Intravascular coagulation in acute liver failure in rats and its treatment with antithrombin III

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SUMMARY Liver damage was induced in rats by injection of dimethylnitrosamine (DMN) or carbon tetrachloride (CCl₄). Fibrin clots were observed in the hepatic sinusoids at 12 hours and soluble fibrin monomer complexes were markedly detected at 24 hours only in the rats given DMN. When antithrombin III concentrate was infused at 12 hours there was a dose dependent improvement of the values of serum total bilirubin, SGPT, prothrombin time, peripheral platelet count, and plasma fibrinogen and coagulation factor VIIIc and of the histological degree of liver injury at 24 hours in the DMN group. The CCl₄-group showed no such improvement. Intravascular coagulation may complicate the course of certain types of acute liver injury and contribute to its aggravation in rats. Under such circumstances, treatment with antithrombin III concentrate would be beneficial.

Both the sinusoidal deposition of fibrin and the increased catabolism of fibrinogen in acute liver failure suggest that intravascular coagulation may contribute to the cell injury in this condition.¹⁻⁷ The efficacy of early treatment of such intravascular coagulation is, however, still unproven.^{2,5-7} It is not clear whether all types of acute liver failure are accompanied by intravascular coagulation and whether in any specific case it contributes to the aggravation of liver damage.

Fibrinogen is converted into fibrin by thrombin. Thrombin activity can be inhibited by various plasma inhibitors.⁸ Of the well known three protease inhibitors: antithrombin III (AT III), α_2 -macroglobulin and α_1 -antitrypsin, AT III, a serine protease inhibitor produced by the liver, is assumed to be the most important.⁹ Heparin, frequently used in the treatment of intravascular coagulation, accelerates the interaction between AT III and thrombin.^{10,11} Heparin is effective, therefore, only when sufficient AT III is present. In acute liver failure, where low concentrations of AT III have been reported, substitution with AT III might be

beneficial for the treatment of intravascular coagulation.¹²⁻¹⁵

In this paper, the therapeutic efficacy of AT III in two different models of acute liver damage is evaluated.

Methods

RATS

INDUCTION OF ACUTE LIVER INJURY

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 170–190 g, received 50 mg/kg body wt dimethylnitrosamine (DMN), ip, as 2.5% solution in saline or 2.0 ml/kg body wt of carbon tetrachloride (CCl₄) as 20% solution in olive oil, and were fed a commercial pelleted diet with water *ad libitum*.

EXPERIMENTS

Experiment 1

In five rats each given DMN or CCl₄, the liver was perfused at 12 hours with saline and then fixative containing 1.5% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.4, and 1% sucrose¹⁶ for several minutes through the portal vein under anaesthesia with pentobarbital sodium and was excised for

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electron microscopic examination. Another 10 intoxicated rats were anaesthetised with ether at 24 hours, and blood was collected through the inferior vena cava with a plastic syringe containing 3.1% sodium citrate solution (1:10 v/v) for determination of soluble fibrin monomer complexes (FM).

Experiment 2

Forty rats given DMN and 40 CCl₄ were divided at 12 hours each into four groups that received infusions of 2 ml/kg body wt of human AT III (Antithrombin III-Concentrate 500, Behringwerke, Marburg, Germany) at 0 (control rats), 100, 200, or 400 U/kg body wt. At 24 hours blood was collected similarly with an empty syringe and a syringe containing 1:10 (v/v) of 3.1% sodium citrate solution to prepare serum and plasma for determinations of liver function and coagulation parameters, and the liver was excised for histological examination.

DETERMINATIONS OF LIVER FUNCTION AND COAGULATION PARAMETERS

Peripheral platelets were counted in platelet rich plasma from the citrated blood using a Thrombocounter-C (Counter Electronics, Inc, Hialeah, Florida, USA).

