Molecular Pathogenesis of Genetic and Inherited Diseases

Cross-Seeding and Cross-Competition in Mouse Apolipoprotein A-II Amyloid Fibrils and Protein A Amyloid Fibrils

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Murine senile [apolipoprotein A-II amyloid (AApoAII)] and reactive [protein A amyloid (AA)] amyloidosis are reported to be transmissible diseases via a seeding mechanism similar to that observed in the prion-associated disorders, although *de novo* amyloidogenesis and the progression of AApoAII or AA amyloidosis remain unclear. We examined the effect of co-injection of AApoAII and AA fibrils and multiple inflammatory stimuli in R1.P1-*Apoa2*^c mice with the amyloidogenic *Apoa2*^c allele. Both AApoAII and AA amyloidosis could be induced in this system, but the two types of amyloid fibrils preferentially promote the formation of the same type of fibrils while inhibiting the formation of the other. Furthermore, we demonstrate that AA or AApoAII amyloidosis could be cross-seeded by predeposited AApoAII or AA fibrils and that the predeposited amyloid fibrils were degraded when the fibril formation was reduced or stopped. In addition, a large proportion of the two amyloid fibrils colocalized during the formation of new fibrils in the spleen and liver. Thus, we propose that AApoAII and AA can both cross-seed and cross-compete with regard to amyloid formation, depending on the stage of amyloidogenesis. These results will aid in the clarification of the mechanisms of pathogenesis and progression of amyloid disorders. (Am J Pathol 2007, 171:172–180; DOI: 10.2353/ajpath.2007.060576)

The amyloidoses are a group of protein misfolding disorders characterized by the accumulation of highly insoluble, β -sheet-rich amyloid fibrils formed from a variety of proteins that, under normal physiological conditions, are harmless and soluble. Twenty-six different human proteins have been found to be amyloidogenic *in vivo*. They are associated with disorders such as the prion diseases, Alzheimer's disease, type 2 diabetes, and various systematic amyloidoses. Nucleation-dependent polymerization or seeding has been postulated to explain the kinetics of amyloid fibrillization, the conversion of the normal to the protease-resistant form of the prion protein (Prp^{Sc}), and the propagation of yeast prions.^{1–4} Despite a lack of similarity in the functional properties or in the primary, secondary, or tertiary structures among these proteins, the amyloid or amyloid-like fibrils formed from them share a common ultrastructure.^{5–7}

There have been infrequent reports of two different amyloid fibrils colocalizing in the same organ or tissue. For example, β -amyloid (A β) and Prp^{Sc} were reported to be colocalized in a single plaque in Gerstmann-Straussler syndrome patients.⁸ Likewise, κ -light chain-derived amyloid and β_2 -microglobulin-derived amyloid were found to be colocalized in a biopsy specimen from a patient with multiple myeloma,⁹ and A β and immunoglobulin light chain λ -amyloid are co-deposited in diseased leptomeningeal and cortical vessels in patients with spontaneous intracranial hemorrhage.¹⁰ This colocalization of multiple amyloid fibrils may be due to the ability of preformed amyloid fibrils to accelerate confor-

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mational changes in other kinds of amyloid precursor protein.^{6,11} In yeast, *[PSI⁺]* and *[URE3]* prions have been demonstrated to interact both positively and negatively in the presence of *[PIN⁺]*,^{12,13} whereas tau and α -synuclein interact synergistically.¹⁴ However, the mechanism of interaction between different types of amyloid fibrils and amyloidogenic proteins remains obscure.

