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Authors:
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Intestinal IgA positive lymphocytes in acute liver necrosis decrease due to lymphocyte homing disturbance and apoptosis

Jinlong Fu, Guodong Li, Jianliang Wu and Zhiyong Wang
Department of Gastroenterology. The Affiliated Hospital. Hangzhou Normal University School of Medicine. Hangzhou. Zhejiang, China

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Correspondence: Zhiyong Wang. Department of Gastroenterology. The Affiliated Hospital. Hangzhou Normal University School of Medicine. 310015 Hangzhou. Zhejiang, China. e-mail: zhiyongwanghznu@163.com

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ABSTRACT
Aim: the number of intestinal IgA+ lymphocytes are decreased in acute liver necrosis and the mechanism remains poorly understood. The purpose of this study was to observe the role of lymphocyte homing and apoptosis associated with decreased intestinal IgA positive lymphocytes in acute liver necrosis.

Methods: the acute liver necrosis mouse model and LTβR pre-treatment were used to assess intestinal mucosal addressin cell adhesion molecule-1 (MAdCAM - 1) expression, cell apoptosis, IgA+ cells and secretory immunoglobulin A (SIgA).

Results: MAdCAM – 1 mRNA and protein expression decreased significantly in the acute necrosis group; 0.57 ± 0.032 fold vs. baseline (p < 0.05) and 0.45 ± 0.072 fold vs. baseline (p < 0.05), respectively. LTβR pre-treatment could significantly improve the decline of MAdCAM – 1 mRNA and protein expression in the intestinal mucosa.
(1.83 ± 0.064 fold vs. baseline, p < 0.05 and 1.75 ± 0.046 fold vs. baseline, p < 0.05, respectively) and partially restore the decline in IgA+ lymphocytes and SIgA levels. There were increased rates of enterocyte apoptosis in both the acute liver necrosis and LTβR pre-treatment group; 0.79% vs. control (p < 0.05) and 0.77% vs. control (p < 0.05), respectively).

**Conclusion:** our results suggest that the dysfunction of lymphocyte homing and apoptosis are both involved with decreased intestinal IgA+ lymphocytes in acute liver necrosis. LTβR pre-treatment can partially restore IgA+ cells and SIgA by increasing MAdCAM – 1 expression, rather than inhibiting lymphocyte apoptosis.

**Key words:** Lymphocyte homing. Liver failure. Mucosal-permeability/secretion. Immunology.

**INTRODUCTION**

The gut is an important place for digestion and absorption and also the largest immune organ in humans. Due to the wide intestinal mucosal area, its structure and function constitute a strong mucosal immune system. Moreover, the high quantity of intestinal microbial flora makes it difficult for foreign bacteria and viruses to enter this powerful line of defense, avoiding eventual damage to the organism (1). The intestinal mucosal immune system includes intestinal epithelial cells (IEC), intestinal intraepithelial lymphocytes (iIEL), lamina propria lymphocytes (LPL), microfold cells (M cells), Peyer's patch and secretory immunoglobulin A (SIgA) among others (2,3). Secretory immunoglobulin A (SIgA) that is synthesized by plasma cells in the lamina propria is considered as the first line of defense in the intestinal mucosal immune system (4). It consists of the secretory component (SC) that links two IgA monomer molecules by the non-covalent bond as well as the J chain (5). In a previous study, we found that intestinal SIgA and IgA positive lymphocytes decreased significantly in an acute liver necrosis model (6). Although, the mechanism of the reduction of IgA positive lymphocytes and SIgA remains poorly understood. There is a growing body of evidence in recent years showing that intestinal lymphocyte homing is closely associated with the incidence and progression of some
intestinal inflammatory diseases (7,8). There are no studies to date that report whether the dysfunction of intestinal lymphocyte homing is involved in the decrease in the number of intestinal IgA-positive lymphocytes in liver necrosis. This study focuses on the evaluation of the role of lymphocyte homing and apoptosis in the decrease of intestinal IgA+ lymphocytes in acute liver necrosis.

MATERIAL AND METHODS

Animals
Male, six to eight-week-old BALB/c mice (Experimental Animal Research Center in Beijing, China) were housed and cared for at a constant room temperature and humidity with free access to food and water. The animals were subjected to a 12-h light/dark cycle. Food was withdrawn overnight before the experiments. All animal experimental procedures were approved by the Ethics Committee of the Hangzhou Normal University before starting the study. All mice were divided randomly into five groups (n = 15 per group) as follows: normal control, acute liver necrosis, lipopolysaccharide (LPS), galactosamine (GalN) or LTβR pre-treatment group. Intraperitoneal injections of GalN (800 mg/kg body weight, Sigma, USA) and LPS (10 μg/kg body weight, Sigma, USA) were used to induce acute liver necrosis, as previously described (6). The LTβR group were treated with LTβR at 10 μg per mouse (R&D, USA) before the induction of acute liver necrosis. LTβR was injected via the vena caudalis 30 min before GalN/LPS administration. Mice were euthanized 9h after the administration of GalN/LPS and 18 cm long strip of intestinal tissues near the ileocecal were subsequently extracted.

