ELSEVIER

Alcohol 43 (2009) 163–172

ALCOHOL

Lactobacillus GG treatment ameliorates alcohol-induced intestinal oxidative stress, gut leakiness, and liver injury in a rat model of alcoholic steatohepatitis

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Received 12 June 2008; received in revised form 31 December 2008; accepted 31 December 2008

Abstract

Because only 30% of alcoholics develop alcoholic liver disease (ALD), a factor other than heavy alcohol consumption must be involved in the development of alcohol-induced liver injury. Animal and human studies suggest that bacterial products, such as endotoxins, are the second key co-factors, and oxidant-mediated gut leakiness is one of the sources of endotoxemia. Probiotics have been used to prevent and treat diseases associated with gut-derived bacterial products and disorders associated with gut leakiness. Indeed, ''probiotic'' *Lactobacillus rhamnosus* has been successfully used to treat alcohol-induced liver injury in rats. However, the mechanism of action involved in the potential beneficial effects of *L. rhamnosus* in alcohol liver injury is not known. We hypothesized that probiotics could preserve normal barrier function in an animal model of ALD by preventing alcohol-induced oxidative stress and thus prevent the development of hyperpermeability and subsequent alcoholic steatohepatitis (ASH). Male Sprague–Dawley rats were gavaged with alcohol twice daily (8 gm/kg) for 10 weeks. In addition, alcoholic rats were also treated with once daily gavage of either 2.5×10^7 live *L. rhamnosus* Gorbach–Goldin (LGG) or vehicle (V). Intestinal permeability (baseline and at 10 weeks) was determined using a sugar bolus and GC analysis of urinary sugars. Intestinal and liver tissues were analyzed for markers of oxidative stress and inflammation. In addition, livers were assessed histologically for severity of ASH and total fat (steatosis). Alcohol + LGG (ALC + LGG)–fed rats had significantly ($P \leq .05$) less severe ASH than ALC + V–fed rats. *L. rhamnosus* Gorbach–Goldin also reduced alcohol-induced gut leakiness and significantly blunted alcohol-induced oxidative stress and inflammation in both intestine and the liver. *L. rhamnosus* Gorbach–Goldin probiotic gavage significantly ameliorated ASH in rats. This improvement was associated with reduced markers of intestinal and liver oxidative stress and inflammation and preserved gut barrier function. Our study provides a scientific rationale to test probiotics for treatment and/or prevention of alcoholic liver disease in man.

Keywords: Alcohol; Alcoholic liver disease; *Lactobacillus* GG; Oxidative stress; Intestinal permeability

Introduction

Alcoholic liver disease (ALD) remains as an important cause of morbidity and mortality with more than 75,000 deaths annually worldwide and the incidence increasing in the last decade (Gramenzi et al., 2006; Tsukamoto, 2007). However, only about 30% of heavy drinkers develop alcoholic steatohepatitis (ASH) and clinically significant ALD (Gramenzi et al., 2006). Thus, heavy alcohol drinking alone does not lead to the development of ASH and other factor(s) are involved. Several animal and human studies have suggested that the gut-derived bacterial product endotoxin is the contributing co-factor. For example, ALD patients and animal models of chronic alcoholism have abnormally high plasma endotoxin levels (Bode et al., 1987; Fukui et al., 1991; Lumsden et al., 1988; Thurman, 1998; Tilg and Diehl, 2000).

The cause of alcohol-induced endotoxemia is not well established, but several studies suggest that alcohol–induced increased intestinal permeability to gut-derived endotoxin is the possible mechanism (Fukui et al., 1991; Keshavarzian et al., 1999; Parlesak et al., 2000; Rao et al., 2004; Tilg and Diehl, 2000; Worthington et al., 1978). If endotoxemia and intestinal hyperpermeability are the key factors in the development of ASH, then intervention aiming at ameliorating endotoxemia and gut

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0741-8329/09/$ – see front matter
doi: 10.1016/j.alcohol.2008.12.009

leakiness should prevent ASH. Studies in animal models of ALD have revealed that treatment with antibiotics to sterilize the gut and thus eliminate this source of endotoxin can prevent alcohol-induced liver injury (Adachi et al., 1995). Moreover, *Lactobacillus rhamnosus* Gorbach–Goldin (LGG) supplementation significantly decreased severity of liver injury in a rat model of ASH (Nanji et al., 1994). We also showed that oats supplementation prevented both increased intestinal permeability and steatohepatitis in chronically alcohol-fed rats (Keshavarzian et al., 2001). These studies strongly support the notion that gut bacteria play a key role in the pathogenesis of alcohol-induced liver injury and that gut leakiness may be one mechanism that allows proinflammatory bacterial products to reach the liver and initiate the proinflammatory cascade for causing ASH.

