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Identification of three isoform patterns of human serum amyloid A protein

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Three pattersn of human apo-SAA (serum amyloid A protein) isoforms have been identified by electrofocusing. In pattern 1, six major apo-SAA isoforms of pI 6.0, 6.4, 7.0, 7.4, 7.5 and 8.0 were found. In pattern 2, the apo-SAA isoforms of pI 7.4 and 8.0 were not detected, whereas in pattern 3 the pI-7.0 and -7.5 isoforms were lacking. Six patients displayed apo-SAA isoform pattern 1, 11 displayed pattern 2 and one displayed pattern 3.

INTRODUCTION

In reactive systemic amyloidosis, fibrils with amyloid A protein (protein AA) as their principal protein component are deposited in body tissues. Protein AA is a product of the partial proteolysis of the 104-residue serum amyloid A protein (apo-SAA), which appears in the plasma during the acute-phase response as an apolipoprotein of high-density lipoproteins (HDL), particularly HDL_3 [1–10]. Apo-SAA is secreted by the liver, probably in response to monocyte-derived factors, and rapidly associates with and remodels existing circulating HDL particles [7,11,12].

In the majority of individuals experiencing an acute or chronic elevation of the plasma acute-phase proteins, apo-SAA appears to be satisfactorily cleared from the circulation. In susceptible individuals, however, partial cleavage of apo-SAA may result in the accumulation of protein AA. When a chronic elevation of the plasma apo-SAA concentration occurs, protein AA may be sequestered in relatively large amounts in sites of amyloid fibrillogenesis [13].

In the mouse model of casein-induced amyloidosis, the involvement of only one apo-SAA isoform in amyloidogenesis has been demonstrated. The preferential sequestration of protein AA derived from mouse apo-SAA_2 in amyloid fibrils is not a reflection of an altered rate of synthesis, secretion or clearance of apo-SAA_2 relative to apo-SAA_1, but is probably a result of differences in the amino acid sequences of the two isoforms [14–16].

We have further characterized apo-SAA isoforms in HDL prepared from the plasma of individuals experiencing an acute-phase response. Our analyses, reported herein, have for the first time revealed that at least three different patterns of apo-SAA isoforms can be identified in humans.

MATERIALS AND METHODS

Subjects

Blood was obtained (with informed consent and Ethical Committee approval) from patients experiencing an acute-phase response. HDL was prepared (see below) from 18 patients (Table 1). Blood was collected into 3.5 mM-EDTA and, after separation of the plasma, aprotinin (Midran; Novo Industrials, Copenhagen, Denmark) and phenylmethanesulphonyl fluoride (Sigma, St. Louis, MO, U.S.A.) were added as previously described [12].

Preparation of HDL

To isolate HDL, the plasma density (ρ) was adjusted to 1.063 g/ml with solid KBr and the plasma centrifuged for 18 h at 50000 rev./min (Ti60 rotor, Beckman Instruments) at 10 °C. The infranatants containing HDL were harvested, re-adjusted to $\rho = 1.25$ g/ml and centrifuged for 20 h at 50000 rev./min (VTi50 rotor, Beckman Instruments) at 10 °C. The pellicles containing HDL were collected and washed by re-centrifugation under the same conditions. The final HDL sample was extensively dialysed against 0.15 M-NaCl/0.01% (w/v) EDTA, pH 7.4, then sterilized by filtration through a Millex-HA, 0.45-μm-pore-size filter unit (Millipore Corp., Bedford, MA, U.S.A.) and stored at 4 °C.

Electrofocusing

Electrofocusing gels were cast on Gel-bond PAG plates (FMC, Rockland, ME, U.S.A.) by using an LKB Ultromould gel-casting unit and a 0.3 mm spacer. The mixture contained 1.28 ml of acrylamide [28.9% (w/v) acrylamide and 1.2% (w/v) methylenebisacrylamide stock), 3.24 g of urea (7 M final concn.), 214 μl of pH 5–7 ampholytes (Pharmacia), 428 μl of pH 6–8 ampholytes (Bio-Rad), all made up to a total volume of 7.7 ml with distilled water. Gels were polymerized with 2.5 μl of *NNN'N'*-tetraethylethylenediamine and 7.7 μl of 40% (w/v) ammonium persulphate solution. A 200 μg portion of HDL was freeze-dried, delipidated with 0.5 ml of chloroform/methanol (2:1, v/v) [17], resuspended in 15 μl of 0.1 M-Tris, 1% (w/v) sodium decyl sulphate, 7 M-urea, 5% (v/v) 2-mercaptoethanol, pH 9.0, and was loaded on 0.5 cm × 1 cm pieces of filter paper applied at the anode. Electrode wicks were wetted with 1 M-NaOH and 0.33 M-H_3PO_4 for the cathode and anode

