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World J Gastroenterol 2005;11(32):4997-5001
World Journal of Gastroenterology ISSN 1007-9327

• *BRIEF REPORTS* •

Impact of antithrombin III on hepatic and intestinal microcirculation in experimental liver cirrhosis and bowel inflammation: An *in vivo* analysis

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Received: 2004-10-29 Accepted: 2004-11-23

Abstract

AIM: To analyze the hepatic and intestinal microcirculation in an animal model of liver cirrhosis and inflammatory bowel disease (IBD) and to characterize the anti-inflammatory action of antithrombin III (ATIII) on leukocyte kinetics and liver damage.

METHODS: Hepatic and intestinal microcirculation was investigated by intravital videomicroscopy. Standardized models of experimental chronic liver cirrhosis and bowel inflammation were employed. Animals were divided into four groups (n = 6/group): controls, animals with cirrhosis, animals with cirrhosis and IBD, animals with cirrhosis and IBD treated with ATIII.

RESULTS: Cirrhosis facilitated leukocyte rolling and sticking in hepatic sinusoids (1.91±0.28 sticker/μm *vs* 0.5±0.5 sticker/μm in controls, P<0.05). The effect enhanced in animals with cirrhosis and IBD (5.4±1.65 sticker/μm), but reversed after ATIII application (3.97±1.04 sticker/μm, P<0.05). Mucosal blood flow showed no differences in cirrhotic animals and controls (5.3±0.31 nL/min *vs* 5.4±0.25 nL/min) and was attenuated in animals with cirrhosis and IBD significantly (3.49±0.6 nL/min). This effect was normalized in the treatment group (5.13±0.4 nL/min, P<0.05). Enzyme values rose during development of cirrhosis and bowel inflammation, and reduced after ATIII application (P<0.05).

CONCLUSION: Liver cirrhosis in the presence of IBD leads to a significant reduction in mucosal blood flow and an increase in hepatic leukocyte adherence with consecutive liver injury, which can be prevented by administration of ATIII.

Key words: Liver cirrhosis; Microcirculation; Bowel inflammation; Liver; Antithrombin III

Maksan SM, Ülger Z, Gebhard MM, Schmidt J. Impact of antithrombin III on hepatic and intestinal microcirculation in experimental liver cirrhosis and bowel inflammation: An *in vivo* analysis. *World J Gastroenterol* 2005; 11(32): 4997-5001
http://www.wjgnet.com/1007-9327/11/4997.asp

INTRODUCTION

Liver cirrhosis is a well-known immunocompromised state, with a high susceptibility to infection and organ dysfunction[1]. Cirrhotic patients are prone to develop a progressive impairment in local and systemic hemodynamics, leading to renal and hepatic failure. The gut mucosa plays an important role in the pathogenesis of complications of cirrhosis. However, little is known about the exact mechanisms promoting severe septic complications and liver failure in cirrhotic patients. Among other pathophysiological mechanisms, hypoperfusion of the gut mucosa has been implicated as an important mechanism contributing to mucosal injury[2]. Likewise changes in the intestinal flora and in the intestinal barrier as well as leukocyte dysfunction are presumed to be responsible for infective complications of cirrhosis[3].

Any inflammatory process is characterized by tissue infiltration of leukocytes mediated by cytokines and cell adhesion molecules expressed on the endothelial surface[4]. By intravital videomicroscopy, leukocyte-endothelial interaction can be visualized and quantitated. Furthermore, the effects of therapeutic agents can be investigated in the same setting.

Patients with progressive liver disease are known to have low plasma concentrations of antithrombin III (ATIII)[5,6]. ATIII is synthesized in liver parenchymal cells and plays a central role in regulating hemostasis[7]. In addition, ATIII has been reported to have some influence on the inflammatory process, which is independent of its effects on coagulation. This effect is thought to be indirect via enhanced prostacyclin release from endothelial cells[8]. We have previously shown that hepatic reperfusion injury could be attenuated by administration of ATIII in an animal model of warm hepatic ischemia and reperfusion[9].

The aim of this study was to analyze the intestinal and hepatic microcirculation in cirrhotic animals with inflammatory bowel disease (IBD) and to evaluate the effects of ATIII application during the onset of bowel

inflammation on microcirculation, leukocyte-endothelium interaction and liver function.

Data of control and cirrhotic animals without bowel inflammation published recently by our group were considered for statistical comparison in the present study.

MATERIALS AND METHODS

All experiments were performed in accordance with the Governmental Animal Protection Committee.

