α_1 -acid glycoprotein concentration and molecular heterogeneity: relationship to oxprenolol binding in serum from healthy volunteers and patients with lung carcinoma or cirrhosis

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1 α_1 -acid glycoprotein (AAG) concentration and molecular heterogeneity, and oxprenolol protein binding were studied in serum of 15 healthy volunteers, 14 patients with lung carcinoma and 17 patients with liver cirrhosis.

2 The AAG serum concentration was increased to 180.7% in patients with lung cancer and decreased to 73.4% in cirrhotic patients as compared with controls ($P < 0.05$).

3 The concanavalin A (conA) dependent heterogeneity of serum AAG was very similar in controls and patients with lung cancer: a ratio of 9/9/2 was obtained for the conA non-reactive, the conA weakly reactive and the conA strongly reactive subfraction respectively; in cirrhotic patients, the ratio shifted to 11/7/1.

4 The heterogeneity in electric charge, demonstrated by isoelectric focusing, was similar in the three groups of subjects: 70–80% of the focussed bands were found in the main three bands.

5 The binding of oxprenolol to serum proteins was increased in lung tumour patients and decreased in liver cirrhotic patients as compared with controls ($P < 0.05$). There was no change in binding affinity and oxprenolol binding was significantly correlated to total AAG serum concentration and to the concentration of each of the conA dependent subtypes, in controls as well as in both patients groups.

Keywords α_1 -acid glycoprotein protein binding oxprenolol liver cirrhosis lung tumour

Introduction

α_1 -acid glycoprotein (AAG) is an acute phase protein (Kushner, 1982). It is the major serum high affinity binding protein for many basic substances such as β -adrenoceptor blocking drugs, antiarrhythmic and antidepressant drugs (Paxton, 1983; Routledge, 1986). When the serum levels of AAG are increased, the free fraction of these drugs decrease, which may lead to a diminished pharmacological response. α_1 -acid glycoprotein is an heterogeneous protein, owing to variability in both its carbohydrate and peptide moieties.

Disease states can affect the relative concentrations of the different molecular forms and this could contribute to the change in protein binding of basic drugs (Tinguely *et al.*, 1985; Pedersen *et al.*, 1987). The purpose of the investigations presented here was to study the influence of lung carcinoma and liver cirrhosis on the serum concentrations and molecular heterogeneity of AAG, and on the consequences thereof for the serum binding of the β -adrenoceptor blocker oxprenolol.

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Table 1 Age, sex and body weight of the subjects (mean $\pm$ s.d.)

	Male/female	Age (years)	Weight (kg)
Control	10/5	34.7 $\pm$ 2.4	67.2 $\pm$ 3.2
Lung tumour	12/2	57.0 $\pm$ 2.3	73.2 $\pm$ 4.3
Cirrhosis	12/5	60.0 $\pm$ 3.3	68.0 $\pm$ 2.3

Methods

Subject selection and serum samples

Three groups of subjects were studied. Data on their age, sex and weight are summarized in Table 1.

The control group consisted of five female and 10 male volunteers without evidence of illness and taking no drugs at the time of sampling; smokers and pregnant women were excluded. The mean age in this group was 34.7 years. A second group consisted of 14 patients with oat-cell carcinoma of the lung with a mean age of 57.0 years. In half of these patients, metastases in liver, bone or brain were noted; all were treated with antimitotics at the time of the study.

The third group consisted of 17 patients in which the diagnosis of hepatic cirrhosis was confirmed by biopsy. The mean age of these patients was 60.0 years. In 10 patients the cirrhosis was due to alcohol abuse, in two cases the diagnosis was post-necrotic cirrhosis, in one case it was primary biliary cirrhosis. In four cases, the exact cause of the liver damage was not known.

Blood was collected by venepuncture using commercial plastic syringes, without anticoagulant, and centrifuged within 30 min. Serum was stored in all glass tubes at -20°C till used (at most 1 month). Preliminary experiments had shown that under these conditions there was no change in the properties of AAG.