Abbreviations used: apo-SAA, serum amyloid A protein; protein AA, amyloid A protein; HDL, high-density lipoprotein(s); ρ, density; BSA, bovine serum albumin.

Table 1. Patient details

Patient	Age (years)	Sex	Condition(s)
A	35	♂	Pneumonia
B	62	♀	Diabetes; cellulitis
C	52	♂	Pneumococcal pneumonia
D	64	♂	Post-abdominal surgery (48 h)
E	26	♀	Post-abdominal surgery (40 h)
F	40	♂	Pneumonia
G	36	♀	Rheumatoid arthritis
H	41	♂	Acute pancreatitis
I	24	♀	Pneumonia
J	49	♀	Pneumonia
K	54	♂	Pneumonia
L	25	♂	Pneumonia
M	50	♂	Diabetes; pneumonia
N	27	♀	Pyelonephritis
O	53	♂	Post-abdominal surgery (50 h)
P	39	♀	Post-abdominal surgery (48 h)
Q	24	♀	Pyelonephritis
R	50	♂	Post-abdominal surgery (56 h)

respectively. Gels were pre-focused at 300 V (constant voltage) for 30 min, and the proteins were then focused at 5 W (constant power) for 2.5 h on an LKB Multiphor II electrophoresis unit with cooling at 10 °C. After electrofocusing, a strip of gel was cut into 0.5 cm × 1 cm pieces, which were placed in 2 ml of degassed distilled water subsequently checked for pH. The pI values of apo-SAA isoforms obtained in the present study (see the Results and discussion section) were found to be reproducible when the abovementioned ampholyte proportions were used. However, small variations in the pI values of the relatively-basic-pI isoforms were obtained by using different ampholyte batches and proportions. The remainder of the gel was soaked in the following solutions: 35% (v/v) methanol/10% (w/v) trichloroacetic acid/ 3.5% (v/w) sulphosalicylic acid for 5 min; 35% (v/v) ethanol/10% (v/v) acetic acid for 5 min; 0.5% (w/v) Coomassie Blue/35% ethanol/10% acetic acid for 5 min; 35% ethanol/10% acetic acid for 10 min (repeated twice); 1% (v/v) glycerol/35% ethanol/10% acetic acid, until a clear background was obtained. Quantification of isoforms was performed by pyridine extraction of Coomassie Blue-stained bands as described previously [12].

Immunochemical analyses

Two separate techniques were used to analyse the apo-SAA isoforms. Firstly, electrofocusing gels were immunoprinted by the procedure described in the Pharmacia application booklet [18], using either goat-anti-(human apo-SAA) (purified apo-SAA was used as antigen and the antibodies were made monospecific over CNBr–Sepharose 4B-coupled normal HDL) or the immunoglobulin fraction of sheep anti-(human apo-A-I) (Boehringer, Mannheim, Germany). Secondly, electrofocusing gels were pressure-blotted on to nitrocellulose membranes for 20 h at room temperature. The membrane was blocked for 30 min at 37 °C by using 5% (w/v) BSA/Tris (20 mM-Tris/HCl buffer, pH 8.2, containing 0.9% NaCl) containing 20 mM-NaN_3 and incubated for 2 h at room temperature with a 1:750 (v/v) dilution of rabbit anti-(human apo-SAA) (Calbiochem–Behring, La Jolla, CA, U.S.A.) in 5% BSA/Tris. The membrane was washed in excess 1% BSA/Tris (see above) and subjected to immunogold silver staining using goat anti-rabbit Auro-Probe BL Plus (Janssen Biotech, Olsen, Belgium) according to the manufacturer's instructions.

