Human fetuin/α2HS-glycoprotein level as a novel indicator of liver cell function and short-term mortality in patients with liver cirrhosis and liver cancer

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ABSTRACT

Objective: Human fetuin/α2HS-glycoprotein (AHSG) is synthesized by hepatocytes. We intended to determine whether liver dysfunction or acute phase reaction is dominant in the regulation of its serum concentrations and to see if decreased AHGS levels are associated with short-term mortality. **Design:** We determined the serum AHSG levels in patients with acute alcoholic, acute A, B, and Epstein-Barr virus hepatitis, alcoholic cirrhosis, and hepatocellular cancer and correlated them to conventional laboratory parameters of inflammation and liver function. Patients were followed for one month. **Methods:** Serum AHSG was determined by radial immunodiffusion. **Results:** Compared to controls, significantly lower levels were found in patients with liver cirrhosis and hepatocellular cancer but not the acute viral hepatitides. Strong positive correlation with serum transferrin, albumin, prothrombin was found. Febrile episodes were not associated with significantly decreased AHSG levels. Concentrations below 300 μg/ml were associated with high mortality rate (52.0%, relative risk: 5.497, 95% C.I.: 2.472-12.23, $p<0.0001$). Of all laboratory parameters studied serum AHSG levels showed the greatest difference between deceased and survived patients with cirrhosis and cancer. Moreover, other acute phase reactants did not differ significantly. The multiple logistic regression analysis indicated that the decrease of serum AHSG patients is independent from all other variables that were found decreased in deceased patients. **Conclusions:** Decreased serum AHSG concentration is due rather to hepatocellular dysfunction than the acute phase reaction and is an outstanding predictor of short-term mortality in patients with liver cirrhosis and liver cancer.

Keywords fetuin/α2HS-glycoprotein, acute phase reaction, transferrin, acute viral hepatitis, liver cirrhosis, liver cancer

INTRODUCTION

Human fetuin/α2HS-glycoprotein (AHSG) is a glycoprotein found in the serum at a mean concentration of 600 μg/ml. AHSG is known to behave as a negative acute phase reactant [1]. It accumulates in bone tissue and it has been postulated that the protein prevents untoward calcification [2, 3]. AHSG is expressed in several tissues of the embryo [4]. In adults, however, this protein is synthesized only by hepatocytes [5]. AHSG has been found to be the natural antagonist of several growth factors, i.e. insulin, transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and hepatocyte growth factor/scatter factor (HGF/SF) [6, 7, 8], and is an inhibitor of the lymphocyte mitogenic response [9]. The regulatory role of AHSG in tissue regeneration has also been postulated [6].

Reduced levels of the negative acute phase reactants transferrin and albumin have been found in patients with cirrhosis and other alcoholic liver diseases [10]. Serum levels of AHSG have been found reduced in alcoholic cirrhosis and fatty liver [11]. These observations and ours on decreased levels of AHSG in primary biliary cirrhosis [12] have prompted us to investigate serum AHSG levels in acute viral hepatitides and liver cancer. By correlating serum AHSG levels to conventional laboratory parameters of liver disease we tried to determine whether decreases of AHSG levels reflect the acute phase reaction or the limited protein synthetic capacity of the liver. Since both processes result in decreased AHSG levels in serum, we also intended to delineate the usefulness of AHSG determinations in short-term follow-up and prognosis of liver diseases.

PATIENTS AND METHODS

Patients

The patients enrolled in the study gave informed consent approved by the local Ethical Committee. Their most important data are listed in *Table 1.* Serum samples of 51 healthy blood donors were used as control. Patients were followed for a 1-month period by blood sampling every 10 days. Besides history and appropriate clinical findings the diagnosis of the subsequent liver diseases was set up as follows: acute A hepatitis: anti-hepatitis A virus (HAV) IgM; acute B hepatitis: anti-hepatitis B virus (HBV) core antigen (HBc) IgM; acute Epstein-Barr virus (EBV) hepatitis: anti-EBV IgM; liver cirrhosis: liver scan, abdominal ultrasonography; hepatocellular cancer: ultrasonography, computer tomography, liver biopsy. We found no correlations between age and serum AHSG concentrations neither in healthy controls nor in patients with any liver disease separately or combined.

