Mouse Senile Amyloid Deposition Is Suppressed by Adenovirus-Mediated Overexpression of Amyloid-Resistant Apolipoprotein A-II

Takuya Chiba,*[§] Kumiko Kogishi,* Jing Wang,*
Chen Xia,* Takatoshi Matsushita,*
Jun-ichi Miyazaki,[†] Izumu Saito,[‡]
Masanori Hosokawa,* and Keiichi Higuchi[§]

From the Field of Regeneration Control,* Institute for Frontier Medical Science, Kyoto University, Kyoto; the Department of Nutrition and Physiological Chemistry,[†] Osaka University Medical School, Osaka; the Laboratory of Molecular Genetics,[‡] Institute of Medical Science, University of Tokyo, Tokyo; and the Department of Aging Angiology,[§] Research Center on Aging and Adaptation, Shinshu University School of Medicine, Matsumoto, Japan

Apolipoprotein A-II (apoA-II), the second most abundant apolipoprotein of serum high density lipoprotein, deposits as an amyloid fibril (AApoAII) in old mice. Mouse strains with a high incidence of senile amyloidosis have the type C apoA-II gene (*Apoa2^c*), whereas the strains with a low incidence of amyloidosis have the type B apoA-II gene (*Apoa2^b*). In this study, to investigate whether the type B apoA-II protein inhibits the extension of amyloid fibrils, we constructed an adenovirus vector bearing the *Apoa2^b* cDNA (Adex1CAT4*Apoa2^b*), which is expressed under the control of a hepatocyte-specific promoter. The mice were infected with Adex1CAT4*Apoa2^b* before induction of amyloidosis by the injection of AApoAII amyloid fibril seeds. Compared with the mice infected with the control virus, amyloid deposition was suppressed significantly in the mice infected with Adex1CAT4*Apoa2^b*. Fluorometry using thioflavine T also revealed that AApoAII fibril extension was inhibited by the addition of type B apoA-II *in vitro*. Thus, we propose that *Apoa2^b* contributes as an active inhibitor of amyloid fibril extension and overexpression of amyloid-resistant gene variant may be an attractive therapeutic target in amyloidosis. (Am J Pathol 1999, 155:1319–1326)

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.¹ ApoA-II, an apolipoprotein in serum high-density lipoproteins (HDL), is a precursor of murine senile

amyloid fibrils (AApoAII) that was first isolated from the Senescence-Accelerated Mouse P1 (SAMP1)^{2,3} and was later found to be present universally in mice.^{4–6} Three variants of apoA-II protein (types A, B, and C) with different amino acid substitutions at four positions have been found in inbred strains of mice.⁷ 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 the apoA-II gene showed that early deposition of AApoAII is linked to *Apoa2^c*.^{7,8}

Previously, using the F1,F2 and backcrossed mice with the variants, we found that early deposition of AApoAII was linked to *Apoa2^c* and that there was much heavy amyloid deposition in mice homozygous for type C apoA-II.^{7,8} Severe spontaneous senile amyloid deposition was also induced in a congenic strain of mice (R1.P1-*Apoa2^c* mice) that had the amyloidogenic *Apoa2^c* transferred from SAMP1 on the genetic background of the SAMR1,^{9,10} but not in R1.P1-*Apoa2^{b/c}* mice having both type B and C apoA-II heterozygously.^{10,11} This suggested that type B apoA-II could inhibit AApoAII amyloidosis.

Nucleation-dependent polymerization is postulated to be a model that explains well the kinetics of fibrilization of amyloid proteins in murine senile amyloidosis (apoA-II protein), Alzheimer's disease (A β protein), scrapie (Prion protein), secondary amyloidosis (AA protein), and familial amyloid polyneuropathy (transthyretin).^{11–15} This model consists of two phases, ie, nucleation and extension phases. Nucleus formation requires a series of association steps of monomers representing the rate-limiting step in amyloid fibril formation. Once the nucleus has been formed, further addition of monomers becomes thermodynamically favorable, resulting in rapid extension of amyloid fibrils. Kinetic analysis of amyloid-fibril polymerization using the amyloid-fibril-specific fluorescence of thioflavine T revealed that type C apoA-II monomers

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Address reprint requests to Dr. K. Higuchi, Department of Aging Angiology, Research Center on Aging and Adaptation, Shinshu University School of Medicine, Matsumoto 390-8621, Japan. E-mail: khiguchi@sch.md.shinshu-u.ac.jp.

attach to the ends of existing fibrils (extension phases) and produce amyloid fibrils *in vitro*.¹⁶ Using mouse senile amyloidosis, we previously showed that the nucleation-dependent polymerization occurs *in vivo*, and demonstrated that nucleation-dependent amyloid fibril extension was suppressed significantly in the mice heterozygous for the type B and type C apoA-II.¹¹ These findings indicated that type B apoA-II might inhibit the pathogenesis of AApoAII amyloidosis.