Serum total bilirubin and glutamic pyruvic transaminase (SGPT) were determined in the sera using an autoanalyser and a commercial kit (Iatron Laboratories, Inc, Tokyo, Japan), respectively. Prothrombin time (PT) was measured in the plasma using Simplastin (Division of Warner-Lambert Co, NJ, USA) and a fibrometer (Division of Becton, Dickinson and Company, Cockeysville, MD, USA). Plasma AT III, fibrinogen, coagulation factor VIIIc (VIIIc) and FM were determined using either Test Team Antithrombin III kit (Kabi Diagnostica, Stockholm, Sweden), Fibrinogen Determination Set (International Reagent Corporation, Kobe, Japan), Coagulation Factor VIII Deficient Substrate Plasma (American Dade, American Hospital Supply del Caribe, Inc, Aguada, USA) or FM-test (Boehringer Mannheim, Mannheim, Germany).

HISTOLOGICAL EXAMINATION

Hepatic histology on light microscope was studied in formalin-fixed and paraffin embedded specimens of the liver stained with haematoxylin and eosin and Masson's trichrome. All slides were evaluated blind by one of the authors.

Electron microscopic examination was carried out as follows: From the excised liver after perfusion, 30 blocks of about 1 cubic mm were made with a blade. They were postfixed in 2% OsO₄ in 0.1 M phosphate buffer, pH 7.4, at 4°C for two hours, dehydrated in ethanol, and embedded in Epon. Ultrathin sections

were observed under a Hitachi H-800 electron microscope at 100 kV.

STATISTICAL ANALYSES

The data for FM levels were analysed by Wilcoxon's two-sample test. The data for liver function and coagulation parameters after AT III infusion were analysed with linear regression analysis; in multiple comparison between the control and three treated groups, corrected *t* according to Bonferroni's method was used, and the histological changes were analysed with Kendall's correlation.

Results

COAGULOPATHY IN DMN- AND CCL₄-INTOXICATED RATS

Figure 1 shows electron micrographs of DMN intoxicated rat liver. Fibrin clots were observed in the sinusoids where the endothelial cells disappeared. The similar results were obtained in every three to four blocks in all five rats. In CCl₄-intoxicated rats, however, there were no fibrin clots in the sinusoids in either block of five rats. They were found only in the necrotic hepatocytes, as shown in Figure 2.

The concentrations of FM were significantly higher in the DMN-group than in the CCl₄ group (Table 1).

EFFECT OF AT III INFUSION ON LIVER FUNCTION AND COAGULATION PARAMETERS AND HEPATIC HISTOLOGY IN DMN- AND CCL₄-INTOXICATED RATS

As shown in Table 2, AT III concentrations were about one fourth of the normal level in both control DMN and CCl₄ groups. The increase of serum total bilirubin and SGPT concentrations in the CCl₄-group was about twice that in the DMN group. Prothrombin time prolongation did not differ, but fibrinogen was reduced more prominently in the DMN-group. The most remarkable difference between both groups was found in reduction of VIIIc

Table 1 Plasma concentrations of soluble fibrin monomer complex (FM) in rats after dimethylnitrosamine (DMN) or carbon tetrachloride (CCl₄) intoxication

Groups	PT* (Mean (SD))	FM†			
		—	‡	§	
		Rats (n)			
DMN	22.1 (6.5)	0	4	2	4¶
CCl ₄	22.1 (3.9)	0	10	0	0**
Normal	9.1 (0.3)	10	0	0	0

*PT indicates prothrombin time. †=no turbidity, ‡=diffuse turbidity, §=normal agglutination, and ||=massive agglutination; ¶p<0.01 v CCl₄ and p<0.001 v normal; **p<0.001 v normal.

Fig. 1 Electron micrographs of rat liver 12 h after a dose of 50 mg/kg body wt of dimethylnitrosamine. (a) Fibrin clots are demonstrated in the sinusoids where endothelial cells disappear. Arrows: fibrins; H: hepatocyte; S: sinusoid. (b) Fibrins show strands with a longitudinal periodicity of 23 nm (arrows). Bar corresponds to 100 nm.

