Induction of AApoAII amyloidosis by various heterogeneous amyloid fibrils

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Abstract Preformed amyloid fibrils accelerate conformational changes of amyloid precursor proteins and result in rapid extension of amyloid fibrils in vitro. We injected various kinds of amyloid fibrils into mice with amyloidogenic apoAII gene (*Apoa2*^{C}). The most severe amyloid depositions were detected in the tissues of mice injected with mouse AApoAII(C) amyloid fibrils. Mild amyloid depositions were also detected in the tissues of mice that were injected with other types of fibrils, including synthetic peptides and recombinant proteins. However, no amyloid depositions were found in mice that were injected with non-amyloid fibril proteins. These results demonstrated that a common structure of amyloid fibrils could serve as a seed for amyloid fibril formation in vivo.

Key words: Amyloid fibril; AApoAII amyloidosis; Induction; Common structure

1. Introduction

Amyloidosis refers to a group of protein folding diseases, including currently prominent diseases such as Alzheimer's, prion diseases, and type 2 diabetes. Amyloidosis is characterized by the deposition of fine and insoluble fibrils from the polymerization of soluble proteins which undergo marked conformational changes. Amyloid fibrils have a high degree of β-sheet structure. To date, more than 30 amyloid fibril proteins have been identified [1]. Prion diseases are highly infectious and transmissible. The current widely accepted prion protein hypothesis suggests that the amyloidogenic and protease-resistant form (PrP^{sc}) of the protein propagates itself by inducing a conformational change in the normal prion protein (PrP^{c}). Nucleation-dependent polymerization or seeding is postulated to explain the propagation of prion diseases [2,3] and pathogenesis of non-prion amyloidosis [4]. However, the molecular nature of the template for fibril formation in vivo is still open for debate.

ApoA-II is the second most abundant apolipoprotein in plasma high-density lipoprotein (HDL) after apoA-I. ApoA-II amyloidosis has been found in patients with hereditary renal amyloidosis [5]. In mice, apoA-II is a precursor of amyloid fibrils (AApoAII) in senile amyloidosis [6,7]. Senescence-accelerated prone (SAMP1) mice having a variant C-type apoA-II (APOAIIC, Gln^{5} and Ala^{38}) spontaneously exhibit a high incidence of AApoAII amyloidosis with aging [8]. Senescence-accelerated resistant (SAMR1) mice with wild-type B apoA-II (APOAIIB, Pro^{5}, and Val^{38}) show few, if any, signs of AApoAII amyloidosis. R1.P1-*Apoa2*^{c} mice are a congenic strain with the amyloidogenic allele of the apoA-II gene from the SAMP1 strain on the genetic background of the SAMR1 strain [9]. These mice spontaneously exhibit a high incidence of amyloidosis and severe amyloid deposition with aging [10]. In previous studies we demonstrated that intravenous injection of AApoAII amyloid fibrils dramatically accelerated the progress of amyloidosis in the R1.P1-*Apoa2*^{c} strain suggesting the transmission of amyloid fibrils via the gastrointestinal tract from mice with severe amyloid deposition to those without [11,12]. Prion-like transmission has also been revealed in a study of inflammation-associated experimental (AA) amyloidosis [13]. Thus it has been shown that amyloid diseases may be transmissible under certain conditions.

All amyloid fibrils share some common features, including a high degree of β-sheet structure in a classical 'cross-β' pattern, a fibrillar morphology seen under electron microscopy, and the ability to bind and alter the spectroscopic properties of the heteroaromatic dyes Congo red and thioflavine T. It has been suggested that the cross-β conformation is dominated by main-chain interactions that are common to different polypeptides [14]. AA amyloidosis has been induced by transthyretine amyloid fibrils (ATTR) [15], synthetic amyloid-like fibrils [16], and modified silk [17] administered intravenously. The synergistic fibrillization of Tau and alpha-synuclein has been demonstrated [18] and the appearance of prions is enhanced by

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heterologous prion aggregates in yeast [19]. Thus the contribution of interactions or cross-talk between various amyloid fibrils and amyloid proteins to the pathogenesis of amyloidosis has been speculated.