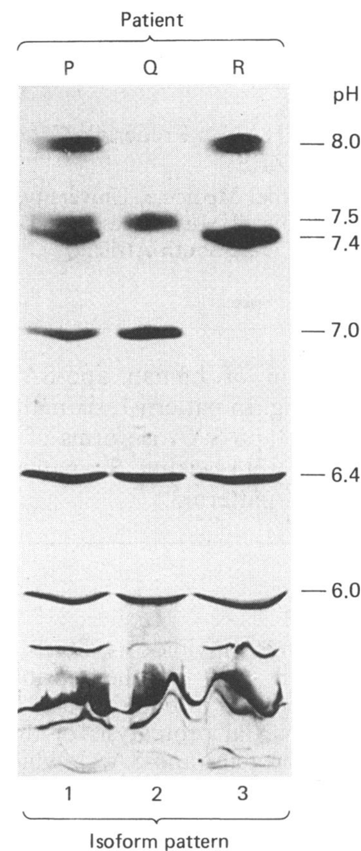

Fig. 1. Electrofocusing of delipidated HDL from three patients (P, Q and R) on a polyacrylamide gel containing 7 M-urea, illustrating the three patterns of the basic apo-SAA isoforms

Two-dimensional electrofocusing and SDS/PAGE

Two-dimensional analysis of apo-SAA isoforms involved using the electrofocusing method described above as the first dimension. For the second dimension, 5–20%-(w/v)-acrylamide gradient gels with a 3% stacking gel were used [19]. All solutions used for the second dimension contained 0.1% SDS. The space above the stacking gel was filled with 1% (w/v) low-gelling-temperature agar (Sigma) and the desired track was cut from the first-dimension gel and immediately lowered, through the agar, on to the surface of the stacking gel. Gels were stained with Coomassie Blue and destained in 10% methanol/10% acetic acid solution.

RESULTS AND DISCUSSION

In the mouse, amyloidosis can be induced by repeated subcutaneous injections of casein [20]. Of the three genes coding for murine apo-SAA, only two products (apo-SAA_1 and apo-SAA_2) are found in the plasma [21–25], and of these, only apo-SAA_2 is the precursor of the monotypic protein AA deposited in murine amyloid fibrils [14,15]. Meek *et al.* [15] concluded that the basis for the preferential deposition of apo-SAA_2 may be

Table 2. Quantification of apo-SAA isoforms

After electrofocusing of delipidated HDL from 18 patients (A–R, see Table 1), Coomassie Blue-stained bands were excised and extracted with 25% (v/v) pyridine solution. The relative contribution of each apo-SAA isoform to the total complement is expressed as a percentage of the total colour yield for the six isoforms detailed (apo-$SAA_{6.0}$–apo-$SAA_{8.0}$). Plasma from patients A–E and patients K–R were processed as two separate batches. Abbreviation: ND, not detectable (< 1.5% of total colour yield of each patient's isoform series).

Patient (see Table 1)		Percentage of total colour yield						Isoform pattern
	Apo-SAA isoform pI...	6.0	6.4	7.0	7.4	7.5	8.0	
A		9	25	10	14	12	30	1
B		15	18	11	17	7	32	1
C		26	37	21	ND	16	ND	2
D		26	35	21	ND	18	ND	2
E		33	31	20	ND	16	ND	2
F		24	19	17	17	9	14	1
G		25	32	14	14	7	8	1
H		29	39	19	ND	13	ND	2
I		36	42	16	ND	6	ND	2
J		31	36	17	ND	16	ND	2
K		35	33	20	ND	12	ND	2
L		26	34	23	ND	17	ND	2
M		29	28	25	ND	18	ND	2
N		29	23	27	ND	21	ND	2
O		31	34	9	14	3	9	1
P		22	38	7	12	5	16	1
Q		33	38	19	ND	10	ND	2
R		24	27	ND	35	ND	14	3

enhanced protein–cell and/or protein–protein affinities resulting from nine conservative amino-acid-residue changes in apo-SAA_2 compared with apo-SAA_1. Thus alterations in phenotypic expression may be associated with amyloidogenesis in the mouse. Although neither the structure nor the transcriptional regulation of the human apo-SAA genes has been fully characterized, they have been located on chromosome 11, and up to six apo-SAA isoforms have been described [26–29]. In contrast with the murine-amyloidosis model, however, the involvement of a particular isoform, or isoforms, of apo-SAA in amyloid-fibril deposition has not been shown, and no disease-associated alteration in apo-SAA phenotypes has been reported [30–32].