Fig. 2 Electron micrograph of rat liver 12 h after a dose of 2.0 ml/kg body wt of carbon tetrachloride. Fibrin clots are demonstrated in necrotic hepatocytes. Arrows: fibrin; H: necrotic hepatocytes; S: sinusoid. The inset shows higher power view of fibrins.

and platelet count. There were negative correlations between concentrations of VIIIC and those of SGPT ($r = -0.884$, $p < 0.01$) and serum total bilirubin ($r = -0.723$, $p < 0.05$) in the DMN group. After AT III treatment, a dose dependent increase in AT III levels was seen in both DMN- and CCl_4 -groups ($p < 0.001$). In the DMN group, the concentration of serum total bilirubin ($p < 0.01$), SGPT ($p < 0.01$), and PT ($p < 0.01$) decreased and those of fibrinogen ($p < 0.01$), VIIIC ($p < 0.001$), and platelet count ($p < 0.1$) increased dose dependently with increasing AT III doses. Similar changes were not found in the CCl_4 group.

There was a dose dependent improvement on hepatic histology in the extent of injury after treatment in the DMN-group (Table 3). In the CCl_4 group, however, no difference was observed between the control and treated groups.

Discussion

The data presented show that treatment with the AT III concentrate can prevent further expression of liver damage in rats intoxicated with DMN where

fibrin clots with typical strands^{17,18} were observed in the hepatic sinusoids and FM concentrations were markedly raised. A comparable benefit was not seen in those with CCl_4 where such changes were not found. Platelet count and VIIIC concentrations were reduced to a greater extent in the DMN model as compared with the CCl_4 model even though serum total bilirubin and SGPT concentrations were reversed.

VIIIC concentrations have been reported to increase in fulminant hepatic failure.^{19,20} Although platelets are decreased in acute liver injury, marked reduction is rare without complications.^{20,21} The differences in these parameters between the two models, therefore, suggest that a consumption coagulopathy occurred in the DMN model, and to a lesser extent in the CCl_4 model. This would be also supported by strongly positive FM and fibrin clots observed in the hepatic sinusoids in the DMN model. According to our preliminary experiments, such a difference seems to be related to sinusoidal cell destruction of the liver (paper in preparation). Dimethylnitrosamine is thought to cause hepatocellular necrosis by blocking liver protein synthesis,

Table 2 Concentrations of plasma antithrombin III (AT III), serum total bilirubin, serum glutamic pyruvic transaminase (SGPT), prothrombin time (PT), plasma fibrinogen and coagulation factor VIII C (VIII C) and peripheral platelet count in rats treated with AT III after dimethylnitrosamine (DMN) or carbon tetrachloride (CCl₄) intoxication

Parameters	Groups							
	DMN				CCl ₄			
	Doses of AT III (U/kg)							
	0	100	200	400	0	100	200	400
AT III (%)	27	37*	43‡	68§	27	48*	79§	130§
(105 (6))	(7)	(9)	(7)	(14)	(16)	(16)	(21)	(45)
Total bilirubin (mg/dl)	0.50	0.29*	0.26‡	0.17§	1.19	0.94	1.16	0.98
(<0.1)	(0.27)	(0.17)	(0.22)	(0.16)	(0.45)	(0.58)	(0.51)	(0.51)
SGPT (Karmen unit)	1410	1340	860*	730†	2500	1990	3090	2540
(19 (5))	(550)	(610)	(360)	(510)	(1980)	(1430)	(1940)	(1290)
PT (s)	40.9	27.4†	28.6*	22.2‡	31.3	29.2	29.1	32.1
(10.2 (0.6))	(16.1)	(7.3)	(12.6)	(4.6)	(17.8)	(17.7)	(10.9)	(18.3)
Fibrinogen (mg/dl)	64.6	71.5	89.4	124.7†	132.3	153.0	104.0	134.2
(245 (20))	(32.6)	(33.3)	(52.1)	(51.0)	(82.6)	(130.0)	(47.7)	(93.2)
VIII C (u/ml×10 ²)	2.0	2.2	2.2	2.8‡	101.1	45.3	51.8	131.1
(290 (90))	(0.4)	(0.3)	(0.4)	(0.8)	(77.6)	(48.8)	(58.3)	(169.5)
Platelet (×10 ⁴ /mm ³)	18.3	19.2	17.5	23.3*	81.6	78.8	69.8	67.8
(100.4 (7.3))	(3.9)	(7.0)	(4.0)	(8.2)	(12.8)	(15.5)	(23.3)	(14.7)