In this study, to evaluate the inhibitory activity of *Apoa2^b* protein on the extension reaction of fibril conformation-dependent polymerization of AApoAII amyloidosis, we constructed a recombinant adenovirus vector harboring the *Apoa2^b* cDNA under control of the hepatocyte-specific CAG promoter.¹⁷ Here, we report that the type B apoA-II protein can effectively inhibit the amyloid deposition induced by the infusion of AApoAII amyloid fibrils used as fibril seeds in senile amyloidosis in the congenic mouse strain, R1. P1-*Apoa2^c* mice.^{10,18}

Materials and Methods

Animals

R1.P1-*Apoa2^c* mice^{9,10} were maintained in our laboratory by sister-brother mating. All mice used in this study (except for amyloid protein isolation) were adult males between 2 and 3 months old, 24 to 32 grams in weight, and were fed a regular chow diet (CE-2, Nihon CLEA, Tokyo) and tap water *ad libitum*. For the mRNA analysis and grading of amyloid deposition, mice were killed by cardiac puncture under diethyl ether anesthesia. Blood samples were collected from the tail vein under diethyl ether anesthesia periodically after adenovirus infection. After left to clot at room temperature, the serum was separated by centrifugation at 4°C and analyzed for the serum apolipoprotein and lipid concentrations.

Construction and Purification of Recombinant Adenoviruses

Adex1CAT*Apoa2^b* and Adex1w1 are E1A-, E1B-, and E3-deleted replication-defective adenovirus vectors derived from adenovirus type 5. Adex1CAT*Apoa2^b* contains the mouse *Apoa2^b* cDNA¹⁹ driven by the cytomegalovirus (CMV) immediate early gene enhancer and chicken β -actin promoter and rabbit β -globin poly(A) signal¹⁷ was created as follows. *MscI* and *PstI* digested full length of mouse *Apoa2^b* cDNA was blunt-ended and cloned into *SmaI* site of the E1-deleted region of expression cassetted cosmid vector, pAdex1CAwt. The resulting cosmid, pAdex1CAT*Apoa2^b*, was cotransfected to the HEK293 (derived from human embryonic kidney) cell line with an *EcoT22I*-digested DNA-TPC (from Ad5dlx)²⁰ to generate the replication-defective adenovirus Adex1CAT*Apoa2^b*. Purified virus stocks were prepared through CsCl step gradient centrifugation as described previously.^{20–22}

Titration of Recombinant Adenoviruses

A total of 50 μ l of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), (5% FBS DMEM) was dispensed into each well of a collagen type-I coated 96-well tissue culture plate. Then eight rows of threefold serial dilutions of the virus starting from $1:10^{-4}$ dilution were prepared. A total of 3×10^5 of HEK293 cells in 50 μ l of 5% FBS DMEM were added to each well. The plate was incubated at 37°C in 5% CO₂ in air, and 50 μ l of 5% FBS DMEM was added to each well every 3 days. Twelve days later, the end point of the cytopathic effect was determined by microscopy, and the 50% tissue culture infectious dose (TCID₅₀) was determined. The titer of recombinant adenovirus vectors was calculated as a plaque forming unit (pfu) from TCID₅₀.²¹

Adenovirus Injection of the Mice

An appropriate volume (100–300 μ l) of the purified recombinant adenovirus containing 3×10^8 or 1.2×10^9 pfu was infused into the tail vein of the mice.