Determination of AHSG concentration

Serum levels of AHSG were determined by radial immunodiffusion (RID) using 10x10 cm slides. Five μl of patient's sera diluted to 1:4 was applied in 11.5 ml of Litex agarose gel (Sigma). Serum samples (1:4 dilution) with known concentrations of AHSG served as standards. The incubation was done at room temperature for 48 hours. We used two types of antibodies against AHSG

as the protein is synthesized is a single chain and is rapidly converted to a dipeptide form following the cleavage of a connecting peptide. The commercially available product (anti-AHSG, IgG fraction, Incstar, Cat No. 81931, 13.7 mg/ml, in a final concentration of 84 μl/11.5 ml gel) recognizes the dipeptide form. The other type of antibody binding to the newly synthesized single chain form of AHSG, was raised by immunizing a rabbit with recombinant human protein (final concentration of 568 μl/11.5 ml gel). There was a strong correlation between data produced by the two assays both in healthy controls and patients.

Production of anti-human AHSG antibodies

Human recombinant AHSG was produced in the baculovirus expression vector system and purified from the supernatant of the Sf9 cells as described elsewhere [8,13]. One hundred μg of purified AHSG was injected subcutaneously in a rabbit with complete Freund's adjuvant. The injection was repeated twice at 14 days' intervals using incomplete Freund's adjuvant. Thereafter the rabbit was bled and the serum was pooled and stored at -20 °C until further use. The AHSG binding capacity of the rabbit serum was confirmed by competitive ELISA.

Other laboratory measurements

The determination of the erythrocyte sedimentation rate (ESR), hematocrit, hemoglobin, white blood cell (WBC), granulocyte and thrombocyte count, serum bilirubin, serum aspartate aminotransferase (ASAT), alanine

aminotransferase (ALAT), serum alkaline phosphatase, γ-glutamyl transpeptidase (γGT), prothrombin activities, total serum protein and albumin concentrations was performed using conventional standardized methods. Serum levels of α2-macroglobulin, transferrin, haptoglobin, and α1-acid glycoprotein were measured by RID.

Statistical analysis

Paired and unpaired data were analysed by the Wilcoxon and Mann-Whitney U tests, respectively. Correlation studies were done by the rank correlation analysis (Spearman). Contingency tables were analysed by the Fisher's exact test. In the follow-up series, the first data of each patient was used in the comparison of groups and the correlation studies, respectively. The multiple logistic regression analysis was performed with the SPSS v.10.0 statistical program. The level of $p < 0.01$ was considered significant.

RESULTS

Serum AHSG concentrations of patients with liver disease

The mean AHSG concentration in sera of patients with various liver diseases is shown in *Figure 1.* Patients with alcoholic cirrhosis and hepatocellular cancer had significantly lower AHSG levels than healthy subjects, whereas AHSG levels did not differ from controls in the acute A, B, and EBV hepatitis. Only data from disease groups with decreased AHSG concentration were analysed further. Since the sample size did not allow for separate statistical analysis in

patients with alcoholic cirrhosis and hepatocellular cancer, in part of evaluations data from these two groups were taken together.

Correlation studies

Serum AHSG levels were correlated to conventional laboratory parameters and to the concentration of some other serum glycoproteins. The serum AHSG concentration correlated positively with prothrombin, albumin, and transferrin levels (*Table 2,* column A).

Serum AHSG levels fail to correlate with fever and infection

We performed two types of calculations in order to estimate the effect of the acute phase reaction generated by fever and inflammation on serum AHSG levels. First, we compared the AHSG values of patients with and without fever at the time of enrolment. In cirrhosis patients, serum AHSG concentrations did not differ significantly between the febrile (381.5 $\pm$ 39.1 μg/ml mean ± SEM, n = 33) and afebrile groups (413.2 ± 24.3 μg/ml, n = 66, p = 0.5108). Second, individual patients were followed for episodes of intercurrent infections. These occurred in 5 patients with liver cirrhosis and in one patient with liver cancer and were proven by positive cultures for blood, sputum, or urine. Serum AHSG levels did not differ when secondary infections were present (371.0 $\pm$ 24.7 μg/ml, mean ± SEM) or absent (447.8 $\pm$ 62.8 μg/ml, n = 6, p = 0.4375).