Results are mean (SD) of 10 rats except for nine rats in the CCl₄-group treated by 100 U/kg of AT III where one rat was killed by anaesthesia at the time of AT III infusion. Results in parentheses are the mean (SD) of five normal rats. *p<0.1; †p<0.05; ‡p<0.01; §p<0.001 v 0 dose of AT III.

while CCl₄ by immediate injury to the plasma membrane.²² The mechanism of liver injury must be further investigated.

Because treatment with AT III did not affect the course of the CCl₄ induced liver injury, the treatment is unlikely to have direct effects on hepatocellular damage. In this model where fibrin clots were present only in the necrotic hepatocytes, coagulation parameters were not changed by the treatment. Infused AT III might not have perfused effectively in these areas, though the reason is obscure. In the

DMN model, reduced fibrinogen concentrations were markedly attenuated by the treatment. The benefit of AT III for liver damage observed in this model would be the result of its effect on formation of fibrin clots in the sinusoids.

Rake *et al*⁴ showed in a CCl₄ rat model that an excessive deposition of ¹²⁵I-fibrinogen in the liver could be delayed by heparin treatment. The coagulopathy and the increased catabolism of fibrinogen observed in this model was similar to the phenomena found in patients with acute hepatic necrosis. The authors did not describe, however, the relation of these changes to the degree of liver damage. Muller-Berghaus *et al*⁵ induced hepatitis and disseminated intravascular coagulation in rabbits by the administration of galactosamine and fibrinolysis inhibitor. The authors could prevent the formation of microclots by treatment with heparin. This treatment did not show any influence on total bilirubin or SGPT. The conclusions drawn from these observations remain equivocal, however, because it is known that heparin does not inhibit thrombin activity in the absence of AT III.¹¹ The experiments presented in this paper provide evidence that intravascular coagulation occurs and varies with the type of injury in rats. Intravascular coagulation may be a contributing factor to the aggravation of the injury. Therapy with AT III concentrate would be of benefit for such types of acute hepatic failure.

Our experimental models may differ in the mech-

Table 3 Histological changes of hepatocellular necrosis and degeneration in rats treated with antithrombin III (AT III) after dimethylnitrosamine intoxication

Doses of AT III (U/kg)	Extent of necrosis and degeneration* (Grades)			
	1	2	3	4
0	0	2	5	3
100	1	1	7	1
200	0	4	5	1
400	2	4	4	0
r _k = -0.350, p<0.05				

Figures indicate number of rats. *The extent was classified according to the extent or numbers of the findings of karyorrhexis (K), cell debris (CD), and spotty necrosis with blood filled spaces (SN) in addition to acidophilic degeneration in the centrilobular areas as follows: Grade 1: K-, CD-, SN-; grade 2: K- CD±, SN±; grade 3: K+, CD+, SN+; grade 4: K++, CD++, SN++.

anism of development of injury from acute liver failure commonly encountered in humans. However, from the fact that acute liver failure takes different courses,^{23,24} and hence the pathological state of disease might vary also in man, the present results would provide useful hints for considering such situation.

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