Here we report the results of induction of AApoAII amyloidosis by various amyloid fibrils extracted from tissues or formed from recombinant protein or synthetic peptides. We found that all of these amyloid proteins accelerated AApoAII amyloidosis. Our results demonstrate that the existence of a generic structure present in many amyloid fibrils might serve as a seed for amyloidogenesis.

2. Materials and methods

2.1. Animals

Amyloidogenic R1.P1-*Apoa2*^{c} and SAMR1 mice were raised in the Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University, under standard conditions at 24 ± 2°C with a light-controlled 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 consent of the Animal Care and Use Committee of Shinshu University School of Medicine.

2.2. Isolation of amyloid fibrils from tissues

Human AA fibrils (hAA) were obtained from an autopsied thyroid of a female patient (49 years old) with rheumatoid arthritis. Human TTR fibrils (hATTR) were obtained from an autopsied heart of a male familial amyloidotic polyneuropathy (FAP) patient (86 years old) with a Val30Met TTR mutation. Two kinds of human AL fibrils (hAL) were obtained from autopsied spleens of two female patients (85 and 68 years old) with primary amyloidosis. β_2-microglobulin fibrils ($A\beta_2M$) were obtained from autopsied tendon sheaths of a patient on long-term dialysis. Mouse AApoAII(C) and AApoAII(A) fibrils were isolated from the liver of an R1.P1-*Apoa2*^{c} mouse and the intestine of a BDF1 mouse, respectively. Mouse AA fibrils (mAA) were isolated from the liver of a C57BL/6 mouse with severe spontaneous AA amyloidosis.

The various amyloid fibrils were isolated as water suspension fractions and further purified by ultracentrifugation as described previously [12].

2.3. Amyloid fibrils of recombinant proteins and synthetic peptide

α-Synuclein cDNA was cloned from human brain tissue, expressed in *Escherichia coli*, and the recombinant protein was purified. Purified α-synuclein was incubated in buffer (25 mM Tris–HCl, 1 M NaCl, pH 7.5) for 17 days at 37°C to make amyloid fibrils (Aα-synuclein). GroES was expressed in *E. coli* and purified. Amyloid fibrils were formed from GroES incubated in buffer (25 mM Tris–HCl, 1.6 M guanidine-hydrochloride, pH 7.5) for 5 days at 37°C (AGroES). Amyloid fibrils were formed from lysozyme (hen egg white lysozyme, Wako Pure Chemical Industries, Ltd., Osaka, Japan) incubated in HCl (pH 2.0) for 7 days at 65°C (ALysozyme). $A\beta_{1-40}$ (Bachem AG, Bubendorf, Switzerland) fibrils were formed after incubation in reaction mixture (50 μM $A\beta_{1-40}$, 50 mM phosphate buffer, pH 7.5, and 100 mM NaCl) for 24 h at 37°C.

2.4. Induction and detection of amyloidosis

2-month-old female R1.P1-*Apoa2*^{c} and SAMR1 mice were injected intravenously with 0.1 mg of various sonicated amyloid fibrils as described previously [12]. Control mice were injected with distilled water (DW), human albumin (Sigma-Aldrich, Tokyo, Japan), or human transthyretin (Wako Pure Chemical Industries). Mice were killed by cardiac puncture under diethyl ether anesthesia at 3, 6, and 12 months after injection. One half of each organ was stored frozen until amyloid fibril isolation. The remaining halves were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4-μm serial sections.

The intensity of amyloid deposition was determined using the amyloid index (AI) as a parameter. AI was the average of grades 0–4 in seven organs (liver, spleen, skin, heart, stomach, small intestine, and tongue) stained with Congo red after immunohistochemical confirmation of the AApoAII deposition [20].

Proteins (5 μg) in the amyloid fraction [12] were separated by 16.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a Tris–tricine buffer system and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad; Hercules, CA, USA) and immunodetected by anti-AApoAII antiserum (1:5000 dilution).

