Serum amyloid A protein (SAA) subtypes in acute and chronic inflammatory conditions

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SUMMARY Serum amyloid A (SAA), a polymorphic high density lipoprotein associated plasma protein, is the putative circulating precursor of tissue AA protein fibrils in reactive (secondary) amyloidosis. In the present study we examined the SAA subtype pattern in various acute and chronic inflammatory states in order to find out whether disease-specific SAA isoform profiles exist. The method used to study the subtype pattern is based on electrofocusing of serum followed by immunoblotting. Our results show that the SAA subtype pattern is similar in patients with rheumatoid arthritis with or without amyloid. In addition, in amyloidotic subjects the SAA subtype response to acute tissue injury (arthroplasty) did not differ from that in patients without amyloidosis. Analysis of patients with acute and chronic infectious diseases and non-rheumatic inflammatory conditions showed similar SAA patterns in all subjects. These results suggest that the SAA subtype response to tissue injury and inflammation is similar irrespective of the initiating stimulus.

Key words: isoforms, amyloidosis.

In reactive (secondary) amyloidosis the major constituent of the protein fibrils accumulating in various tissues is an 8.5 kD polypeptide designated protein AA. This protein shows amino acid sequence homology with the NH₂ portion of the 12 kD serum amyloid A protein, SAA.¹ In the circulation SAA is associated with high density lipoproteins (HDL)^{2,3} and shows characteristics of a sensitive acute phase reactant.^{1,4}

Recent studies have shown the heterogeneity of SAA in several mammalian species. In humans six forms of SAA have been identified.^{3,5,6} It is not clear whether amyloid-specific or tissue-specific subtypes of SAA exist. In a recent study of a murine model of reactive amyloidosis Hoffman *et al.*⁷ showed that the AA protein deposited in tissues shared NH₂ terminal amino acid sequence with only one of the two SAA isotypes found in murine plasma. In familial amyloidotic polyneuropathy it has been shown that a variant prealbumin represents

the biochemical cause of the disease and that this mutated protein deposits in tissues as amyloid.^{8,9} The purpose of the present study was to examine the SAA subtype pattern in various inflammatory conditions. In particular, the question of whether patients with amyloidosis have an altered SAA subtype profile was evaluated.

Patients and methods

PATIENTS

Reactive systemic amyloidosis

This group consisted of 10 patients, (seven female, three male, mean age 45 years, range 30–68 years) with biopsy-proved amyloidosis. They all had rheumatoid arthritis as underlying disease. In these patients the concentration of SAA ranged from 58 to 146 mg/l.

Rheumatoid arthritis

This group consisted of 10 patients (six female, four male, mean age 54 years, range 35–76 years) with no clinical signs of amyloidosis. Their concentration of SAA ranged from 93 to 405 mg/l.

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Other diseases

This group consisted of two patients with juvenile rheumatoid arthritis (SAA 375 and 575 mg/l respectively), two patients with leprosy (SAA 42 and 55 mg/l respectively), one patient with bacterial sepsis (SAA 900 mg/l), one patient with glomerulonephritis (SAA 75 mg/l), and one patient with intracerebral and subdural bleeding (SAA approximately 800 mg/l).

Studies during the acute phase reaction

These studies were carried out in two patients with reactive systemic amyloidosis undergoing arthroplasty, one patient with rheumatoid arthritis without amyloidosis undergoing arthroplasty, one renal transplant recipient having an acute allograft rejection,¹⁰ one liver transplant recipient having acute graft rejection,¹¹ and one subject receiving injections of leucocyte interferon.¹²

Effect of enzyme inhibitors

To exclude the possible effect of proteolytic enzymes in serum on the SAA subtype pattern serum samples from a patient with rheumatoid arthritis were drawn into tubes containing aprotinin (Aprotinin) and trypsin inhibitor (Sigma, St Louis, Missouri, USA) and into control tubes without enzyme inhibitors.

ELECTROFOCUSING

Pretreatment of sample

5 µl serum sample was incubated for one hour at room temperature with 50 µl of 10 mM trometamol hydrochloride (TRIS-HCl) pH 8.2 containing 1% decyl sulphate (w/v), 1% ampholyte (LKB Ampholine 4-6 (w/v)), 20% sucrose (w/v), and 10% 2-mercaptoethanol (v/v).