Experimental protocol

Twenty-four male Wistar rats weighing 200-220 g were eligible for analysis in this study. Two rats died during induction of liver cirrhosis. Animals were divided into four groups, six animals each: controls, animals with cirrhosis, animals with cirrhosis and IBD, and animals with cirrhosis and IBD treated with ATIII.

Induction of liver cirrhosis

Chronic progressive liver cirrhosis was induced by gavage with carbon tetrachloride (CCl_4) as previously described[10]. In brief, animals had free access to tap water containing barbitural sodium (100 mg/dL) 2 wk before the first CCl_4 dose. The CCl_4 doses were calibrated weekly from an initial dose of 0.04 mL, to a maximum dose of 0.4 mL after 10 wk.

Induction of bowel inflammation

Bowel inflammation was induced by the protocol of Yamada, described in detail elsewhere[11]. Indomethacin at a dose of 7.5 mg/kg body weight (Sigma, Germany) was injected subcutaneously in the lower abdomen. Drug application was repeated after 24 h, and intravital videomicroscopy was performed 7 d, after induction of bowel inflammation.

Antithrombin III application

A group of six animals received intravenously 250 IU/kg body weight ATIII (Kybernin HS 1000, Centeon Pharma, Germany) 24 h, after the last indomethacin application.

Monitoring

Mean arterial blood pressure and heart rate were recorded continuously via an arterial catheter placed in the left carotid artery. In addition, arterial blood samples were obtained to perform blood-gas analysis (ABL 5, Radiometer GmbH, Willich, Germany) and venous samples to measure liver enzymes (AST, ALT, GGT, and alkaline phosphatase) and clotting (ATIII levels and prothrombin time) using commercial assays.

Intravital videomicroscopy

Animals were fasted overnight, but received water *ad libitum*. Experiments were performed under general anesthesia with ketamine/pentobarbital (Ketanest, Park-Davis, Germany and Narcoren, Merial, Germany), administered via a catheter (B. Braun, Melsungen, Germany) placed in the left jugular vein. A midline incision was made and intravital fluorescence microscopy was performed using a Leitz orthoplan microscope (Leitz, Wetzlar, Germany). With different excitation filters (wavelengths 450-490 and 530-560 nm), selective visualization of FITC-labeled erythrocytes and rhodamine 6G stained leukocytes was possible. For contrast enhancement, FITC-labeled albumin was administered intravenously.

Liver microcirculation

The left liver lobe was exteriorated onto a specially designed stage and videomicroscopy was performed according to the technique described by Menger *et al*.[12]. Ten liver lobules were observed for 30-60 s. Vessels with a diameter between 20 and 40 μm were eligible for analysis.

Small intestine microcirculation

In the same animal, the terminal ileum was exteriorized and placed on a glass slide. Videomicroscopy of mean mesenteric vessels was performed. Then the bowel was opened along the anti-mesenteric border and fixed at the incision margins. Continuous superfusion with buffered Ringer's solution was provided. Mesenteric microcirculation was investigated in 10 fields of ileal arteries and corresponding veins following the technique described in detail elsewhere[13]. Mucosal microcirculation was measured in the main arteriole of five single villi. Each vessel was observed for 30 s.

Data analysis

Microcirculatory parameters were quantified by off-line analysis of video-recorded microscopic images using the computer-assisted image analysis system Capimage (Capimage, Zeintl, Heidelberg, Germany)[14]. Vessel diameter and erythrocyte velocity were evaluated. Leukocyte-endothelium interaction (LEI) was characterized by leukocyte adherence measurements as the number of leukocytes that remained stationary or temporary on the vessel wall. Leukocytes that adhered for at least 30 s were considered as stickers, whereas leukocyte rolling was based on endothelial lining that was less than 66% of the erythrocyte velocity. Microvascular blood flow was derived from erythrocyte velocity (V_e) and vessel diameter (D) using the following equation[15]: $V_b = V_e \times \pi \times (D/2)^2$.

Histology

After microscopy, animals were killed. One part of the liver and one part of the small intestine were obtained for histopathological investigations. Specimens were fixed in buffered formalin (4%), embedded in paraffin and stained with hematoxylin and eosin.

Statistical analysis

All data were expressed as mean±SD. Inter-group comparisons were made using the Mann-Whitney U test. $P<0.05$ was considered statistically significant.

RESULTS

Hemodynamics and laboratory data

There were no significant differences in hemodynamic variables between the groups. Signs of sepsis were not observed in any of the animals (Table 1). Analysis of laboratory data demonstrated significant differences in all groups (Table 4). In summary, transaminase values rose

during development of cirrhosis and bowel inflammation. This effect was withdrawn after ATIII treatment. Prothrombin time showed comparable characteristics.