2.5. Electron microscopy

10 μl of amyloid fractions (0.4 mg/ml) and 10 μl of 2% phosphotungstic acid (pH 7.0) were mixed. Half of a carbon-coated plastic grid (Ouken, Tokyo, Japan) was immersed in this mixture for 1 s. The negatively stained samples were observed with a JEOL 1200 EX electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

2.6. Statistical analysis

The AI of AApoAII deposition in the different groups was compared with results of the Mann–Whitney *U*-test.

3. Results

3.1. Transmission electron microscopy of amyloid fibrils

We observed various types of negatively stained amyloid fibrils using transmission electron microscopy. All of the samples exhibited amyloid-characteristic unbranched fibril images (Fig. 1). The diameter and length of the various fibrils were as follows: AApoAII(C), 9.02 ± 1.25 and 439.28 ± 43.38 nm; AApoAII(A), 9.05 ± 1.28 and 87.58 ± 10.39 nm; mAA, 10.03 ± 1.30 and 90.12 ± 9.77 nm; hAA, 13.01 ± 1.17 and 380.25 ± 36.59 nm, hATTR, 12.04 ± 1.08 and 100.06 ± 20.01 nm; hAL-1, 13.01 ± 1.95 and 150.43 ± 18.45 nm; hAL-2, 13.02 ± 1.34 and 145.22 ± 16.23 nm; $A\beta_2M$, 10.01 ± 1.56 and 305.21 ± 19.56 nm; Aα-synuclein, 11.01 ± 1.07 and 401.20 ± 21.04 nm; AGroES, 12.05 ± 1.32 and 108.42 ± 13.72 nm; ALysozyme, 10.03 ± 1.20 and 441.28 ± 24.60 nm; and $A\beta_{1-40}$, 9.02 ± 1.23 and 442.06 ± 18.75 nm, respectively.

3.2. Induction of mouse AApoAII amyloidosis by mouse amyloid fibrils

3 months after treatment, AApoAII deposition was observed in all AApoAII(C)-injected mice (Fig. 2). At 6 months severe amyloid deposition was seen throughout the entire body with the exception of bone and brain parenchyma. AApoAII depositions were also induced by mAA and mouse AApoAII(A) amyloid fibrils. The intensity of amyloid depositions was significantly lower than that seen in mice 3 and 6 months after AApoAII(C) injection ($P < 0.05$ by Mann–Whitney *U*-test) (Fig. 2). mAA deposits were not detected by immunohistochemistry (data not shown).

3.3. Induction of mouse AApoAII amyloidosis by human amyloid fibrils

We detected slight AApoAII deposition in the interstitium of the myocardium, the lamina propria of the tongue (Fig. 3a, A and B), the lamina propria and submucosa of the small intestine, and the glandular portion and squamous glandular junction of the stomach in all hAA amyloid fibril-injected mice at 3 months after injection. At 6 months after injection there was severe AApoAII deposition in the tongue (Fig. 3a, D and E), and moderate AApoAII deposition in the liver, spleen, heart, skin, stomach and small intestine. AApoAII deposits were also induced by hATTR, AL, and $A\beta_2M$ fibrils. However, the degree of amyloid deposition was significantly lower than that seen in mice at 3 and 6 months after hAA injection ($P < 0.05$ by Mann–Whitney *U*-test) (Fig. 2). Immunohistochemical staining did not detect mAA deposits (Fig. 3a, C and F).

