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Study of the expression of the genetic variants of human α_1-acid glycoprotein in healthy subjects using isoelectric focusing and immunoblotting

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Abstract

Human α_1-acid glycoprotein (AAG) exists as an heterogeneous population of two or three genetic variants (ORM1 F1 and/or S and ORM2 A) in the plasma of most individuals. The ORM1 and ORM2 variants have a separate genetic origin. AAG belongs to the acute-phase proteins, which, under conditions of inflammation, increase several-fold in concentration. Additionally, there is evidence to suggest that it is not only the concentration but also the distribution of the two gene products of AAG (ORM1 and ORM2) that alter in such conditions. Variations of the relative concentrations of the AAG variants in certain diseases, such as cancer, can only be shown by reference to data collected in healthy people. In this study, we have investigated a group of 74 healthy subjects (42 men and 32 women) for AAG concentrations, AAG phenotypes and relative proportions of genetic variants in plasma. The specific assay of AAG was carried out by an immunonephelometric method and the phenotyping was performed, after desialylation of AAG, by analytical isoelectric focusing. Detection of the AAG variants was made by immunoblotting and their relative proportions were determined by laser densitometry analysis. The AAG plasma concentrations in the healthy group ranged between 0.28 and 0.92 g/l (mean value 0.50 ± 0.14 g/l). The relative proportions of the variants derived from the two genes of AAG were variable, depending on the individual, but the amount of ORM1 variants almost always exceeded that of the ORM2 variant. No sex-related differences were observed in respect either in the total AAG level nor the relative proportions of the ORM1 and ORM2 variants. The data collected in this study may serve as a reference towards the investigation of possible changes in the expression of the genetic variants of AAG in chronic inflammatory diseases.

Keywords: Genetic variants; α_1-Acid glycoprotein

1. Introduction

α_1-Acid glycoprotein (AAG or orosomucoid) is a heavily glycosylated component of blood plasma. Its normal concentration is approximately 0.7 g/l, although it can vary greatly both between and within healthy individuals [1,2]. AAG presents a genetic polymorphism which is demonstrated when the protein is in the desialylated state [3]. Isoelectric focusing (IEF) methods using carrier ampholytes or immobilized pH gradients [4] and subsequent immunoblotting or immunoprinting with anti-human AAG antibodies allow the detection of various genetic variants of AAG in neuraminidase-treated plasma [5–7]. Population studies indicate that the AAG polymorphism is controlled by various alleles

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at two loci [7]. The first locus (ORM1) has two main variants (ORM1 F1 and ORM1 S), while the second locus (ORM2) is mainly monomorphic (ORM2 A variant). Three phenotypes are most frequently observed for AAG in the general population, ORM1 F1S/ORM2 A, ORM1 F1/ORM2 A and ORM1 S/ORM2 A, depending on the presence of two or three of the F1, S and A variants in plasma (ditto). It is worth noting that the relative proportions of the ORM1 and ORM2 variants vary according to the AAG phenotype, and also vary between individuals of the same phenotype [8].

The existence of two AAG loci, as postulated by population studies, is in agreement with the studies by Dente et al. [9] who have cloned and sequenced two different genes coding for AAG in a human haploid genome, the AAG-A and the AAG-B/B′ genes. The AAG-A gene is structurally similar to the AAG-B/B′ gene, but contains 22 base substitutions. A study using transgenic mice has shown that the AAG-A gene encodes the variants ORM1 and the AAG-B/B′ gene the variants ORM2 [10].

The plasma concentration of AAG is seen to increase up to five-fold during conditions of inflammation or tissue repair [11]. Additionally, the relative proportions of the gene products of the AAG-A and AAG-B/B′ genes (the variants ORM1 and ORM2, respectively) have been found to alter during acute-phase reactions [12,13].

The investigation of possible changes in the relative occurrence of the genetic variants of AAG under chronic pathological conditions (e.g., tumour growth) requires sufficient data on the expression of these variants in the normal situation. In this study, we determined the relative concentrations of the AAG variants in the plasma of 74 healthy volunteers, using analytical methods by IEF and immunonephelometry. The healthy group may serve as a reference group towards investigations into possible changes in the expression of the AAG variants in pathological situations.

2. Experimental

2.1. Source of plasma

Seventy-four healthy volunteers (31.7±9.7 years; range 20–58 years), of whom 42 were men (28.6±7.8 years; range 20–51 years) and 32 were women (35.7±10.7 years; range 20–58 years), took part in the study. Blood was obtained by venepuncture and collected into glass tubes containing heparin. Heparinized blood samples were centrifuged at 600 *g* for 20 min and at 4°C and the plasma fractions were frozen at −20°C until use.

