that pro-apoA-II has greater amyloidogenicity than mature apoA-II protein. We detected aggregated proteins which migrated in smears at the top of gels by PAGE of purified pro-apoA-II, but not in that of mature apoA-II (Figures 1 and 4). Our finding that a specific antibody reacts with pro-apoA-II suggests that attachment of propeptide induces a considerable structural change to apoA-II protein. The involvement of the N-terminal region of amyloid protein in fibrilization was also suggested for AA amyloid proteins [43].

ApoA-II protein is exclusively synthesized in the liver in mice, and propeptide is cleaved intra- and extra-cellularly. Thus pro-apoA-II may be abundant in the amyloid fibrils in the liver because (1) newly synthesized pro-apoA-II may be trapped by the amyloid deposits around the hepatic cells before capture and entry into HDL particles in the plasma, or (2) pro-apoA-II in the plasma may be polymerized more preferentially than mature apoA-II and is concentrated in the amyloid fibrils. Compared with the liver, the high concentration of pro-apoA-II in the amyloid-fibril fraction from the spleen, in which apoA-II is not synthesized, supported the higher amyloidogenicity of pro-apoA-II. Since the yields of the extraction procedure were unknown, the absolute levels have not been determined. However, the amyloid-fibril fraction contained almost 10-fold more pro-apoA-II protein than the serum. Although the concentration of pro-apoA-II in serum was not different between the amyloidogenic R1.P1-*Apoa2^c* and amyloid-resistant SAMR1 strains, the metabolism of pro-apoA-II might be an important factor in the initiation and development of senile amyloidosis.

In Alzheimer's-disease brain, heterogeneity in the C-terminus of β -amyloid protein was revealed by sequencing and immuno-histochemical analysis. The two-amino-acid-longer protein, A β 42, which has a greater propensity to aggregate, might initiate nucleation followed by elongation by the shorter A β 40 [25,26]. The production of A β 42 may be a critical event in Alzheimer's disease [43]. In this study, we isolated pro-apoA-II protein from

amyloid deposition in murine senile amyloidosis. The determination of the physical and structural properties of type C pro-apoA-II may help clarify the mechanism of initiation and elongation of amyloid fibril proteins of other types of amyloidosis.

We are grateful to Dr. A. Okumura and Dr. M. Yamaguchi at Shimadzu Corporation for helpful comments, and E. Deguchi and S. Yasuoka for technical assistance. This work was supported in part by a grant from the Ministry of Health and Welfare of Japan and Grant-in-Aid for Scientific Research (C) from Ministry of Education of Japan.