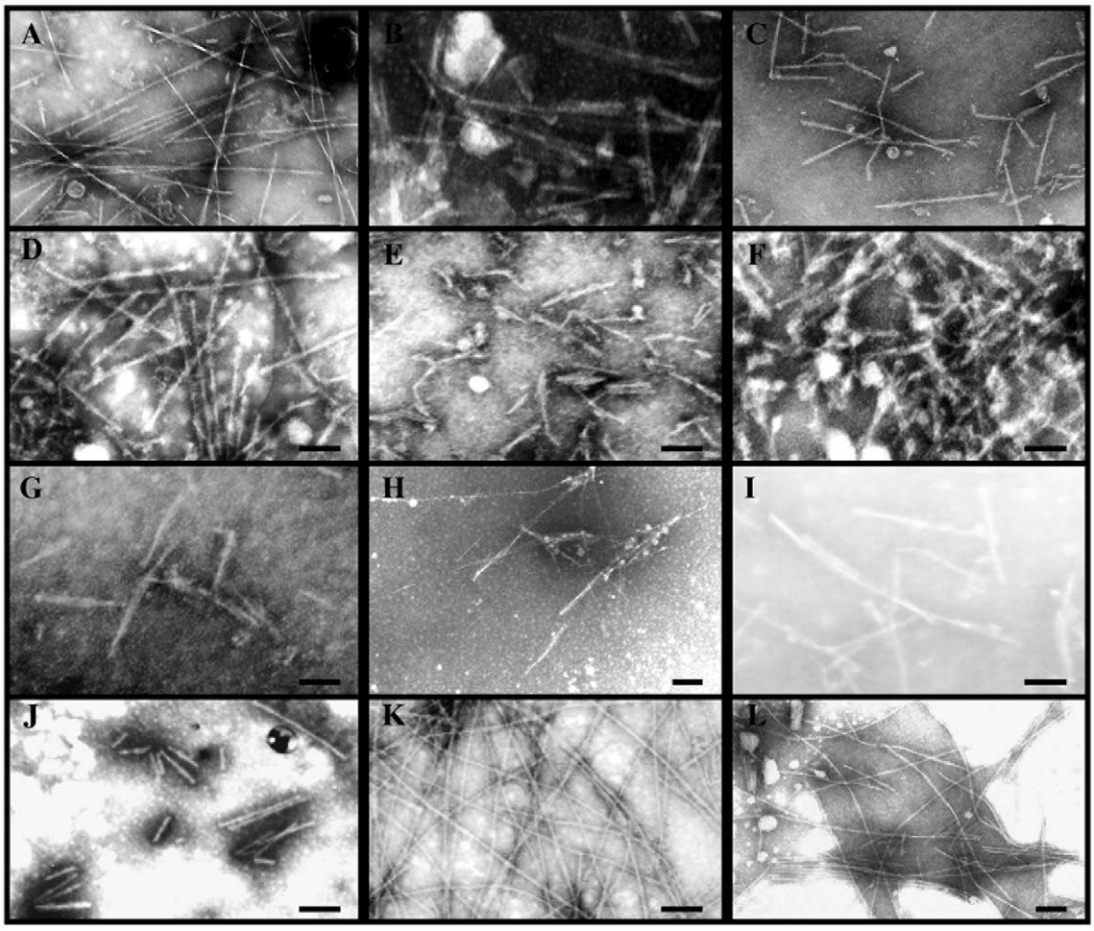

Fig. 1. Transmission electron microscopy images of amyloid fibrils. Amyloid fibrils were negatively stained. A: AApoAII(C); B: AApoAII(A); C: mAA; D: hAA; E: hATTR; F: hAL-1; G: hAL-2; H: hAβ_2M; I: Aα-synuclein; J: AGroES; K: ALysozyme; L: Aβ_{1-40} fibrils. The scale bar indicates 100 nm.

