Metabolism of antithrombin III in cirrhosis and carcinoma of the liver

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(Received 14 October 1980; accepted 23 January 1981)

Summary
1. The metabolism of human antithrombin III (ATIII) was studied by using $^{125}$I-labelled tracer.
2. The plasma half-life ($t_{0.5}$) was $2.71 ± 0.26$ days in normal subjects and was similar in patients with cirrhosis or primary carcinoma of liver.
3. Patients with cirrhosis had low ATIII levels, decreased intravascular mass, total body mass and decreased absolute catabolic rate, suggesting decreased synthesis. The positive correlation of ATIII level with fractional catabolic rate ($K_{10}$) indicated that the decreased catabolism might exert a positive inhibition on ATIII production.
4. These abnormalities were more exaggerated in patients with macronodular cirrhosis associated with hepatitis surface antigen or in those with ascites.
5. In cirrhotic patients with ascites an additional extravascular pool of ATIII was present which did not turn over at the same rate as the intravascular pool.
6. Patients with primary carcinoma of liver had moderately low ATIII, but normal intravascular mass and total body mass because of the increased plasma volume and normal absolute catabolic rate.
7. The negative correlation of ATIII levels with $K_{10}$ suggested that the low levels could be due to increased catabolism or consumption.
8. One patient with disseminated malignancy and active superficial thrombophlebitis had normal ATIII metabolism.

Key words: antithrombin III, carcinoma of liver, cirrhosis, metabolism, turnover.

Introduction
Antithrombin III (ATIII), a natural inhibitor of coagulation, plays a major role in the modulation of haemostasis (Abilgaard, 1967; Yin, Wessler & Stoll, 1971; Harpel & Rosenberg, 1976). Its importance can be adduced from the increased incidence of deep vein thrombosis in patients with congenital ATIII deficiency (Egeberg, 1965; Filip, Eckstein & Veltkamp, 1976). In the nephrotic syndrome urinary protein loss resulted in an acquired ATIII deficiency associated with thrombotic episodes (Kauffmann, Veltkamp, Van Tilburg & Van Es, 1978; Jorgensen & Stoffersen, 1979). In liver dysfunction the pathogenesis for the decrease in ATIII level remains undefined (Abilgaard, Fagerhol & Egeberg, 1970; Chan, Chan, Wong, Tso & Todd, 1979). Although it has been suggested that ATIII is synthesized by the liver (Koj, Regoezzi, Toews, Leveille & Gareldio, 1978), immunohistochemical techniques failed to localize ATIII in liver cells (Lee, Chan & Chan, 1979). The finding of ATIII in the vascular endothelium (Chan & Chan, 1979) and the more recent confirmation of its synthesis by endothelial cells (V. Chan & T. K. Chan, unpublished work) disprove the theory of hepatic cell synthesis. To examine the aetiology of the decreased ATIII levels in chronic liver diseases, we studied ATIII turnover in patients with cirrhosis and carcinoma of liver.

Materials and methods

Subjects
A total of 21 patients with histologically proven chronic liver diseases were studied, of whom seven had cirrhosis due to chronic alcoholism and seven had macronodular cirrhosis with
positive hepatitis-B surface antigen (HBsAg) (Lam, Lai, Wu & Todd, 1980); six had primary hepatocellular carcinoma (Lai, Lam, Wong, Wu & Todd, 1981) and one had carcinoma of pancreas with secondaries in the liver complicated by superficial thrombophlebitis. All were males, apart from the last patient, and none was receiving oral anticoagulant therapy. Seven healthy male volunteer subjects acted as controls. All subjects gave their informed consent and research was carried out according to the Declaration of Helsinki.

Preparation of radioactive tracer

Highly purified ATIII (NIBSC 77/618) was 125I-labelled enzymatically by lactoperoxidase as described previously (Chan et al., 1979). The 125I-labelled protein was separated from free iodine by gel filtration with Sephadex G-100 and further purified by affinity chromatography on a heparin/Sepharose column. The coupling of heparin to cyanogen bromide (CNBr)-activated Sepharose (Pharmacia Fine Chemicals, Sweden) was performed by the method of Miller-Andersson, Borgh & Andersson (1974) with minor modifications. Covalent binding of heparin (Weddel Heparin: 10 000 units, 0.4% solution in deionized water) to CNBr-Sepharose (2 g) was achieved by mixing for 3 h at room temperature, and the unchanged material removed by washing with deionized water. The gel was further equilibrated with six alternate wash cycles of acetic acid/sodium acetate buffer (0.1 mol/l, pH 4.8) and sodium bicarbonate (0.5 mol/l). The 125I-labelled tracer was applied on to the freshly prepared heparin/Sepharose column (1 cm x 10 cm). Non-heparin-binding tracer was removed by washing with Tris/citrate buffer [Tris/HCl 0.02 mol/l, trisodium citrate 0.01 mol/l (pH 8.5), NaCl 0.15 mol/l] and the heparin-binding 125I-labelled ATIII eluted with Tris/HCl buffer (0.15 mol/l, pH 7.8) containing NaCl (1 mol/l) (Fig. 1). These fractions were pooled, sterilized by Millipore filtration (0.045 μm), and stored in portions at −70°C, in the presence of trace amounts of 1% human serum albumin.

