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Changes in expression and microheterogeneity of the genetic variants of human α_1-acid glycoprotein in malignant mesothelioma

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Abstract

Human α_1-acid glycoprotein (AAG), an acute-phase plasma protein, is heterogeneous in the native state and polymorphic in the desialylated state. The AAG heterogeneity is mainly explained by a variable glycan chain composition in its five glycosylation sites. The AAG polymorphism is due to the presence of genetic variants. Three main variants are observed for AAG, ORM1 F1, ORM1 S and ORM2 A, which have a separate genetic origin. In this paper, we have used different isoelectric focusing (IEF) methods and chromatography on immobilized metal affinity adsorbent to study the relative occurrence of the genetic variants of AAG in relation to changes in microheterogeneity, in plasma and pleural effusions of patients with malignant mesothelioma (MM). The results were compared to those obtained with the variants in plasma of healthy individuals. Significant changes in variant distribution were observed in the MM samples, that corresponded to a rise in the proportion of the ORM1 variants and a fall in that of the ORM2 variant. However, the concentration in MM plasma increased for both variants. The AAG in MM plasma and effusion fluids was found to be more heterogeneous on IEF than AAG of healthy plasma. The evidence of stronger concentrations of both the high and low p*I* forms of AAG in the MM samples suggested two kinds of changes in charge heterogeneity. These two changes were shown to be attributed to different variants — i.e. the high p*I* forms to ORM1 F1 and S and the low p*I* forms to ORM2 A, after fractionation of AAG by chromatography on immobilized copper(II) ions. These results indicate specific changes in both the expression and glycosylation for each AAG variant, according to its separate genetic origin, in MM.

Keywords: Genetic variants; Malignant mesothelioma; α_1-Acid glycoprotein

1. Introduction

Human α_1-acid glycoprotein (AAG or orosomucoid) is an extensively glycosylated plasma protein with glycosylation accounting for some 45% of its molecular mass of 41 kDa [1]. AAG is heterogeneous in the native (sialylated) state. Different electrophoretic patterns of either 5, 6, 7 or more AAG bands can be observed after the separation of individual plasma by isoelectric focusing (IEF) [2–5]. Isoforms may result from amino acid substitutions and due to glycoforms which contain varying ratios of the five types of oligosaccharide chains found in AAG [1]. These chains differ in the degree of branching (di-, tri- or tetra-antennary) and in the extent of sialylation and fucosylation [6,7].

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AAG is an acute-phase reactant and as such, its plasma concentration increases several-fold during acute-phase reactions, e.g. trauma, and also under chronic pathological conditions, such as tumour growth [8]. Additionally, the expressed glycosylation pattern of AAG [9,10] and its degree of microheterogeneity [2,11,12] alter in such conditions. Whilst there has been much interest over changes in the proportions of the microheterogeneous forms of AAG in inflammation and cancer, little attention has been paid to the changes in the expression and glycosylation of its genetic variants.

AAG has three main genetic variants, ORM1 F1, ORM1 S and ORM2 A, which can be detected in plasma by IEF after desialylation of AAG [13,14]. These variants determine three main phenotypes for AAG in the human population, ORM1 F1S/ORM2 A, ORM1 F1/ORM2 A and ORM1 S/ORM2 A. This polymorphism is explained by the presence of two different AAG genes [15], of which the AAG-A gene encodes the variants ORM1 F1 and S and the AAG-B/B′ gene the variant ORM2 A [16]. The AAG-B/B′ gene is structurally similar to the AAG-A gene but contains 22 translationally significant base substitutions [15] for a total of 181 amino acid residues [1]. Besides, the ORM1 F1 and S variants encoded by two alleles of the AAG-A gene [13,14] differ by less than five amino acids [15].

We have recently developed a fractionation method for the AAG variants, according to their separate genetic origin [17–19]. Studies with the ORM1 F1 and S and ORM2 A variants separated in the native (sialylated) form, have shown that these variants differ in their glycosylation [18]. Genetic studies have shown that the two different genes which code for the ORM2 A variant separately, and for the ORM1 F1 and S variants, are differently transcribed under acute-phase conditions [20]. Accordingly, each AAG variant may be changed individually in both its concentration and glycosylation, under conditions of inflammation.

In this paper, we have used different IEF methods and chromatography on immobilized metal affinity adsorbent to study the occurrence of the various genetic variants of AAG in relation to changes in microheterogeneity, in plasma and pleural effusions of patients with diffuse malignant mesothelioma, a primary tumour of the pleura. The results were compared to those obtained with the variants in plasma of healthy individuals.

2. Experimental

2.1. Source of plasma and pleural effusions

The plasma and pleural effusion samples of individual patients with diffuse malignant mesothelioma (MM) (seven men; 57.5±7.3 years; range 51–66 years) were a gift from Professor J. Bignon, Service de Pneumologie at the Hôpital Intercommunal (Créteil, France). MM was histologically proven by pathological and histochemical studies and confirmed by the French Mesothelioma Panel. Blood was obtained by venepuncture and pleural effusion by needle puncture, and were collected into glass tubes containing EDTA. The samples were collected before any treatment was given to the patients. After centrifugation (600 *g*, 10 min, 4°C), small volumes of the plasma and effusion fluid fractions were saved for this study and stored frozen at −20°C until use.

