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Effect of amino acid variations in the central region of human serum amyloid A on the amyloidogenic properties

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ABSTRACT

Human serum amyloid A (SAA) is a precursor protein of the amyloid fibrils that are responsible for AA amyloidosis. Of the four human SAA genotypes, SAA1 is most commonly associated with AA amyloidosis. Furthermore, SAA1 has three major isoforms (SAA1.1, 1.3, and 1.5) that differ by single amino acid variations at two sites in their 104-amino acid sequences. In the present study, we examined the effect of amino acid variations in human SAA1 isoforms on the amyloidogenic properties. All SAA1 isoforms adopted α-helix structures at 4 °C, but were unstructured at 37 °C. Heparin-induced amyloid fibril formation of SAA1 was observed at 37 °C, as evidenced by the increased thioflavin T (ThT) fluorescence and β-sheet structure formation. Despite a comparable increase in ThT fluorescence, SAA1 molecules retained their α-helix structures at 4 °C. At both temperatures, no essential differences in ThT fluorescence and secondary structures were observed among the SAA1 isoforms. However, the fibril morphologies appeared to differ; SAA1.1 formed long and curly fibrils, whereas SAA1.3 formed thin and straight fibrils. The peptides corresponding to the central regions of the SAA1 isoforms containing amino acid variations showed distinct amyloidogenicities, reflecting their direct effects on amyloid fibril formation. These findings may provide novel insights into the influence of amino acid variations in human SAA on the pathogenesis of AA amyloidosis.

1. Introduction

Human serum amyloid A (SAA) is a precursor protein of AA amyloidosis, which is among the most severe complications that occur secondary to chronic inflammatory diseases such as rheumatoid arthritis [1]. Although persistently high concentrations of SAA proteins comprise a plausible risk factor for AA amyloidosis, a detailed molecular mechanism has not been elucidated. AA amyloidosis occurs in only 5–10% of humans during persistent chronic inflammatory conditions [2], indicating the existence of disease-modifying factors [3]. Instead of full-length SAA proteins (104 amino acids), the N-terminal fragments mainly consisting of 76-amino acid SAA molecules known as AA fragments have been detected in amyloid deposits [4]. Although several enzymes are known to be involved in SAA degradation, it has not been revealed whether enzymatic SAA cleavage is an essential prerequisite for amyloid deposition in AA amyloidosis. In addition, because heparan sulfate proteoglycan has been found in amyloid deposits, glycosaminoglycans are presumed to facilitate amyloid fibril formation as a scaffold for protein aggregation [5]. These biomolecules can act as disease-modifying factors through a currently unknown mechanism.

At least 20 different amyloid-forming proteins have been identified and associated with human diseases, including amyloid-β peptide with Alzheimer's disease and α-synuclein with Parkinson's disease [6]. Amyloid fibrils formed by these different proteins share some common structural features [7]. For example, amyloid fibrils are typically straight, with unbranched morphology. Another feature is a β-sheet secondary protein structure, in which β-strands run perpendicular to the fibril axis. Fluorescent dyes such as Congo red and thioflavin T (ThT), which bind to β-sheet structures, are used to indicate fibril formation. In some cases, the ThT emission intensity selectively increases by several orders in the presence of amyloid fibrils [8]. These characteristics allow us to determine amyloid fibril formation and are employed as common experimental techniques in amyloid research.

There are four human SAA genes (*SAA1–4*), of which *SAA3* is a pseudogene. SAA1 and SAA2 are synthesized during acute-phase inflammatory reactions, whereas SAA4 is a constitutively

Abbreviations: CD, circular dichroism; SAA, serum amyloid A; ThT, thioflavin T; TEM, transmission electron microscopy.

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expressed protein. SAA1 and SAA2 are allelic, giving rise to three distinct SAA1 isoforms (SAA1.1, 1.3, and 1.5) and two SAA2 isoforms (SAA2.1 and SAA2.2). SAA1 is most commonly associated with AA amyloidosis, probably because the circulating concentration of SAA1 is much higher than that of SAA2. The SAA1 isoforms differ by single amino acid variations at two sites, which are considered to affect the susceptibility to AA amyloidosis [9]. Thus, it is conceivable that the SAA1 isoforms could be disease-modifying factors. However, currently there is no direct molecular evidence to demonstrate that amino acid variations in the SAA1 isoforms affect fibril formation.

Using synthetic fragment peptides, we previously revealed that not only the N-terminal region but also the central region of the SAA molecule is involved in amyloid fibril formation [10]. The central region of SAA1 contains the isoform-associated amino acid variations, specifically SAA1.1 (52Val, 57Ala), SAA1.3 (52Ala, 57Ala), and SAA1.5 (52Ala, 57Val). Thus, in the present study, the effects of amino acid variations on the SAA molecule fibril formation were investigated using full-length SAA1 proteins that contain the amyloidogenic N-terminal region common to the three SAA1 isoforms as well as fragment peptides derived from the central regions of the SAA1 isoforms.