Evaluation of 125I-labelled ATIII tracer in vitro

Crossed immunoelectrophoresis (Laurell, 1965) of a mixture of the purified 125I-labelled ATIII and normal pooled plasma in the presence of heparin (16 units/ml of gel), against anti-(human ATIII)serum and subsequent radioautography showed a single radioactive peak with the same electrophoretic mobility as plasma ATIII (Fig. 2). The radioactive tracer behaved like plasma ATIII during coagulation by calcium chloride, as evidenced by a second radioactive peak corresponding to the second serum ATIII peak which represents ATIII–procoagulant complexes (Andersson, Engman & Henningsson, 1977; Chan & Chan, 1979b) (Fig. 2).

The absence of pyrogens was tested in rabbits for each batch of label according to the British Pharmacopoeia recommendations (British Pharmacopoeia, 1968, Appendix XV H). Immunological activity of the 125I-labelled ATIII was assessed by binding to an excess of monospecific anti-(human ATIII)serum. The specific binding to ATIII antibody was 90%, after correction for non-specific binding, which amounted to 2.5%.
The test for HBsAg was made in an aliquot of ATIII (NIBSC 77/618) and it was undetectable by radioimmunoassay.

**Turnover studies**

To avoid thyroidal uptake of $^{125}$I each subject received Lugol's solution (20 drops) 1 day before the injection of labelled tracer and daily throughout the course of the study. $^{125}$I-labelled ATIII (10μCi, $3.7 \times 10^5$ Bq, 12 μg) was given intravenously and blood (5 ml) taken into heparinized tubes at 5, 10 and 15 min for the determination of plasma volume. Further sampling was made at 30, 60, 90 and 120 min and at 3, 6, 12 and 24 h, then twice daily for 7 days. In the control and cirrhotic subjects total urine output was collected at 6, 12 and 24 h, then daily for 7 days. Ascitic fluid (2 ml) was removed from one subject with HBsAg-positive cirrhosis and chronic gross ascites at similar time intervals as blood sampling. At the end of the study, the radioactivity of each sample was counted in an Autogamma counter with 80% efficiency for $^{125}$I. The amount of non-trichloroacetic acid-precipitable $^{125}$I was also assessed for all the plasma samples.

**Results**

**Analysis based on plasma radioactivity**

Fig. 3 shows a representative turnover study in one control subject. The plasma radioactivity was plotted against time and the subsequent curve graphically resolved into three exponential components, with a curve-peeling procedure described previously (Matthews, 1957). The metabolic parameters can then be derived mathematically as follows:

**Fractional catabolic rate**

\[
(K_{10}) = \frac{1}{b_1 + \frac{c_2}{b_2} + \frac{c_3}{b_3}}
\]

**Transcapillary permeability**

\[
(K_{12}) = c_1b_1 + c_2b_2 + c_3b_3 - K_{10}
\]

**Intravascular fraction**

\[
= \frac{(c_1b_1 + c_2b_2 + c_3b_3)^2}{c_1/b_1^2 + c_2/b_2^2 + c_3/b_3^2}
\]

where $c_1$, $c_2$ and $c_3$ were the intercepts of the three curves and $b_1$, $b_2$ and $b_3$ were the respective slopes.