Systemic senile amyloidosis and reactive amyloidosis are two naturally occurring types of amyloid disease that have been described in mice. These amyloidoses involve the formation of fibrils from apolipoprotein A-II (apoA-II) and serum amyloid A (SAA) protein, respectively. Formation of fibrils in apolipoprotein A-II amyloid (AApoAII) and protein A amyloid (AA) amyloidosis can be promoted by seeding or cross-seeding.^{6,11,15–18} In mice, AApoAII fibrils are derived from apoA-II proteins that circulate in the blood constitutively without degradation or modification. Senescence-accelerated prone-1 mice, which have the C-type variant of apoA-II (Gln⁵ and Ala³⁸), have a high incidence of spontaneous AApoAII amyloidosis as they age.¹⁹ In contrast, senescence-accelerated resistant-1 mice, which have wild-type B apoA-II (Pro⁵ and Val³⁸) show few, if any, signs of AApoAII amyloidosis.²⁰ Another mouse line, R1.P1-*Apoa2^c*, is congenic for the amyloidogenic *c* allele of the apoA-II gene from the senescence-accelerated prone-1 strain in the genetic background of the senescence-accelerated resistant-1 strain.²¹ These mice have a high incidence of spontaneous amyloidosis and show severe deposition of amyloid as they age.²² In previous studies, we demonstrated that AApoAII amyloidosis can be transmitted by intravenous or intraperitoneal and intragastric injection of AApoAII fibrils and to offspring of mice with AApoAII amyloidosis.^{15,16,23,24}

SAA protein is an acute-phase apolipoprotein reactant produced mainly by hepatocytes under control of interleukin-1, interleukin-6, and tumor necrosis factor- α .²⁵ The plasma concentration of SAA is normally very low but can increase to >1000 mg/L after an inflammatory stimulus.^{26–28} This protein can be proteolytically processed into an N-terminal cleavage product of approximately 44 to 100 residues that is deposited as amyloid in vital organs, including the spleen, liver, and kidneys.²⁹ AA amyloidosis occurs in patients with rheumatoid arthritis and other chronic inflammatory diseases. AA can also be induced experimentally in mice by injecting them with silver nitrate, casein, or lipopolysaccharide, all of which greatly increase the concentration of circulating SAA.^{30,31} The lag phase of AA amyloidogenesis can be dramatically shortened by a co-injection of amyloid-enhancing factor. There is evidence that the amyloid-enhancing factor is AA itself and that AA amyloidosis might be transmitted by a prion-like mechanism.¹⁷

The purpose of this study was to characterize any interactions that may occur between AApoAII/AA fibrils and precursor apoA-II/SAA proteins in R1.P1-*Apoa2^c* mice that were co-injected with AApoAII and AA fibrils and that received multiple inflammatory stimuli. In addition, we tested whether AA or AApoAII amyloid could be cross-seeded by predeposited AApoAII or AA fibrils. These results may help clarify the pathogenesis and progression of amyloid disorders.

Materials and Methods

Animals

Amyloidogenic R1.P1-*Apoa2^c* mice were raised in the Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University, under specific-pathogen-free conditions at 24 $\pm$ 2°C with a 12-hour light/dark cycle. A commercial diet (MF; Oriental Yeast, Tokyo, Japan) and tap water were provided ad libitum. All experiments were performed with the consent of the Animal Care and Use Committee of Shinshu University School of Medicine.

Amyloid Fibrils Isolated from Tissues

AApoAII fibrils were isolated from the liver of an R1.P1-*Apoa2^c* mouse. AA fibrils were isolated from the liver of a C57BL/6J mouse with severe AA amyloidosis. Both the amyloid fibril fractions were isolated by Pras' method with some modification.^{32,33} Both isolated amyloid fibrils were suspended in distilled deionized water (DDW) at a concentration of 1.0 mg/ml.

Induction of Amyloidosis in R1.P1-*Apoa2^c* Mice

Two-month-old male R1.P1-*Apoa2^c* mice were injected with AApoAII or AA fibrils or with a mixture of both in the presence or absence of inflammatory stimuli. Control mice were injected with DDW in place of the amyloid fibrils. The number of mice, a detailed schedule, and combinations of AApoAII and AA fibrils are described in Table 1. The mice were sacrificed by cardiac puncture under diethyl ether anesthesia, and major tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4- μ m sections for Congo Red staining and immunohistochemistry. One half of the spleen, liver, and lungs were stored at –70°C for later biochemical analysis.