The plasma of healthy individuals used as control samples in this study were obtained as described previously [21].

2.2. AAG determination

The specific determination of AAG was carried out by an immunonephelometric method described previously [21].

2.3. Isoelectric focusing

IEF was run with a LKB 2117 Multiphor II electrophoretic apparatus equipped with a Pharmacia EPS 3500XL power supply.

2.3.1. Analytical IEF with carrier ampholytes in the pH range 2.5–5

IEF was carried out in 0.5-mm thin-layer polyacrylamide gels (5% T, 3% C). Gels contained 6.4% (v/v) Pharmalyte carrier ampholytes in the pH range 2.5–5 (Pharmacia, Uppsala, Sweden) and were prepared according to the instructions of the manufacturer. The electrode solutions were 0.1 *M* NaOH for

the cathode and 0.1 *M* H_2SO_4 for the anode. Plasma, pleural effusions, and protein fractions isolated after chromatography of these samples were separately applied at the cathodic end of the gel, using small pieces of filter paper which were removed after 1 h of focusing. Focusing was carried out at 10 W constant power. A maximum voltage of 2000 V with unlimited current was applied. IEF was carried out for a total of 3.5 h at a cooling temperature of 10°C. After focusing, the proteins were stained with silver (Merck, Darmstadt, Germany) according to [22], or with Coomassie Brilliant Blue R-250 (Merck) using a previously described method [12]. Determination of pH gradients was performed by using the low p*I* calibration kit from Pharmacia [i.e. p*I* markers: pepsinogen (p*I* 2.80), amyloglucosidase (p*I* 3.50), methyl red (p*I* 3.75), glucose oxydase (p*I* 4.15) and soybean trypsin inhibitor (p*I* 4.55)].

2.3.2. Analytical IEF on a 4.5–5.4 immobilized pH gradient

Genetic variants of human AAG were determined according to the method of Eap and Baumann [23]. After desialylation with neuraminidase, the plasma, pleural effusions, and protein fractions isolated after chromatography of these samples were separately subjected to analytical IEF on Immobiline polyacrylamide gels in the pH range 4.5–5.4, supplemented with 8 *M* urea and 2% 2-mercaptoethanol. Detection of the desialylated variants was made by immunoblotting. The desialylation, IEF and immunoblotting procedures were carried out as described previously [21]. The type of variant of the AAG bands was identified by running control plasma.

2.3.3. Laser densitometry

Densitometric measurements were performed with a LKB 2202 Ultroscan laser densitometer. Each track was determined in two positions.

2.4. Affinity chromatography of plasma and pleural effusion samples on immobilized copper(II) ions

Iminodiacetate (IDA)–Sepharose (45–165 μm mean particle size, 22–30 μmol Cu^{2+} per ml gel; Pharmacia) was equilibrated with copper(II) ions [copper(II) chloride, ACS reagent; Sigma, St. Louis, MO, USA] as described previously [17]. The loaded IDA–Cu(II) gel was packed into small polyethylene columns (1.4 cm I.D; Econo-Pac chromatography columns, Bio-Rad, Richmond, CA, USA) to a final volume of 2.5 ml and the gel was equilibrated with 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.5 *M* NaCl (equilibration buffer E). Chromatography was performed at room temperature (20°C). The plasma and pleural effusion samples (~2 ml) were first dialyzed for 36 h at 4°C against column equilibration buffer E with several buffer changes. Then, a 1.8-ml volume of each dialyzed sample was applied to the affinity column at a flow-rate of 5 ml/h. Fractions of 0.5 ml were collected and their respective absorbances were measured by spectrophotometry at 280 nm. After several bed volumes of buffer E had been applied to remove the nonbound proteins, a second elution buffer, also pH 7, consisting of 20 m*M* imidazole (Merck) in buffer E, was applied to remove the high-affinity proteins. The columns were regenerated after each run by removing the metal with several volumes of 50 m*M* EDTA (Sigma) in buffer E.

After completion of the chromatographic separation, the appropriate peak fractions were collected and concentrated on a YM 10 membrane filter (Amicon, Danvers, MA, USA). They were then dialyzed against distilled water and finally lyophilized before studying by analytical IEF.

2.5. Statistical analysis of the data

Data were analyzed for statistical significance using paired Student's *t*-test for within-group analysis, and unpaired nonparametric Mann–Whitney two sample test for between-group analysis. A P value <0.05 was considered to be statistically significant.

3. Results

3.1. Occurrence of the genetic variants of AAG in plasma and pleural effusion of patients with malignant mesothelioma (MM)

The results for the AAG concentrations in the plasma and effusion fluids of the MM patients are

Table 1
AAG concentration and relative occurrence of the ORM1 F1, ORM1 S and ORM2 A variants in the plasma of patients with malignant mesothelioma