2.2. Determination of AAG concentration

AAG concentrations were measured by an immunonephelometric method with a Beckman assay kit (''AAG reagent'' Ref. No. 449440) and nephelometer analyzer (Model 7571 ARRAY Protein System, Beckman Instruments, Fullerton, CA, USA).

2.3. AAG phenotyping in desialylated whole plasma

This was performed by analytical IEF, as described by Eap and Baumann [14], using (0.5 mm thick) Immobiline polyacrylamide gels in the pH range 4.5–5.4 (Pharmacia, Uppsala, Sweden) supplemented with 8 *M* urea (ACS reagent; Sigma, St. Louis, MO, USA), 2% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol (87% (w/v) (Merck, Darmstadt, Germany). Prior to IEF, the individual plasma were desialylated with neuraminidase (from *Clostridium perfringens*; Boehringer, Mannheim, Germany) as follows: a 5-μl volume of each plasma was added to 20 μl of a neuraminidase solution (1 U/ml) in a 5 m*M* sodium acetate buffer (pH 5.5), containing 0.9 m*M* $CaCl_2$ and 15.4 m*M* NaCl. The mixtures were incubated for 24 h at 37°C. They were then analyzed by IEF after serial dilutions to avoid an excess of AAG antigen [12]. IEF was run with a LKB 2117 Multiphor II electrophoresis apparatus equipped with a 2297 Macrodrive 5 constant-power supply. The diluted samples (20 μl) were applied at the cathodic end of the Immobiline gel using small pieces of filter paper which were removed after 1 h of focusing. IEF was carried out at 10°C, using electrode strips soaked in distilled water. The conditions were set to 5 W, 5 mA and 500 V for 1 h, and then 5 W, 5 mA and 2000 V, overnight.

The desialylated AAG variants were probed, after blotting onto nitrocellulose membrane (0.45 μm)

(Sartorius, Göttingen, Germany), with rabbit anti-(human AAG) immunoglobulins (Dakopatts, Glostrup, Denmark) and with the use of goat anti-rabbit IgG conjugated with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitroblue tetrazolium (Merck) in order to visualize the AAG bands [14].

The relative intensities of the bands (ORM1 F1, ORM1 S and ORM2 A) in each individual plasma were determined by scanning with a LKB 2202 Ultroscan laser densitometer. These intensities were multiplied by the total AAG level in corresponding plasma to obtain the relative concentrations of the variants.

2.4. Statistics

The data obtained for the plasma of the men and of the women were compared for statistical significance using the unpaired non-parametric Mann–Whitney two sample test. A P value less than 0.05 was considered to be significant.

3. Results

The results for AAG plasma concentrations in the whole group of the healthy subjects ($n=74$) are summarized in Table 1. Dot plots of the AAG plasma concentrations in the men ($n=42$) and women groups ($n=32$) taken separately, are shown in Fig. 1A. The corresponding results are summarized in Table 2. The AAG plasma levels in the whole group of the subjects ranged from 0.28 to 0.92 g/l with a mean value of 0.50±0.14 g/l (Table 1). No significant differences were observed when the AAG concentrations measured in the plasma of the men were compared to those measured in the plasma of the women (Table 2).

Fig. 2 shows the results from typical experiments, after AAG phenotyping in the desialylated plasma of the healthy individuals by analytical IEF and subsequent immunoblotting. Among a total of 74 subjects, 43 were of the ORM1 F1S/ORM2 A phenotype (lane 2 in Fig. 2), 26 of the ORM1 F1/ORM2 A phenotype (lane 1) and five of the ORM1 S/ORM2 A phenotype (lane 3). The calculated allele frequencies were 0.642 for ORM1 F1 and 0.358 for ORM1 S.

The results for the relative proportions of the gene products of the AAG-A and the AAG-B/B′ genes (the variants ORM1 and ORM2, respectively), as determined by laser densitometry analysis of the AAG patterns, are summarized in Table 1. Examples of densitometric determination in three individuals classified as being ORM1 F1S/ORM2 A, ORM1 F1/ORM2 A and ORM1 S/ORM2 A, respectively, are shown in Fig. 2. The average proportion of the ORM1 variants in the whole group of the subjects amounted to 67.3±8.5% (range 43.9–81.8%) and

Table 1
Total AAG concentrations, relative concentrations and proportions of the ORM1 and ORM2 variants in the plasma of healthy volunteers ($n=74$)