Table 1 gives the mean ± SD values of ATIII turnover parameters for all three groups of
patients with carcinoma of liver compensated for their low ATIII, so that the intravascular mass and total body mass of ATIII were normal, whereas, in patients with cirrhosis of liver, the increase in plasma volume (44.91 ± 9.20 ml/kg) was insufficient to compensate for their even lower ATIII levels (13.8 ± 4.4 mg/dl). Thus intravascular mass and total body mass were both significantly decreased ($P < 0.001$ and $< 0.01$ respectively compared with normal values). There was a good correlation between ATIII levels and intravascular mass for all subjects ($r = 0.8058$, $P < 0.0001$) and for patients with cirrhosis ($r = 0.8315$, $P = 0.0004$). The mean fractional catabolic rate ($K_{10}$) was not significantly different in the three groups. However, $K_{10}$ was found to be positively related to the ATIII level in cirrhosis of liver ($r = 0.06047$, $P = 0.0471$) but negatively related to the ATIII level in carcinoma of liver ($r = -0.07208$, $P = 0.0426$) (Fig. 4), and whereas the absolute catabolic rate was normal in the latter, it was decreased in the former group ($P < 0.01$). The transcapillary rate ($K_{11}$) was elevated in the patients, particularly in those with malignancy (5.62 ± 1.81 vs 2.75 ± 1.46 day$^{-1}$ in normal subjects).

The patient with secondary carcinoma of liver and thrombophlebitis had normal turnover parameters.

Table 2 gives a more detailed analysis of the turnover data in patients with cirrhosis. When they were classified according to the aetiology of their disease, those associated with positive HBsAg had significantly lower values for all the parameters listed, compared with those of patients associated with chronic alcoholism. Similarly, cirrhotic patients with ascites were found to have significantly lower ATIII, and both fractional and absolute catabolic rates lower than those without ascites.

![Fig. 3. Analysis of plasma 125I-labelled ATIII radioactivity data in one normal subject by Matthew's multi-exponential method. The curve is graphically resolved into three exponential components by a curve peeling procedure.](image-url)

**TABLE 1. ATIII turnover parameters from plasma radioactivity curves**

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>Plasma volume (ml/kg)</th>
<th>ATIII (mg/dl)</th>
<th>Intravascular mass (mg/kg)</th>
<th>Catabolic rate $K_{10}$</th>
<th>$K_{11}$ (day$^{-1}$)</th>
<th>$i_{c}$ (days)</th>
<th>Intravascular fraction</th>
<th>Total body mass (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>38.50</td>
<td>23.0</td>
<td>8.79</td>
<td>0.720</td>
<td>6.32</td>
<td>2.75</td>
<td>2.71</td>
<td>0.462</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 4.36</td>
<td>3.2</td>
<td>1.00</td>
<td>0.116</td>
<td>1.22</td>
<td>1.46</td>
<td>0.26</td>
<td>0.081</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>14</td>
<td>44.91***</td>
<td>13.8***</td>
<td>6.10***</td>
<td>0.683</td>
<td>4.15***</td>
<td>3.89</td>
<td>2.88</td>
<td>0.462</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 9.20</td>
<td>4.4</td>
<td>2.22</td>
<td>0.144</td>
<td>1.57</td>
<td>1.69</td>
<td>0.50</td>
<td>0.047</td>
</tr>
<tr>
<td>Primary carcinoma</td>
<td>6</td>
<td>48.02*</td>
<td>16.1**</td>
<td>7.78</td>
<td>0.862</td>
<td>6.68</td>
<td>5.62**</td>
<td>2.46</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 8.06</td>
<td>2.4</td>
<td>1.85</td>
<td>0.163</td>
<td>2.13</td>
<td>1.81</td>
<td>0.38</td>
<td>0.060</td>
</tr>
<tr>
<td>Secondary carcinoma with thrombophlebitis</td>
<td>1</td>
<td>40.45</td>
<td>17.5</td>
<td>7.49</td>
<td>0.812</td>
<td>6.08</td>
<td>2.01</td>
<td>2.38</td>
<td>0.477</td>
</tr>
</tbody>
</table>

(continued)
Analysis based on both plasma and urine radioactivity

Fig. 5 illustrates the calculation of $^{125}$I-labelled ATIII turnover by the clearance method, from both plasma and urine radioactivity measurements (Pearson, Veall & Vetter, 1958). If $X_p = ^{125}$I-labelled ATIII in plasma, $X_u = ^{125}$I excreted in urine, then $X_r$, the $^{125}$I-labelled ATIII retained in the body = $1 - X_u$. $X_r$, the fraction of the administered radioactivity in the extravascular space = $X_r - X_p$. After equilibrium, in control subjects, the decrease of $^{125}$I-labelled ATIII from plasma ($X_p$) and from the extravascular space ($X_e$) were almost parallel to each other. Whereas, in the cirrhotic patients with mild ascites, the clearance of $^{125}$I-labelled ATIII from the extravascular space was delayed, as denoted by a slower decline in the radioactivity of the $X_e$ curve. This retention of $^{125}$I-labelled ATIII was even more evident in those patients with gross ascites, when 30–60% of the total injected dose was retained on day 7 (Fig. 6).