MM patient	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
1	0.93	84.6	–	84.6	15.4	0.79	–	0.79	0.14
2	0.64	43.6	33.7	77.3	22.7	0.28	0.21	0.50	0.14
3	2.95	35.9	41.3	77.2	22.8	1.06	1.22	2.28	0.67
4	0.83	36.2	34.8	71.0	29.0	0.30	0.29	0.59	0.24
5	2.09	43.5	37.9	81.4	18.6	0.91	0.79	1.70	0.39
6	1.31	43.5	31.1	74.6	25.4	0.57	0.41	0.98	0.33
7	1.08	–	64.4	64.4	35.6	–	0.70	0.70	0.38
Mean±S.D.	1.40±0.83[a]	47.9±18.4	40.5±12.2	75.8±6.7[b]	24.2±6.7 [c]	0.65±0.32	0.60±0.38	1.08±0.66[d]	0.33±0.18[e]

Each AAG concentration is the mean of two determinations; the coefficients of variation did not exceed 1.6%. Each proportion of variant is the mean of two determinations; the coefficients of variation did not exceed 6.7% and 7.3% for the ORM1 F1 and S variants, respectively, and 8.1% for the ORM2 A variant. The relative concentrations of the variants were obtained by multiplying their respective proportions by the concentration of total AAG in plasma.
Comparison of the mesothelioma patients ($n=7$) to healthy individuals ($n=74$) for AAG concentrations[a] and for relative proportions and concentrations of the ORM1[b,d] and ORM2 variants [c,e] in plasma, respectively, by an unpaired nonparametric method

[a] $P<0.0001$.

[b] $P=0.01$.

[c] $P=0.01$.

[d] $P<0.0001$.

[e] $P=0.003$.

See [21] (this issue) for the AAG concentrations, relative proportions and concentrations of the ORM1 and ORM2 variants in plasma of healthy individuals.

Table 2
AAG concentration and relative occurrence of the ORM1 F1, ORM1 S and ORM2 A variants in the effusion fluids of patients with malignant mesothelioma

MM patient	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
1	0.64	81.9	–	81.9	18.1	0.52	–	0.52	0.12
2	0.52	41.3	40.5	81.8	18.2	0.21	0.21	0.42	0.10
3	1.16	38.8	43.5	82.3	17.7	0.45	0.50	0.95	0.21
4	0.47	41.7	34.1	75.8	24.2	0.20	0.16	0.36	0.11
5	0.81	44.6	33.5	78.1	21.9	0.36	0.27	0.63	0.18
6	0.93	49.7	29.5	79.2	20.8	0.46	0.27	0.74	0.19
7	1.00	–	67.8	67.8	32.2	–	0.68	0.68	0.32
Mean±S.D.	0.79±0.26 a)	49.7±16.2	41.5±13.9	78.1±5.1[b]	21.9±5.1[c]	0.37±0.14	0.36±0.22	0.62±0.20[d]	0.17±0.08[e]

Each AAG concentration is the mean of two determinations; the coefficients of variation did not exceed 1.8%. Each proportion of variant is the mean of two determinations; the coefficients of variation did not exceed 5.4 and 6.6% for the ORM1 F1 and S variants, respectively, and 8.9% for the ORM2 A variant. The relative concentrations of the variants were obtained by multiplying their respective proportions by the concentration of total AAG in effusion fluid. Comparison of the plasma and pleural effusions of the mesothelioma patients for AAG concentrations[a] and for relative proportions and concentrations of the ORM1[b,d] and ORM2 variants[c,e], respectively, by a paired method.

[a] $P=0.04$.

[b] $P=0.14$.

[c] $P=0.15$.

[d] $P=0.05$.

[e] $P=0.03$.

presented in Tables 1 and 2, respectively. The AAG concentrations in the plasma ranged from 0.64 to 2.95 g/l, while those in the corresponding effusions fluids were lower, ranging between 0.47 and 1.16 g/l. A statistical analysis of the data showed that the decrease of the total AAG level in pleural effusion was significant (Table 2).

Fig. 1 shows representative results for the AAG patterns obtained when the plasma and pleural effusions that had been desialylated with neuraminidase, were subjected to analytical IEF followed by immunoblotting. Fig. 1 also shows examples of the densitometric determination in one patient. Depending on the patient investigated, two or three genetic variants of AAG were detected in plasma, namely ORM1 F1 and/or S and ORM2 A. The variants detected in each effusion fluid were identical to those detected in the corresponding plasma. The observed AAG phenotypes in the whole group of the patients were ORM1 F1S/ORM2 A ($n=5$), ORM1 F1/ORM2 A ($n=1$) and ORM1 S/ORM2 A ($n=1$) (Tables 1 and 2), which correspond to the three main AAG phenotypes.

The results for the relative proportions of the genetic variants ORM1 F1, ORM1 S and ORM2 A in plasma and pleural effusion are shown in Tables 1 and 2, respectively. For all samples, the proportion of the variants derived from the AAG-A gene (the variants ORM1 F1 and S) was higher than that of the variant derived from the AAG-B/B′ gene (the variant ORM2 A). The ratio of AAG-A over AAG-B/B′ gene products was found to be up to 5.5. Only small differences were observed between the relative proportions of each variant in the plasma and corresponding effusion fluid. Moreover, these differences were statistically not significant (Table 2). The existence of a similar distribution of AAG variants in the plasma and pleural effusion of each patient indicates that both the ORM1 and ORM2 variants contribute to the decreased level of total AAG in pleural effusion, compared to plasma. The calculation and comparison of the relative concentrations of

