Intra- and extracellular α_1 -antitrypsin in liver disease with special reference to Pi phenotype

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SUMMARY In order to study the relation between intra- and extrahepatocellular α_1 -antitrypsin (α_1 -AT) concentrations in patients with various Pi phenotypes, a prospective series of needle liver biopsies was stained with both periodic acid-Schiff (PAS) and a specific immunoperoxidase technique to demonstrate intracellular α_1 -AT. Concomitant blood samples from all patients were analysed for α_1 -AT. Pi phenotypes were determined by isoelectric focusing.

Non-globular intrahepatocellular α_1 -AT can be seen in biopsies from Pi M patients with increased plasma α_1 -AT concentrations and active liver disease. No evidence was found in this study of 250 patients (including 22 controls) for predisposition toward liver disease in any phenotypic group. PAS or immunoperoxidase staining (or both) for α_1 -AT demonstrated characteristic globular inclusions in 11 of 15 cases having the Z allele, one case being diffusely positive and three negative. Biopsies from 3 of 207 patients with liver disease and lacking the Z allele had globular inclusions seen with both PAS and immunoperoxidase techniques. α_1 -AT globules in absence of the Z allele are most often found in elderly patients with severe disease and high plasma α_1 -AT concentrations.

Alpha₁-antitrypsin (α_1 -AT, α_1 -protease inhibitor) is an acute phase reactant plasma protein with broad spectrum antiprotease activity synthesised by the hepatocytes. A selective increase of its plasma concentration is often seen in liver disease and this level generally seems to reflect the activity of the disease.¹ Low plasma concentrations are seen in some phenotypes. Of special interest is the Pi Z phenotype in which increased frequencies of fibrosis, cirrhosis and hepatoma^{2,3} have been reported. Neonatal hepatitis occurs in 10% of homozygous Pi Z children and increased hepatic enzyme activity is found in approximately 50% of Pi children.⁴ Typical PAS-positive inclusion bodies, first described by Sharp *et al.*,⁵ and consisting of aggregated α_1 -AT lacking sialic acid and with an amino acid substitution⁶ are seen in Pi Z hepatocytes. Such inclusions were first thought to be pathognomonic for the Z allele⁷ but several reports during the past few years have disputed this assumption. Heterozygous Pi MZ individuals also have globular inclusions and one case of hepatocellular carcinoma in a 16-year-old MZ boy has been reported,⁸ but most investigators have found no evidence of increased liver disease frequency in such individuals.

α_1 -AT may have an important protective role as a protease inhibitor in liver disease. The mechanisms regulating the plasma concentration in both normal and other phenotypes are, however, incompletely known. To gain insight into these mechanisms we have studied (i) the relation between intrahepatocellular immunoreactive α_1 -AT and plasma concentrations in various liver diseases; (ii) the frequency and some clinical conditions associated with globular inclusions (α_1 -AT globules) in absence of the Z allele and (iii) the distribution of phenotypic groups among patients with liver disease. The study was performed prospectively using an α_1 -AT-specific immunoperoxidase staining in addition to routine PAS-staining of 250 liver biopsies (including 22 controls) for visualisation of intracellular α_1 -AT and isoelectric focusing for phenotype determination in all cases.

Patients, material and methods

The material comprises 250 consecutive needle liver biopsies obtained from patients admitted to Malmö General Hospital. It includes 22 control biopsies obtained with informed consent from patients with normal liver function undergoing elective cholecystectomy, according to the principles of the Ethical