Isolation of Amyloid Fibrils and Induction of Amyloidosis

The amyloid-fibril fraction was isolated as a water suspension from the liver and spleen of an 18-month-old R1.P1-*Apoa2^c* mouse as described by Pras et al.²³ The isolated amyloid-fibril fraction was further purified by ultracentrifugation as described previously.²⁴ Pellets after ultracentrifugation were resuspended in distilled water by mixing thoroughly with an ultradisperser (Ultra-Turrax T25, Janke and Kunlel GmbH, Staufen, Germany). For amyloidosis induction, amyloid fibrils (AApoAII) were diluted in distilled water to a concentration of 1 mg/ml. Before injection, AApoAII amyloid fibril suspensions were sonicated for 30 seconds 3 times on ice. Then 0.1 mg of amyloid fibrils was injected into the tail vein 3 days after infection with adenovirus vector.

mRNA Analysis

Total RNA was extracted from mouse livers using the RNeasy Midi Kits (Qiagen Inc., Valencia, CA). After denaturation, 10 μ g of total RNA was electrophoresed in 1% formaldehyde-agarose gel, transferred to a nylon membrane (Gene Screen Plus, NEN Products, Boston, MA), hybridized with the ³²P-labeled mouse *Apoa2^b* cDNA probes, and exposed to KODAK XAR film at –80°C. Filters were stripped and rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a control.

Apolipoprotein Quantitation

The serum concentrations of apoA-I and apoA-II were determined by a quantitative immunoblotting method.¹⁰ Serum aliquots (20 nl) were subjected to a 15 to 25%

gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 15 mA for 2.5 hours. After electrophoresis, the proteins were electrophoretically transferred to a polyvinylidene difluoride (PDVF) membrane (Bio-Rad Laboratories, Richmond, CA) using a semidry apparatus (Nihon Eido, Tokyo) at 150 mA for 2 hours. The blot was then incubated with primary antibody solution, either with monospecific rabbit anti-mouse apoA-I (diluted 1:2000) and apoA-II antisera (diluted 1:4000)^{2,25} in 1% skim milk in PBS containing 0.1% Tween-20 (T-PBS) for 1 hour at room temperature with gentle shaking. Blots were washed in T-PBS and incubated for 1 hour with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Daiichi Pure Chemicals, Tokyo) solution in T-PBS (1:500). Apolipoproteins were detected by the enhanced chemiluminescence (ECL) method (Amersham International, Buckinghamshire, UK) and quantitated using a densitometric image analyzer (Molecular Imager, Bio-Rad).

Serum Lipid and Lipoprotein Levels and HDL Size Distribution

Serum concentrations of total cholesterol were determined using enzymatic procedures employing colorimetric end points (Cholesterol C-Test, Wako Pure Chemical Industries, Osaka, Japan). HDL cholesterol was estimated according to a modified heparin-manganese precipitation procedure (HDL-Cholesterol-Test, Wako). Non-denaturing gradient PAGE was performed to determine the size distribution of HDL. Gels containing a 4 to 15% linear polyacrylamide gradient were electrophoresed in 25 mmol/L Tris, 192 mmol/L glycine. Before electrophoresis, serum samples (3 μ l) were stained for lipids by incubation for 3 hours at room temperature with 2.4 μ l freshly prepared Sudan Black B dye solution (5 parts 1% Sudan Black B in ethyleneglycol to 3 parts 40% sucrose). Electrophoresis was carried out at 15 mA for 2 hours.

Detection of Amyloid Deposition

Amyloid deposition was identified by green birefringence in Congo red-stained sections under a polarizing microscopy. The amyloid fibril protein AApoAII, was also immunohistochemically identified by the avidin-biotinylated horseradish peroxidase complex (ABC) method with specific antiserum against AApoAII as described.¹⁰ The Amyloid Index (AI) was graded 0 (no amyloid) to 4 (heaviest deposits) from the degree of AApoAII deposits in the liver, spleen, skin, heart, stomach, intestines, tongue, and kidneys in sections stained with Congo red. One observer who had no information about the tissue examined each tissue and assigned it an AI grade. AI was averaged for each mouse.