Detection of Amyloid Deposition

Amyloid deposition in each mouse was identified by green birefringence in Congo red-stained sections under a polarizing microscope.³⁴ AApoAII and AA amyloid proteins were also identified immunohistochemically by specific anti-apoA-II or anti-AA antiserum, respectively.²⁰ In addition, amyloid fibrils were isolated from the spleen, liver, and lungs of an individual mouse from each group. Thawed organs (0.1 g) were sonicated twice for 30 seconds with a 30-second interval in 1.0 ml of 0.15 mol/L NaCl on ice using an ultrasonic homogenizer VP-5S (Tietech Co., Ltd., Tokyo, Japan) at power level 4. The homogenate was centrifuged at 40,000 $\times$ g for 20 minutes at 4.0°C, after which the supernatant was discarded, and the pellet was suspended in 1.0 ml of DDW. The sonication and centrifugation were repeated once more, and the pellet was suspended in 1.0 ml of DDW. After centrifugation at 30,000 $\times$ g for 20 minutes at 4.0°C, the supernatant containing amyloid fibrils was collected. Iso-

Table 1. Schedule of Injection of Amyloid Fibrils and 1% AgNO₃ to Induce Amyloidosis *in Vivo*

Group	Mice	Intravenous injection of amyloid fibrils at day 0	Day of subcutaneous injection of 1.0 ml of 1% AgNO ₃											
			0	14	28	42	56	60	70	80	90	100	110	130
Induction of AApoAll and AA deposition by co-injection of AApoAll and AA fibrils														
1	5	100 μg of AApoAll						Sac						
2	3	100 μg of AApoAll	+					Sac						
3	4	50 μg of AApoAll + 50 μg of AA						Sac						
4	7	50 μg of AApoAll + 50 μg of AA						Sac						
5	4	50 μg of AApoAll + 50 μg of AA	+	+	+	+	+	Sac						
Induction of AA deposition by predeposited AApoAll fibrils														
6	6	100 μg of AApoAll						+	Sac					
7	3	100 μg of AApoAll						+	+	+	Sac			
8	3	100 μg of AApoAll						+	+	+	+	Sac		
9	4	100 μg of AApoAll										Sac		
10	6	100 μl of DDW						+	+	+	+	+	Sac	
Induction of AApoAll and AA deposition by injection of AA fibrils														
11	4	100 μg of AA						Sac						
12	3	100 μg of AA	+					Sac						
13	3	100 μg of AA						+	Sac					
14	5	100 μg of AA	+	+	+	+	+	Sac						
15	3	100 μl of DDW	+					Sac						
Induction of AApoAll deposition by predeposited AA fibrils														
14	5	100 μg of AA	+	+	+	+	+	Sac						
16	5	100 μg of AA	+	+	+	+	+	.						Sac
17	6	100 μl of DDW												Sac

Sac, sacrifice; +, injection of 1% AgNO₃.

lated amyloid fibril fractions (10 µg) from the spleen, liver, and lungs were separated on two Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel [16.5% (w/v) acrylamide] electrophoreses.³⁵ Proteins on the duplicated part of the gels were transferred electrophoretically to Immuno-Blot polyvinylidene difluoride membrane (0.2-µm pore size; Bio-Rad, Hercules, CA). Proteins on the membrane were detected with rabbit anti-mouse apoA-II antiserum (1:3000) and rabbit anti-AA antiserum (1:3000), followed by peroxidase-conjugated goat IgG against rabbit immunoglobulin (1:1000; ICN Pharmaceuticals, Inc., Aurora, OH). Immunoreactive proteins were visualized with enhanced chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK).

Immunofluorescence Confocal Microscopy

Immunofluorescent staining of the liver of G8 animals (Table 1), which had previously been demonstrated to possess both AApoAII and AA deposits by Western blotting, was performed. Sections were deparaffinized, and nonspecific binding was blocked by incubation for 1 hour with 10% normal goat serum. The sections were then incubated with rabbit anti-apoA-II antiserum (1:3000) for 1 hour at room temperature. After three 10-minute washes with 0.01 mol/L phosphate-buffered saline, the sections were incubated with Alexa-488-labeled secondary antibody (1:400; Molecular Probes, Eugene, OR) for 30 minutes. The green fluorescent images were obtained using a DAS microscope (Leica Microsystems AG, Wetzlar, Germany). Thereafter, the section was heated in

DDW for 10 minutes at 95°C. The section was then stained a second time by treating it as described above, but replacing the primary antiserum and secondary antibody with rabbit anti-AA antiserum and Alexa-568-labeled anti-rabbit immunoglobulin, respectively. Red fluorescent images of exactly the same area were then obtained. The red and green fluorescent images for the same areas were merged using Adobe Photoshop 7.0 digital imaging software (Adobe Systems Inc., San Jose, CA). To confirm the findings, the combination of primary and fluorescence-labeled secondary antibodies was interchanged on an adjacent section.