In one patient with gross ascites the radioactivity present in the ascitic fluid varied from 2.35 to 5.99% of the injected dose, which was insufficient to account for the retained $X_e$ (50%) on day 7.

The fractional clearance ($k_e$) can be calculated from daily urine radioactivity ($X_u$) and mean plasma radioactivity ($X_p$) after the equilibrium time ($t_e$), which for the present study was attained on day 2. For normal subjects and cirrhotic patients without ascites the daily clearance values

![Graph showing correlation between fractional catabolic rate ($K_{10}$) and circulating ATIII level.](image)

**Fig. 4.** Correlation between fractional catabolic rate ($K_{10}$) and circulating ATIII level. △, Normal control; ○, cirrhosis of liver ($r = 0.6047; P = 0.0471$); ●, hepatocellular carcinoma ($r = -0.7208; P = 0.0426$).

**Fig. 5.** Analysis of $^{125}$I-labelled ATIII turnover by the clearance method by both plasma and urine radioactivity measurements (see the Results section).

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Albumin (g/l)</th>
<th>ATIII (mg/dl)</th>
<th>Intravascular mass (mg/kg)</th>
<th>Catabolic rate</th>
<th>$K_{12}$ (day$^{-1}$)</th>
<th>Intravascular fraction</th>
<th>Total body mass (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$K_{10}$</td>
<td>Absolute</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>42.00</td>
<td>16.98</td>
<td>7.66</td>
<td>0.695</td>
<td>5.24</td>
<td>3.80</td>
<td>0.481</td>
</tr>
<tr>
<td>± 4.90</td>
<td>3.54</td>
<td>2.24</td>
<td>0.684</td>
<td>1.34</td>
<td>1.92</td>
<td>0.043</td>
<td>4.65</td>
</tr>
<tr>
<td>HBsAg +</td>
<td>30.29*</td>
<td>11.83*</td>
<td>5.01*</td>
<td>0.619</td>
<td>3.13**</td>
<td>4.10</td>
<td>0.457</td>
</tr>
<tr>
<td>± 9.95</td>
<td>2.94</td>
<td>1.08</td>
<td>0.092</td>
<td>0.97</td>
<td>1.40</td>
<td>0.039</td>
<td>2.88</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No ascites</td>
<td>42.40</td>
<td>17.08</td>
<td>6.98</td>
<td>0.739</td>
<td>5.13</td>
<td>3.52</td>
<td>0.466</td>
</tr>
<tr>
<td>± 5.35</td>
<td>3.32</td>
<td>1.96</td>
<td>0.051</td>
<td>1.39</td>
<td>1.42</td>
<td>0.045</td>
<td>4.03</td>
</tr>
<tr>
<td>With ascites</td>
<td>31.50*</td>
<td>12.41*</td>
<td>5.77</td>
<td>0.601**</td>
<td>3.46*</td>
<td>4.24</td>
<td>0.469</td>
</tr>
<tr>
<td>± 9.82</td>
<td>5.22</td>
<td>2.16</td>
<td>0.078</td>
<td>1.29</td>
<td>1.76</td>
<td>0.041</td>
<td>4.52</td>
</tr>
</tbody>
</table>
Fig. 6. $X_e$ curves showing the amount of $^{125}$I-labelled ATIII in the extravascular space in cirrhotic patients with and without ascites. The normal limits are represented by the shaded area.

$(X_u/X_p)$ from day 2 to 7 were averaged, such that $k_e = \sum (X_u/X_p)/n$, where $n =$ number of days. In cirrhotic patients with moderate and gross ascites, in whom there was significant retention of $X_e$ radioactivity, the clearance rate $(X_u/X_p)$ from the intravascular compartment was also constant from day 2 to 7 and $k_e$ could be similarly derived. Hence it could be concluded that the catabolism of ATIII in these subjects occurred only in the intravascular compartment. Furthermore the fractional catabolic rate $(K_{10})$ derived from the plasma radioactivity analysis was linearly related to the fractional clearance rate $(k_e)$ in the subjects studied and can be represented by $(K_{10}) = 2.722 + 6.00 (k_e)$ ($r = 0.7115, P = 0.0045$).