Blood biochemistry assay
Serum levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) were determined using an automatic analyzer (Cobas C501; Roche, USA).

Western blotting analysis of MAdCAM - 1
Intestinal tissue samples were homogenized in lysis buffer (20 mmol Tris-HCl [pH 7.5], 1% Triton X 100, 0.2 mol NaCl, 2 mmol EDTA, 2 mmol EGTA, 1 M DTT and 2 mol
aprotinin) for the detection of the MAdCAM - 1 protein. After 20 min, the tissues were centrifuged and the supernatants were collected for protein content quantification using an ultraviolet spectrophotometer DU800 (Beckman). Proteins (50 μg) were analyzed via electrophoresis using SDS-PAGE (12%) and subsequently transferred to a nitrocellulose membrane. The membranes were blocked with non-fat dried milk in TBS containing 0.05% Tween-20 (TTBS) for one hour at room temperature and incubated with a rabbit anti-mouse polyclonal MAdCAM - 1 antibody (diluted 1:400; Invitrogen, USA) at 4 °C overnight. After three washes in TTBS, the membranes were treated with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology) for two hours at room temperature. The immunoreaction was detected using the ECL Plus chemiluminescence detection system (Pierce, Rockford, USA), and quantified by densitometry.

RNA isolation and quantitative real-time PCR analysis of MAdCAM - 1 mRNA
Total RNA was extracted from intestinal tissue using the RNase Mini Kit (Takara Biotechnology Corp, Japan). The quality of extracted RNA was determined by agarose gel electrophoresis and cDNA was synthesized using 100 ng of RNA. The concentrations of individual RNA transcripts were quantified by real-time polymerase chain reaction (PCR). Oligonucleotide primers for the genes MAdCAM - 1 (forward 5’-CCCATGGCCACAGCTACCTCA-3’ and reverse 5’-CCCTGGCCCTAGTACCCTAC-3’) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: forward 5’-TGTGTCCGTCGTGGATCTGA-3’ and reverse 5’-TTGCTGTGAAGTCGCCAGGAG-3’) in mice were used. PCR was performed at 5°C for 30 s, followed by 45 cycles of 95 °C for 5 s, 57 °C for 20 s and 72 °C 30 s. The extension was performed at 72°C for 2 min. The concentration of mRNA was calculated according to the standard curve and then normalized to the levels of GAPDH. All experiments were performed in triplicates.

TUNEL analysis of cell apoptosis
Apoptosis in the intestinal samples was detected and quantified using the situ
terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method with a commercial a kit according to the manufacturers´ instructions. Three fields of vision under microscopy were chosen at random to analyze the positive rate of apoptotic cells using the ‘multi-system color/RGB monitor’ computer image processing system. The counting formula used was stained cells (rate) = the area of brown cells/total area×100%.

**Immunohistochemistry analysis of IgA**

Frozen sections of proximal small intestine (5 μm thick) were fixed on glass slides by incubation with acetone for 10 min at 4°C and antigen retrieval was performed via pressure cooker boiling for 2 min in 10 mmol/L citrate buffer (pH 6.0). Slides were then blocked in skimmed milk for 30 min at 37 °C and incubated with goat anti-mouse IgA (Zymed, USA, diluted 1:50) for 12 h at 4 °C. The secondary antibody (rabbit anti-goat IgG) was diluted 1:200 and was applied for two hours at 37°. Sections were rinsed in PBS and distilled water. The slides were stained with 3, 3'-diaminobenzidine and counterstained with hematoxylin.

**Double-antibody sandwich immunoradiometric assay analysis of intestinal SIgA content**

Intestinal SIgA was measured as previously described (9,10). A tissue sample of 10 cm in length was obtained from the small intestine and carefully dissected and washed with normal saline. Intestine mucus was collected and centrifuged at 3000 r/min for 10 min (environmental temperature: 0 °C) and 1 mL 0.01 mol/L PBS was added. The supernatant was harvested and SIgA levels were measured using the double-antibody sandwich immunoradiometric assay (Rapidbio Lab, Calabasas, USA). The total protein in intestine mucus was assayed simultaneously using the Bradford brilliant blue method. The SIgA content of the total protein in one milligram small intestine mucus was determined.