We recently observed that, of the apo-SAA isoforms, two (apo-$SAA_{6.0}$ and apo-$SAA_{6.4}$) constituted approx. 80% of the total apo-SAA complement, and the contribution of the individual isoforms to the total complement was, with the exception of the higher-pI isoforms, relatively constant in various individuals [33]. In that study, the pI > 6.4 isoforms were poorly resolved. However, it was noted that, in two of eight HDL preparations, the most basic isoform of apo-SAA accounted for up to 26% of the total apo-SAA [31]. The possibility exists that this variation may be important in amyloidogenesis. Thus in the present study we have analysed in detail the apo-SAA isoforms in the relatively-high-pH region.

Electrofocusing, in the presence of 7 M-urea, was performed by using a pH gradient designed to amplify the high-pH region (Fig. 1). By using this gradient, apo-A-II, apo-A-I and apo-$SAA_{5.6}$ remained at, or to the acidic side of, the point of application and were not clearly resolved. The latter peptides can be resolved by using appropriate pH gradients and a second dimension

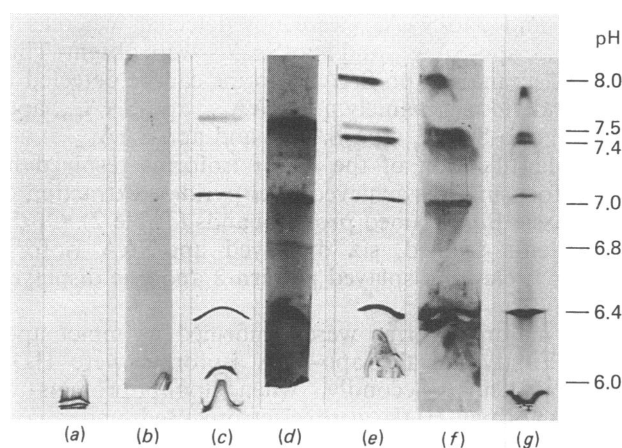

Fig. 2. Immunochemical analyses of electrofocused delipidated HDL from normal and acute-phase plasma

(*a*) Electrofocused HDL from normal plasma, Coomassie Blue-stained, with apo-A-I, apo-A-II and apo-C visible at the extreme acid end of the gel. (*b*) Immunoprint of a section of a track of electrofocused HDL from acute-phase plasma developed with sheep anti-(human apo-A-I). (*c*) Electrofocused HDL from acute-phase plasma showing apo-SAA isoform pattern 2 after Coomassie Blue staining. (*d*) Immunoprint of duplicate track (*c*) developed with immunopurified goat anti-(human apo-SAA). (*e*) Electrofocused HDL from acute-phase plasma showing apo-SAA isoform pattern 1 after Coomassie Blue staining. (*f*) Immunoprint of duplicate track (*e*) developed with immunopurified goat anti-(human apo-SAA). (*g*) Nitrocellulose blot of electrofocused HDL from acute-phase plasma (pattern 1) developed with rabbit anti-(human apo-SAA) and immunogold silver staining (gold-labelled goat anti-rabbit immunoglobulins).

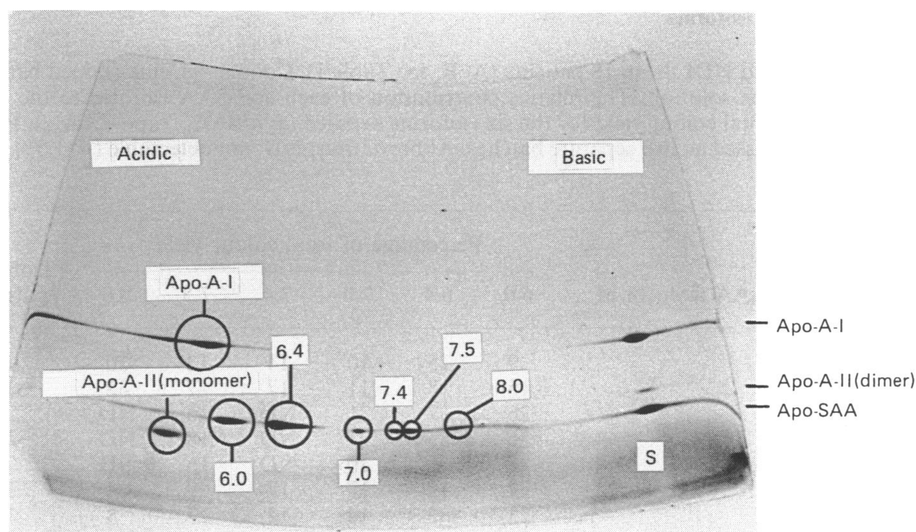

Fig. 3. Two-dimensional electrofocusing and SDS/5–20%-(w/v)-polyacrylamide-gel electrophoresis of delipidated HDL from acute-phase plasma of an individual with apo-SAA isoform pattern 1, illustrating size homogeneity of the apo-SAA isoforms