Committee of the hospital. The diagnoses were alcoholic liver disease in 88 cases (cirrhosis in 20), chronic active hepatitis (CAH) 15; chronic persistent hepatitis 5; primary biliary cirrhosis 10; venous congestion 6; toxic liver disease 6; hepatoma 6; viral hepatitis 4; systemic disease with liver involvement in 52 cases and diverse or complex diagnoses in the remaining cases. Only one biopsy from each patient was included. Blood samples were obtained from all patients within 24 h of needle liver biopsy for quantitative α_1 -AT and phenotype determinations. Plasma α_1 -AT concentrations were determined at the Department of Clinical Chemistry by an electro-immunoassay technique using a mono-specific antiserum available at the laboratory. Phenotypes were analysed by isoelectric focusing in polyacrylamide gels using a modification of the method of Pierce *et al.*⁹ We thank Dr Jan-Olof Jeppsson for reviewing phenotype designations. All liver biopsies were routinely stained with haematoxylin and eosin, Gordon-Sweet's reticulin, van Gieson's stain and PAS-staining after diastase digestion. Biopsies were judged using only the internal code from the Department of Pathology for identification. Scores were given for the pathological parameters: bridging necrosis, piece-meal necrosis, fibrosis, cirrhosis, portal inflammation, centrilobular necrosis, bile-duct replication, Kupffer-cell activity, steatosis, cholestasis and subjective impression of inflammatory activity.¹⁻¹⁰ Untreated paraffin sections of 242 biopsies were examined separately and independently using the indirect peroxidase conjugate method reported by Taylor.¹¹ Rabbit antiserum, found to be monospecific for human α_1 -AT by crossed immunoelectrophoresis¹² was used in a dilution 1/20 instead of human immunoglobulin components and carbazol was substituted for diaminobenzidine.¹³ Peroxidase-conjugated swine antirabbit IgG was used in a 1/50 dilution. All biopsies which stained positively with this technique were also stained with rabbit antisera against albumin, fibrinogen and IgG. Antisera were obtained from DAKO, Copenhagen, Denmark. Results of the immunoperoxidase examin-

ation were compared with the diastase-treated PAS-stained biopsies.

Results

Distribution of phenotypes and gene frequencies among patients with liver disease compared to Scandinavian normal populations is shown in Table 1. A slight but statistically insignificant ($p > 0.10$) overrepresentation of the Z and S alleles was found among patients with liver disease. No significant differences were found in mean age, total biopsy score, inflammatory activity, degree of fibrosis or cirrhosis between phenotypic groups (Table 2). Two of 11 patients with phenotype MS had chronic active hepatitis (13% of all CAH patients) whereas only 6% of all patients with liver disease had this diagnosis. Subtypes of the M phenotype could easily be distinguished in 14% of M phenotypes after electrofocusing. No evidence was present for any case of M Malton or M Duarte in this material.

Immunoperoxidase staining of α_1 -AT was performed on all 242 biopsies with sufficient material for investigation (including all controls). Hepatocytes in a minority of biopsies (28/227) from patients lacking the Z allele stained diffusely positive for α_1 -AT. Mean plasma α_1 -AT concentrations, biopsy scores and values for inflammatory activity in biopsies were calculated and compared to those for negative biopsies. Among Pi M patients in the positive group the mean α_1 -AT concentration was 1.9 ± 0.7 (SD) and 0.2 g/l higher ($0.32 > p > 0.10$) than for patients with negative biopsies. Mean biopsy score was 7.6 ± 5.1 (SD) compared to 5.3 ± 4.4 ($0.05 > p > 0.01$). Inflammatory activities were 1.4 ± 1.0 and 1.0 ± 0.9 ($0.05 > p > 0.01$) for the two groups, respectively. Biopsies from 102 patients showed positive α_1 -AT staining in leucocytes, Kupffer's cells or round cells, but no differences were found between mean plasma α_1 -AT concentrations, biopsy scores or activities in these and patients with negative biopsies.

Table 1 Distribution of phenotypes and gene frequencies

Phenotype	MM	M-	MS	MZ	FM	SS	SZ	ZZ
2830 healthy Norwegians (14)	2540		116	81	72	4	4	2
228 biopsied patients with liver disease	199	1	12	11	3	0	1	1
Gene frequencies	Pi M		Pi S	Pi Z	Pi F	Other		
2830 healthy Norwegians (14)	0.946		0.023	0.0157	0.0133	0.002		
6995 adult Swedes (15)				0.0240				
228 patients with liver disease	0.932		0.0285	0.0307	0.0066	0.002		

The probability that the frequency of the Z gene in liver patients is the same as that among normal Norwegians and Swedes is $0.50 > p > 0.10$ using the χ^2 test. The major contribution to the difference arises in the Z gene group.