2. Materials and methods

2.1. Materials

Heparin sodium and ThT were purchased from Celsus Laboratories (Cincinnati, OH) and Sigma–Aldrich (St. Louis, MO), respectively. Fmoc amino acid derivatives were obtained from the Peptide Institute, Inc. (Minoh, Japan) and used without further purification. The buffers used were 10 mM sodium phosphate (pH 7.4), 10 mM sodium citrate (pH 5.6), or 10 mM sodium acetate (pH 4.0). All other reagents were special or peptide synthesis grade.

2.2. Preparation of SAA proteins and peptides

The recombinant human SAA1 isoforms SAA1.1 (52Val, 57Ala), SAA1.3 (52Ala, 57Ala), and SAA1.5 (52Ala, 57Val) were produced in *Escherichia coli*. Each plasmid corresponding to an SAA1 isotype was constructed and transformed into *E. coli* BL21 cells as previously described [11]. Recombinant proteins were prepared from expression-induced *E. coli* lysates according to the methods described [12]; these proteins contained an extra Met residue at the N-terminus.

The primary sequences of the synthetic fragment peptides used in the present study are listed in Table 1. Peptide synthesis and purification were performed according to the methods described [13]. Peptides were synthesized by the solid-phase method using Fmoc chemistry. The N- and C-termini were each capped with an acetyl group and an amide group, respectively. The peptide molecular masses were determined by matrix-assisted laser desorption ionization mass spectrometry using an Applied Biosystems Voyager-DE PRO (Table 1).

The SAA proteins were freshly dialyzed from 4 M urea into ultrapure water, and the SAA peptides were dissolved in ultrapure water. Protein or peptide solutions were centrifuged to remove any insoluble or aggregated matter before use. The protein solutions were maintained at 4 °C throughout the preparation. Protein or peptide concentrations were determined by the Lowry method, using bovine serum albumin (Bio-Rad, Hercules, CA) as a standard.

Table 1
Amino acid sequences and molecular masses of synthetic fragment peptides.

Peptide	Sequence	Found: *m/z* (calculated: M+H)
SAA1.1 (43–63)	DAAKRGPGGVWAAEAISDARE	2169.2 (2168.1)
SAA1.3 (43–63)	DAAKRGPGGAWAAEAISDARE	2140.5 (2140.1)
SAA1.5 (43–63)	DAAKRGPGGAWAAEVISDARE	2168.4 (2168.1)

2.3. Circular dichroism (CD) spectroscopy

Far-ultraviolet CD spectra were recorded at 4 °C or 37 °C with an Aviv 62ADS spectropolarimeter (Lakewood, NJ). The results were corrected by subtracting the baseline of an appropriate blank sample. The mean residual ellipticity ([θ]) was calculated using the equation $[\theta] = (\mathrm{MRW})\,\theta/10lc$, where θ is the measured ellipticity in degrees, l is the cuvette path length (0.2 cm), c is the protein or peptide concentration in g/mL, and the mean residue weight (MRW) is obtained from the molecular weight and the number of amino acids. The protein or peptide concentrations were 50 μg/mL. For fibril formation experiments, the measurements were performed 1 day after sample preparation.

2.4. Fluorescence spectroscopy

All fluorescence measurements were performed at 4 °C or 37 °C on a Hitachi F-7000 spectrophotometer (Tokyo, Japan). ThT fluorescence spectra were recorded in a 4 × 4 mm cuvette from 450 to 600 nm at an excitation wavelength of 440 nm. The ThT, heparin, and protein or peptide concentrations were 10 μM, 41 μg/mL, and 50 μg/mL, respectively.

2.5. Transmission electron microscopy (TEM)

Samples were applied to carbon-coated copper grids (400-mesh) and negatively stained with 2% (w/v) uranyl acetate. These samples were examined under a JEM-1200EX transmission electron microscope (JEOL, Akishima, Japan) with an acceleration voltage of 80 kV. The observations were performed at least 6 days after sample preparation.

3. Results

3.1. Secondary structure of the SAA1 isoforms

Secondary structures of SAA were analyzed by CD spectroscopy at pH 7.4. Fig. 1 illustrates the typical CD spectra for SAA1.1 at 4 °C and 37 °C. At 37 °C, the CD spectrum showed a single minimum of approximately 200 nm, indicating that the SAA1.1 molecule had formed a random coil structure. In contrast, at 4 °C, the CD spectrum exhibited double minima at approximately 208 and 222 nm, representing the potential propensity of the SAA1.1 molecule to form an α-helical structure. SAA1.1 reversibly formed an α-helical structure at 4 °C, even after measurement at 37 °C. SAA1.3 and SAA1.5 showed similar results, indicating that all SAA1 isoforms adopted α-helix structures at 4 °C, but were unstructured at 37 °C.

3.2. Amyloid fibril formation of the SAA1 isoforms

Amyloid fibril formation of the SAA1 isoforms was investigated using a fluorescent dye (ThT), which is well-known to specifically bind to ordered β-sheet aggregates [14]. Fig. 2A and B illustrate the pH-dependent increase in ThT fluorescence after 1 day of incubation following the addition of heparin to SAA1.1 at 37 °C and 4 °C, respectively. Heparin, an analog of heparan sulfate, has been shown to stimulate the fibril formation of many amyloidogenic polypeptides [15,16]. In the absence of heparin, no ThT fluores-