REFERENCES

- 1 Sipe, J. D. (1992) *Annu. Rev. Biochem.* **61**, 947–975
- 2 Higuchi, K. and Takeda, T. (1986) in *Amyloidosis* (Marrink, J. and Van Rijswijk, M. H., eds.), pp. 283–290, Martinus Nijhoff Publishers, Dordrecht
- 3 Higuchi, K., Yonezu, T., Kogishi, K., Matsumura, A., Takeshita, S., Higuchi, K., Kohno, A., Matsushita, M., Hosokawa, M. and Takeda, T. (1986) *J. Biol. Chem.* **261**, 12834–12840
- 4 Yonezu, T., Higuchi, K., Tsunawasa, S., Takagi, S., Sakiyama, F. and Takeda, T. (1986) *FEBS Lett.* **203**, 149–52
- 5 Higuchi, K., Naiki, H., Kitagawa, K., Hosokawa, M. and Takeda, T. (1991) *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **60**, 231–238
- 6 HogenEsch, H., Niewold, T. A., Higuchi, K., Tooten, P. C., Gruys, E. and Radl, J. (1993) *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **64**, 37–43
- 7 Shimizu, K., Morita, H., Niwa, T., Maeda, K., Shibata, M., Higuchi, K. and Takeda, T. (1993) *Acta Pathol. Jpn.* **43**, 215–221
- 8 Higuchi, K., Kitagawa, K., Naiki, H., Hanada, K., Hosokawa, M. and Takeda, T. (1991) *Biochem. J.* **279**, 427–433
- 9 Naiki, H., Higuchi, K., Shimada, A., Takeda, T. and Nakakuki, K. (1993) *Lab. Invest.* **68**, 332–337
- 10 Higuchi, K., Naiki, H., Kitagawa, K., Kitado, H., Kogishi, K., Matsushita, T. and Takeda, T. (1995) *Lab. Invest.* **72**, 75–82
- 11 Higuchi, K., Wnag, J., Kitagawa, K., Matsushita, T., Kogishi, K., Naiki, H., Kitado, H. and Hosokawa, M. (1996) *J. Gerontol.* **51A**, B295–B302
- 12 Naiki, H., Higuchi, K., Nakakuki, K. and Takeda, T. (1991) *Lab. Invest.* **65**, 104–110
- 13 Jarrett, J. T. and Lansbury, P. J. (1993) *Cell* **73**, 1055–1058
- 14 Bessen, R. A., Kocisko, D. A., Raymond, G. J., Nandan, S., Lansbury, P. T. and Caughey, B. (1995) *Nature (London)* **375**, 698–700
- 15 Niewold, T. A., Hol, P. R., van Andel, A. C., Lutz, E. T. and Gruys, E. (1987) *Lab. Invest.* **56**, 544–549
- 16 Ganowiak, K., Hultman, P., Engstrom, U., Gustavsson, A. and Westermark, P. (1994) *Biochem. Biophys. Res. Commun.* **199**, 306–312
- 17 Kelly, J. T. and Lansbury, Jr., P. T. (1994) *Amyloid* **1**, 186–205
- 18 Pepys, M. B., Dyck, R. F., de Beer, F. C., Skinner, M. and Cohen, A. S. (1979) *Clin. Exp. Immunol.* **38**, 284–293
- 19 Inoue, S. and Kisilevsky, R. (1996) *Lab. Invest.* **74**, 670–682
- 20 Snow, A. D., Willmer, J. and Kisilevsky, R. (1987) *Lab. Invest.* **57**, 687–698
- 21 Kisilevsky, R. (1990) *Lab. Invest.* **63**, 589–591
- 22 Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E. and Ikeda, K. (1991) *Brain Res.* **541**, 163–166
- 23 Kindy, M. S., King, A. R., Perry, G., de Beer, M. C. and de Beer, F. C. (1995) *Lab. Invest.* **73**, 469–475
- 24 Miyata, T., Inagi, R., Iida, Y., Sato, M., Yamada, N., Oda, O., Maeda, K. and Seo, H. (1994) *J. Clin. Invest.* **93**, 521–528
- 25 Fukumoto, H., Asami, O. A., Suzuki, N., Shimada, H., Ihara, Y. and Iwatsubo, T. (1996) *Am. J. Pathol.* **148**, 259–265
- 26 Jarrett, J. T., Berger, E. P. and Lansbury, P. J. (1993) *Ann. N.Y. Acad. Sci.* **695**, 144–148
- 27 Pras, M., Zucker-Franklin, D., Rimon, A. and Franklin, E. C. (1969) *J. Exp. Med.* **130**, 777–791
- 28 Naiki, H., Higuchi, K., Hosokawa, M. and Takeda, T. (1989) *Anal. Biochem.* **177**, 244–249
- 29 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- 30 Swank, R. T. and Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477
- 31 Higuchi, K., Matsumura, A., Honma, A., Takeshita, S., Hashimoto, K., Hosokawa, M., Yasuhira, K. and Takeda, T. (1983) *Lab. Invest.* **48**, 231–240
- 32 Kunisada, T., Higuchi, K., Aota, S., Takeda, T. and Yamagishi, H. (1986) *Nucleic Acids Res.* **14**, 5729–5740
- 33 LeBoeuf, R. C., Puppione, D. L., Schumaker, V. N. and Lusis, A. J. (1983) *J. Biol. Chem.* **258**, 5063–5070
- 34 Lackner, K. J., Edge, S. B., Gregg, R. E., Hoeg, J. M. and Brewer, H. B. J. (1985) *J. Biol. Chem.* **260**, 703–706

-
- 35 Remaley, A. T., Wong, A. W., Schumacher, U.K., Meng, M. S., Brewer, H. J. and Hoeg, J. M. (1993) *J. Biol. Chem.* **268**, 6785–6790
- 36 Hussain, M. M. and Zannis, V. I. (1990) *Biochemistry* **29**, 209–217
- 37 Gordon, J. I., Budelier, K. A., Sims, H. F., Edelstein, C., Scanu, A. M. and Strauss, A. W. (1983) *J. Biol. Chem.* **258**, 14054–14059
- 38 Gordon, J. I., Sims, H. F., Edelstein, C., Scanu, A. M. and Strauss, A. W. (1984) *J. Biol. Chem.* **259**, 15556–15563
- 39 Folz, R. J. and Gordon, J. I. (1986) *J. Biol. Chem.* **261**, 14752–14759
- 40 De-Coen, J., Deboeck, M., Delcroix, C., Lontie, J. F. and Malmendier, C. L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5669–5672
- 41 Folz, R. J. and Gordon, J. I. (1987) *J. Biol. Chem.* **262**, 17221–17230
- 42 Rost, B. (1996) *Methods Enzymol.* **266**, 525–599
- 43 Gonnerman, W., Elliott-Bryant, R., Carreras, I., Sipe, J. D. and Cathcart, E. S. (1995) *J. Exp. Med.* **181**, 2249–2252
- 44 Suzuki, N., Cheung, T. T., Cai, X.-D., Okada, A., Otvos, Jr., L., Eckman, C., Golde, T. E. and Youkin, S. G. (1994) *Science* **264**, 1336–1340
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Received 4 December 1996/11 March 1997; accepted 19 March 1997