Preparation of antibodies

The method of Krotosky & Weimer (1966) was followed with minor modifications. Human AAG (Sigma) was desialylated by gentle acid hydrolysis (0.05M H_2SO_4 for 60 min) followed by overnight dialysis against phosphate buffered saline (PBS). Male albino rabbits were immunized by repeated subcutaneous injections of 1 mg of desialylated protein in a mixture of PBS and Freund's Adjuvant (1/1, v/v), every 2 weeks. The first emulsion was prepared in complete Freund's Adjuvant, the following in incomplete

Freund's Adjuvant. The development of antibodies was monitored by radial immunodiffusion. After eight injections, the titre, defined as the highest dilution of antiserum resulting in a sharp precipitation arc when diffused against 1 mg ml^{-1} AAG, remained constant irrespective of further injections. The rabbits were bled and the serum was stored in aliquots at -20°C .

Measurement of AAG and albumin concentrations

Serum AAG concentrations were determined with the Laurell rocket immunoelectrophoresis (Laurell, 1966). The antiserum was used at $20\text{ }\mu\text{l ml}^{-1}$ gel. Standard curves were constructed using commercial AAG (Sigma). Peak heights were linear with the concentration of AAG in a range of 0.01 to 0.1 g l^{-1} . The inter- and intra-assay coefficients of variation, determined on a serum sample with an average concentration of 0.70 g l^{-1} , were 4.6% ($n = 15$) and 3.4% ($n = 30$), respectively. For a control serum (Boehringer) containing 0.50 g l^{-1} , a value of $0.567 \pm 0.011\text{ g l}^{-1}$ (mean $\pm$ s.d., $n = 6$) was found, which is, according to the manufacturer, within an acceptable range. Serum albumin concentration was determined by the bromocresol green method (Doumas *et al.*, 1971).

Concanavalin A gel electrophoresis

In order to determine the heterogeneity of AAG in its carbohydrate composition, the serum samples were analysed by electrophoresis in 1% agarose gel, containing 1.6 g l^{-1} conA in buffer A (75 mM Tris, 25 mM 5,5-diethylbarbituric acid, 0.35 mM Ca-lactate and 2 mM sodium azide, pH 8.6) according to Nicolle *et al.* (1981). At this pH, conA mobility in the electric field is minimal and AAG, which migrates to the anode in the conA containing gel is separated according to its reactivity with the lectin. Subsequently, at right angles to the first, a second electrophoresis was performed in 1% agarose gel containing antiserum and 70 g l^{-1} α -methyl mannoside in buffer A. After drying and staining, three major fractions became visible which are referred to as 'non-reactive' (NR), 'weakly reactive' (WR) and 'strongly reactive' (SR). The total area under the precipitation arc, measured with manual planimetry, was taken as 100% and the relative amount of each subtype was expressed as percentage of the total area. The concentration of AAG in each form was calculated from the percentage area under each precipitation arc and the total concentration in the sample.

Isoelectric focusing

Isoelectric focusing (IEF) was performed in pH gradients 2.5 to 5 (Farmalyt) according to Atland *et al.* (1982). Scanning of the gels was performed with a laser densitometer (LKB Ultrosan with computer program Scanmain adapted for IBM). The sum of the optical densities of all bands was taken as 100% and the relative amount of each band was expressed as percentage of the total optical density.

Oxprenolol serum binding

Oxprenolol binding to serum was estimated with equilibrium dialysis at 25°C at an initial concentration of 1.5 $\mu\text{g ml}^{-1}$ oxprenolol, as described previously (Belpaire *et al.*, 1982). In three serum samples, randomly chosen from each of the three subject groups, the binding characteristics (affinity and capacity) were analysed using Rosenthal plots (Rosenthal, 1967). In these experiments oxprenolol concentration varied between 0.25 and 51.25 $\mu\text{g ml}^{-1}$. The binding data were fitted by the following equation using the program Multi (Yamaoka *et al.*, 1981) for non-linear regression analysis

$$\frac{C_b}{C_u} = \frac{N_1 k_1}{1 + k_1 C_u} + \frac{N_2 k_2}{1 + k_2 C_u}$$

where C_b and C_u denote the bound and free concentration, k the association constant and N the total binding site concentration of the two classes of binding sites. The binding site with the highest affinity for oxprenolol represents the binding to AAG. The number of binding sites per molecule, represented in n_1 , was calculated assuming a molecular weight of 41,000 D for human AAG (Schmid, 1975).

