Intra- and extracellular alpha_1-antitrypsin in liver disease with special reference to Pi phenotype

JOYCE CARLSON, STEN ERIKSSON, INGA HÄGERSTRAND

From the Department of Medicine, University of Lund, Malmö General Hospital, Malmö, Sweden and the Institute of Pathology, University of Odense, Odense, Denmark

SUMMARY In order to study the relation between intra- and extrahepatocellular alpha_1-antitrypsin (alpha_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 alpha_1-AT. Concomitant blood samples from all patients were analysed for alpha_1-AT. Pi phenotypes were determined by isoelectric focusing.

Non-globular intrahepatocellular alpha_1-AT can be seen in biopsies from Pi M patients with increased plasma alpha_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 alpha_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. alpha_1-AT globules in absence of the Z allele are most often found in elderly patients with severe disease and high plasma alpha_1-AT concentrations.

Alpha_1-antitrypsin (alpha_1-AT, alpha_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. Low plasma concentrations are seen in some phenotypes. Of special interest is the PiZ phenotype in which increased frequencies of fibrosis, cirrhosis and hepatoma 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. Typical PAS-positive inclusion bodies, first described by Sharp et al., and consisting of aggregated alpha_1-AT lacking sialic acid and with an amino acid substitution are seen in Pi Z hepatocytes. Such inclusions were first thought to be pathognomonic for the Z allele 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, but most investigators have found no evidence of increased liver disease frequency in such individuals.

alpha_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 alpha_1-AT and plasma concentrations in various liver diseases; (ii) the frequency and some clinical conditions associated with globular inclusions (alpha_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 alpha_1-AT-specific immunoperoxidase staining in addition to routine PAS-staining of 250 liver biopsies (including 22 controls) for visualisation of intracellular alpha_1-AT and isoelectric focusing for phenotype determination in all cases.

Patients, material and methods

The material comprises 250 consecutively 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 α1-AT and phenotype determinations. Plasma α1-AT concentrations were determined at the Department of Clinical Chemistry by an electro-immunoassay technique using a mono-specific antisera 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 α1-AT by crossed immuno-electrophoresis12 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 examination 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 electro-focusing. No evidence was present for any case of M Malton or M Duarte in this material.

Immunoperoxidase staining of α1-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 α1-AT. Mean plasma α1-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 α1-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 α1-AT staining in leucocytes, Kupffer’s cells or round cells, but no differences were found between mean plasma α1-AT concentrations, biopsy scores or activities in these and patients with negative biopsies.

Table 1 Distribution of phenotypes and gene frequencies

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>MM</th>
<th>M-</th>
<th>MS</th>
<th>MZ</th>
<th>FM</th>
<th>SS</th>
<th>SZ</th>
<th>ZZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2830 healthy Norwegians (14)</td>
<td>2540</td>
<td>116</td>
<td>81</td>
<td>72</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>228 biopsied patients with liver disease</td>
<td>199</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene frequencies</th>
<th>Pi M</th>
<th>Pi S</th>
<th>Pi Z</th>
<th>Pi F</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>2830 healthy Norwegians (14)</td>
<td>0.946</td>
<td>0.023</td>
<td>0.0157</td>
<td>0.0133</td>
<td>0.002</td>
</tr>
<tr>
<td>6995 adult Swedes (15)</td>
<td></td>
<td></td>
<td>0.0240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>228 patients with liver disease</td>
<td>0.932</td>
<td>0.0285</td>
<td>0.0307</td>
<td>0.0066</td>
<td>0.002</td>
</tr>
</tbody>
</table>

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 x² test. The major contribution to the difference arises in the Z gene group.
Fifteen cases were found to have $\alpha_1$-AT specific globules in the hepatocytes including 10 cases of PiMZ (two controls) and one PiZZ. Of these 11 cases, three MZ biopsies were PAS-negative. Three cases of PiMZ and 1 PiSZ 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 $\alpha_1$-AT concentrations are summarised in Table 3 together with similar cases from published reports. Globules in our patient with adenocarcinoma had typical appearance for $\alpha_1$-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 $\alpha_1$-AT of both diffuse and globular appearance were found peripherally 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 PiZ 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 immunohistochemical staining nor determination of plasma $\alpha_1$-AT concentrations was performed and, therefore, viewing the occurrence of $\alpha_1$-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. Blenkinson and Haffenden\(^21\) find