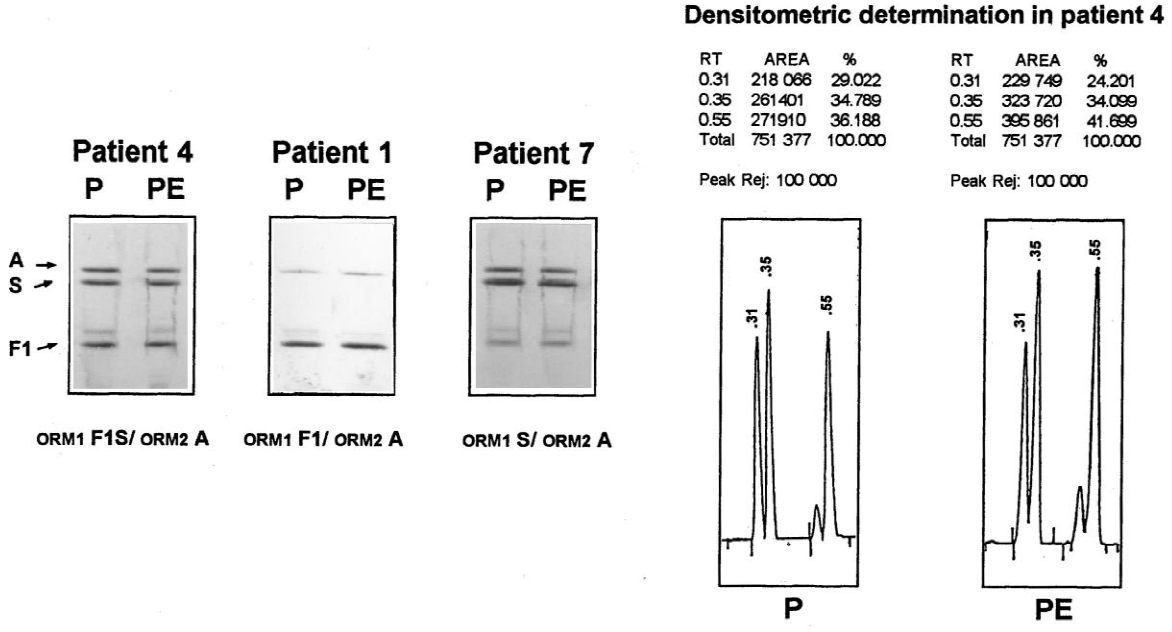

Fig. 1. IEF analysis after desialylation of AAG in plasma (P) and corresponding pleural effusion (PE) from three mesothelioma patients on a 4.5–5.4 immobilized pH gradient. For all samples, the same amount of AAG was applied (0.08 μg). The numbers indicated for the patients correspond to those used in Tables 1 and 2. The position of the variants is indicated by the arrows. The AAG phenotypes are indicated under the patterns. The other faint bands appearing on the blot are probably due to incomplete desialylation. All other details are described in Section 2. Examples of the densitometric determination in one patient are shown on the right.

each variant in plasma and in pleural effusion further confirmed this result (Table 2).

3.2. Comparison of the mesothelioma patients with healthy individuals for the occurrence of the AAG variants in plasma

In order to investigate possible changes in the expression of the genetic variants of AAG in MM, we compared the group of patients to another group of healthy individuals for AAG concentrations and relative proportions of the variants in plasma. The healthy individuals of the reference group ($n=74$), as well as the results obtained for AAG and its variants in the plasma of these individuals, have been described earlier [21].

In comparison to the healthy group, there was a significant increase in the total AAG level in the MM group (Table 1). On average, the AAG level increased some three-fold. Significant differences in the distribution of the AAG variants were also observed between the two groups. There was a rise in the relative proportion of the ORM1 variants (and reciprocally a fall in that of the ORM2 variant) in the MM plasma (Table 1). The ratio of ORM1 over ORM2 variants in these samples was, on average, 1.5-times higher than that determined in the healthy plasma [21]. However, a comparison of the relative concentrations of each variant in the MM plasma with those in the healthy plasma showed that there was a significant increase in the concentration of both the ORM1 and ORM2 variants in the MM samples (Table 1). From this data, it follows that the gene products of both the AAG-A and AAG-B/B′ genes contribute to the increased plasma level of AAG in MM. Nevertheless, the rise in the relative proportion of the ORM1 variants, compared to the ORM2 variant, indicates that the contribution of the former variants is more important than that of the latter.

3.3. Charge heterogeneity of AAG in plasma and pleural effusions of the mesothelioma patients and in plasma of healthy individuals

In order to investigate possible changes in the degree of microheterogeneity of (native) AAG, the plasma and pleural effusions of the MM patients and the plasma of healthy individuals were analyzed by IEF on an acidic pH gradient. The healthy plasma samples used in this study were obtained from individuals from the reference group previously studied [21].

Previous investigators have reported the existence of a correlation between the electrophoretic patterns of the native and desialylated forms of AAG [5,19,24]. This can further complicate a comparison of the microheterogeneity of (native) AAG in samples from different individuals. Therefore, the IEF study of AAG in the mesothelioma and healthy samples was performed according to the phenotype of the protein.