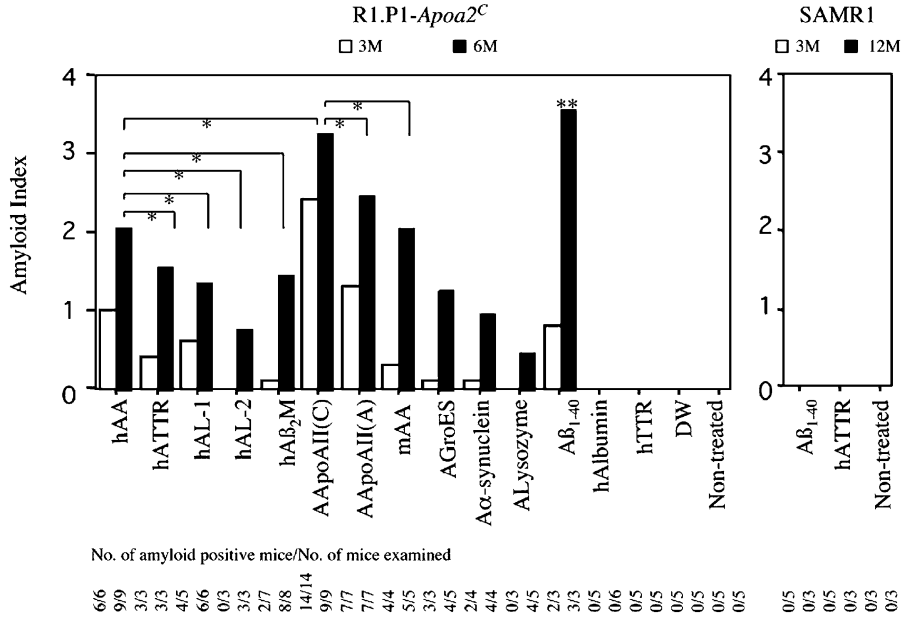

Fig. 2. Comparison of the AI in various amyloid fibrils injected into R1.P1-*Apoa2*^{c} and SAMR1 mice. In R1.P1-*Apoa2*^{c} mice, a significant difference in AI was found between the hAA, hATTR, hAL-1, hAL-2, hAβ_2M fibril groups, and the mouse AApoAII(C) and mouse AApoAII(A) or mAA fibril groups. Non-treated R1.P1-*Apoa2*^{c} mice are 5, and 8 months old. Non-treated SAMR1 mice are 5, and 14 months old. *$P < 0.05$ by the Mann–Whitney *U*-test. **12 months after injection.

To investigate the kinetics of amyloid fibril formation, R1.P1-*Apoa2*^{c} mice were injected intravenously with 0.2 mg hAA fibrils and euthanized at 2, 4, and 6 weeks, and 2 and 3 months after injection. ApoA-II proteins in the amyloid fibril fractions were then detected in the tissues. AApoAII deposits were first detected in the lungs at 4 weeks after injection. At 6 weeks after injection, AApoAII deposits extended into the tongue and stomach. At 3 months after injection, AApoAII deposits were detected in the lungs, stomach, liver, and tongue (Fig. 3b). However, we failed to detect injected human amyloid fibrils in the tissues (data not shown).