Statistics

Differences between groups were calculated with the Mann-Whitney U-test; correlations were calculated by linear regression analysis.

Results

AAG and albumin serum concentration and AAG heterogeneity

The concentrations of AAG and albumin in the serum of healthy volunteers and of the two groups of patients, are given in Table 2. In cirrhotic patients, total serum AAG concentrations were significantly decreased ($P < 0.05$) to 73.4%, whereas in patients with lung carcinoma they were significantly increased ($P < 0.05$) to 180.7% as compared with controls. In both groups of patients, the serum albumin levels were significantly ($P < 0.05$) lower than in the controls.

In Figure 1, a typical example of the conA dependent distribution pattern is given for serum of two controls with different serum concentration of AAG, one cirrhotic and one lung tumour patient; in Table 3, the mean percentage of each AAG form for all serum samples is given. The distribution of AAG forms in the serum of lung carcinoma patients was not significantly different from that in controls while for the cirrhosis patients, both conA reactive forms were significantly decreased and the conA non-reactive form was significantly increased. The concentration of all three AAG forms in serum of lung carcinoma patients was significantly increased as compared with controls. In cirrhotic patients, however, the serum concentration of both conA reactive forms of AAG was decreased and the concentration of the conA non-reactive form remained unchanged.

Table 2 Serum concentrations of AAG and albumin and oxprenolol free fraction in serum of healthy subjects and of patients with lung tumour and cirrhosis. (mean $\pm$ s.d. the range is given between brackets)

Serum samples	n	AAG (g l^{-1})	Albumin (g l^{-1})	Free fraction oxprenolol (%)
Controls	15	0.91 $\pm$ 0.04 (0.70–1.23)	50 $\pm$ 3 (37–75)	13.1 $\pm$ 0.7 (8.9–17.6)
Lung tumour	14	1.65 $\pm$ 0.16* (1.05–2.85)	37 $\pm$ 1* (31–47)	8.0 $\pm$ 0.8* (4.4–14.1)
Cirrhosis	17	0.67 $\pm$ 0.05* (0.28–1.06)	34 $\pm$ 2* (24–50)	22.8 $\pm$ 2.2* (9.9–45.4)

* $P < 0.05$ (vs control subjects)

Figure 1 Representative example of the precipitation pattern in conA crossed immunoelectrophoresis obtained with serum of (a) and (d) healthy volunteers; (b) lung tumour patient and (c) cirrhotic patient. The serum AAG concentrations were (in g l^{-1}): for (a): 0.5, for (b): 1.4, for (c): 0.5 and for (d): 1.5.

Subjecting the serum samples to IEF revealed the pH-dependent microheterogeneity. Typically, seven to nine bands became visible after staining. Densitometric scanning of the samples and calculation of the percentage AAG in each stained band yielded the result shown in Figure 2. Almost 80% of the total amount of the IEF separated proteins was found in the three major bands. No significant difference in distribution pattern was found between the samples tested.

Oxprenolol binding

In the patients with lung tumours, the free fraction of oxprenolol was decreased while in cirrhotic patients it was increased ($P < 0.05$ in both cases) as shown in Table 2. The correlation between

total serum concentration of AAG and oxprenolol binding, expressed as the ratio of bound to free, is presented for the three groups of patients in Figure 3. A highly significant correlation between the two variables was found. In Table 4, the correlation coefficients between the ratio of bound to free oxprenolol, and either the total serum concentration of AAG, or the calculated serum concentration of one of its subtypes, are shown for each of the groups of individuals. Again, a highly significant correlation was obtained. No significant correlation was found between serum albumin concentration and oxprenolol binding.