In Vitro AApoAII Fibril Extension Assay by Fluorescence Spectroscopy

The type C apoA-II monomer was purified from crude AApoAII as described previously.²⁴ The type B apoA-II

monomer was purified from the serum HDL in the SAMR1 mice with type B apoA-II, as described previously.¹¹ AApoAII amyloid fibril extension in the presence of type B apoA-II was assayed by the fluorometric method, using the fluorescent dye thioflavine T (ThT).^{16,24} The reaction mixture was prepared on ice and contained 25 ng/ μ l of sonicated AApoAII and 200 μ mol/L type C apoA-II protein in 25 mmol/L phosphate buffer, pH 7.5, 300 mmol/L urea. Type B apoA-II protein was added to yield the reaction mixture containing type B apoA-II 0, 1, 10, and 30% of type C apoA-II. The reaction mixture (40 μ l) was put into Eppendorf tubes and the reaction was initiated at the same time in a heat block set at 37°C. At each designated incubation time (0 to 240 minutes), the reaction of one corresponding tube was stopped by cooling on ice and the preparation was subjected to fluorescence spectroscopy, using a spectrofluorophotometer RF-1500 (Shimadzu Co., Tokyo). The assay volume was 1.0 ml with excitation at 450 nm and emission at 482 nm.²⁴ Excitation and emission slits were set at 5 and 10 nm, respectively. The reaction mixture contained 250 nmol/L ThT (Nakalai Tesque, Kyoto, Japan) and 50 mmol/L glycine-NaOH buffer, pH 9.0.

Statistical Analysis

A Statview software package (Abacus Concepts, Berkeley, CA) was used to analyze the data. All data are presented as the mean $\pm$ SD. Student's *t*-test was used for all data except for AI. The AI of AApoAII deposition was assigned consecutive integral values from 0 to 4 and compared by Mann-Whitney's *U* test.

Results

Apoa2^b Gene Transfer to the Liver and its Expression

The recombinant adenovirus Adex1CATApoa2^b was constructed by homologous recombination between the expression cosmid cassette and the parental adenovirus type 5 genome, as described in Materials and Methods. The transcriptional orientation of the *Apoa2^b* expression cassette is opposite to the original orientation of E1A and E1B gene. Figure 1A depicts the construct of the expression cassette in the adenoviral vector. Adex1w1 is the control virus, which has no recombinant protein gene. Total RNA was prepared from the infected liver of R1.P1-*Apoa2^c* mice at Days 0 (before virus injection), 1 (24 hours), 3 (72 hours), and 28 after injection of 1.2×10^9 pfu of Adex1CATApoa2^b or Adex1w1 via tail vein. Then it was subjected to RNA blot hybridization analysis using mouse *Apoa2^b* cDNA as a probe. Two major transcripts of 0.65 and 0.5 kb were detected in the liver infected with Adex1CATApoa2^b. In contrast, a single transcript of 0.5 kb was detected in the liver infected with Adex1w1 (Figure 1B). The 0.5-kb transcript was endogenous *Apoa2^c* mRNA and the 0.65-kb transcript was derived from Adex1CATApoa2^b. The *Apoa2^b* mRNA derived from Adex1CATApoa2^b had rabbit β -globin 3' untranslated

Figure 1. Construct of recombinant adenovirus, which has *Apoa2^b* cDNA (*Adex1CAT^bApoa2^b*) and apoA-II mRNA expression in the liver infected with adenovirus vector. **A:** Schematic representation of recombinant adenovirus vector. *Adex1CAT^bApoa2^b* and *Adex1w1* are E1A-, E1B-, and E3-deleted replication-defective adenovirus vectors derived from adenovirus type 5. *Adex1CAT^bApoa2^b* contains the mouse *Apoa2^b* cDNA driven by the cytomegalovirus (CMV) immediate early gene enhancer and chicken β -actin promoter and rabbit β -globin poly(A) signal (CAG promoter). *Adex1w1* is the control virus, which does not encode a recombinant protein. **B:** Expression of Apo2 mRNA after infection with adenovirus vector. Total RNA was prepared from the infected liver of the mice at Days 0 (before), 1, 3, and 28 after injection of 1.2×10^9 pfu of *Adex1CAT^bApoa2^b* or *Adex1w1* via tail vein. Then it was subjected to RNA blot hybridization using mouse *Apoa2^c* cDNA as a probe. The 0.5-kb transcript was endogenous *Apoa2^c* and 0.65 kb was *Apoa2^b* mRNA derived from *Adex1CAT^bApoa2^b*. *Apoa2^b* mRNA derived from *Adex1CAT^bApoa2^b* had rabbit β -globin 3' untranslated sequences, and was about 0.15 kb longer than endogenous *Apoa2^c* mRNA.

sequences, and was 0.15 kb longer than endogenous *Apoa2^c* mRNA. *Apoa2^b* mRNA expression increased to twofold more than endogenous *Apoa2^c* mRNA at 24 hours after injection of *Adex1CAT^bApoa2^b*. *Apoa2^b* mRNA expression lasted until Day 7 (data not shown). Endogenous *Apoa2^c* mRNA expression was not affected by the infection with *Adex1CAT^bApoa2^b* or *Adex1w1*.