**Statistical analysis**

The SPSS version 11.5 Software was used for statistical analysis. Each parameter was
expressed as the mean ± SE and compared using a one-way ANOVA test, followed by the Tukey test. A p-value < 0.05 was set as statistical significance.

RESULTS
Serum ALT, AST
ALT and AST serum levels were only slightly elevated in the LPS control (65 ± 19U/L) (58 ± 12U/L) and GalN control (62 ± 15U/L) (47 ± 18U/L) compared to the saline control (23 ± 11U/L) (20 ± 9U/L) (p > 0.05). However, the levels increased significantly in acute necrosis models (7563 ± 790 U/L) (6982 ± 455 U/L) and the LTβR pre-treatment group (6185 ± 528 U/L) (5627 ± 783 U/L) (p < 0.05).

Semi-quantitative analysis of MAdCAM - 1 protein expression by western blot
The ratio of the absorbance of the β-actin protein band compared to the MAdCAM-1 protein was used to calculate the relative expression. The protein level of the saline group was set to 1 as a baseline level (11). MAdCAM - 1 expression was strongly decreased in animal models with acute liver necrosis; 0.45 ± 0.072 fold vs. baseline (p < 0.05). This value was significantly lower than that of MAdCAM - 1 in the LTβR pre-treatment group (1.75 ± 0.046 fold vs. baseline, p < 0.05) and was statistically significant (p < 0.05). The protein expression in the LPS-treated group and GalN-treated group was close to the normal range; 0.94 ± 0.055 fold vs. baseline (p > 0.05) and 0.95 ± 0.091 fold vs. baseline (p > 0.05) (Figure 1, n = 3).

Expression of MAdCAM - 1 mRNA
The mRNA level in the saline group was set to 1 as the baseline level. A reasonable amplification curve and standard curve for MAdCAM - 1 and GAPDH RNAs were obtained. The correlation coefficients of all standard curves were 0.9997. Real-time PCR quantitative analyses showed that there were marked decreases in MAdCAM - 1 mRNA expression in the acute necrosis group; 0.57 fold ± 0.032 vs. baseline (p < 0.05). This value was significantly lower than that of MAdCAM – 1 mRNA in the LTβR pre-treatment group (1.83 ± 0.064 fold vs. baseline, p < 0.05) and was statistically significant (p < 0.05). The mRNA expression in the LPS-treated group and GalN-
treated group was close to the normal range; 0.97 ± 0.025 fold vs. baseline (p > 0.05) and 0.95 ± 0.093 fold vs. baseline (p > 0.05) (n = 3) (Fig. 1).

**Apoptosis of enterocytes**

TUNEL positive enterocytes cells were significantly higher in the acute liver necrosis group compared to the control (p < 0.05). Moreover, the rate of TUNEL positive cells in the LTβR pre-treatment group was 4-fold higher than the control. Interestingly, there was no significant difference between the LTβR pre-treatment group and the acute liver necrosis group (Fig. 2).

**Immunohistochemical staining for IgA positive plasma cells**

Brown particles were observed in the IgA positive cells, which dispersed into the lamina propria. Immunohistochemical analysis revealed a high density of IgA expression in the saline control group. IgA expression was weaker in the intestinal tissue of the LTβR pre-treatment, LPS and GalN groups. The lowest expression was observed in the acute necrosis group (Fig. 3).

**Intestinal SIgA Levels**

Small intestine sections were homogenized and SIgA was measured by a double-antibody sandwich immunoradiometric assay. A remarkable decrease of SIgA expression was observed in the acute necrosis group vs. the saline group (p <0.05). The content of intestinal SIgA in the LPS, GalN and LTβR pre-treatment groups was only slightly reduced compared to the saline group (p > 0.05). When compared with acute necrosis group, SIgA expression was significantly higher in the LTβR pre-treatment group (*p < 0.05) (Fig. 3).

**DISCUSSION**

The mechanism of the liver necrosis is complex and includes some influential factors, thus a multi-aspect intervention is required. Intestinal mucosal immune barrier injury is crucial for intestinal endotoxemia, which is considered to be a “second hit” to aggravate the liver injury. Recent evidence suggests that mucosal immune barrier
injury, especially the number of IgA positive lymphocyte and SlgA expression, are reduced during acute liver necrosis, severe acute pancreatitis and severe burns (6,12-14). The specific damage mechanism of the mucosal immune barrier is not clear. Whether it can improve liver injury by protecting against intestinal mucosal immune damage is a subject worth exploring.