Serum AHSG levels and short-term mortality

During the 1-month follow-up period, 23 out of 99 patients died: 19 patients with alcoholic cirrhosis, and 4 with hepatocellular cancer, respectively. Three patients died of intercurrent illnesses unrelated to liver disease, namely chronic pancreatitis, purulent meningitis, acute left ventricular failure. Only values of the remaining 20 patients, who were considered dying of liver failure, were analysed further. For comparison, a survivor group was formed from patients who survived the follow-up period and had the same liver disease as those in the deceased group. As *Table 3A* shows patients with AHSG concentrations constantly below 300 μg/ml had a significantly higher risk of mortality than those with values above this level.

Serum transferrin, prothrombin, and albumin levels and short-term mortality

We made calculations regarding the risk of mortality for transferrin, as a strong correlation with this negative acute phase protein had been found in several disease groups. Only patients with transferrin levels below 124 mg/dl had higher but not statistically significant mortality rates associated with a high relative risk (*Table 3B.*).

The comparison of the laboratory parameters of the deceased and survived groups is shown in column B of *Table 2.* Significant differences were found with hematocrit, WBC, granulocyte count, α2-macroglobulin. The AHSG values showed the strongest difference between the two groups - the difference in transferrin was much less pronounced. The deceased and survived groups

showed no statistical difference in prothrombin activity and serum albumin concentration whereas these parameters correlated with AHSG values strongly.

Thus low serum AHSG levels were associated with short-term mortality of patients with liver cirrhosis and liver cancer. However, factors such as hematocrit or serum transferrin (with marginal significance) were also associated with the short-term mortality of the patients. Therefore, in order to adjust for the effect of these confounding variables, we have analysed the data by using multiple logistic regression (*Table 4*). When data were adjusted for age, gender, and the confounding variables enlisted above, a highly significant regression coefficient was found between AHSG levels and short-term mortality. This association was independent of the association between low hematocrit levels and mortality while no significant association was found in the case of other variables tested. When low AHSG levels were defined as $\leq$ 300 μg/ml (limit of the lowest quartile), according to logistic regression adjusted for the variables in Table 4, patients with low serum AHSG concentrations compared to those with normal AHSG had a 88.7 (95% C.I.: 3.4 – 2310, p = 0.007) odds ratio to not survive 3 months after blood sampling.

DISCUSSION

Apart from a study in alcoholic cirrhosis and fatty liver [11] and our previous work on primary biliary cirrhosis [12] no other study has investigated serum AHSG levels in enough patients for statistical analysis in liver diseases. No correlation studies have been performed. In accordance with one study on

children hepatitis A [14] serum AHSG levels did not differ from controls in acute A, B and EBV hepatitis. Although each disease we have investigated is characterized by a considerable degree of liver cell dysfunction, low serum AHSG concentrations were detected only in the cirrhosis and hepatocellular cancer groups. Either acute phase reaction or decreased liver function or both can result in decreased serum concentration of AHSG.

The role of the acute phase reaction is favoured by the strong positive correlation with transferrin levels. Proinflammatory cytokines such as interleukin (IL)-1, IL-6, tumour necrosis factor α (TNFα) are known to induce the synthesis of the positive and down-regulate that of the negative acute phase reactants including AHSG in rat liver and human HepG2 cells [15, 16]. Their determinant role, however, can be questioned as elevated levels of these cytokines have been reported in patients with all diseases we have investigated, including acute hepatitis A [17], acute hepatitis B [18], hepatitis C [17, 19], acute hepatitis and liver failure [20, 21, 22]. The elevation of the serum concentration of the positive acute phase reactants is, however, often masked by the overall reduced protein synthesizing capacity in liver diseases [23]. Conversely, decreased level of the negative acute phase reactants would be expected to occur due to both inflammation and hepatocyte dysfunction and may serve as a good prognostic marker in liver diseases. The relationship between the levels of these cytokines and negative acute phase proteins has not been studied in clinical setting, nor has their effect on mortality in liver diseases. IL-6, however, has been found the strongest predictor of mortality in patients with end-stage kidney disease [24].

On the other hand, several findings strongly argue against the major determinant role of the acute phase reaction and inflammation in regulation of AHSG levels in patients with liver diseases. First, we did not find inverse relationship with the positive acute phase reactants and several laboratory parameters of inflammation (ESR, WBC, granulocyte count). Second, serum AHSG was not markedly reduced further during febrile states or episodes of intercurrent infections, which was a common finding in bacterial sepsis, pneumonias and in infections occurring in haematological malignancies [1,25]. Third, serum AHSG levels correlated strongly and consistently with prothrombin activity, which is dependent solely on hepatocyte function. Although the positive correlation with albumin can be interpreted in both ways as this protein is also a negative acute phase reactant. In clinical practice, prothrombin and albumin levels are used as the best indicators of protein synthetic reserve in liver disease.