Apoa2^b Overexpression Modulates the Apolipoprotein Concentrations and Lipid Metabolism

No significant differences were observed in apoA-I concentrations between the *Adex1CAT^bApoa2^b* and *Adex1w1* infected mice. However, the concentrations of serum apoA-II in the mice infected with *Adex1CAT^bApoa2^b* were significantly higher than those in the *Adex1w1*-infected

Figure 2. Immunoblot analysis of apoA-I and apoA-II in the serum of mice infected with adenovirus vectors. Expression of apoA-I (upper band) and apoA-II (lower band) in the serum of mice infected with *Adex1CAT^bApoa2^b* or *Adex1w1*. The concentrations of apoA-II were significantly higher at Day 3.

mice at Day 3 (Figure 2). The concentration of serum apoA-II in the *Adex1CAT^bApoa2^b*-infected mice was increased at Day 1 and reached its peak at Day 3 (Figure 3). Because our antibodies did not distinguish between type B and type C apoA-II, we could not estimate the exact concentration of type B apoA-II in the serum derived from *Adex1CAT^bApoa2^b*. However, compared to the control mice, the concentrations of apoA-II were more than twice as high at Day 3, and this increase was attributed to the overexpression of type B apoA-II mRNA. Then we considered that more than half of total apoA-II were type B apoA-II at Day 3. Significant differences in apoA-II concentrations were observed from Day 1 to Day 7. Like the apoA-II concentration, both total cholesterol and HDL cholesterol were increased and peaked at Day 3 in the mice infected with *Adex1CAT^bApoa2^b* (Figure 4). Although these lipid concentrations were slightly increased in the mice infected with control virus, the concentrations were significantly lower at Days 1, 3, and 7 than those in the mice infected with *Adex1CAT^bApoa2^b*. Serum HDL resolved by nondenaturing gradient PAGE revealed a marked difference in size, with larger HDL particle sizes in the mice infected with *Adex1CAT^bApoa2^b* at Day 3 (Figure 5).

Figure 3. Serum apoA-II concentration in the mice infected with adenovirus vectors. Mice were infected with *Adex1CAT^bApoa2^b* or *Adex1w1*. The difference was significant from Day 1 to Day 7 ($*P < 0.0001$). The number of mice used for the experiments is shown at the bottom of the figure. Error bars are means $\pm$ SD.

Figure 4. Serum concentration of lipids in mice infected with adenovirus vectors. As the serum lipid parameters in the mice infected with Adex1CATApoa2^b or Adex1w1, total cholesterol (A) and HDL cholesterol (B) were analyzed. Differences at Days 1, 3, and 7 between the two groups was significant (**P* < 0.05, ***P* < 0.01). The number of mice used for the experiments are shown at the bottom of the figure. Error bars are mean ± SD.

AApoAll Amyloid Deposition Is Suppressed by Adex1CATApoa2^b Infection

One month after injection of AApoAll in the mice infected with Adex1w1 had mild deposition on the whole body particularly on the small intestine, whereas the mice infected with Adex1CATApoa2^b had a slight deposition only on the small intestine and tongue (Figure 6). The AI was graded 0 to 4 according to the degree of AApoAll deposits in the liver, spleen, skin, heart, stomach, intestines, tongue, and kidneys, then averaged. The AI indicated a significant inhibition of amyloid deposition in the mice infected with Adex1CATApoa2^b (Figure 7). The lower AI in the mice infected with the larger dose of virus implied that the inhibitory effect of type B apoA-II was dose-dependent.

Figure 5. Serum HDL size in mice infected with adenovirus vectors. Serum HDL was stained with Sudan Black B and subjected to nondenaturing gradient PAGE (4–15%). The results are the representative of three separate experiments.