Fig. 2 shows the results taken from typical experiments after separation of MM plasma and effusion fluids and of healthy plasma by IEF. Using a 2.5–5 pH gradient and applying the specimens at the cathode-end of the gel, the AAG bands can be seen to run in the opposite direction to the other plasma or effusion fluid proteins. All the samples gave multiple AAG bands over the pH range from ~3.6 to 2.8. However, a cursory inspection of the figures shows that the IEF patterns yielded by AAG in the MM samples were different from those in the healthy plasma. More specifically, the MM samples had more of the high p*I* forms of AAG, and also contained more of the low p*I* forms, but the former change was more apparent. One can equally note that the AAG pattern in MM plasma mimics that of the corresponding effusion fluid, which suggests a similar distribution of the AAG isoforms in each pair of samples.

3.4. Separation of the AAG variants in the native (sialylated) form by affinity chromatography of mesothelioma effusion fluid and of healthy plasma on immobilized copper(II) ions

A fractionation method has been developed for the AAG variants in the native (sialylated) form from purified AAG samples, by chromatography on an immobilized affinity copper(II) adsorbent [18,19]. In this method, the ORM2 A variant was found to strongly bind to immobilized copper(II) ions and was separated from the ORM1 F1 and S variants which showed no significant binding affinity for the transition metal ions.

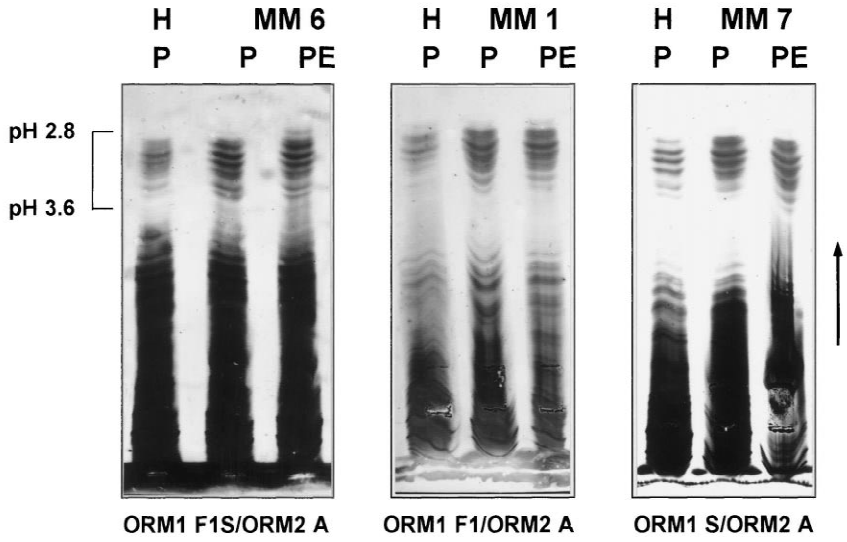

Fig. 2. Analytical IEF with carrier ampholytes in the pH range 2.5–5 of AAG in plasma and corresponding pleural effusion from three mesothelioma patients and in plasma from three healthy individuals. Equal volumes of plasma (P) or pleural effusion (PE) were loaded (10 μl; 5–13 μg AAG). On each figure, the specimen order is healthy (H) plasma, mesothelioma (MM) plasma and pleural effusion, respectively. The numbers indicated for the MM samples correspond to those used in Tables 1 and 2. The results are presented according to the AAG phenotype. The pH scale is indicated. All other details are described in Section 2.

Here, we investigated the possibility of using this chromatographic method for the separation of the AAG variants from small samples of MM pleural effusions and of healthy plasma, without prior isolation of AAG, in order to then study the charge heterogeneity of the individual variants by analytical IEF. The amount of plasma or pleural effusion which could be processed by chromatography without affecting the efficiency of the AAG fractionation, was determined in preliminary experiments (data not shown). Fig. 3 shows representative results for the elution profiles obtained after affinity chromatography of these samples. Irrespective of the individual sample used, the chromatography resolved only two protein fractions (1 and 2) under our experimental conditions: a nonbound minor fraction (1) eluted with several volumes of the equilibration buffer and a bound major fraction (2) eluted after introduction of 20 m*M* imidazole in buffer. The elution volumes measured for each fraction in the different chromatographic experiments were similar. The yield of total protein in fractions 1 and 2, as measured by spectrophotometry, was about 80% in both the experiments with MM effusion fluids and with healthy plasma. Integration showed that fractions 1 and 2 consisted respectively of approximately 5 and 95% of the eluted proteins. The rest of the bound proteins was finally removed from the affinity columns by subsequent washing with 50 m*M* EDTA in buffer E.