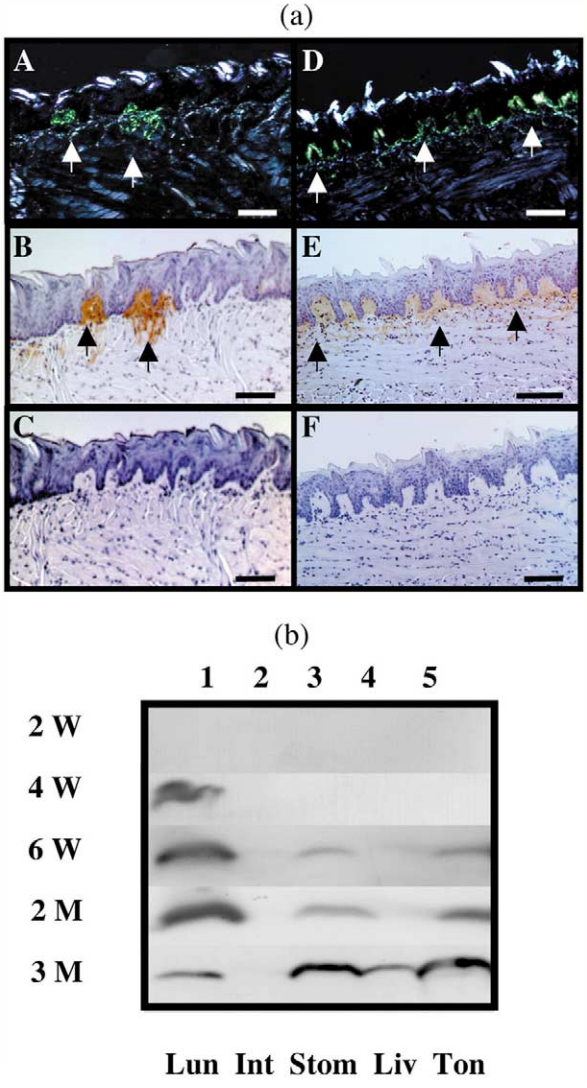

Fig. 3. AApoAII deposition in tissues of hAA-treated R1.P1-*Apoa2*^{c} mice. a: Amyloid deposits in the papillary layer of the tongue of a mouse at 3 and 6 months (grades 2 and 4 respectively) after hAA injection were identified by green birefringence in Congo red-stained sections using polarizing microscopy (A and D). AApoAII deposits were identified by immunohistochemical staining using anti-AApoAII antiserum (1:5000) (B and E). mAA deposits were excluded from amyloid deposits by immunohistochemical staining using anti-mAA antiserum (1:3000) (C and F). Arrows show the AApoAII deposits in tissues. Scale bar equals 100 μm. b: Western blot analysis of AApoAII amyloid protein. Lane 1, lung; lane 2, small intestine; lane 3, stomach; lane 4, liver; lane 5, tongue. The AApoAII amyloid protein was detected in tissues at 2, 4, 6 weeks, and 2 and 3 months after hAA injection.

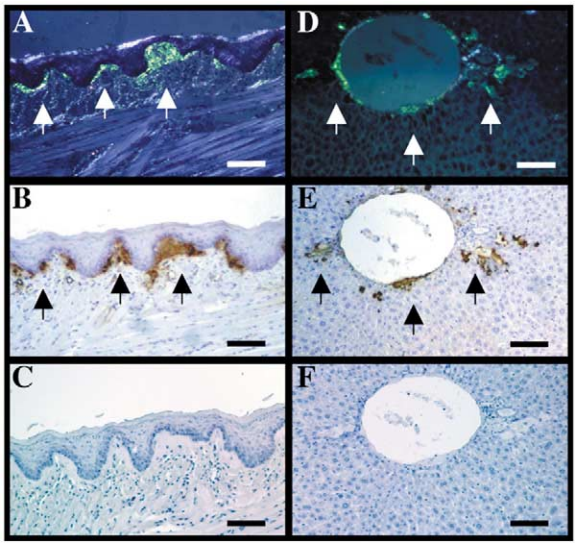

Fig. 4. AApoAII deposition in tissues of an AGroES-injected R1.P1-*Apoa2*^{c} mouse. Amyloid deposits in the papillary layer of the tongue (grade 3) and around the central vein in the liver (grade 2) of a mouse at 6 months after AGroES injection were identified by green birefringence in Congo red-stained sections using polarizing microscopy (A and D). AApoAII deposits were identified (B and E) and mAA deposits were excluded from amyloid deposits by immunohistochemical staining (C and F). Arrows show the AApoAII deposits in tissues. Scale bar equals 100 μm.

3.4. Induction of AApoAII amyloidosis by recombinant and synthetic fibrils

At 3 months after treatment with recombinant and synthetic fibrils, slight AApoAII deposits were detected in the tissues of the Aβ_{1-40}-, Aα-synuclein-, and AGroES-injected mice. No amyloid deposits were detected in the tissues of ALysozyme-injected mice. At 6 months after treatment the amyloid deposits became more extensive. The most severe amyloid deposits were detected in the tissues of AGroES-injected mice. AApoAII deposits were detected in the tongue, liver (Fig. 4B and E), spleen, heart, stomach, and small intestine. No mAA deposits were observed (Fig. 4C and F).

To study the effect of amyloid fibril structure on amyloid induction, we injected DW and two kinds of non-amyloid proteins including human albumin and hTTR proteins. We did not observe amyloid deposition in any tissues as late as 6 months after injection (Fig. 2).

3.5. Induction of AApoAII amyloidosis in SAMR1 mice

To investigate the effect of the mouse *Apoa2* allele on amyloid induction, hATTR and synthetic Aβ_{1-40} fibrils were injected into SAMR1 mice which have the less amyloidogenic *Apoa2*^{b}. No amyloid deposition was observed at 3 or 12 months after injection (Fig. 2).