AApoAll Amyloid Fibril Extension Was Inhibited by Type B apoA-II

As shown in Figure 8, when AApoAll was incubated with type C apoA-II in the presence or absence of type B apoA-II, a linear increase in the fluorescence was observed until 240 minutes. When AApoAll and type C apoA-II were incubated with increasing concentrations of type B apoA-II, the initial rate of fibril extension showed a dose-dependent decrease. In the presence of 1, 10, and 30% type B apoA-II in the reaction mixture, the extension rate of AApoAll fibril formation was inhibited to about 95, 60, and 45% of the extension rate without type B apoA-II, respectively, indicating the strong inhibition of amyloid fibril extension by a small amount of type B apoA-II.

Discussion

In the present study, we showed that the amyloid deposition in mouse senile amyloidosis was suppressed by adenovirus-mediated overexpression of the amyloid-resistant gene variant type B apoA-II *in vivo*. We also showed that type B apoA-II suppressed AApoAll amyloid fibril extension *in vitro*.

To investigate whether type B apoA-II inhibits the extension of AApoAll fibrils *in vivo*, and if so, for use in basic research in amyloidosis gene therapy, we have constructed a replication-defective adenovirus vector that contains murine *Apoa2^b* cDNA under the control of hepatocyte-specific CAG promoter (Adex1CATApoa2^b). R1.P1-*Apoa2^c* mice were infected with Adex1CATApoa2^b or Adex1w1 at two doses, 1.2×10^9 pfu and 3×10^8 pfu. To examine the effects of the overexpression of type B apoA-II protein, we measured the concentrations of serum apolipoproteins and lipids. The higher dose of

Adex1CATApoa2^b

Adex1w1

Figure 6. Congo red staining of amyloid depositions on the small intestine. Amyloid deposits were identified by green birefringence under a polarizing microscopy. Mild deposition was observed 1 month after injection of AApoAII in the mouse infected with Adex1w1, but no deposition was observed in the mice infected with Adex1CATApoa2^b. Scale bar, 100 μ m.

Adex1CATApoa2^b infection did not modulate the concentration of serum apoA-I, but the serum apoA-II and lipid concentrations, and HDL particle size were increased from Day 1 to Day 7 compared to those in the mice infected with Adex1w1. These results were consistent with those observed in the transgenic mice that overexpressed the mouse apoA-II gene.²⁶ In the mice infected with Adex1w1, serum total and HDL cholesterol were increased from Day 1 to Day 7. Such an increase in the Adex1w1-infected mice would result from the inflammation by the adenovirus vectors. In mice, total cholesterol and free HDL cholesterol were increased in the presence of acute inflammation.²⁷ Acute phase serum amyloid A (AP-SAA) would direct the reverse cholesterol transport by HDL to the inflammatory cells during the acute inflam-

mation, and this function of AP-SAA might be the collection of macrophage-sequestered cholesterol. However, in the present study, serum total cholesterol and HDL cholesterol concentrations were significantly higher in Adex1CATApoa2^b-infected mice than in Adex1w1-infected mice from Day 1 to Day 7. This implied that newly synthesized type B apoA-II in the liver was incorporated in the HDL particles in the plasma, and circulated as type B apoA-II-rich HDL particles. This change of HDL particles produced by the increased type B apoA-II might suppress the extension of amyloid fibrils. The HDL component and its size might be critical factors for the pathogenesis of AApoAII amyloidosis, because such changes might modify the affinity of the HDL to the receptor and/or its stability. However, this does not fully explain the fibril extension inhibitory effect of overexpression of type B apoA-II, because (i) in the mouse strains homozygous for the type A apoA-II (C57BL/6J, B10.BR and AKR/J) the size and concentration of HDL particles are decreased, but amyloid deposition is much lower in these strains than

Figure 7. Amyloid index (AI) of amyloid deposition. Amyloids were identified by green birefringence in sections stained with Congo red under a polarizing microscopy. Adenovirus vectors were injected at the doses of 3×10^8 or 1.2×10^9 pfu. As a control, 0.2 ml of PBS was injected into the tail vein instead of the virus vectors. The AI indicated significant inhibition of amyloid deposition by the infection with Adex1CATApoa2^b in a dose-dependent manner. * $P < 0.0005$. The number of mice used for the AI is shown at the bottom of the figure.