Serum apoA-II Concentration after Induction of Acute Inflammation in Mice

Six 2-month-old male R1.P1-Apoa2^c mice were injected subcutaneously with 1.0 ml of 0.5% (w/v) AgNO₃ to induce the acute-phase reaction. Serum obtained from mice tail veins 0, 24, 48, 72, 96, and 168 hours after induction was used for quantitative comparison of apoA-II and SAA by Western blot analysis. Serum (0.1 µl) was separated under reduced conditions by 16.5% Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane to quantify apoA-II and SAA. ApoA-II and SAA were detected by the enhanced chemiluminescence (ECL) method (Amersham International, Buckinghamshire, UK) and quantified using a densitometric image analyzer with NIH Image version 1.61 (Bethesda, MD).

Negative Staining and Immunoelectron Microscopy

Aliquots (20 μ l; 0.5 μ g/ μ l) of resuspended amyloid fibrils were applied to 400-mesh collodion-coated copper grids (Nissin EM Co., Ltd., Tokyo, Japan) for 1 minute and subjected to negative staining with 1% phosphotungstic acid (pH 7.0) for 1 minute. Immunoelectron microscopy was conducted as described previously.³⁶ Aliquots (20 μ l; 0.05 μ g/ μ l) were loaded onto grids for 15 minutes. The grids were transferred to 1:100 rabbit anti-apoA-II or anti-AA antiserum in phosphate-buffered saline containing 0.5% bovine serum albumin. After 3 hours, the grids were then immersed in Biotinylated Swine Anti-Rabbit Immunoglobulins (1:300; Dako Denmark A/S, Glostrup, Denmark) in phosphate-buffered saline containing 0.5% bovine serum albumin. After 1.5 hours, the grids were then immersed in 1:4 streptavidin-conjugated 10-nm colloidal gold in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.05% Tween 20. After 3 hours, the grids were washed and then negatively stained with 1% phosphotungstic acid. All incubations and washes were performed at room temperature by depositing 50- μ l droplets on sheets of Parafilm. The negatively stained samples were viewed with a JEOL 1200 EX electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

Statistical Analysis

Statistical analyses were performed using the Statview software package (Abacus Concepts, Berkeley, CA).

Results

AApoAII Formation Is Induced Preferentially by AApoAII Fibrils but Inhibited When AA Formation Is Dominant

In R1.P1-*Apoa2*^c mice, AApoAII amyloid can be seeded by AApoAII fibrils or by various heterogeneous amyloid fibrils.⁶ It is also possible to seed AA in these mice with AA fibrils in conjunction with an inflammatory stimulus.¹¹ Therefore, we used male R1.P1-*Apoa2*^c mice to study the interaction among AApoAII and AA fibrils and apo-AII and SAA precursor proteins. Mice injected with 100 μ g of AApoAII fibrils alone (Table 1, G1) exhibited abundant deposition of AApoAII in the spleen, liver, and lungs after 2 months (Figure 1A, G1). These tissues stained positive with Congo red in all mice examined (data not shown). Deposition of AApoAII in the spleen and liver was decreased to an undetectable level in mice (zero of three mice) injected with 100 μ g of AApoAII fibrils and given a single inflammatory stimulus simultaneously, with deposition of AApoAII in the lungs greatly decreased (Figure 1A, G2). Interestingly, abundant AApoAII was deposited in the spleen and liver when co-injected with equal amounts of AApoAII and AA fibrils (all four mice had AApoAII deposition), whereas AA was not deposited in