S is the second-dimension acute-phase HDL standard containing apo-A-II dimer, apo-A-I and apo-SAA.

of SDS/polyacrylamide-gel electrophoresis [33]. When HDL from 18 individuals was electrofocused, three different isoform patterns were observed, with marked differences in the more basic isoforms (examples of the three patterns are shown in Fig. 1). In pattern 1, four pI > 6.4 isoforms could be identified. In pattern 2, apo-$SAA_{7.4}$ and apo-$SAA_{8.0}$ were not detected, whereas in pattern 3, apo-$SAA_{7.0}$ and apo-$SAA_{7.5}$ were absent. Thus at least six major apo-SAA isoforms can be detected in some individuals, namely apo-$SAA_{6.0}$, apo-$SAA_{6.4}$, apo-$SAA_{7.0}$, apo-$SAA_{7.4}$, apo-$SAA_{7.5}$ and apo-$SAA_{8.0}$.

A quantification of the major isoforms resolved by electrofocusing was achieved after pyridine extraction of Coomassie Blue-stained protein bands (Table 2). Of the 18 patients studied, six displayed apo-SAA isoform pattern 1, eleven displayed pattern 2 and one displayed pattern 3.

The major isoforms were confirmed as intact apo-SAA. Firstly, all the apo-SAA isoforms were HDL apolipoproteins. Secondly, when apolipoproteins of pooled normal HDL were electrofocused under conditions identical with those used previously, no bands could be seen in the region of the gel occupied by apo-SAA isoforms after electrofocusing of acute-phase HDL (Fig. 2). Thirdly, all the major apo-SAA isoforms were detected using specific polyclonal antibodies (from two sources) to apo-SAA (Fig. 2). None of the putative apo-SAA isoforms were immunofixed with anti-(apo-A-I), but all the Coomassie Blue-stained apo-SAA isoforms and some very minor isoforms were immunofixed with the goat anti-(apo-SAA). This result was confirmed using the rabbit antibody to apo-SAA and the technique of immunogold silver staining of nitrocellulose-blotted, electrofocused acute-phase HDL. Finally, to exclude the possibility that these isoforms were partially degraded apo-SAA, two-dimensional electrofocusing and SDS/5–20%-(w/v)-polyacrylamide-gel electrophoresis was performed, confirming the intact nature of the major isoforms (Fig. 3). This result also indicated that none of the apo-SAA isoforms was a glycosylated derivative of another.

The isoform patterns reported here were not sex-related, nor did they appear to be related to any disease. None of the patients had clinical evidence of amyloidosis. The basis for the different patterns could be at the level of the structural genes, could reflect different rates of synthesis or metabolism of the same gene products in different subjects [34], or it could be the result of variable post-translational modifications other than glycosylation. None of these possibilities need, of course, be mutually exclusive. Since it is known that there are multiple human apo-SAA alleles [35–37] and we have now described three apo-SAA phenotypic patterns in acute-phase patients, there is a definite possibility that all the bands in pattern 2 arise from a single allele subject to a particular pattern of post-translational modification and that all the bands of pattern 3 similarly reflect systematically modified versions of a second allele. Pattern 1 would simply represent the heterozygote situation in such a model. Alternatively, there may in fact be a large number of different alleles coding for different peptides which, in the extreme case, would not vary in their electrophoretic mobility as a result of post-translational modification, but would do so only on the basis of different amino acid sequences (a smaller number of alleles would be compatible with some of the observed electrophoretic differences being due to post-translational modifications). In such a model we would have to postulate systematic *cis* linkages of different alleles arranged in such a manner that patterns 2 and 3 would respectively represent two sets of genes, and pattern 1 would reflect the heterozygotic situation for the two sets.

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