Table 2 Correlation between phenotype, liver biopsy characteristics, age and sex among 228 patients with liver disease

Phenotype	n	Mean age	% male	Biopsy score	Biopsy activity	Fibrosis	Cirrhosis
Pi M	199	53 ± 14	59	6.1 ± 4.5	1.1 ± 0.9	0.7 ± 0.9 (107)	0.4 ± 0.8 (48)
Pi M-	1	27	0	0	0	0 (0)	0 (0)
Pi FM	3	52 ± 19	33	7.2 ± 7.4	1.2 ± 0.8	1.0 ± 1.7 (1)	0.7 ± 1.1 (1)
Pi MS	12	51 ± 17	45	6.5 ± 4.7	1.2 ± 1.2	0.8 ± 0.7 (8)	0.1 ± 0.3 (2)
Pi MZ	11	50 ± 15	56	5.9 ± 3.7	1.1 ± 1.0	0.9 ± 1.0 (5)	0.4 ± 1.0 (2)
Pi SZ	1	46	100	0.5	0	0.5 (0)	0 (0)
Pi ZZ	1	39	100	5.5	1.5	0 (0)	0 (0)

Biopsy score is based on the sum of characteristics listed in the text (see ref. 14). Maximal possible score was 30. Maximal degree of the single characteristics activity, fibrosis and cirrhosis is 3 points each. The number of patients within each phenotypic group having biopsy signs of fibrosis or cirrhosis is shown in parentheses.

No statistically significant differences were found between any of the phenotypic groups.

Fifteen cases were found to have α_1 -AT specific globules in the hepatocytes including 10 cases of Pi MZ (two controls) and one Pi ZZ. Of these 11 cases, three MZ biopsies were PAS-negative. Three cases of Pi MZ and 1 Pi SZ were negative to both immunoperoxidase and PAS-staining. Of the 207 cases studied with liver disease and lacking the Z allele, 3 had immunoperoxidase and PAS-positive globules in the hepatocytes. Their clinical data, phenotypes, biopsy scores and plasma α_1 -AT concentrations are summarised in Table 3 together with similar cases from published reports. Globules in our patient with adenocarcinoma had typical appearance for α_1 -AT globules and were present in approximately 10 normal hepatocytes adjacent to metastatic tissue. In the case with venous congestion 50-100 hepatocytes with immunoreactive α_1 -AT of both diffuse and globular appearance were found periportally as well as centrilobularly in one lobulus (Fig. 1). In this case alone staining was also positive for IgG,

fibrinogen and albumin. The third case (rheumatoid arthritis) had globules with appearance and location typical for those seen in Pi Z deficiency (Fig. 2).

Discussion

Opinions on the occurrence of liver disease among MZ individuals are divided. Eriksson *et al.*⁹ reported an increased incidence of cirrhosis in MZ patients in a necropsy study where the presence of PAS-positive material was used as a marker of the Z-gene. Neither immunochemical staining nor determination of plasma α_1 -AT concentrations was performed and therefore, viewing the occurrence of α_1 -AT globules unrelated to the Z allele in severe disease, one cannot exclude a certain overdiagnosis of the MZ state in that study. Morin *et al.*²⁰ have found no increased frequency of the MZ phenotype among patients with liver cirrhosis. Blenkinsopp and Haffenden²¹ find

Table 3 Summary of cases with immunoperoxidase demonstrable α_1 -AT containing globules in hepatocytes in absence of the Z gene

Author (ref)	Phenotype	PAS-staining	Plasma α_1 -AT (g/l)	Age (yr)	Clinical data
Bradfield <i>et al.</i> ^{16*}	M	+	2.3	male 79	Acute pancreatitis patient survived 12 days after biopsy
Fischer <i>et al.</i> ^{17†}	M-	++	1.8	female —	Alcoholic hepatitis no further clinical information reported
Kelly <i>et al.</i> ^{18‡}	3MS S	+— ++	0.9	male 56	Alcoholic hepatitis no further information reported patient survived 5 months after biopsy
Present study §	M	+	> 4.0	male 50	Metastatic adenocarcinoma of the pancreas. Patient survived 6 months after biopsy
	M	+	2.3	male 67	Severe cardiac decompensation with venous congestion. Patient still alive
	M	+	2.7	female 76	Highly active rheumatoid arthritis. Patient survived 7 months after biopsy

* Plasma α_1 -AT determined by immunoprecipitation (reference 2-4 g/l) Pi typing by isoelectric focusing.

† M-phenotype deduced in absence of family studies, phenotyping done by starch gel and crossed immunoelectrophoresis. Plasma α_1 -AT by the Mancini technique, reference 2.1 g/l.

‡ As for * with reference for P- α_1 -AT 1.8-3 g/l.

§ Plasma α_1 -AT determined by electroimmunoassay (reference 0.9-1.70, average value 1.32 g/l), Pi typing by isoelectric focusing.