Figure 8. AApoAII fibril extension kinetics in the presence of type B apoA-II in vitro. Time course of the fluorescence after the initiation of the polymerization reaction. The reaction mixture contained 25 ng/ μ l of AApoAII, 200 μ mol/L type C apoA-II, 25 mmol/L phosphate buffer, pH 7.5, 300 mmol/L urea and 0 (■); 0, 2 μ mol/L (◆; 1%), 20 μ mol/L (●; 10%) or 60 μ mol/L (▲; 30%) type B apoA-II. This is a representative pattern of three independent experiments.

in mouse strains homozygous for type C apoA-II (SAMP1, SM/J, and SJL/J),⁷ and (ii) low-titer treatment suppressed the amyloid fibril extension without any significant increase of either size or concentration of HDL (data not shown). Moreover, purified type B apoA-II could inhibit AApoAII fibril extension *in vitro*. This indicates that the HDL concentration is not important to delay the pathogenesis of AApoAII amyloidosis, and that the type B apoA-II primary structure (proline at position 5) is the critical factor inhibiting the amyloidosis. Only one-eighth of the expression of wild-type prion protein (PrP^C) was found to be sufficient to delay the onset of spontaneous neurological dysfunction involving PrP amyloid plaques in the transgenic mice overexpressing mutant PrP in familial prion protein disease.²⁸ Proline residues are β -sheet blockers in A β protein.^{29,30} Treatment of familial amyloid polyneuropathy (FAP) with thyroxine and the treatment of Alzheimer's disease with β -sheet breaker peptides have been recently reported.^{31,32} The whole type B apoA-II protein is needed to inhibit AApoAII fibril extension; whether a shorter peptide containing the region homologous to the N-terminus of type B apoA-II can effectively inhibit amyloid fibril extension remains to be examined to establish a new therapeutic strategy against the amyloidosis.

The present results suggest that the pathogenesis of amyloidosis may be delayed by overexpression of amyloid-resistant variants of amyloid precursor protein (type B apoA-II in the mouse senile amyloidosis) by the suppression of fibril extension even after the amyloid nucleus has been formed. In mouse amyloid A (AA) amyloidosis, the amyloid-resistant isoform of precursor protein, apoSAA_{CE/J}, has been shown to inhibit the pathogenesis of AA amyloidosis by inbred crossing of mice.³³ ApoSAA_{CE/J} has a different amino acid residue at position 11 from the amyloidogenic isoform of apoSAA. Protein isoforms that have one or two amino acid substitutions of a amyloid precursor protein at the crucial residues in the determination of amyloid formation might serve as an inhibitor of the fibril formation. Using an *in vitro* polymerization assay, we are currently examining three hypotheses for the inhibition of amyloid fibril extension by the type B apoA-II: (i) type B apoA-II may attach to the ends of the injected AApoAII fibrils; (ii) type B apoA-II bind to type C apoA-II and suppress fibril extension because of blocking of the active site of type C apoA-II; or (iii) type B apoA-II might disturb the AApoAII fibril conformation stability.

Adenovirus-mediated gene transfer is feasible across a broad spectrum of eukaryotic cells and in whole animals. It has also been evaluated as a vector for gene therapy in a variety of metabolic disorders, neurodegenerative disorders, and tumors.³⁴⁻³⁷ One of the advantages of adenoviral vectors is that there are few restrictions in the selection of promoters that drive the gene to be expressed. Thus, strong promoters that express foreign genes in targeted organs will reduce the amount of vector required for treatment and would be expected to prevent adverse effects of the vector. The adenovirus vector is useful for the screening of the amyloid-resistant variants for gene therapy of amyloidosis, and analysis of

the modulation factor for amyloid fibril formation to develop a new treatment for amyloidosis.

Here we showed that hepatic overexpression of *Apoa*^b resulted in a significant decrease of amyloid deposition even though the expression was transient. Type B apoA-II protein was expressed for only 7 days, but its inhibitory effect was sustained for 1 month. We considered that type B apoA-II would interact with the fibril seeds and inhibit its extension. However, because the inhibition of the amyloid fibril extension was not complete, the remaining fibril seeds might extend later. To clarify the inhibitory effect of the type B apoA-II, we must identify the characteristics of the type C apoA-II protein when it interacts with AApoAII fibrils, type C apoA-II itself and type B apoA-II, and with potential pathological molecular chaperones such as apoE during fibril formation. In this study, we demonstrated that type B apoA-II inhibited the extension phase of fibrillogenesis, but the nucleation phase is also critical for the pathogenesis of senile amyloidosis and other amyloid diseases. Thus, more investigation of the effects of type B apoA-II on the nucleation phase in the AApoAII fibril extension is needed.

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