We conclude that decreased serum AHSG levels indicate rather dysfunction of hepatocytes than an acute phase response. This concept has been supported by several observations. In patients with hepatitis C-related diseases elevated serum levels of IL-1, IL-6, and TNFα correlated with prothrombin time better than with parameters of hepatic inflammation (ASAT and ALAT) [19]. In accordance with our observation a positive relationship between AHSG levels and the severity of alcoholic cirrhosis was found [11]. Similarly, serum IL-6 levels did not correlate to the gradual decrease of AHSG in advanced stages of primary biliary cirrhosis [12, 26].

The regression studies suggest that hematocrit value may be almost as good as AHSG as a predictor of short-term mortality. Clearly, massive gastrointestinal bleeding was not a cause of death in our patients, mild microscopic bleeding, however, could not be ruled out. Since the aetiology of anaemia in these diseases is variable and is often independent of hepatocyte function low hematocrit levels cannot be regarded as good as AHSG in predicting short-term mortality in liver cirrhosis and hepatocellular cancer. In addition, red blood cell transfusion can also alter the haematological status independently from liver cell function.

Our results suggest that at least at short-term, AHSG concentration has an outstanding predictivity of lethal outcome in patients with alcoholic cirrhosis and liver cancer even after adjustment to other confounding variables. In this respect AHSG was the best of all other laboratory parameters we studied. Compared to AHSG the alteration of transferrin concentration was much less pronounced and was associated with lower mortality rate and a slightly lower equal risk of fatal outcome. To our knowledge no other glycoprotein has been considered as short-term predictor of fatal outcome in liver disease. A longer follow-up is already under way.

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Table 1. The number, age, and gender of patients

	Number	**Gender Male/Female**	**Age years, mean ± SD (min-max)**	**Correlation with age* r (p)**
Acute viral hepatitis A	50	37/13	28 ± 10 (15-53)	0.0251 (0.8625)
Acute viral hepatitis B	23	15/8	47 ± 17 (20-80)	0.0077 (0.9709)
Acute EBV hepatitis	19	11/8	27 ± 13 (15-68)	0.1603 (0.5122)
Liver cirrhosis	92	54/38	53 ± 12 (28-88)	-0.1138 (0.2802)
Hepatocellular cancer	7	6/1	58 ± 8 (50-71)	0.6724 (0.1389)
Healthy controls	51	27/24	47 ± 6 (33-59)	-0.1728 (0.2253)

n: number of observations; *: Spearman's rank correlation coefficient; r: regression coefficient; p: value of significance.

Table 2. Correlation between serum fetuin/α2HS-glycoprotein levels and laboratory parameters (A) and comparison of parameters of deceased and survived patients (B) with liver cirrhosis and hepatocellular cancer

	A.		**B.**				
	Correlation with AHSG levels*		**Comparison of laboratory parameters in**				
			Deceased patients		**Survived patients**		
Parameter	**n**	**p**	**n**	**mean ± SEM**	**n**	**mean ± SEM**	**p**
ESR	90	0.0980	11	52.8 ± 9.6	79	47.7 ± 3.3	0.5668
Hematocrit	56	0.9627	9	0.29 ± 0.02	47	0.37 ± 0.01	0.0040
Hemoglobin	56	0.8553	9	101.2 ± 6.5	47	121.4 ± 2.6	0.0435
WBC	92	0.4224	13	11280 ± 1404	79	8266 ± 416	0.0505
Granulocyte count	53	0.5344	8	8788 ± 1653	45	6295 ± 552	0.2096
Platelet count	88	0.1865	13	163.8 ± 38.3	76	191.7 ± 10.1	0.2723
Serum bilirubin	96	0.0332	18	189.3 ± 40.1	78	142.2 ± 14.3	0.2278
ASAT	97	0.8851	18	93.1 ± 14.7	79	352.9 ± 101.9	0.7772
ALAT	95	0.8974	18	40.4 ± 5.8	78	395.4 ± 109.6	0.7927
Serum alkaline phosphatase	95	0.7873	18	481.1 ± 85.8	77	325.1 ± 26.1	0.0621
γGT	95	0.5129	18	380.2 ± 124.8	77	361.4 ± 66.4	0.7174
Prothrombin	86	0.0003	13	53.9 ± 6.3	73	65.5 ± 2.5	0.2648

