Alcohol 48 (2014) 787–793

Contents lists available at ScienceDirect

Alcohol

journal homepage: http://www.alcoholjournal.org/

Abnormal ultrastructure of intestinal epithelial barrier in mice with alcoholic steatohepatitis

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ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form
8 August 2014
Accepted 26 August 2014

Keywords:
Alcoholic steatohepatitis
Intestinal epithelial barrier dysfunction
Tight junction
Adherens junction
Transmission electron microscope

ABSTRACT

Intestinal barrier dysfunction caused by chronic alcohol consumption is closely associated with disruption of the intestinal epithelial apical junction complex. The present study was undertaken to directly display by transmission electron microscopy the abnormal ultrastructure of the intestinal epithelial barrier in mice with alcoholic steatohepatitis. The results showed that chronic alcohol consumption could induce obvious liver injury, with diffuse lipid accumulation and focal inflammatory cell infiltration in the liver, assessed by hematoxylin and eosin staining. The indicators of intestinal barrier dysfunction, D-lactic acid and lipopolysaccharide, were significantly higher in alcohol-fed mice than in control mice. Alcohol exposure obviously caused high permeability in the ileum to fluorescein isothiocyanate-dextran (FD-4; molecular weight 4000). Transmission electron microscopy demonstrated that tight junctions and adherens junctions expanded noticeably in alcohol-fed mice. Although the tight junction (TJ) length of alcohol-fed mice had no significant difference compared with control mice, the adherens junction (AJ) length of alcohol-fed mice significantly decreased compared with control mice. Additionally, the ratios of both TJ_{max}/TJ_{min} and AJ_{max}/AJ_{min} were significantly larger in alcohol-fed mice than in control liquid-fed mice. In conclusion, high intestinal permeability caused by alcohol attributes to the irregular ultrastructure of the intestinal epithelial barrier.

Introduction

Chronic alcohol consumption is a major cause of chronic liver disease around the world; this chronic liver disease can eventually progress to fibrosis and cirrhosis. Among many factors that contribute to the pathogenesis of alcoholic liver disease (ALD), gut-derived endotoxemia plays a critical role in the development and progression of ALD (Keshavarzian et al., 2009; Purohit et al., 2008; Rao, Seth, & Sheth, 2004). Accumulated endotoxin/lipopolysaccharide (LPS) in the systemic circulation can activate parenchymal cells and nonparenchymal cells (e.g., Kupffer cells, hepatic stellate cells) by combining with Toll-like receptor 4 (TLR4), cause the release of various pro-inflammatory cytokines and chemokines, and cause "two-hit" to liver (Szabo & Bala, 2010).

Chronic alcohol consumption could lead to gut-derived endotoxemia by reducing the ability of Kupffer cells to detoxify LPS, inducing bacterial overgrowth to produce excessive LPS (Mutlu et al., 2009), and disturbing the intestinal mucosal barrier and increasing permeability to LPS (Elamin et al., 2012; Elamin, Masclee, Dekker, & Jonkers, 2014; Rao, 2008). The gut is a habitat for billions of microorganisms, so it is the biggest and most important source of LPS derived from gram-negative bacteria. Disruption of the intestinal epithelial barrier (IEB) function and diffusion of luminal LPS into the systemic circulation are central to gut-derived endotoxemia (Rao, 2009). The IEB function is regulated by the apical intercellular junctional complex referred to as the apical junctional complex (AJC). The major constituents of this complex are tight junctions (TJs) and adherens junctions (AJs). Both TJs and AJs incorporate several transmembrane and cytosolic proteins, such as occludin, claudin, junctional adhesion molecule-A (JAM-A), zonula occludens (ZO), and E-cadherin (Blaschuk & Rowlands, 2002; González-Mariscal, Betanzos, Nava, & Jaramillo, 2003). Transmembrane proteins interact with cytosolic plaque and cytoskeletal proteins, which are responsible for assembling and anchoring of the AJC and establishing an effective paracellular barrier (Anderson & Van Itallie, 1995; Turner, 2006). Thus, abnormal structure and function of intestinal AJC will lead to IEB dysfunction and subsequent gut-derived endotoxemia.

Several recent experimental studies have demonstrated that alcohol and its metabolic product of acetaldehyde can disturb intestinal AJC and increase intestinal permeability by different mechanisms (Chang, Sang, Wang, Tong, & Wang, 2013; Elamin, Masclee, Dekker, et al. 2014; Elamin, Masclee, Troost, Dekker, &

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http://dx.doi.org/10.1016/j.alcohol.2014.08.012
0741-8329/

Jonkers, 2014; Rao et al., 2004; Tong, Wang, Chang, Zhang, Liu, et al., 2013; Tong, Wang, Chang, Zhang, & Wang, 2013). Most of the research has focused mainly on the mechanisms of IEB dysfunction; research focusing on IEB ultrastructure is rare. The present study was undertaken to directly display the detailed abnormal ultrastructure of IEB in mice with alcoholic steatohepatitis by transmission electron microscopy.