Table 1 Cardiorespiratory parameters at the beginning of intravital videomicroscopy (mean±SD)

	Cirrhosis	Cirrhosis and IBD	ATIII treatment group (cirrhosis and IBD)	Controls
Mean arterial blood pressure (mmHg)	107±8.4	102±8.7	106±7.8	112±7.3
Heart rate (bpm)	310±9.2	318±7.4	312±8.2	304±8.4
paO_2 (mmHg)	89.1±5.3	86.4±4.3	88.6±5.4	92.3±4.8

Microcirculation

Table 2 compares the vessel diameter, RBC velocity and blood flow of the liver, mesentery, and ileal mucosa. Blood flow analysis showed significant differences between the groups. Liver cirrhosis was associated with a reduction of volumetric blood flow in mesenteric vessels, compared to control animals (135.1±3.56 nL/min *vs* 156.5±4.3 nL/min). However, an inverse effect was observed in the presence of bowel inflammation: mesentery blood flow increased significantly (149.8±2.41 nL/min) and rose further in the ATIII treatment group (243.1±3.71 nL/min). Mucosal blood flow in central arterioles showed no differences in cirrhotic animals and controls (5.3±0.31 nL/min *vs* 5.4±0.25 nL/min), but was significantly attenuated in the inflammation group (3.49±0.6 nL/min). This effect was normalized in the treatment group (5.13±0.4 nL/min). Hepatic blood flow increased in the IBD group and remained stable under ATIII substitution.

Table 2 Microcirculatory parameters of the liver, mesentery and ileal mucosa (mean±SD)

	Cirrhosis	Cirrhosis and IBD	ATIII treatment group (cirrhosis and IBD)	Controls
Vessel diameter (μm)				
Liver	27.86±2.03[a]	28.15±1.72	28.08±1.27	23.03±0.62
Mesentery	32.06±8.91	33.51±5.16	42.51±7.04[e]	33.46±11.66
Mucosa	7.51± 0.30	6.32±0.41	7.21±0.52	7.20±0.23
RBC velocity (mm/s)				
Liver	0.93±0.09[a]	1.08±0.13[c]	1.16±0.11	1.22±0.18
Mesentery	2.81±0.49	3.28±0.42	2.93±0.41	3.11±0.35
Mucosa	2.03±0.15	1.81±0.13[c]	2.11±0.12	2.18±0.16
Volumetric blood flow (nL/min)				
Liver	32.1±0.43	39.5±0.43[c]	40.9±0.62	31.2±0.6
Mesentery	135.1±3.56[a]	149.8±2.41[c]	243.1±3.71[e]	156.5±4.3
Mucosa	5.3±0.31	3.49±0.6[c]	5.13±0.4[e]	5.4±0.25

[a]P<0.05 cirrhosis *vs* controls; [c]P<0.05 cirrhosis+IBD *vs* cirrhosis; [e]P<0.05 ATIII *vs* cirrhosis+IBD.

Leukocyte-endothelium interaction

Analysis of leukocyte kinetics showed marked differences in hepatic leukocyte adherence. Cirrhosis facilitated leukocyte rolling and sticking in hepatic sinusoids (1.91±0.28 sticker/100 μm *vs* 0.5±0.5 sticker/100 μm in control, P<0.05). The effect was enhanced in bowel inflammation (5.4±1.65 sticker/100 μm), but reversed in the ATIII group (3.97±1.04 sticker/100 μm, P<0.05). LEI in mesenteric vessels was characterized by a significant increase of adherent leukocytes during bowel inflammation (5.24±1.23 sticker/100 μm *vs* 2.54±1.19 sticker/100 μm, P<0.05) with no substantial difference in the ATIII treatment group (Table 3).

Table 3 Results of intravital videomicroscopy: LEI in the liver and mesentery (mean±SD)

	Cirrhosis	Cirrhosis and IBD	ATIII treatment group (cirrhosis and IBD)	Controls
Adherent leukocytes (n/100 μm)				
Liver	1.91±0.28[a]	5.40±1.65[c]	3.97±1.04[e]	0.5±0.5
Mesentery	2.54±1.19	5.24±1.23[c]	4.36±1.19	1.62±0.85
Rolling leukocytes (n/100 μm)				
Liver	4.80±0.90[a]	5.32±1.29	3.40±0.54[e]	2.33±0.75
Mesentery	7.68±3.18	10.34±8.94	10.82±7.29	6.88±1.94

[a]P<0.05 cirrhosis *vs* controls; [c]P<0.05 cirrhosis+IBD *vs* cirrhosis; [e]P<0.05 ATIII *vs* cirrhosis+IBD.

