The impact of probiotic *Lactobacillus casei* and *Lactobacillus paracasei* on zonula occludens 1 production in enterocytes of gnotobiotic mice

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Abstract

Intestinal permeability plays a role in pathogenesis of several disorders, in which probiotics are successfully used. The regulation of intestinal barrier integrity is dependent on zonula occludens (ZO) proteins which link cytoskeleton actin filaments and intercellular tight junctions of epithelial cells. The aim of the study was to evaluate the influence of the probiotic mixture of *Lactobacillus casei* LOCK 0900, *Lactobacillus casei* LOCK 0908 and *Lactobacillus paracasei* LOCK 0919 on expression of ZO-1 protein in ileum of gnotobiotic mice. ZO-1 was visualized by the immunohistochemical technique and quantitatively analyzed by morphometric Cell P program in germ-free (GF) mice associated with the mixture of probiotics, GF mice and mice reared in conventional conditions (CONV). The largest amounts of ZO-1 were found in epithelial cells of CONV mice (mean 41.58%), and the quantity of this protein was significantly higher in comparison with GF mice (mean 13.54%). Association of GF animals with *Lactobacillus* strains induced significantly higher synthesis of ZO-1 in intestinal epithelium in comparison with GF mice (27.4% versus 13.54%). We conclude that probiotic *Lactobacillus casei* and *Lactobacillus paracasei* strains, which were shown to induce clinical improvement in children with cow’s milk protein allergy could exhibit beneficial effects by modulation of the intestinal barrier function.

Key words: intestinal barrier, ZO-1, *Lactobacillus casei*, *Lactobacillus paracasei*, probiotics, allergy

Introduction

The intestinal barrier is formed by the epithelial cells and the junctional complex, consisting of tight junctions (TJ), adherents junctions and desmosomes. In the maintenance of barrier integrity TJ complexes play a decisive role [12]. TJ are intricate macromolecular protein structures located at the most apical regions of the junctional complex sealing the spaces between the epithelial cells. The maintenance of TJ competence prevents movements of potentially harmful environmental factors such as bacteria, viruses, toxins, food allergens and macromolecules across the intestinal barrier [16]. The appropriate regulation of intestinal TJ by modulators such as zonulin (ZO) is essential to the structural and functional intestinal integrity. The first junction associated protein identified was zonula occludens 1 (ZO-1) [14]. Together with ZO-2 and ZO-3 they form a cytoplasmic plaques that function as the structural link between the cytoskeleton and the TJ by binding to both actin filaments and the TJ protein occludin [17]. Acting together, they open...
and close the paracellular junctions. Breakdown of this structure leading to intestinal hyperpermeability (so-called "leaky gut") plays a role in the pathogenesis of several diseases such as allergies, asthma, inflammatory bowel disease [6]. Reduction of the increased permeability is an interesting target for improvement of the clinical status of gastrointestinal diseases.

It is known fact that microbiota, including probiotic bacteria affect the intestinal barrier. Clinical applications of probiotics contain primary and secondary prevention of allergy, treatment of inflammatory bowel diseases, diarrhea, irritable bowel syndrome, and Helicobacter pylori infection [1, 2, 5, 20]. To exert beneficial roles on the host, probiotics regulate intestinal epithelial homeostasis, such as promotion of cell survival and barrier function, improve intestinal microbiota ecology and regulate immune functions [9, 18, 19].

There is only limited knowledge about the effects of primary colonization of originally germ-free (GF) animals with probiotic bacteria on the development of the intestinal barrier. Gnotobiotic mice (GF or colonized with known microbiota) used as the experimental model allow us to elucidate the role of probiotic bacteria in activation of protein production playing role in creation of intraepithelial junctions.

The aim of the study was to evaluate the influence of probiotic Lactobacillus casei and Lactobacillus paracasei strains on expression of ZO-1 in ileum of gnotobiotic mice.

Material and methods

Animals

Two-month-old GF inbred BALB/c mice were used for our experiments. GF mice were born and housed under sterile conditions, and fed a sterile standard pellet diet (ST1, Bergman, Kocanda, Czech Republic, 59 kGy irradiated for 30 minutes) and sterile water ad libitum. Animals were kept in a room with a 12 h light-dark cycle at 22°C. Faecal samples were weekly evaluated for the presence of aerobic and anaerobic bacteria, moulds and yeast by standard microbiological methodology. Control Balb/c mice were associated with physiological pathogen free microbiota and reared in conventional conditions (CONV mice). All experiments were approved by the Animal Experimentation Ethics Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic and conducted in accordance with the “European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS No.: 123)”.

Bacteria

Lactobacilli were obtained from Pure Culture Collection of Technical University, Lodz, Poland (LOCK). The strain identification of L. casei LOCK 0900, L. casei LOCK 0908 and L. paracasei LOCK 0919 was done by 16S rRNA gene sequencing with universal primers and subsequent BLAST software analysis [4]. The strains were kindly provided by prof. Z. Libudzisz, Technical University, Lodz, Poland.

Experimental design

Experimental GF animals were divided into two groups. The first group of mice was kept germ-free (n = 5). The second group of mice (n = 6) was at the age of two months colonized by intragastric tubing with 2 x 10^8 colony forming units (CFU) of equal parts of L. casei LOCK 0900, L. casei LOCK 0908 and L. paracasei LOCK 0919 in 0.2 ml of buffered saline. Faecal samples were weekly evaluated for the presence of lactobacilli using MRS agar (Oxoid, UK). Six weeks after colonization, lactobacilli-colonized mice as well as age-matched GF and CONV (n=5) controls were sacrificed and ileum was removed. Ileum sections were fixed in 4% formalin and embedded in paraffin.