	AAG (g/l)	Relative proportions of genetic variants (%)				Relative concentrations of genetic variants (g/l)			
		AAG-A gene products			AAG-B/B′ gene product	AAG-A gene products			AAG-B/B′ gene product
		ORM1 F1	ORM1 S	Total ORM1 variants	ORM2 A variant	ORM1 F1	ORM1 S	Total ORM1 variants	ORM2 A variant
Mean±S.D.	0.5±0.14	47.6±14.7	35.3±12.8	67.3±8.5	32.7±8.5	0.24±0.11	0.18±0.08	0.34±0.12	0.16±0.05
Median value	0.46	45.5	31.3	68.9	31.1	0.22	0.16	0.31	0.14
Minimal value	0.28	16.7	11.8	43.9	18.2	0.05	0.06	0.17	0.07
Maximal value	0.92	81.8	75.2	81.8	56.1	0.60	0.46	0.69	0.31

The mean values of concentrations and proportions are shown with their standard deviations. The minima and maxima, as well as the medians, are also shown.

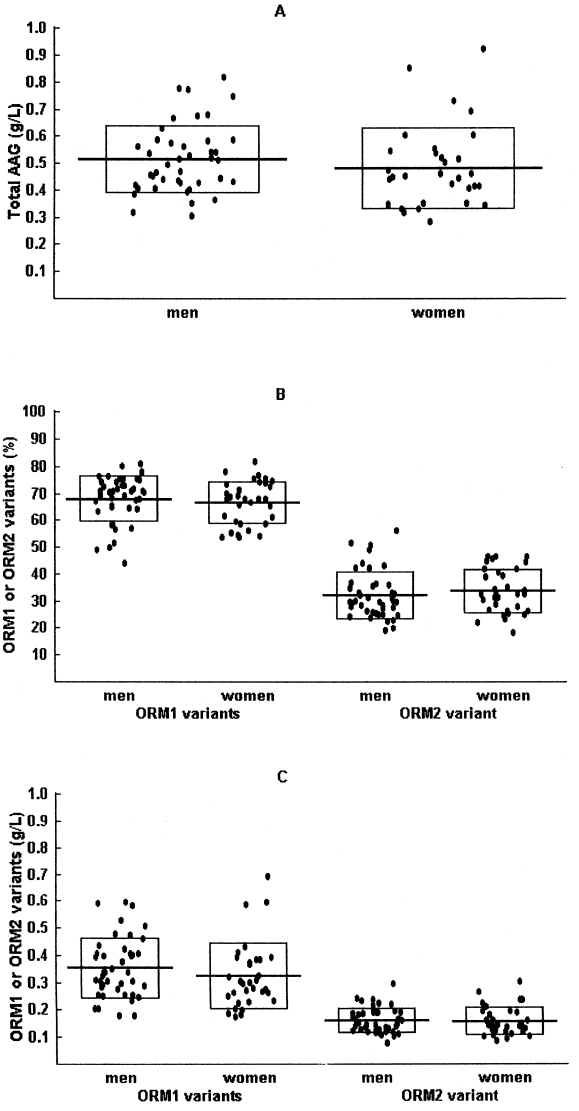

Fig. 1. Dot plots of the total AAG concentrations (A) and of the relative proportions (B) and concentrations (C) of the ORM1 and ORM2 variants in the plasma of the healthy men ($n=42$) and in those of the healthy women ($n=32$). The concentrations of total AAG were determined by an immunonephelometric method; the coefficients of variation of the data were less than 5%. The relative proportions of the AAG variants were determined by laser densitometry analysis of AAG patterns (cf. Fig. 2); the coefficients of variation were less than 4 and 2.5% for the ORM1 F1 and S variants, respectively, and less than 4% for the ORM2 A variant. See Section 2 for further details.

that of the ORM2 variant to 32.7±8.5% (range 18.2–56.1%) (Table 1). We also calculated the relative concentrations of each AAG variant in the plasma of the healthy individuals. The mean concentration of the ORM1 variants amounted to 0.34±0.12 g/l, while that of the ORM2 variant was 0.16±0.05 g/l (Table 1). In addition, we compared the results obtained for each variant in the plasma of the men to those obtained in the plasma of the women (Fig. 1B and 1C). With regard to the relative proportions of the ORM1 and ORM2 variants and their relative concentrations, no sex-related differences were observed (Table 2).