Isoelectric focusing

The focusing gel was prepared as follows: 2 g acrylamine (Bio-Rad) and 45 g *N,N'*-methylenebis(acrylamide) (Kodak) were dissolved and the volume adjusted to 40 ml with deionized 8 M urea. To this 1.2 ml of Ampholine pH 4-6 (LKB) and 0.8 ml of Ampholine pH 3.5-10 (LKB) were added, and the solution was deaerated under reduced pressure. 75 µl *N,N,N',N'*-tetramethylenediamine and 2 ml of fresh 1% ammonium persulphate solution were added. Glasses, 1.5 mm spacers, and 10 well combs of the LKB 2001 vertical electrophoresis unit were used. 50 µl aliquots of sample were used. The slots were filled with a solution containing 25 µl LKB Ampholine pH 4-6 per ml and 7.5% sucrose. The gel was focused for 16 hours at 250 V in an LKB 2001 vertical electrophoresis apparatus at 4°C. 0.2% sulphuric acid was

used as anodal buffer and 0.4% ethylenediamine as cathodal buffer.

IMMUNOBLOTTING

After isoelectric focusing the gel to be blotted was washed briefly in distilled water and put on a glass plate. A Bio-Rad nitrocellulose membrane sheet was wetted in water and laid on the gel to make an airtight seal. Two pieces of filter paper and a layer of cellulose wad 1 cm thick were put on top of the membrane. The pocket was then evenly pressed for 1 h under another glass plate and a weight of 1 kg. After transfer the nitrocellulose paper was immersed for six hours in 10 mM TRIS, 0.9% sodium chloride buffer pH 7.4 (TBS) containing 3% gelatin (Bio-Rad EIA grade). The membrane was then incubated overnight with rabbit anti-SAA, diluted

Fig. 1. SAA subtype patterns in acute inflammatory conditions. A=application line. The arrows show the positions of the five SAA isomers: 1=reference SAA purified from HDL; 2=serum samples from patients with intracerebral and subdural bleeding; 3=active rheumatoid arthritis; and 4=bacterial sepsis.

1:50 in 1% gelatin-TBS. After incubation the membrane was washed for 40 minutes in TBS containing 25 μ l Tween 20 (Bio-Rad EIA grade) in 100 ml TBS. The washed membrane was then incubated with affinity purified goat antirabbit IgG horseradish peroxidase (HRP) conjugated second antibody (Bio-Rad) in a dilution of 1:2000 in 1% gelatin-TBS for two hours and then washed as earlier. Finally, the membrane was immersed in colour developing solution: 60 mg HRP colour development reagent (Bio-Rad) in 20 ml cold (-20°C) methanol, 100 ml TBS, and 60 μ l of 30% hydrogen peroxide.

ANTISERUM TO SAA

SAA was purified from the plasma of a patient with glomerulonephritis caused by glomerular basement membrane antibodies,¹³ and antiserum was raised in rabbits as described elsewhere.¹³

IMMUNOCHEMICAL QUANTIFICATION OF SAA

Radial immunodiffusion was used to measure SAA.¹⁰

PLASMA LIPOPROTEIN ISOLATION

Plasma was obtained after an overnight fast, and the lipoproteins were isolated by sequential ultracentrifugation with KBr to adjust the density.¹⁴

Results

Two major and three minor isoforms of SAA could be shown with the present method (Fig. 1). Monitoring of the two major isoforms was possible in serum samples with moderately raised SAA levels, whereas monitoring of all five isoforms was possible only in samples containing more than 900 mg of SAA/l. No difference in the SAA subtype pattern was observed between serum samples obtained with or without protease inhibitors (Fig. 2), suggesting that the SAA subtypes were not the result of the action of proteolytic serum enzymes after the withdrawal of the sample.

In order to study whether the SAA subtypes distributed evenly between the lipoproteins and the lipoprotein-free plasma fraction plasma was subjected to ultracentrifugal fractionation. As is evident from Fig. 2 SAA could be shown in the HDL and in the lipoprotein free infranant fraction. No difference in the subtype distribution was observed.