Figure 1. Western blot analysis of AApoAII (top) and AA (bottom) amyloid proteins in the amyloid fibril fractions. **A:** AApoAII and AA amyloid were induced by co-injecting AApoAII and AA fibrils followed by one or five repeated injections of 1.0 ml of 1% AgNO₃ (Table 1). **B:** Predeposited AApoAII cross-seeded the formation of AA in the spleen and liver (Table 1). **C:** AA fibrils induce both AA and AApoAII by co-injection of AA fibrils and one subcutaneous injection of 1.0 ml of 1% AgNO₃, but AA is preferentially induced in the presence of AA fibrils and 1.0 ml of 1% AgNO₃ five times (Table 1). **D:** Predeposited AA cross-seeded the formation of AApoAII with aging. **Lane 1,** spleen; **lane 2,** liver; **lane 3,** lungs. G, group.

the absence of an inflammatory stimulus (zero of four mice had AA amyloid deposition; Figure 1A, G3). Both AApoAII and AA were deposited in the spleen when mice (six of seven) were co-injected with an equal amount of AApoAII and AA fibrils and given a single inflammatory stimulus (Figure 1A, G4). Abundant AA was deposited

Figure 2. Colocalization of AApOII and AA fibrils in the liver. Amyloid fibrils in the liver of G8 mice were identified by green birefringence in Congo red-stained sections under polarized microscopy (A). Distribution of AApOII (B) and AA (C) was visualized with rabbit anti-mouse apoA-II or AA antisera and Alexa-488- or Alexa-568-conjugated anti-rabbit polyclonal antibodies, respectively. The red (B) and green (C) fluorescent images were merged using imaging software (D). The majority of AApOII and AA immunoreactivity colocalizes in the periportal areas and is depicted in yellow. Bar = 40 μ m.

predominantly in the spleen and liver when the mice received a co-injection of AApOII and AA fibrils and a series of five injections with inflammatory stimuli (Figure 1A, G5). This corresponded with inhibition of AApOII deposition in the spleen and liver to an undetectable level (zero of four mice had AApOII deposition in the liver and spleen). AApOII amyloid formation in the lungs was not affected by this regimen (four of four mice; Figure 1A, G5). AA amyloid deposition was not observed in the lungs of mice in any of the groups.

Predeposited AApOII Could Cross-Seed AA Formation and Was Progressively Degraded in the Spleen and Liver

When mice were injected with 100 μ g of AApOII fibrils, after 2 months, abundant AApOII was deposited in the

spleen, liver, and lungs (Figure 1A, G1). AApOII deposition in the spleen, liver, and lungs became more severe after 40 days (Figure 1B, G9). To determine whether predeposited AApOII could cross-seed the formation of AA fibrils, we performed a 2-month incubation of AApOII fibrils in mice, and then the mice were injected with 1.0 ml of 1% AgNO₃ on day 60 (G6); days 60 and 70 (data not shown); days 60, 70, and 80 (G7); or days 60, 70, 80, and 90 (G8). The deposition of AApOII and AA was then analyzed over time. Deposition of AApOII did not vary significantly within the first 10 days nor did AA deposition appear (Figure 1B, G6). In contrast, deposition of AApOII decreased in the spleen after 20 days, whereas AA could not be detected at this time point (data not shown). AA was deposited initially in the spleen and liver within 30 days (Figure 1B, G7 and G8), whereas AApOII deposition decreased sharply in the spleen and some-

Figure 3. Colocalization of AApoAII and AA fibrils in the spleen. Amyloid fibrils in the spleen were identified by green birefringence in Congo red-stained sections examined by polarized microscopy (A). Distribution of AApoAII (B) and AA (C) in the serial sections was determined by immunohistochemical staining with rabbit anti-mouse apoA-II or AA antiserum as the first antibody and 3,3-diaminobenzidine as the chromogen, respectively. Magnification, $\times 200$.

what in the liver (Figure 1B, G8). Finally, to determine whether predeposited AApoAII was a prerequisite for AA fibril formation in R1.P1-*Apoa2^c* mice, we injected the mice with 100 μ l of DDW and then administered a series of five injections of 1.0 ml of 1% AgNO₃. This treatment did not result in the formation of AA (zero of six mice in G10 had AA deposition in any tissues; Figure 1B, G10).