One mechanism whereby alcohol induces intestinal permeability in vivo may be oxidative stress. Antioxidants have been shown to normalize intestinal permeability in alcoholic patients (Varella Morandi Junqueira-Franco et al., 2006). We have also shown that alcohol increases intestinal epithelial cell permeability in vitro through an oxidant stress mediated mechanism (Banan et al., 1998, 1999). In our earlier studies, we demonstrated that alcohol stimulates activation of the key transcription factor NF-κB which in turn stimulates upregulation of inducible nitric oxide synthase (iNOS) (Banan et al., 2000, 2007). Our data support iNOS production of reactive nitrogen species (e.g., peroxynitrite) as the principal source of ethanol-induced oxidative stress. The resulting oxidative stress causes increased carbonylation and nitrotyrosination of cellular proteins, including the actin and microtubule cytoskeletons, which when disrupted results in loss of tight junction integrity and increased paracellular permeability (Farhadi et al., 2006). This model has recently been supported independently by studies examining alcohol-induced epithelial permeability in the lung (Brown et al., 2004; Polikandriotis et al., 2007). Thus, we propose that oxidative stress is the key element driving alcohol-induced intestinal hyperpermeability and that antioxidants may be effective therapeutic agents to prevent ASH.

Probiotics are microorganisms that when taken by the host have beneficial effects on the host beyond their simple nutritive value (Ewaschuk and Dieleman, 2006). They can change the gut microbiota profile and thereby change the gut lumen favoring an anti-inflammatory milieu, resulting in decreased production of proinflammatory bacterial products and also improved barrier integrity. One widely studied probiotic bacterium is LGG (Di Caro et al., 2005; Mattar et al., 2001; Nanji et al., 1994; Zhang et al., 2006). Probiotics, including LGG, have been shown to have several beneficial effects on intestinal function, including stimulating intestinal development and mucosal immunity, ameliorating diarrhea, prolonging remission in ulcerative colitis and pouchitis, and maintaining and improving intestinal barrier function (Bruzzese et al., 2004; Ewaschuk and Dieleman, 2006; O'Hara and Shanahan, 2006; Resta-Lenert and Barrett, 2003; Sartor, 2004; Versalovic, 2007). *L. rhamnosus* GG has also been shown to reduce intestinal oxidative stress (Tao et al., 2006).

To determine whether we can exploit these favorable characteristics of LGG to prevent alcohol-induced leaky gut and liver injury, we studied the effects of daily LGG on intestinal permeability, intestinal and liver oxidative stress, and severity of steatohepatitis in our rat model of alcohol-induced leaky gut and steatohepatitis. We hypothesized that LGG would reduce alcohol-induced oxidative stress and preserve barrier function and thus prevent liver disease in alcohol-fed rats. Using our model of chronic alcohol gavage, we show in this study that treatment with LGG significantly reduces markers of intestinal oxidative stress, normalizes barrier function, and ameliorates ASH.

Materials and methods

Culture of L. rhamnosus GG

L. rhamnosus GG (ATCC 531030) were cultured in *Lactobacillus* De Man, Rogosa, and Sharpe broth (MRS broth; Difco, BD, Sparks, MD) at 37°C in accordance with ATCC guidelines. Bacteria were harvested from MRS broth by centrifugation and CFU counted by dilution and streaking on MRS agar plates (Difco) at 37°C overnight. LGG were then centrifuged and resuspended at a dilution of 2.5×10^7/mL in PBS and 1 mL gavage was used for once-a-day daily treatment.

Animal subjects

Male Sprague–Dawley rats (Zivic-Miller Laboratories, Zelienople, PA; $n = 17$; 250–300 g, initial body weight) were acclimatized for 6–7 days, at 22 ± 1°C with a 12:12-h dark:light cycle. During acclimatization, rats were given water and standard laboratory food (rat chow) ad libitum. During the experiment, alcohol or dextrose were administered intragastrically by gavage twice daily (6 h between doses). A 12-gauge gavage needle was used (Popper & Sons, New Hyde Park, NY). Alcohol-fed rats received alcohol gavage (~2–3 mL) twice daily starting with an initial dose of 2 g/kg/day. This dose was progressively increased during weeks 1 and 2 to a maintenance dose of 8 g/kg/day (solutions maximally contained 50–60% alcohol with each gavage containing 4 g/kg that is 1/2 the daily dose) that was continued for 8 more weeks. Control rats received an isocaloric amount of dextrose, also by gavage. Rats also received intragastric feedings of a slurry of either powdered rat chow (vehicle [V]) or LGG (ATCC, #53103; $2.5 \times 10^{7\ \text{live}}$/once daily). All rats also had regular rat chow available (ad libitum) throughout the 10-week period. Rats were weighed daily. Four treatment groups were studied: (1) ALC + V alone ($n = 11$); (2) ALC + LGG ($n = 9$); (3) dextrose control (CON; $n = 5$); and (4) dextrose + LGG (CON + LGG; $n = 3$). Gut permeability was measured (as described in the