Immunohistochemistry of ZO-1

Four µm sections were de-paraffined and rehydrated using a graded alcohol series. Antigen retrieval was done using 0,01 M citrate buffer pH 6,0. Slides were boiling for 30 minutes and washed in deionized water and then washed in Tris buffer pH 7,4. After incubation for 30 minutes with 10% rabbit serum, primary polyclonal goat anti-mouse ZO-1 (C-19, sc-8146 Santa Cruz Biotechnology) in dilution 1:50 diluted in Tris buffer pH 7,4 were applied for overnight in 4°C. Then slides were washed in Tris buffer and secondary antibody rabbit anti-goat (Jackson ImmunoResearch) in dilution 1:250 diluted in Tris buffer was used. Reactions were developed using AEC-chromogen (Dakocytomation). Finally, slides were counterstained with hematoxylin. Negative controls were also prepared by replacing the primary antibody with Tris buffer.

Morphometric analysis of ZO-1 in epithelial cells

Quantitative analysis of ZO-1 expression was done by morphometric program Cell P. Apical parts of villi were analyzed in magnification X20. Results are expressed as % of area in which reaction with antibody against ZO-1 was visualized.

Results

ZO-1 was detected in enterocytes of all examined groups of animals, and presented cytoplasmic pattern. In CONV mice ZO-1 was steadily diffused in the whole cytoplasm of epithelial cells (Fig. 1A). In GF mice the amount of ZO-1 was much lower in comparison with CONV mice (Fig. 1B), and the location of ZO-1 was similar to that found in CONV mice, i.e. dispersed pattern in cytoplasm of enterocytes. Lactobacillus strains induced expression of ZO-1 in epithelial cells in comparison with GF mice, and location of ZO-1 was similar to that found in CONV mice, i.e. dispersed pattern in cytoplasm of enterocytes. Lactobacillus strains induced production of ZO-1 in most epithelial cells covering intestinal villi. The highest protein expression was observed in enterocytes located in apical part of villi.

Morphometric analysis of ZO-1 expression in intestinal epithelium correlated with results obtain by immunohistochemical studies (Table 1). The largest amounts of ZO-1 were found in epithelial cells of CONV mice (mean 41.58%), and the quantity of this protein was significantly higher in
comparison with GF mice (mean 13.54%). Association of GF animals with *Lactobacillus casei* and *Lactobacillus paracasei* strains induced significantly higher synthesis of ZO-1 in intestinal epithelium in comparison with GF mice (27.4% versus 13.54%).

**Discussion**

In this study we have presented that colonization of GF mice with probiotic *Lactobacillus casei* and *Lactobacillus paracasei* strains up regulates the expression of ZO-1. Previous studies showed that the TJ and adherens junctions proteins such as occludin, ZO-1, E-cadherin and β-catenin are associated with F-actin-reach fractions of an intact epithelium and these fractions correlate well with integrity of TJ [7]. Thus, our observation that probiotic *Lactobacillus* strains induce higher expression of TJ associated protein - ZO-1 in epithelial cells of intestinal villi indicates that those strains are able to affect the gut barrier permeability. The effect of probiotic bacteria on barrier function was observed in many in vitro studies using epithelial cell line monolayer [13, 21]. However, there is limited number of studies presenting in vivo effect of probiotic bacteria on the stabilization of the intestinal barrier under healthy conditions. Such beneficial effect was only described in gnotobiotic mice for *Escherichia coli* Nissle 1917 [15]. Ukena et al have demonstrated that *E. coli Nissle* 1917 is able to mediate up-regulation of ZO-1 expression in murine intestinal epithelial cells at both mRNA and protein levels. As *E. coli* Nissle 1917 was showed to have beneficial clinical effect in patients with ulcerative colitis [8], they also analyzed the impact of per oral administration of those probiotics to Balb/c mice with experimentally induced acute colitis. *E. coli* administration to dextran sodium sulphate treated mice reduced clinical syndromes of colitis, i.e. the loss of body weight and colon shortening as well as the histological features of inflammation. In *E. coli* colonized mice infiltration of the colon with leucocytes was ameliorated. Concomitant administration of *E. coli* Nissle 1917 to DSS treated animals resulted in significant protection against intestinal barrier dysfunction measured by the mucosal uptake of Evans blue in vivo and intestinal epithelial cells isolated from these mice exhibited a more pronounced expression of ZO-1 [15].

*Lactobacillus* strains used in our study represent a probiotic bacteria with possible use in prophylaxis and/or therapy of allergic diseases [4]. Impairment of the intestinal mucosal barrier appears to be involved in the pathogenesis of allergy, especially food allergy. Rosenfeldt et al presented by using lactulose-mannitol test that in children with atopic eczema (dermatitis) the integrity of the intestinal barrier is disturbed [11]. After probiotic (*Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 12246) treatment the lactulose to mannitol ratio was lower and this finding was positively associated with severity of the eczema [11, 12]. We presented that administration of the mixture of *Lactobacillus* strains used in this study to children suffering form cow’s milk protein allergy induced significant decrease in clinical symptoms of atopic dermatitis [3]. As these strains induced production of ZO-1 responsible for epithelial cell integrity, we suggest that probiotic *Lactobacillus* could exhibit beneficial effects on health by modulation of the epithelial barrier function.

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References