AA Fibrils Induce Both AA and AApoAII Deposition, Although AA Is Induced Preferentially

AApoAII was induced in the lungs by injecting AA fibrils in the absence of an inflammatory stimulus (Figure 1C, G11). Furthermore, AA and AApoAII were both induced in the spleen (AA) and lungs (AApoAII) by injection of 100 μ g of AA fibrils followed by an injection of 1.0 ml of 1% AgNO₃ at day 0 (Figure 1C, G12) or day 60 (Figure 1C, G13). Injection of AA fibrils at day 0 with five concomitant injections of 1.0 ml 1% AgNO₃ at intervals of 14 days resulted in preferential AA formation, whereas AApoAII deposition did not occur (Figure 1C, G14). As a control, we injected mice with DDW and 1.0 ml of 1% AgNO₃. This treatment did not result in the formation of AA or AApoAII deposition (Figure 1C, G15).

Predeposited AA Fibrils Can Cross-Seed the Deposition of AApoAII and Are Progressively Degraded in the Liver

Injection of mice with 100 μ g of AA fibrils followed by a series of five injections with 1.0 ml 1% AgNO₃ resulted in deposition of AA but not AApoAII in the spleen, liver, or

lungs after 60 days (Figure 1C, G14). We also examined whether predeposited AA could cross-seed AApoAII formation as the mice aged. We prolonged the incubation for an additional 70 days (130 days total) and then assessed AApoAII deposition (G16). Abundant AApoAII deposition was seen in the spleen, with a lower level observed in the liver that could be cross-seeded by predeposited AA (Figure 1D, G6). At the same time, the deposition of AA was reduced to an undetectable level in the liver. AApoAII was not formed in R1.P1-*Apoa2^c* mice injected with DDW even after 130 days (Figure 1D, G17).

AApoAII Fibrils Colocalized with AA Fibrils

Both AApoAII and AA were present in the same fibril fraction isolated from the liver of G8 mice as well as from the spleens of G4, G7, and G16 mice. We therefore tested whether newly formed AA fibrils colocalize with predeposited AApoAII fibrils. The amyloid fibrils in the liver (Figure 1B, G8) and spleen (Figure 1B, G7) were first identified by green birefringence under polarized microscopy in Congo red-stained sections (Figures 2A and 3A, respectively). The vast majority of AApoAII (Figure 2B) and AA (Figure 2C) fibrils colocalized in the periportal areas of the liver (Figure 2D), as demonstrated by double immunofluorescent microscopy. Immunohistochemical staining of serial sections showed that the vast majority of AApoAII (Figure 3B) and AA (Figure 3C) fibrils localized in the same perfollicular region of the spleen. Furthermore, we analyzed the amyloid fibril fraction isolated from the liver of G8 mice by negative-staining electron microscopy to confirm the formation of amyloid fibrils (Figure 4A). To determine the composition of the fibrils isolated from the liver, we performed immunoelectron microscopy

Figure 4. AApoAII and AA fibrils in the amyloid fibril fraction isolated from the liver of G8 mice. Amyloid fibrils composed of both AApoAII and AA were negatively stained with 1% phosphotungstic acid (A). AApoAII (B) and AA (C) fibrils were identified by immunogold staining using rabbit anti-mouse apoA-II or AA antiserum, respectively. Bars = 100 nm.

Figure 5. Serum levels of apoA-II and SAA after induction of acute inflammation. Male 2-month-old R1.P1-*Apoa2^c* mice ($n = 6$) were injected with 1.0 ml of 1% AgNO₃. Serum apoA-II (●) and SAA (○) levels were quantified by Western blot analysis using anti-apoA-II and AA antibodies. The mean value and SE were determined from changes in the relative ratios of protein levels at different time points versus the initial and peak protein levels (apoA-II at 0 hours and SAA at 24 hours, respectively).

with rabbit antiserum to apoA-II and AA. We found that some fibrils were labeled with antibodies to apoA-II (Figure 4B) and some with antibodies to AA (Figure 4C).