The AAG variants of the different chromatographic fractions were identified by analytical IEF on a 4.5–5.4 immobilized pH gradient, followed by immunoblotting. Prior to IEF, a small amount of each fraction was desialylated with neuraminidase. Representative results for the IEF patterns obtained with these fractions are shown in Fig. 4. Depending on the AAG phenotypes of the samples used in chromatography, fraction 1 was found to contain only the ORM1 F1 or the ORM1 S variant (the samples with AAG of the ORM1 F1/ORM2 A phenotype and those with AAG of the ORM1 S/ORM2 A phenotype, respectively), or both variants (the samples with AAG of the ORM1 F1S/ORM2 A phenotype). However, for all samples, fraction 2 was found to contain essentially the ORM2 A variant. Furthermore, no AAG variant was detected in the 50 m*M*

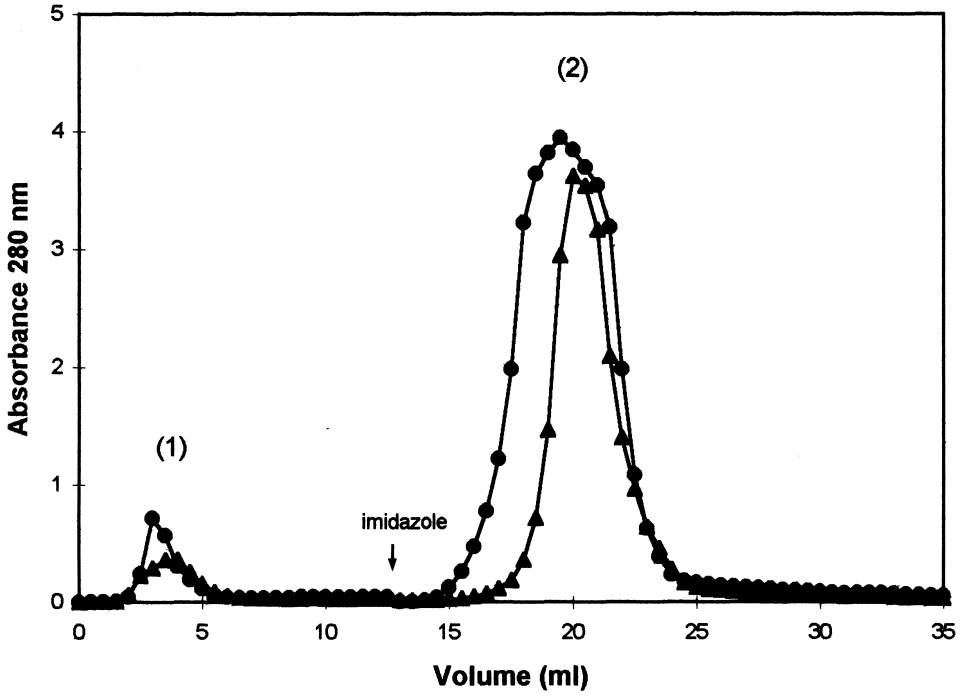

Fig. 3. Elution profiles of the proteins from affinity chromatography of mesothelioma pleural effusion (▲) and of healthy plasma (●) on IDA–Cu(II) gel. The nonbound protein fraction (1) was removed by application of several bed volumes of the equilibration buffer (20 m*M* sodium phosphate buffer, pH 7, with 0.5 *M* NaCl). The bound protein fraction (2) was eluted after 20 m*M* imidazole was introduced (indicated by an arrow). The elution profiles correspond to those obtained with samples containing AAG of the ORM1 F1S/ORM2 A phenotype, but similar profiles were obtained with samples containing AAGs of the other two phenotypes. All other details are described in Section 2.

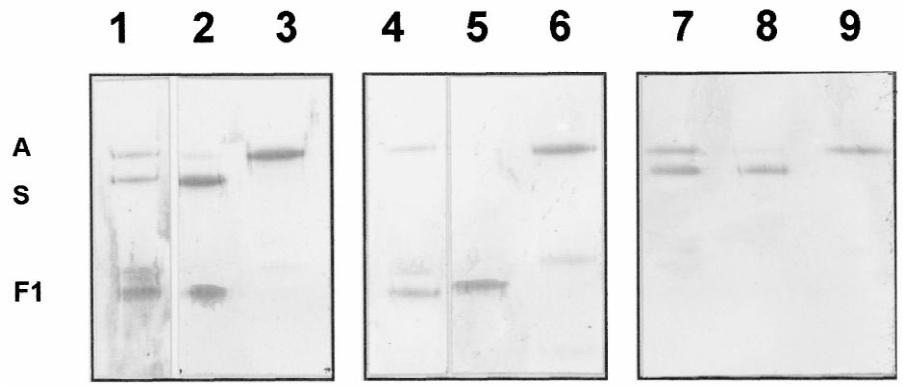

Fig. 4. IEF analysis after desialylation of the AAG variants in fractions 1 and 2 separated by affinity chromatography of mesothelioma pleural effusion or healthy plasma, on a 4.5–5.4 immobilized pH gradient. For all samples, about the same amount of AAG or variant was applied (~0.1 μg). The IEF patterns correspond to those obtained with protein fractions isolated from mesothelioma samples, but the IEF of fractions isolated from healthy samples yielded similar results. Lanes 1, 4 and 7, pleural effusions of mesothelioma patient 5 (ORM1 F1S/ORM2 A-AAG), patient 1 (ORM1 F1/ORM2 A-AAG) and patient 7 (ORM1 S/ORM2 A-AAG), respectively. Lane 2, fraction 1 (variants ORM1 F1 and S) and lane 3, fraction 2 (variant ORM2 A), separated from pleural effusion of patient 5. Lane 5, fraction 1 (variant ORM1 F1) and lane 6, fraction 2 (variant ORM2 A), separated from pleural effusion of patient 1. Lane 7, fraction 1 (variant ORM1 S) and lane 9, fraction 2 (variant ORM2 A), separated from pleural effusion of patient 7. All other details are described in Section 2.