The binding characteristics of oxprenolol for the high affinity binding site are summarized in Table 5 and it is clear that the total number of

Table 3 Occurrence of the three conA dependent molecular forms of AAG expressed as percentage of the total area under the precipitation arc. (mean $\pm$ s.d.)

Subjects	n	ConA-NR	ConA-WR	ConA-SR
Control	15	46.3 $\pm$ 1.4	42.0 $\pm$ 0.7	11.6 $\pm$ 0.8
Lung tumour	14	47.3 $\pm$ 1.5	40.3 $\pm$ 0.6	12.4 $\pm$ 1.2
Cirrrosis	17	56.7 $\pm$ 1.9*	36.4 $\pm$ 1.3*	7.0 $\pm$ 0.7*

* $P < 0.01$ vs control subjects

Figure 2 Occurrence of the IEF dependent molecular forms of AAG in healthy volunteers ($\square$); lung tumour patients (▨) and cirrhotic patients (▤) expressed as percentage of the total amount measured with laser densitometry (see Method section). For each group serum samples of six subjects were used (mean $\pm$ s.d.).

Figure 3 Correlation between the amount of bound oxprenolol, expressed as bound/free, and the total serum concentration of AAG (g l⁻¹). All samples of healthy volunteers ($\bullet$), lung tumour patients ($+$) and cirrhotic patients ($\blacktriangle$) were used ($r = 0.959$, $P < 0.001$, $n = 46$).

Table 4 Correlation coefficients between the ratio bound/free of oxprenolol and the total AAG concentration, the calculated serum concentrations of each conA dependent subtype and the serum albumin concentration, in the three groups of individuals.

AAG fraction	Controls	Lung tumour	Cirrhosis
Total AAG	0.69 ⁺⁺⁺	0.94 ⁺⁺⁺	0.88 ⁺⁺⁺
ConA-NR	0.55 ⁺	0.96 ⁺⁺⁺	0.68 ⁺⁺
ConA-WR	0.66 ⁺⁺	0.92 ⁺⁺⁺	0.93 ⁺⁺⁺
ConA-SR	0.52 ⁺	0.80 ⁺⁺⁺	0.62 ⁺⁺
Albumin	0.45NS	-0.13NS	0.14NS

+++ $P < 0.001$, ++ $P < 0.01$, + $P < 0.05$

NS: not significant

Table 5 Binding characteristics of oxprenolol in the sera of three healthy volunteers, three lung tumour patients and three cirrhotic patients.

Subject		N_1	k_1	n_1
Control	1	17.9	0.44	1.05
	2	19.5	0.39	1.07
	3	14.4	0.59	0.86
Lung tumour	1	28.4	0.41	1.06
	2	24.2	0.43	0.92
	3	23.6	0.43	0.86
Cirrhosis	1	7.3	0.43	0.84
	2	16.1	0.37	1.28
	3	12.4	0.38	0.98

N_1 : total binding capacity (μM); k_1 : affinity constant (μM^{-1}); n_1 : number of binding sites per molecule AAG. The molecular weight of AAG was assumed to be 41,000 Da.

binding sites, i.e. capacity constant (N_1) is higher in patients with lung tumours and lower in cirrhotic patients compared with controls. No difference in affinity constant (k_1) was seen between the different groups; the number of binding sites (n_1) per AAG molecule equalled unity in all cases.

Discussion

In this study, we compared serum AAG from normal healthy volunteers, from patients with lung carcinoma and from patients with cirrhosis.

In a first series of experiments, we determined the AAG serum concentration. For the control group our results are comparable with what is described in the literature (Shibata *et al.*, 1975; Piafski *et al.*, 1978; Wood & Wood, 1981; Belpaire *et al.*, 1982). There was a significant

increase for the lung tumour patients and a significant decrease for cirrhosis patients. These observations are compatible with the data from the literature for lung tumour patients (Adan & Farber, 1982), while for cirrhotic patients, a decreased (Chio & Oon, 1979; Barre *et al.*, 1984, 1987; Pedersen *et al.*, 1987), or an unchanged concentration (Piafski *et al.*, 1978; Teirlynck *et al.*, 1982) have been reported. These discrepancies are not fully understood although the use of different methods for the determination of the serum concentration of AAG could yield different results (Bordas *et al.*, 1982). Differences in clinical status could also contribute to these discrepancies. One might indeed expect that, since the liver is the major site of biosynthesis of AAG, the more the liver is injured, the more the serum AAG would be decreased.