4. Discussion

In this study, we demonstrated the induction of mouse AApoAII amyloidosis from various types of amyloid fibrils. These results suggest that a generic conformation present in these amyloid fibrils may serve as a seed to accelerate conformational changes of amyloid proteins. Despite this promiscuity, the degree of induced amyloid deposition was highest when injected amyloid fibrils had the same sequence as the

endogenous amyloid protein. The high sequence specificity of the propagation has also been revealed in prion diseases and yeast prions [21,22]. These phenomena cause 'species barriers' that limit transmission. Our findings suggest that the generic and specific conformation of amyloid fibrils may serve to modify the transmission of non-prion amyloidosis.

Although all amyloid fibrils share common biochemical and morphologic features, the generic and specific conformations remain to be elucidated. Monoclonal antibodies, which bind to many disease-related amyloid fibrils but not to non-fibrillar amyloid proteins, have been generated [23]. In one study it was found that an anti-AL monoclonal antibody could react with many other amyloid fibril proteins and could inhibit not only AL amyloidomas but also AA amyloidosis [24]. Recently, the common structure relating to the cell toxicity of fibrillar amyloid was found in the soluble oligomer of various amyloid proteins using an oligomer-specific antibody [25,26]. These results support our hypothesis of the existence of a common structure present in many amyloid fibrils.

The range of induction of amyloidosis should be sensitive to the primary structure and other components of amyloid fibrils. Because we do not have a method for further purification at this time, it is possible that contaminant molecules such as ApoE, amyloid P, and other proteoglycans in the isolated amyloid fibril fractions from mouse and human tissues might serve to modify amyloid deposition. In this study we made amyloid fibrils using recombinant proteins or synthetic amyloid peptide. We induced AApoAII amyloid deposition successfully using in vitro formed amyloid fibrils of Aα-synuclein, AGroES, and ALysozyme, and even $A\beta_{1-40}$ amyloid peptide. These results confirm our hypothesis that the structure of amyloid fibrils is essential for the acceleration of amyloid formation.

In humans, large variations in the occurrence and progression of amyloidosis have been observed in several populations. This suggests that endogenous and environmental agents may play a significant role in the pathogenesis of amyloid-related diseases by affecting the rate of amyloid fibril formation. Many kinds of non-disease-related proteins have been found that are able to form amyloid fibrils under certain conditions. These proteins include myoglobin [14], phosphatidyl-inositol-3′-kinase [26], apoC-II [27], the curli subunit in *E. coli* [28], and over 100 potential prion proteins in yeast *Saccharomyces cerevisiae* [26]. Our results and these findings suggest the possibility of cross-acceleration of amyloidosis among different forms of amyloid proteins. In our study, amyloid deposition was induced by injection of relatively large amounts of amyloid fibrils (0.1 mg/mouse). Thus a more careful evaluation of the practical importance of this cross-acceleration in amyloidosis and further investigation of the minimum amount of amyloid injection needed to induce deposition and the molecular basis of seeding of heterologous amyloid fibril formation remain.

The induction or acceleration of amyloid disease by amyloid fibril proteins may require either an elevated level of amyloid precursor proteins, since inflammation and hemodialysis elevate plasma AA and β_2M protein levels and immunoglobulin light chains are monoclonally increased in AL amyloidosis, or amyloidogenic mutants of precursor proteins. Almost all hereditary amyloidoses are associated with mutant amyloid proteins. In this study, heterologous amyloid fibrils could not induce amyloidosis in SAMR1 mice which do not have elevated or amyloidogenic precursor proteins. However, normal prion protein (PrP^c) develops the conformational change into PrP^{sc} seeded by PrP^{sc}. Thus, general amyloidosis should be considered a distinct process from the prion diseases.

In conclusion, our results demonstrate the existence of a common structure in amyloid fibrils that serves as a seed for the acceleration of amyloidosis. This feature should contribute to the understanding of amyloid pathology and may benefit the development of therapeutic agents for amyloid diseases.

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