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Mean age</th>
<th>% male</th>
<th>Biopsy score</th>
<th>Biopsy activity</th>
<th>Fibrosis</th>
<th>Cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi M</td>
<td>199</td>
<td>53 ± 14</td>
<td>59</td>
<td>6.1 ± 4.5</td>
<td>1.0 ± 0.4</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Pi M-</td>
<td>1</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pi FM</td>
<td>3</td>
<td>52 ± 19</td>
<td>33</td>
<td>7.2 ± 4.1</td>
<td>1.2 ± 0.8</td>
<td>1.0 ± 0.6</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>Pi MS</td>
<td>12</td>
<td>51 ± 12</td>
<td>45</td>
<td>6.5 ± 4.7</td>
<td>1.2 ± 0.2</td>
<td>0.8 ± 0.7</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>Pi MZ</td>
<td>11</td>
<td>50 ± 15</td>
<td>56</td>
<td>5.9 ± 3.7</td>
<td>1.1 ± 0.0</td>
<td>0.9 ± 0.5</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Pi SZ</td>
<td>1</td>
<td>46</td>
<td>100</td>
<td>0.5</td>
<td>0</td>
<td>0.5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pi ZZ</td>
<td>1</td>
<td>39</td>
<td>100</td>
<td>5.5</td>
<td>1.5</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

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 in 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.

### Table 3  Summary of cases with immunoperoxidase demonstrable $\alpha_1$-AT containing globules in hepatocytes in absence of the Z gene

<table>
<thead>
<tr>
<th>Author (ref)</th>
<th>Phenotype</th>
<th>PAS-staining</th>
<th>Plasma $\alpha_1$-AT (g/l)</th>
<th>Age (yr)</th>
<th>Clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradfield et al.(^14)*</td>
<td>M</td>
<td>-</td>
<td>2.3</td>
<td>male 79</td>
<td>Acute pancreatitis patient survived 12 days after biopsy</td>
</tr>
<tr>
<td>Fischer et al.(^13)†</td>
<td>M-</td>
<td>-</td>
<td>1.8</td>
<td>female —</td>
<td>Alcoholic hepatitis no further clinical information reported</td>
</tr>
<tr>
<td>Kelly et al.(^13)*</td>
<td>3MS</td>
<td>+</td>
<td>0.9</td>
<td>male 56</td>
<td>No further information reported Alcoholic hepatitis patient survived 5 months after biopsy</td>
</tr>
<tr>
<td>Present study §</td>
<td>M</td>
<td>+</td>
<td>&gt; 4.0</td>
<td>male 50</td>
<td>Metastatic adenocarcinoma of the pancreas Patient survived 6 months after biopsy</td>
</tr>
<tr>
<td>Present study §</td>
<td>M</td>
<td>+</td>
<td>2.3</td>
<td>male 67</td>
<td>Severe cardiac decompensation with venous congestion Patient still alive Highly active rheumatoid arthritis Patient survived 7 months after biopsy</td>
</tr>
<tr>
<td>Present study §</td>
<td>M</td>
<td>+</td>
<td>2.7</td>
<td>female 76</td>
<td>Highly active rheumatoid arthritis Patient survived 7 months after biopsy</td>
</tr>
</tbody>
</table>

*Plasma $\alpha_1$-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 $\alpha_1$-AT by the Mancini technique, reference 2.1 g/l.
‡As for * with reference for P- $\alpha_1$-AT 1.8-3 g/l.
§Plasma $\alpha_1$-AT determined by electroimmunoassay (reference 0.9-1.70, average value 1.32 g/l), Pi typing by isoelectric focusing.
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In prospective series we find a trend towards over-representation 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 α₁-AT, and since α₁-AT is often raised in active liver disease,1 these figures may be an underestimation. Kueppers et al.28 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-
staining showed diffuse or granular intracellular α1-AT deposits in a minority of the biopsies studied. This corresponded to slight increases in plasma α1-AT concentrations, total score and inflammatory activity seen in biopsies. Our hypothesis has been that in situations with high plasma α1-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 α1-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 α1-AT in leucocytes, Kupffer’s cells (30 cases) or round cells. This may represent degradation products of complex-bound α1-AT after phagocytosis but de novo synthesis in these cells, in similarity to that reported in other cells of histiocytic origin, cannot be excluded.

The immunoperoxidase method could correctly identify intrahepatocellular α1-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 α1-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 α1-AT globules unrelated to the Z gene can be calculated to be 3/207 or 1.4%.

“Congestive globules” are well known and are usually easily distinguished from “true” α1-AT globules by localisation and appearance. Our case with venous congestion (Table 3 and Fig. 1) probably had such globules, but they were present peripertorially as well as centrilobularly and could not with certainty be distinguished from α1-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 α1-AT globules unrelated to Z gene occur in elderly or terminal patients with high plasma α1-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.

This study was supported by the Elsa and Thorsten Segerfalks Stiftelse.

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Requests for reprints to: Dr Joyce Carlson, Department of Medicine, University of Lund, Malmö General Hospital, Malmö, Sweden.