MAdCAM-1 is an immunoglobulin superfamily adhesion molecule for lymphocytes, which is expressed by mucosal venules and helps direct lymphocyte traffic into Peyer's patches (PP) and the intestinal lamina propria (15,16). Leukocyte trafficking to the small and large intestine is tightly controlled in order to maintain intestinal immune homeostasis, mediate immune responses and regulate inflammation (17).

An increased expression of MAdCAM-1 in inflammatory sites of various disease has also been previously reported (18,19). The present study demonstrates that the expression of the MAdCAM-1 protein is decreased in acute liver necrosis, which was positively related with a decrease in IgA+ lymphocytes and SlgA expression levels in acute liver necrosis models. All these findings suggest that the dramatic decline of MAdCAM-1 is involved in the intestinal mucosal immune injury in acute liver necrosis. However, an accurate mechanism of MAdCAM-1 reduction in acute liver necrosis remains to be further defined.

There is an important triggering factor, the lymphocyte toxin receptor (LTβR), upstream of the signaling pathway that mediates the expression of MAdCAM – 1 that mainly exists in the PP stroma and the intestinal lamina propria mucous membrane. An important study found that the signaling pathway of LTβR is crucial for the regulation of intestinal epithelial cell homeostasis in order to limit mucosal damage (20,21). After LTβR is combined with ligand LTβ1β2 (LT), the expression of MAdCAM - 1 and IL-4 is stimulated via the activation of NF-κB (22). This is important for the formation of lymph nodes and lymphoid tissues, differentiation of lymphocytes and the generation of IgA (23). Therefore, we used LTβR in this study to stimulate the production of MAdCAM - 1 and to evaluate its effect on mucosal immunity in liver necrosis. There was a significant increase in the intestinal lymphocyte homing factor MAdCAM - 1 expression (Fig. 1), IgA+ cells and SlgA level (Fig. 3) within the lymphoid structures in the intestinal mucosa of mice with acute
liver necrosis after LTβR treatment. Moreover, we also analyzed apoptosis of enterocytes in acute liver necrosis. There was an increased number of TUNEL positive enterocyte cells in the acute liver necrosis model and there was no obvious difference between the number of TUNEL enterocyte positive cells in the LTβR pre-treatment group compared to the acute liver necrosis group (Figure 2). These findings indicate that both the dysfunction of lymphocyte homing and apoptosis may be involved in the decrease of intestinal IgA positive lymphocytes in acute liver necrosis. The present study demonstrates that LTβR pre-treatment triggered a 1.5-2 fold upregulation of MAdCAM-1 that only partially restored IgA+ cells and SIgA. Our data suggest that LTβR pretreatment may have a protective effect against intestinal mucosal immunity injury. This effect may occur via the up-regulation of MAdCAM – 1 expression, rather than inhibiting lymphocyte apoptosis. In the current study, we also observed that after injection of LPS/GalN, serum levels of ALT and AST increased remarkably. Furthermore, the degree of elevation of ALT and AST in the LTβR pre-treatment group was slightly increased. These findings indicate that liver injury can be improved by protecting against intestinal mucosa immune damage. In summary, the dysfunction of lymphocyte homing and apoptosis are both involved in a decrease in the levels of intestinal IgA+ lymphocytes in acute liver necrosis. This in turn may lead to the “second hit” to aggravate the liver injury. Moreover, LTβR pretreatment alleviates intestinal IgA+ lymphocyte and SIgA decreases in acute liver necrosis by increasing MAdCAM – 1 expression, rather than inhibiting lymphocyte apoptosis.

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Fig. 1. The expression of the MAdCAM-1 protein and MAdCAM-1 mRNA. MAdCAM-1 protein and mRNA expression decreased significantly after GaIN/LPS injection and was significantly higher in the LTβR pre-treatment group (*p < 0.05, vs. baseline, #p < 0.05, vs. the acute necrosis group).
**Fig. 2.** TUNEL Apoptosis Detection Analysis. The TUNEL assay shows a positive enterocyte apoptosis rate in mice (*p < 0.05, vs. saline control).

**Fig. 3.** Intestinal IgA positive cells and SIgA levels. Saline control (A), liver acute necrosis (B), LTβR pre-treatment (C), LPS (D) and GalN groups (E). Intestinal SIgA in the acute necrosis group was significantly lower, whereas the content of intestinal SIgA in LTβR pre-treatment, LPS and GalN groups was only slightly reduced (*p < 0.05, vs. saline control; #p < 0.05, vs. acute necrosis group).