Materials and methods

Alcohol feeding

Wild-type C57BL/6 mice were purchased from Vital River Laboratory (Beijing, China). The animal experiments were approved by the Animal Use and Care Committee of Capital Medical University and were conducted according to the regulations set by Institutional Animal Care and Use Committees. According to the classical method of the Lieber-DeCarli model with alcoholic liver disease, 8-week-old mice were pair-fed with Lieber-DeCarli alcohol or isocaloric maltose-dextrin control liquid diet for 8 weeks with a stepwise feeding procedure. The percentage of alcohol in the diet (%, wt/vol) was 4.8% (34% of total calories) at the beginning of the study and was gradually increased to 5.4% (38% of total calories). At the end of the 8th week of alcohol treatment, mice were fasted for 8 h, and then anesthetized with pentobarbital (50 mg/kg), which was immediately followed by carotid artery blood collection and laparotomy for the collection of intestinal and liver tissues.

Assessment of liver injury

Isolated liver tissue was fixed in 10% formalin and stained with hematoxylin and eosin (H&E). Histopathological changes in the liver were examined by light microscopy. Serum alanine aminotransferase (ALT) activity was detected by an Automatic Biochemical Analyzer (Hitachi-7180).

Intestinal mucosal permeability measurement

For *ex vivo* detection of intestinal permeability, the procedure was modified and carried out according to the method described in a previous study (Zhong, McClain, Cave, Kang, & Zhou, 2010). Briefly, the ilea were freshly isolated and placed in modified Krebs-Henseleit bicarbonate buffer containing 8.4 mM HEPES, 119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose (KHBB, pH 7.4). Intestinal contents were rinsed thoroughly using a gavage needle to avoid mucosal injury. The distal ileum was first ligated with suture line, and 100 μL FITC-dextran (FD-4, molecular weight 4000, 40 mg/mL) was injected into the lumen by a gavage needle to prevent mucosal injury. Then the other end of the ileum segment was ligated with suture line to form a 4-cm gut sac. Next, the gut sac was washed repeatedly in the KHBB buffer to avoid a nonspecific effect produced by FD-4 left outside the gut sac. In the last step, the gut sac was placed into 1 mL of KHBB and incubated at 37 °C for 60 min. The FD-4 that diffused from the lumen into the incubation buffer was measured spectrofluorometrically with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

ELISA assessment

Blood samples harvested from the carotid artery were allowed to naturally coagulate in a room temperature state, centrifuged at 1500 rpm for 10 min, and the supernatant serum was collected and stored at −80 °C until analysis. Endotoxin/LPS and D-lactic acid (D-LA) were measured with a mouse endotoxin kit and a D-LA ELISA kit (Cusabio, Wuhan, China), respectively. The procedure was executed according to the kit instructions.

Ultrastructure assessment of intestinal injury

Isolated intestinal tissues were respectively fixed in 10% formalin or 2.5% glutaraldehyde. The specimens fixed in 10% formalin were stained with hematoxylin and eosin (H&E). The specimens fixed in 2.5% glutaraldehyde were rinsed in 0.1 mol/L phosphate buffer. After hydration by graded ethanol, specimens were infiltrated and embedded with epoxy resin. Ultra-thin slices were cut with a diamond knife and then examined with a transmission electron microscope (Hitachi-7650). For morphological assessment of the intestinal epithelial barrier, 10 tight junctions or adherens junctions of intestinal epithelial cells were examined in each sample as described in a previous method (Hara, Ishida, Cangara, & Hirata, 2009). The lengths of TJ and AJ were measured. We also measured maximal and minimal widths of TJ and AJ, which are respectively referred to as TJ_{max}, TJ_{min}, AJ_{max}, or AJ_{min}. Quantification of the ratios of TJ_{max}/TJ_{min} and AJ_{max}/AJ_{min} served as statistical reference points for contrast analysis.

Statistical analysis

All data were expressed as mean ± standard error. Data between groups were analyzed by *t* tests. $p < 0.05$ was considered statistically significant.

Results

Alcohol-induced liver injury

At the end of the 8th week of pair-feeding with Lieber-DeCarli alcohol or control liquid diet, the control mice were energetic, flexible, and active with normal appetite, tidy fur, and had experienced gradual weight gain. In contrast, alcohol-fed mice were slow, sluggish, and easily irritable with untidy and unpolished fur, and had experienced slow weight gain. The body weight of alcohol-fed mice (22.8 ± 0.25 g) at the end of the 8th week was significantly lower than that of control mice (27.5 ± 1.16 g). The liver/body weight ratio of alcohol-fed mice (0.052 ± 0.001) was significantly higher than that of control mice (0.036 ± 0.002) (Fig. 1A). The serum ALT activities were significantly elevated in alcohol-fed mice (Fig. 1B). H&E staining demonstrated diffuse lipid accumulation and focal inflammatory cell infiltration in the liver tissue of alcohol-fed mice (Fig. 1C), but Masson staining did not show any abnormal fibrosis in the liver tissue (Fig. 1D).

Alcohol-induced disruption of intestinal barrier function

The levels of serum D-LA and LPS are the indicators of gut leakiness. ELISA showed that serum levels of both D-LA and LPS in the alcohol-fed mice were significantly higher than those of control mice (Fig. 2A). The effect of alcohol on the intestinal barrier function was determined by *ex vivo* measurement of ileum permeability to FD-4 (Fig. 2B). As shown in Fig.2C, alcohol exposure caused a significant increase in permeability of ileum to FD-4.

Alcohol-induced abnormal ultrastructure of intestinal epithelial barrier

As mentioned above, alcohol could induce intestinal barrier dysfunction. However, light microscopy of intestinal tissue with H&E staining did not reveal remarkable pathological differences between alcohol-fed mice and control mice (Fig. 3A). Further,