It should be stressed that significant differences were found in spite of the large inter-variability indicated by the relative standard deviation of the average serum AAG concentrations: 17.5% for controls, 36.4% for lung tumour patients and 31.6% for cirrhotic patients.

This again is compatible with data from the literature (Edwards *et al.*, 1982; Pedersen *et al.*, 1987). Our own results in three healthy subjects, from which serum samples were taken every month during at least 6 months for determination of AAG, show an inter- and intra-individual variability of the same magnitude (results not shown).

Another factor which has to be considered is the influence of age differences between the different groups. Although the average age is lower in the control group than in both patient groups, it is clear from data in the literature, that only part of the changes we observe can be explained by the influence of age (Wallace & Verbeeck, 1987).

We can thus conclude that the changes in AAG serum concentration are the consequence of the disease and not secondary phenomena. With respect to the heterogeneity of AAG, we found a distribution in the control serum samples which is comparable with the data from the literature (Bøg-Hansen *et al.*, 1978; Nicolle *et al.*, 1981; Wells *et al.*, 1981; Hansen *et al.*, 1984; Pedersen *et al.*, 1987) with, roughly, a 9/9/2 ratio for conA non reactive, conA weakly reactive and conA strongly reactive fractions. This ratio remains essentially unchanged in lung tumour patients, as also found by Bleasby *et al.* (1985), but changed significantly in patients with cirrhosis to about 11/7/1. A shift to the conA non-reactive and weakly reactive form was also reported recently by Pedersen *et al.* (1987) for cirrhotic patients. The decrease in total serum concentration in

liver cirrhotic patients is mainly due to the decrease in the concentration of the conA weakly reactive and conA strongly reactive form, while the concentration of the conA non-reactive form is nearly unchanged. Whether this decrease is due to impaired synthesis or to degradation is not clear and cannot be deduced from our results.

The heterogeneity in charge of serum AAG in healthy volunteers has been described before (Schmid, 1975). Between 70 and 80% of all the focused bands are found in the main three bands; the distribution remains essentially unchanged in both patient groups, except for a slight shift to higher pH in the cirrhotic patients.

Finally, we studied the binding of oxprenolol to the serum samples of the three groups of individuals. Oxprenolol binds mainly to AAG in serum (Belpaire *et al.*, 1982), and changes in the AAG serum concentration are reflected by changes in binding. Indeed, in patients with lung carcinoma binding increases, and in patients with cirrhosis binding decreases, and these changes are paralleled by changes in AAG serum concentration. In the disease states studied, the binding affinity however remains in the same range seen in the healthy subjects. Similarly, Pedersen *et al.* (1987) found in cirrhotic patients a decreased binding capacity and an unchanged binding affinity as compared with controls. Jackson *et al.* (1982) however demonstrated an increased affinity of AAG for lignocaine in the

serum of patients with various types of cancer and suggested that this is caused by changes in the physicochemical properties of the molecule due to the disease.

Correlations between AAG subtype and oxprenolol binding were not changed in both groups of patients, suggesting that the changes in oxprenolol binding are due to the change in total concentration of AAG, and not in the concentration of a particular subtype. This is in keeping with the fact that the binding sites for the drug are probably located on the polypeptide chain of AAG rather than on the sugar part as suggested by Tinguely *et al.* (1985).

We can thus conclude that there are changes in serum concentration of AAG in lung cancer and cirrhotic patients, which are accompanied by corresponding changes in binding of oxprenolol. We suggest that the change in binding can be explained completely by a change in concentration of AAG, the presence of subtypes being of lesser importance in this respect.

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