Table 4 Data of blood samples taken at the end of experiments (mean±SD)

	Cirrhosis	Cirrhosis and IBD	ATIII treatment group (cirrhosis and IBD)	Controls
ASOT (U/L)	63.67±10.42[a]	109.5±39.18[c]	29.17±7.62[e]	28.67±4.85
ALT (U/L)	31.83±4.84[a]	84.67±32.95[c]	21.17±13.61[e]	16.67±1.11
GGT (U/L)	5.33±1.49	14.83±3.48[c]	7.67±1.25[e]	3.17±0.69
AP (U/L)	130.5±35.75[a]	268.83±53.25[c]	69.83±12.21[e]	56.83±2.67
Prothrombin Time (%)	75.3±5.4[a]	58.7±4.3[c]	74.6±4.9[e]	102.4±2.7
ATIII (IU)	108.5±14.59	71.67±8.94[c]	138.50±2.29[e]	125.0±8.96

[a]P<0.05 cirrhosis *vs* controls; [c]P<0.05 cirrhosis+IBD *vs* cirrhosis; [e]P<0.05 ATIII *vs* cirrhosis+IBD.

Histology

At the time of videomicroscopy, all animals had evidence of ascites. Light microscopy examination showed that features of cirrhosis were evident in the liver of all rats. The overall impression of the liver was nodular with extensive deposits of fibrous tissue. Sections of ileum revealed macroscopic inflammation. Necrotic areas were not assessed in any group and network of lymphatic vessels was extended in cirrhotic animals and influenced markedly by ATIII treatment.

DISCUSSION

Portal hypertension caused by cirrhosis is characterized by multiple complications, including development of ascites, disturbance of the mucosal barrier, thrombotic, and intestinal derangements[16,17]. Microcirculation is of

heterogeneous nature in cirrhosis[18,19]. As portal hypertension develops, local production of vasodilators, mainly nitric oxide, increases, leading to splanchnic arterial vasodilatation[20]. Clinical observations revealed that there was a close relationship between circulatory dysfunction in portal hemodynamic and the impairment in hepatic function. The results of our study indicate that liver cirrhosis is associated with a significant increase of liver enzymes and hepatic vessel diameter, but has no influence on hepatic blood flow. There was a marked decrease in mesenteric blood flow and this effect was significantly reversed by bowel inflammation. This observation has led to the hypothesis that increased portal pressure may in part depend upon an increased splanchnic inflow and may be related to the overactivity of the endogenous vasoconstrictor systems[21]. Mucosal blood flow on the other hand, showed a significant decrease in animals with cirrhosis and bowel inflammation. Our data are consistent with the hypothesis that mucosal inflammation may induce shunting in the villus microcirculation, with a depression in capillary perfusion, though the levels of central arteriolar blood flow are normal[22]. However, the magnitude of mucosal hemodynamic alterations in rats treated with ATIII was not as pronounced as in cirrhotic rats with IBD without this treatment.

LEI and microvascular perfusion changes are known to play a crucial role in the development of organ dysfunction and failure. Leukocyte-endothelial cell adhesion is modulated by a variety of adhesion glycoproteins expressed on the surface of leukocytes and endothelial cells[23,24]. It has been shown that both leukocyte and endothelial cell adhesion molecules contribute to the granulocyte accumulation in a chronic model of intestinal inflammation[25]. As venous drainage of the intestine occurs via the portal vein in the liver, intestinal inflammation may lead to an upregulation of the liver LEI and contribute to liver injury. This phenomenon is also described for gut ischemia and reperfusion[26].

In addition, endothelium plays a key role in the pathogenesis of inflammation and coagulation disorders in infectious diseases[27]. Though the exact mechanism is not known, there is evidence that intervention in the coagulation pathway may have some beneficial influence on the course of infections[28]. It has been shown that small intestine injury and LEI are significantly reduced in endotoxemic rats after application of ATIII[29]. Our group has previously described the same effect in an animal model of hepatic ischemia-reperfusion injury[9], which is in agreement with the observation of the present study, indicating that application of ATIII reduces both intestinal and hepatic microcirculation failure in bowel inflammation with coexisting liver cirrhosis. The extent of leukocyte adherence to the endothelium and the course of liver enzymes can significantly improve.

In conclusion, though vessel morphology is changed during liver cirrhosis, systemic effects of bowel inflammation in cirrhosis can be attenuated by substitution of dropped ATIII levels subsequently to the onset of inflammation. Maintenance of gut perfusion seems to prevent enhancement of LEI and hepatic damage.

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Science Editor Wang XL and Guo SY **Language Editor** Elsevier HK