Serum apoA-II and SAA Concentrations after Induction of Acute Inflammation

To evaluate the effect of AgNO₃ injection on the concentrations of precursor proteins, serial serum samples were obtained successively after induction (Figure 5). At 24 hours after injection, serum apoA-II levels had been reduced to 55% of the level before stimulation (44.7 mg/dl at 0 hour), showed a subsequent increase to 80% at 48 hours, but then returned to the pre-injection level at 96 hours. The highest serum SAA level was observed at 24 hours, with SAA levels decreasing gradually until they were undetectable at 168 hours.

Discussion

The seeding/nucleation model of amyloid fibril growth has been proposed to explain the pathogenesis of various amyloidosis,^{11,37} the transmissible spongiform encephalopathies in animals,^{38,39} and the *de novo* appearance of yeast prion states.⁴⁰ The cross-seeding model postulates that there is a direct interaction between newly forming and pre-existing heterologous amyloid fibrils. An example of such behavior is presented by the transmission and propagation of yeast prion states. *Saccharomyces cerevisiae* [PSI⁺] and [URE3] are the amyloid forms of the translational termination factor Sup35 and the nitrogen catabolite repressor Ure2 proteins, respectively.^{41,42} [PIN⁺] is the amyloid form of Rnq1.⁴³ [PIN⁺] can enhance the *de novo* appearance of [PSI⁺] and [URE3].^{12,44,45} Conversely, [PSI⁺] and [URE3] facilitate the *de novo* formation of Rnq1 prion aggregates.⁴⁶ However, [PSI⁺] and [URE3] repress propagation and *de novo* appearance of one another in the presence of [PIN⁺].^{12,13}

Figure 6. Models illustrating the self-seeding (a), cross-seeding (b), and cross-competition (c) of AApoAII and AA fibrils. When AApoAII (green oblique rectangles) and AA (red triangles) fibrils are both present, the majority of the fibrils seed more of their own type of fibrils, and the newly formed amyloid fibrils will undergo fragmentation and extension (self-seeding, **thick solid arrow**). Fibrils no longer growing due to being blocked by other amyloid proteins are ultimately degraded (blocking, **thick dotted arrow**; brown squares and light blue rectangles). Likewise, a small portion of the fibrils cross-seed the formation of fibrils by other amyloid proteins (cross-seeding, **thin solid arrow**). In other words, the blocking and degrading of amyloid fibrils contributes to the cross-competition of AApoAII and AA amyloid fibrils (dotted blue frame). G1 to G16 are mouse groups in Table 1.

In this study, we investigated whether AApoAII and AA fibrils cross-seed and cross-compete *in vivo*. AApoAII deposition was induced in R1.P1-*Apoa2^c* mice by injection with AApoAII fibrils (G1 and G6), and AA amyloid deposition was induced by injection with AA fibrils when serum SAA level was increased (G12, G13, and G14). These represent homologous self-seeding events (Figure 6a, thick solid arrow). A single inflammatory stimulus inhibited the seeding effect of AApoAII fibrils (G2). It is possible that increased SAA might bind to AApoAII fibrils and block the binding of these to apoA-II. However, when R1.P1-*Apoa2^c* mice were co-injected with equal amounts of AApoAII and AA fibrils and were given simultaneous inflammatory stimuli, the vast majority of the amyloid fibrils formed were self-seeded by the respective amyloid fibrils (G3, G4, and G5). Increased SAA may preferentially bind with AA fibrils, and AApoAII fibrils could bind to AApoAII without competition. This preference may be due to the much greater capacity of the amyloid fibrils to convert their respective precursor proteins, relative to other proteins to amyloid fibrils. The rate of fibril formation depends on the concentration of amyloid protein both *in vitro* and *in vivo*. Several studies have reported reduced apoA-II synthesis, mRNA, and serum concentrations associated with inflammation.^{47–49} Reduction of the serum apoA-II concentration was also observed here 24 to 72 hours after injection of AgNO₃ (Figure 5). This reduction may explain in part the inhibited AApoAII deposition in G2 and the disappearance of AApoAII deposition in G7, G8, and G9.