Fig. 1 α_1 -AT containing globules in hepatocytes surrounding the central vein from the Pi M patient with venous congestion (Table 3). Immunoperoxidase staining as described in text $\times 720$.

it probable that Pi MZ is associated with increased fibrosis, cirrhosis and hepatocellular carcinoma (HCC), whereas Kelly *et al.*²² have found no increase in HCC among MZ individuals. In the present

prospective series we find a trend towards overrepresentation of the MZ phenotype among patients with liver disease but it does not reach statistical significance. It should be stressed that absolute comparisons are difficult to perform because of relatively small sample size and diagnostic heterogeneity within phenotypic groups. No increase in mean biopsy score for fibrosis or cirrhosis could be seen in patients with phenotype MZ compared to other phenotypes at similar age (Table 2). Of the 2 MZ patients having cirrhosis, both had a history of alcoholism. No MZ patient lacking history of alcohol overconsumption (3 patients) or cardiac failure (1 patient) had signs of fibrosis. Interestingly an increased frequency of CAH was seen in the Pi MS group. Fisher *et al.*¹⁷ have reported diagnosis CAH among 5 of 18 (28%) Pi MS patients with liver disease. In their study 5/87 (5.75%) of patients with CAH had Pi MS and 18/567 (3.17%) of all patients with liver disease had this phenotype. As they had determined phenotype only in patients with low plasma concentrations of α_1 -AT, and since α_1 -AT is often raised in active liver disease,¹ these figures may be an underestimation. Kueppers *et al.*²³ found the phenotype MS among 8.5% of patients with chronic active liver disease at the Mayo Clinic compared to 3% of healthy blood donors there. These observations together may suggest a predisposition for chronic active liver disease among patients with Pi MS. The plasma concentration is very moderately decreased in these individuals and the mechanism behind such an association is unknown.

The immunoperoxidase staining as well as PAS-

Fig. 2 α_1 -AT globules in hepatocytes of a Pi M patient with rheumatoid arthritis (Table 3). α_1 -AT specific immunoperoxidase staining as described in text $\times 720$.

staining showed diffuse or granular intracellular α_1 -AT deposits in a minority of the biopsies studied. This corresponded to slight increases in plasma α_1 -AT concentrations, total score and inflammatory activity seen in biopsies. Our hypothesis has been that in situations with high plasma α_1 -AT concentrations one should expect high intracellular concentrations reflecting increased synthesis. The regulation of synthesis and secretion, however, may well vary in different diseased states and the intracellular presence of precursor proteins, incompletely glycosylated forms or complexes remains to be studied. Many biopsies probably contain α_1 -AT in concentrations below the threshold of sensitivity for the immunoperoxidase method. In addition, intracellular proteins may be extracted or antigenically altered during treatment of biopsy sections, possibly giving false-negative immunoperoxidase results.

Biopsies from 102 patients stained positively with the immunoperoxidase method for intracellular α_1 -AT in leucocytes, Kupffer's cells (30 cases) or round cells. This may represent degradation products of complex-bound α_1 -AT after phagocytosis but de novo synthesis in these cells, in similarity to that reported in other cells of histiocytic origin,²⁴ cannot be excluded.

The immunoperoxidase method could correctly identify intrahepatocellular α_1 -AT globules in the majority of patients with phenotype MZ and Z, and in that respect was more sensitive than the PAS-staining. Some biopsies from MZ patients had no visible globules with either method. This is not unexpected due to small sample size. More interesting is the occurrence of α_1 -AT globules in 3 cases with the normal M phenotype. Plasma protein analysis, phenotyping and biopsies were evaluated independently in this prospective study of unselected liver biopsied patients. Thus the chance of finding α_1 -AT globules unrelated to the Z gene can be calculated to be 3/207 or 1.4%.

"Congestive globules"²⁵ are well known and are usually easily distinguished from "true" α_1 -AT globules by localisation and appearance. Our case with venous congestion (Table 3 and Fig. 1) probably had such globules, but they were present periportal as well as centrilobularly and could not with certainty be distinguished from α_1 -AT globules. In this case alone globules stained positively even with antisera for fibrinogen, IgG, and albumin.

It appears from our cases and those presented in Table 3 that α_1 -AT globules unrelated to Z gene occur in elderly or terminal patients with high plasma α_1 -AT concentrations reflecting high disease activity. This accumulation may reflect a rate of synthesis exceeding the hepatocyte's capacity for glycosylation or other steps in the secretory process.

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