Total protein	83	0.1111	13	67.3 ± 3.1	70	68.6 ± 1.1	0.4907
Albumin	83	0.0003	13	29.6 ± 1.5	70	33.2 ± 0.7	0.2465
α2-macroglobulin	62	0.1650	14	268.3 ± 14.2	48	342.0 ± 20.8	0.0180
Transferrin	62	0.0024	14	150.1 ± 13.6	48	194.9 ± 10.1	0.0973
Haptoglobin	59	0.5219	13	159.7 ± 49.4	46	160.9 ± 15.3	0.5462
α1-acid glycoprotein	61	0.7232	14	97.8 ± 14.3	47	90.9 ± 4.8	0.9248
AHSG			20	297.8 ± 29.6	79	459.0 ± 16.4	< 0.0001

n: number of observations; *: Spearman's rank correlation coefficient; p: value of significance.

Table 3. The mortality rates and relative risk of low serum levels of fetuin/α2HS-glycoprotein AHSG (A) and transferrin (B) in patients with liver disease

A.

AHSG concentration	**Died**	**Survived**	**Total**	**Mortality rate (%)**
Below 300 μg/ml	13	12	25	52.0
Over 300 μg/ml	7	67	74	9.5
Total	20	79	99	

p < 0.0001, Relative Risk: 5.497, 95% C.I.: 2.472 – 12.23

B.

Transferrin concentration	**Died**	**Survived**	**Total**	**Mortality rate (%)**
Below 124 mg/dl	6	10	16	37.5
Over 124 mg/dl	8	38	46	17.4
Total	14	48	62	

p = 0.1619, Relative Risk: 2.156, 95% C.I.: 0.8829 – 5.266

Table 4. Association of low fetuin/α2HS-glycoprotein levels with short-term (within 3 months after blood sampling) mortality of patients with liver cirrhosis and liver cancer calculated by adjusted multiple logistic regression*

Variable	**P value of regression coefficient***
Age, years	0.4883
Gender, male/female	0.5966
Hematocrit	0.0095
Granulocyte count	0.8497
Transferrin, $\leq$ 124 mg/dl / > 124 mg/dl	0.1053
AHSG $\leq$ 300 μg/ml / > 300 μg/ml	0.0070

*adjusted for age, gender, and variables significantly associated with short-term mortality at univariate analysis (Table 2 column B).

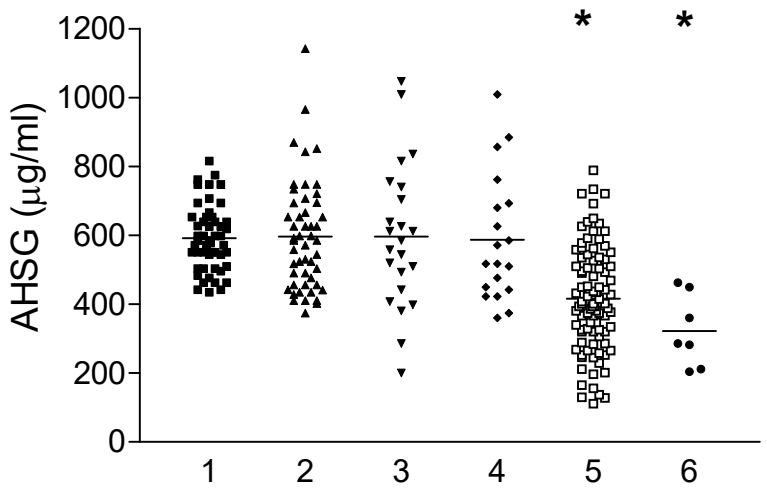

Figure legend:

Figure 1. Serum fetuin/α2HS-glycoprotein concentration in patients with liver disease. 1: Healthy controls (n = 51), 2: Acute hepatitis A (n = 50), 3: Acute hepatitis B: (n = 23), Acute Epstein-Barr virus hepatitis (n = 19), 4: Alcoholic cirrhosis (n = 92), 5: Hepatocellular cancer (n = 7). *: $p < 0.001$.