4. Discussion

Human AAG exists as an heterogeneous population of two or three genetic variants (ORM1 F1 and/or S and ORM2 A) in the plasma of most individuals [7]. The ORM1 and ORM2 variants are encoded by two different genes, the AAG-A and AAG-B/B′ genes, respectively [9,10]. As one of the acute-phase proteins, AAG is chiefly affected during acute-phase reactions (e.g., trauma) and also under chronic pathological conditions (e.g., tumour growth) (for a review see Ref. [11]). There is evidence to suggest that not only the concentration of AAG but also the distribution of its genetic variants alters during conditions of inflammation [12,13]. In certain diseases, such as cancer, variations in the relative concentrations of AAG variants can only be shown by reference to data collected in healthy people. However, this data must be large enough to reflect the variability in both the AAG level [1,2] and relative proportions of AAG variants [8] observed between healthy individuals.

In the present paper, we describe an investigation into the occurrence of the various genetic variants of AAG in the plasma of 74 healthy individuals. As far as we know, it is the first report that describes as much information on the relative distribution of the AAG variants in healthy people.

In accordance with previous studies (reviewed in Refs. [1,2]), we observed large variations in AAG concentration between the healthy individuals. The normal value of the average AAG plasma concentration determined in our study was 0.50±0.14 g/l.

Table 2
Plasma AAG concentrations, relative concentrations and proportions of ORM1 and ORM2 variants in the groups of the men (n=42) and the women (n=32) taken separately

	AAG (g/l)	Relative proportions of genetic variants (%)				Relative concentrations of genetic variants (g/l)			
		AAG-A gene products			AAG-B/B′ gene product	AAG-A gene products			AAG-B/B′ gene product
		ORM1 F1	ORM1 S	Total ORM1 variants	ORM2 A variant	ORM1 F1	ORM1 S	Total ORM1 variants	ORM2 A variant
Men	0.51±0.13	48.5±14.7	33.9±11.0	67.9±8.8	32.1±8.8	0.25±0.11	0.18±0.09	0.35±0.11	0.16±0.05
Women	0.48±0.15[a]	46.4±15.0	37.2±15.0	66.5±8.2[b]	33.5±8.2[c]	0.23±0.12	0.18±0.07	0.32±0.12[d]	0.16±0.05[e]

See the legend to Table 1 for further details.
Comparison of the men (n=42) to the women (n=32) for AAG concentrations[a], and for relative proportions and concentrations of the ORM1[b,d] and ORM2 variants[c,e], respectively in plasma by an unpaired non-parametric method.
[a] P=0.18; [b] P=0.34; [c] P=0.57; [d] P=0.12; [e] P=0.62.

This value is somewhat lower than the average normal value reported by other investigators [15], but the method used for AAG determination in the present study (i.e., immunonephelometry) was, however, different from that used in the former study (i.e., single radial immunodiffusion). Small differences were observed between the men and the women included in the present study – the mean concentration of AAG in the men being slightly higher than that in the women – but these differences were statistically non-significant. Despite this, previous investigators have reported sex-related differences in AAG plasma level [15], yet the number of subjects investigated was greater than the number of those studied here.

The observed AAG phenotypes in the whole group of subjects were the three main AAG phenotypes. Their frequencies were 58.1% for ORM1 F1S/ORM2 A, 35.1% for ORM1 F1/ORM2 A and 6.8% for ORM1 S/ORM2 A. The distribution of AAG phenotypes in this French population was not statistically different (χ^2=3.1; P=0.21) from that described by Eap and Baumann in a Swiss population of 500 subjects [6].

The relative proportions of ORM1 and ORM2 variants were variable depending on the phenotype and also between individuals of the same phenotype. Nevertheless, the proportion of ORM1 variants always exceeded that of the ORM2 A variant (Fig. 1B), with the exception of two individuals for which the proportion of ORM2 A amounted to 51.2 and 56.1%, respectively. Our results concur with those of a former study in the plasma of 45 healthy volunteers, in which ORM2 A was found to represent between 16.9 and 47.8% of the total of the AAG variants [8]. The former and present results are consistent with genetic studies which show that in human liver, the concentration of mRNA transcribed from the AAG-A gene is higher than that transcribed from the AAG-B/B′ gene [9]. However, while the ratio of AAG-A mRNA over AAG-B/B′ mRNA was at least one hundred in human liver, the mean ratio of ORM1 over ORM2 variants found in the former and present studies was approximately two. All together, these studies suggest important post-transcriptional factors in the regulation of the expression of the AAG genes.