Simple cross-seeding was observed in G11 (Figure 6) as reported in our previous study.⁶ We also examined whether predeposited amyloid formed from one protein can cross-seed the deposition of amyloid formed from a

different protein, and we studied the interaction of the newly formed and predeposited amyloid fibrils. The predeposited AApoAll fibrils cross-seeded the formation of AA (G7 and G8). Newly formed AA fibrils colocalized with predeposited AApoAll fibrils (Figure 2). However, the levels of predeposited AApoAll amyloid in the spleen and liver were decreased to an undetectable level (G7 and G8). In addition, we found that predeposited AA fibrils can cross-seed AApoAll (G16). Normally, AApoAll amyloidosis does not arise without exogenous amyloid fibrils, but it was accelerated in the presence of predeposited AA fibrils. At the same time, the deposition of AA in the spleen and liver was reduced. We suggest that a small portion of the predeposited fibrils acted as seeds for the initiation of amyloidogenesis after which the newly formed amyloid fibrils seeded their precursor proteins (Figure 6b, thin solid arrow). The vast majority of the predeposited amyloid fibrils were blocked and ultimately can be degraded (Figure 6c, thick dotted arrow). That is, the blocking by other amyloid precursors and degrading of amyloid fibrils may contribute to cross-competition between two species of amyloid fibril (Figure 6, dotted blue frame). We hypothesized that blocking and degrading reactions might occur during aggregation of fibrils in the tissue-specific environments in which AA amyloid is preferentially deposited. Cross-competition of AApoAll by SAA occurred in the spleen and liver but was much weaker in the lungs, where AA amyloid deposition was generally not observed (G2, G4, G5, G7, and G8).

A cross-competing/seeding model may also have applications with regard to other experimental findings. For example, amyloid-like fibrils made from ¹²⁵I-labeled synthetic transthyretin peptide (115–124) are first trapped within murine AA fibrils in the lungs and spleen, but at later stages, radioactivity is not detected in the organs where there is substantial AA deposition.¹⁸ In addition, exogenous human AA fibrils cross-seed AApoAll amyloid in R1.P1-Apoa2^c mice, but human AA fibrils could not be detected in the same tissues after 3 months.⁶

Recently, Soto et al⁵⁰ summarized that amyloid fibrils associated with Alzheimer's disease, mouse AApoAll and AA amyloidosis, and other amyloid-related disorders could have prion-like seeding properties *in vivo*. The vast majority of type 2 diabetes patients have pancreatic amyloid deposits composed of islet amyloid polypeptide, which shares a striking primary sequence similarity with A β .⁵¹ Cross-seeding of A β fibrils by islet amyloid polypeptide amyloid may also explain the elevated risk of Alzheimer's disease observed in patients with diabetes, although the inability of islet amyloid polypeptide to seed the formation of A β (1–40) fibrils *in vitro* argues against this.⁴⁶ Despite the fact that coexistence of two amyloid species derived from different precursors is an atypical pathological observation,⁵² we could not exclude the existence of a limited or undetectable amount of other amyloid. Exposure to naturally occurring amyloid-like protein fibrils has been proposed to explain AA amyloidogenesis in some patients.¹¹ Thus, like predeposited amyloid, amyloid fibrils in the environment may act to seed other amyloid species *in vivo*.

In summary, we have verified that AApoAll and AA fibrils could both cross-seed and cross-compete at different stages of amyloidogenesis using an amyloidogenic mouse model and considering principally the possibility of direct protein-protein interaction. In addition, we provide the first evidence that in systemic amyloidosis, a predeposited amyloid can cross-seed the formation of another amyloid after which the predeposited amyloid is degraded. It is important to consider the possibility that many of the results obtained here *in vivo* might also be interpreted as being due to indirect and complex mechanisms, for example gene regulation, chaperone activity, or protein clearance. However, these findings and the proposed model should provide insight into the mechanisms that underlie the pathogenesis and progression of amyloid disease, and these data suggest that therapeutic agents that directly or indirectly inhibit the formation of one form of amyloid might be effective for preventing other forms of amyloidogenesis.

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