Discussion

The $^{125}$I-labelled ATIII used in the present study was found to be similar to plasma ATIII by biological and immunological tests, including heparin-Sepharose affinity chromatography, binding to activated procoaguants on clotting and precipitation by monospecific antiserum. Hence the turnover data obtained with this label can be assumed to reflect that of the total body ATIII pool.

The plasma radioactivity $t_{0.5}$ of ATIII was $2.71 \pm 0.26$ days in normal control subjects, and this was similar in patients with cirrhosis and carcinoma of liver ($2.88 \pm 0.50$ and $2.46 \pm 0.38$ days respectively). Collen, Schetz, DeCock, Holmer & Verstraete (1977) found similar values in normal subjects and patients with peripheral arterial disease and venous thromboembolism. In addition ATIII turnover was normal in the patient with secondary carcinoma of the liver and active superficial thrombophlebitis, suggesting that the turnover parameters are not grossly disturbed during venous thrombosis. Collen et al. (1977) had shown that these parameters were increased only with heparin treatment.

In patients with cirrhosis of liver ATIII levels were decreased and in many cases lower than that in congenital ATIII deficiency (about 50% normal), yet no clinical evidence of venous thromboembolism was noted, probably because of the low prothrombin and other vitamin K-dependent factors in these patients (Deutsch, 1965). They tend to be more prone to bleeding from thrombocytopenia and increased fibrinolysis (Kwan, McFadzean & Cook, 1956). Even though the plasma volume was increased, the intravascular mass and total body mass were still significantly decreased. The decreased absolute catabolic rate indicates decreased production of ATIII. These abnormalities of ATIII metabolism were more pronounced in those patients with
positive HBsAg and those with ascites. Further insight into the aetiology of low ATIII was obtained when it was shown that the fractional catabolic rate ($K_{10}$) was directly related to ATIII level, which means that the low ATIII level was associated with decreased fractional clearance or catabolism. It can be concluded that, in the steady state, the decreased demand for ATIII in cirrhotic patients resulted in a positive feedback on ATIII production and hence low circulating level. However, this very low ATIII could be conducive to acute disseminated intravascular coagulation, which is known to occur in those patients with chronic liver disease progressing to acute liver failure (Verstraete, Vermylen & Collen, 1974).

Clinically detectable ascites occurred in eight out of 14 cirrhotic patients. In these patients there was a marked retention of $^{125}$I-labelled ATIII in the extravascular space. Removal of ascitic fluid at various intervals after injection of the labelled tracer in one patient showed that leakage into the ascitic fluid could not account for the total amount of radioactivity which was retained. There appeared another extravascular compartment where the tracer might accumulate. Previous study of albumin exchange between plasma and ascitic fluid had shown that the protein diffused into an adjacent extraperitoneal space other than the peritoneal cavity (Dykes & Jones, 1968). It seemed that the open two-compartment mamillary system, which explains the metabolism of ATIII in normal subjects adequately (Fig. 7a), has to be modified for patients with cirrhosis when ascites is present. Analysis by urine clearance ($k_c$) suggests that catabolism occur only in the intravascular compartment in both normal subjects and cirrhotic patients and thus the calculation of absolute catabolic rate from $K_{10}$ values are valid for both groups. For the cirrhotic patients a different compartment system is proposed (Fig. 7b), where there are two extravascular pools, $EV_1$ and $EV_2$, in which $EV_1$ equilibrates with $EV_2$ rapidly but the latter ($EV_2$) exchanges at a much slower rate with $EV_1$ and the intravascular pool (IV).

In primary carcinoma of liver the ATIII level was moderately decreased, and the increased plasma volume resulted in normalization of the intravascular mass and total body mass in this group. It is of interest to note that the fractional transcapillary ATIII transfer rates were greater in these patients. Contrary to patients with cirrhosis of liver, the absolute catabolic rate was normal, indicating normal ATIII production. However, the fractional catabolic rate ($K_{10}$) varied inversely with ATIII level, which would suggest that the low circulating level was associated with increased catabolism. Since it is well known that hepatic malignancy is associated with hypercoagulability (Kwaan, Lo & McFadzean, 1959; Nusbacher, 1964) an increased consumption or catabolism of ATIII may be required to counteract chronic disseminated intravascular coagulation in these patients.

**Acknowledgments**

We thank the National Institute of Biological Standards and Control, U.K. for the generous supply of ATIII and Mr A. Wong for skilful technical assistance. This work was presented in part at the 18th Congress of the International Society of Haematology, Montreal, 1980.

**References**