In another study [16], some of us investigated the relative occurrence and glycosylation of the genetic variants of AAG in plasma and pleural effusions of patients with malignant mesothelioma. Determination of the AAG phenotypes and relative concentrations of the variants was performed using the same analytical methods as those described here and the data collected in the healthy group was used as a reference to investigate possible changes in the relative distribution of the AAG variants in the mesothelioma patients.

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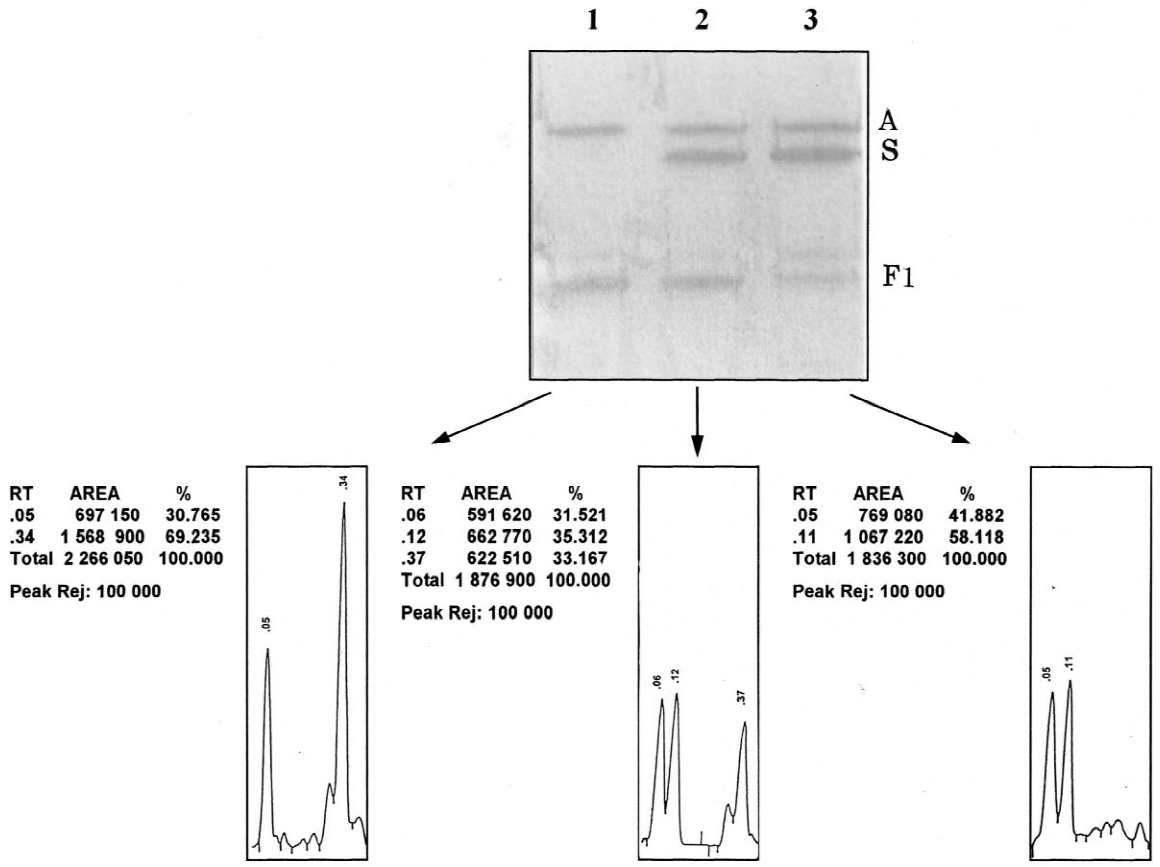

Fig. 2. IEF analysis after desialylation of AAG in the plasma of three different healthy individuals, on an immobilized pH 4.5–5.4 polyacrylamide gel gradient supplemented with 8 *M* urea and 2% (v/v) 2-mercaptoethanol and subsequent immunoblotting. Lanes 1, 2 and 3, individual plasma samples with AAG of the ORM1 F1/ORM2 A, ORM1 F1S/ORM2 A and ORM1 S/ORM2 A phenotype, respectively. For each plasma, about the same amount of AAG was applied (0.15 μg AAG in 20 μl diluted sample). The dilutions used corresponded to 0.27, 0.36 and 0.25 μl plasma in lanes 1, 2 and 3, respectively. The position of the bands corresponding to the ORM1 F1, ORM1 S and ORM2 A variants is indicated. The other faint bands appearing on the blot are probably due to incomplete desialylation of AAG. See Section 2 for details of the desialylation, IEF and immunoblotting procedure. Densitometric determinations in these three individuals are shown underneath.

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