EDTA eluates, which indicates that nearly all of the ORM1 and ORM2 variants were recovered in fractions 1 and 2, respectively.

3.5. Study of the charge heterogeneity of the separate ORM1 and ORM2 variants in fractions of mesothelioma effusion fluid and of healthy plasma by analytical IEF on an acidic pH gradient

The charge heterogeneity of the AAG variants separated in the native (sialylated) form was studied by analytical IEF with carrier ampholytes in the pH range 2.5–5. Fig. 5 shows the results from typical experiments after IEF analysis of fractions 1 [the variant(s) ORM1 F1 and/or S] and 2 (the variant ORM2 A) separated from MM effusion fluids and from healthy plasma. For a better comparison of the results obtained with the MM and healthy samples, equal amounts of AAG variants were analyzed. The IEF patterns showed that the ORM1 F1 or S variant or a mixture of these variants were resolved into several bands between pH ~2.7 and 3.6 (Fig. 5A). However, it was observed that the ORM1 variants separated from the MM effusion fluids had a less acidic distribution of bands than the ORM1 variants separated from the healthy plasma.

The ORM2 A variant was also heterogeneous, consisting of several isoforms that focused at pH ~2.9–3.5 (Fig. 5B). Small changes in the charge heterogeneity of the ORM2 A variant were observed, but interestingly, these changes were different from those observed with the separate ORM1 variants. Of the various bands corresponding to the ORM2 A variant there was an average shift towards more acidic pH values for the patterns obtained with the MM samples. Densitometric evaluation of the respective IEF patterns of ORM1 and ORM2 variants agreed with these observations (data not shown).

4. Discussion

Malignant pleural mesothelioma (MM) is a rare tumour which is principally related to occupational

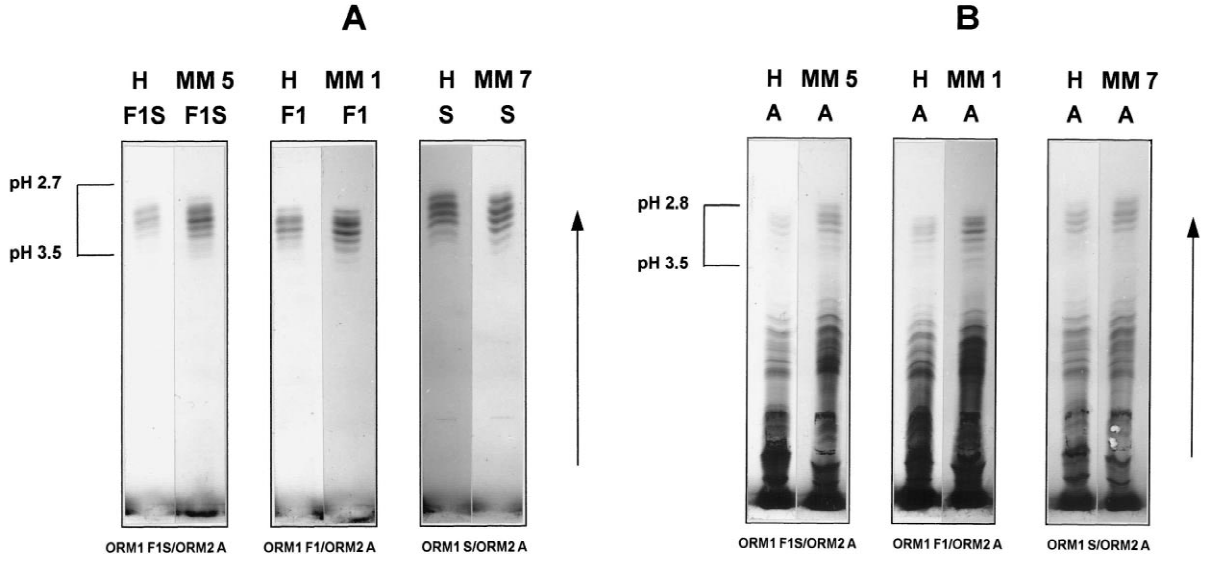

Fig. 5. Analytical IEF with carrier ampholytes in the pH range 2.5–5 of the AAG variants in fractions 1 and 2 isolated after affinity chromatography of mesothelioma (MM) pleural effusions and of healthy (H) plasma. In (A) and (B), the IEF patterns obtained with fraction 1 [ORM1 F1 and/or S variant(s)] and fraction 2 (ORM2 A variant), respectively. The loaded amounts of variants isolated from the mesothelioma and healthy samples were identical (15 or 20 μg). The loaded amount of ORM1 or ORM2 variant was estimated from its relative concentration in the pleural effusion or plasma sample used in affinity chromatography and assuming that the recovered variants were 90% [18]. The numbers indicated for the MM samples correspond to those used in Table 2. The identity of the AAG variants is indicated on top of the IEF patterns; the AAG phenotypes of the samples used in chromatography are indicated underneath. All other details are described in Section 2 and the legend to Fig. 2.