Fig. 3 shows the SAA subtype profiles in patients with reactive systemic amyloidosis and patients with rheumatoid arthritis without amyloidosis. Similar subtype patterns were seen in both patient groups. The SAA pattern was also unaltered during acute phase reactions induced by surgery in amyloidotic and non-amyloidotic subjects (Fig. 2), during acute renal and liver allograft rejections (Fig. 3), and during interferon administration. The SAA subtype

Fig. 2. SAA subtypes in lipoprotein fractions and lipoprotein-free plasma and the effect of enzyme inhibitors and surgery on the SAA subtype pattern. 1=lipoprotein-free infranant fraction; 2=HDL; 3=LDL (no SAA bands visualised); 4=VLDL (no SAA bands visualised); 5=fresh serum sample without inhibitors; 6=fresh serum sample + protease inhibitors; 7-9=arthroplasty.

Fig. 3. SAA subtype patterns in patients with and without reactive amyloidosis. 1, 2=rheumatoid arthritis; 3, 4=rheumatoid arthritis + amyloidosis; 5=reference SAA; 6, 7=juvenile rheumatoid arthritis; 8=renal allograft rejection; 9=renal transplantation (effect of operation); 10, 11=liver allograft rejection; 12=liver transplantation (effect at operation).

patterns in patients with bacterial sepsis (Fig. 1), leprosy, and glomerulonephritis were also similar.

Discussion

Several lines of evidence suggest that in AA-type amyloidosis the circulating precursor of the amyloid fibril protein is SAA. First, the SAA and AA proteins are structurally closely related,¹⁵⁻¹⁹ and SAA purified from plasma can be cleaved to an AA-sized fragment by serine proteases.^{20, 21} Secondly, in conditions associated with AA-type amyloidosis persistently raised SAA levels are found,^{4, 22} and thirdly, the progression of amyloidosis correlates with raised SAA levels.²³ Finally, in a recent report Husebekk *et al.*²⁴ presented evidence that SAA can be deposited as amyloid A fibrils in vivo. At present a raised SAA level can be regarded as a prerequisite for the development of reactive amyloidosis, but since only a small percentage of patients with chronic inflammatory diseases and raised SAA

level develop amyloidosis, additional factors are obviously needed for amyloidogenesis to occur.²⁵

In humans two major²⁶ and at least four minor^{5, 13} SAA isotypes have been identified, but the role of the different forms of SAA in the pathogenesis of amyloid deposits is unclear. It is known that the two main isoforms, which both display NH₂ terminal amino acid sequence homology with the tissue amyloid protein AA, differ from each other in that one of them is lacking the NH₂ terminal arginine.²⁶

To study the SAA subtype profiles in various diseases we developed a clinically applicable method which is based on electrofocusing of a small serum sample followed by immunoblotting. The sensitivity of this method is such that an SAA concentration of about 20 mg SAA/l can be readily detected. Monitoring of the different isoforms of SAA is quantitative. At a SAA concentration of 500 mg SAA/l three or four of the isoforms can be seen, whereas at concentrations lower than 100 mg SAA/l only the two main SAA bands are visible.

Our results show that the SAA subtype pattern is similar in patients with rheumatoid arthritis with or without amyloidosis. Moreover, in amyloidotic subjects the SAA subtype response to arthroplasty did not differ from that of rheumatic patients without amyloidosis. Analysis of patients with various acute and chronic infectious diseases and non-rheumatic inflammatory conditions showed similar SAA subtype patterns in all subjects, suggesting a non-specific character of the SAA subtype response. These results are in conformity with those of previous studies in which SAA was first isolated by preparative ultracentrifugation and then subjected to ion exchange chromatography^{5 13} or electrofocusing.^{3 27} The present method of separating the SAA subtypes is based on charge differences of the molecular species. Therefore it should be pointed out that our finding of similar SAA subtype patterns in various diseases including reactive amyloidosis does not exclude the possible existence of amyloid-specific SAA molecules having differences in amino acid sequence but not different isoelectric points.

In conclusion, our results based on electrofocusing and immunoblotting do not show amyloid-specific SAA subtype patterns or any disease-specific patterns, suggesting that the SAA subtype response to tissue injury and inflammation is similar irrespective of the initiating stimulus. With respect to amyloidogenesis our results suggest that though a persistently raised SAA level is a prerequisite for amyloid formation, other factors like tissue uptake mechanisms and degradation processes²⁵ are likely to be important additional factors.

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