and perioccupational asbestos exposure [25]. This tumour arises from the neoplastic transformation of mesothelial cells. An acute-phase reaction is commonly associated with MM. This reaction, as well as other systemic manifestations of this tumour, may be related to the production of interleukin 6 (IL-6) by malignant mesothelial cells [26]. IL-6 is one of the mediators involved in the regulation of altered synthesis of human AAG within hepatocytes [27,28]. In this study, we observed a significant increase of the AAG plasma level in the MM patients as a whole. Additionally, the distribution of the variants derived from the AAG-A gene and from the AAG-B/B′ gene was significantly modified. These changes corresponded to a rise of the proportion of the ORM1 variants, compared to the ORM2 variant. Nevertheless, the concentration in MM plasma was increased for both variants. These results suggest that the two different genes coding for AAG are induced in MM, but the AAG-A gene would be more strongly induced than the AAG-B/B′ gene. Genetic studies have shown that, in human liver, the level of mRNA derived from the AAG-A gene is always higher than that derived from the AAG-B/B′ gene [15] and that only the AAG-A gene is strongly expressed in human hepatoma or transgenic mice liver cells after induction by inflammatory stimuli [20]. On the basis of these studies, one would expect that increased synthesis of AAG variants during acute-phase conditions would only concern the ORM1 variants of the protein. Our results, therefore, suggest a strong influence of post-transcriptional factors in the expression of the AAG genes. This is supported by previous investigations in which increases in the plasma level of both the ORM1 and ORM2 variants were observed during acute-phase reactions, such as surgical trauma [29] and burn injury [30]. One of these studies also gave evidence for significant changes in the distribution of the ORM1 and ORM2 variants [29], but these changes were less important than those observed in our mesothelioma studies. Considering these results, it would be worthwhile to study the relative occurrence of the genetic variants of AAG in various malignant and nonmalignant inflammatory conditions, in order to see whether the determination of the AAG variants may help to differentiate between these two conditions.

MM almost always produces exudative effusions (95% of cases) [31]. Effusion fluids usually have a high protein content. Most of the proteins involved are circulating blood proteins, but they are less concentrated in the effusion fluid. In this study, we observed a lower concentration of both AAG and total protein (data not shown) in the effusion fluids of patients, compared to that of corresponding plasma. However, the existence of a similar distribution of AAG variants, and also of AAG isoforms, in the plasma and effusion fluid of each patient indicates an equilibrium between the circulating forms of AAG and those present in effusion fluid.

The expressed glycosylation pattern of AAG alters during acute-phase reactions. These changes correspond to variations in the number of branches on *N*-linked glycans and/or in the sialic acid or fucose content [9,10]. They result from alterations in the biosynthesis within hepatocytes owing to cytokine (e.g. IL-6) and hormone effects [32,33]. Our IEF study showed that (native) AAG in the MM plasma and effusion fluids was more heterogeneous and contained greater amounts of the least and highly charged forms, compared to AAG in healthy plasma. These different changes were shown to be attributed to different variants — i.e. ORM1 F1 and S in the less acidic region and ORM2 A in the more acidic region — after fractionation of AAG by affinity chromatography on IDA–Cu(II) gel. Our results indicate specific changes in the charge heterogeneity of each AAG variant, according to its separate genetic origin. Because the isoforms corresponding to each AAG variant can only result from variations in the structure of the carbohydrate moiety, any change in the charge heterogeneity of the variants should, therefore, indicate changes in their glycosylation. It has been previously reported that AAG-derived biantennary glycans contain less of the charged sugar–sialic acid than more branched glycans of this protein [7]. Accordingly, the changes observed in our study with the separate AAG variants may be interpreted by increased proportions of biantennary glycans in the ORM1 variants and decreased proportions in the ORM2 variant. In line with this hypothesis, it is worth noting that the relative amount of biantennary glycans over more branched glycans is increased in AAG during the acute-phase response [9,10]. Nevertheless, this inter-

pretation needs to be confirmed. Indeed, the electrophoretic behaviour of AAG forms would depend not only on the total amount of sialic acid associated with these forms, but also on the particular types of sialic acid-containing glycans [1,11].

Previous observations have indicated that the individual native ORM1 and ORM2 variants separated from the plasma of healthy individuals, displayed different microheterogeneities on IEF and reactivities towards immobilized concanavalin A [18]. The fact that these variants differ in their glycosylation under normal conditions could lead one to expect different changes in their microheterogeneity/glycosylation under inflammatory conditions, such as the changes found here. As a whole, these results suggest that the amino acid substitutions between the two gene products of AAG may influence the glycosylation of the final products. It is recognized that a polypeptide can exert control over its own glycosylation [34]. In this respect, even subtle variations in the primary amino acid sequence of glycoproteins can cause changes in the oligosaccharide chains attached to these polypeptides [35].

The exact physiological function of AAG is not yet known, although diverse activities have been described for this glycoprotein. The most well-known activities of AAG are its ability to bind a variety of endogeneous (e.g. steroids) and exogeneous compounds (e.g. drugs) [36], and its ability to modulate various immunological reactions [9]. The former activity would be related to the polypeptide moiety of AAG, while the second seems to be glycosylation-dependent. Evidence has been found that the ORM1 and ORM2 variants have different ligand-binding properties [37,38] and that individual AAG glycoforms may have functional diversity in the processes of immunomodulation [39]. Accordingly, it appears that the different changes in expression and glycosylation observed for the two gene products of AAG in mesothelioma may be of functional significance.

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