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Lactobacillus salivarius reverse diabetes-induced intestinal defense impairment in mice through non-defensin protein☆

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Abstract

Altered intestinal microbiota and subsequent endotoxemia play pathogenic roles in diabetes. We aimed to study the mechanisms of intestinal defense impairment in type 1 diabetes and the effects of *Lactobacillus salivarius* as well as fructooligosaccharides (FOS) supplementation on diabetes-induced bacterial translocation. Alterations in the enteric microbiome, expression of mucosal antibacterial proteins and bacteria-killing activity of the intestinal mucosa in streptozotocin (STZ)-induced diabetic mice and $Ins2^{Akita}$ mice were investigated. The effects of dead *L. salivarius* (2×10^8 CFU/ml) and FOS (250 mg per day) supplementation for 1 week on endotoxin levels and *Klebsiella pneumoniae* translocation were also examined. Finally, germ-free mice were cohoused with wild-type or $Ins2^{Akita}$ mice for 2 weeks to examine the contribution of microbiota on the antibacterial protein expression. STZ-induced diabetic mice developed intestinal defense impairment as demonstrated by decreased mucosal bacteria-killing activity; reduction of non-defensin family proteins, such as Reg3β, Reg3γ, CRP-ductin and RELMβ, but not the defensin family proteins; and increased bacterial translocation. Intestinal bacteria overgrowth, enteric dysbiosis and increased intestinal bacterial translocation, particularly pathogenic *K. pneumoniae* in STZ-induced diabetic mice and $Ins2^{Akita}$ mice, were noted. Treating diabetic mice with dead *L. salivarius* or FOS reversed enteric dysbiosis, restored mucosal antibacterial protein and lessened endotoxin levels as well as *K. pneumoniae* translocation. Moreover, germ-free mice cohoused with wild-type mice demonstrated more intestinal Reg3β and RELMβ expression than those cohoused with $Ins2^{Akita}$ mice. These results indicate that hyperglycemia induces enteric dysbiosis, reduction of non-defensin proteins as well as bacteria-killing activity of the intestinal mucosa and intestinal defense impairment. Reversal of enteric dysbiosis with dead *L. salivarius* or FOS supplementation decreases diabetes-induced *K. pneumoniae* translocation and endotoxin levels through the induction of non-defensin proteins.

Keywords: Fructooligosaccharides; Enteric dysbiosis; Hyperglycemia; Reg3β; *K. pneumoniae*; Endotoxin

1. Introduction

Diabetes mellitus (DM) is a chronic, progressive, medically incurable disease and is characterized by a component of intestinal dysfunction [1]. Increased intestinal permeability, altered intestinal microbiota and subsequent metabolic endotoxemia have been suggested to be causal factors in diabetes [2]. Type 1 DM is referred to as insulin-dependent DM, and type 2 DM is referred to as non-insulin-dependent DM. The incidence of type 1 diabetes is increasing worldwide [3]. Recent studies indicate that an aberrant gut microbiota is also associated with the development of type 1 diabetes [4]. Gut-derived endotoxin is a contributing factor for the development of low-grade inflammation that seems to be a hallmark for the development of type 2 DM [5]. However, the correlation between intestinal defense impairment and endotoxin in diabetes has not yet been defined.

The connection between an altered gut microbiota and metabolic disorders such as obesity and diabetes is well established [6,7]. The intestinal epithelial cells express pattern recognition receptors that protect against microbial invasion while maintaining epithelial barriers in the presence of commensal microflora [8]. Paneth cells are important contributors to the small intestinal antimicrobial barrier through the synthesis and release of antimicrobial peptides and proteins such as enteric α-defensins (called cryptdins in mice), cryptdin-related sequence peptides, lysozyme, Reg3β, Reg3γ, RELMβ and CRP-ductin [9]. Previously, we demonstrated that commensal microflora was critical in maintaining intestinal non-defensin protein expression and the intestinal barrier [10]. Understanding the factors regulating the intestinal barrier function will provide an important

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insight into the interactions between luminal bacteria and low-grade inflammation in diabetes.

Prebiotics are non-digestible short-chain oligosaccharides that can be used as food additives. They work indirectly by altering the gastrointestinal tract environment to favor proliferation of *Bifidobacteria* and *Lactobacillus* [11]. Fructooligosaccharides (FOS) are one of the most extensively studied prebiotics. FOS were used in this study to examine the effect of prebiotics on diabetes-induced intestinal defense impairment. Probiotics are live microorganisms that are ingested to promote beneficial effects on health by altering indigenous microflora. Recently, probiotic supplementation (*Lactobacillus casei* Shirota) has been proved to prevent high fat diet-induced insulin resistance in human subjects but with undefined mechanisms [12]. Probiotic bacteria have multiple and diverse influences on the host. In addition to the inhibition of growth of potential pathogens, probiotics can influence mucosal cell–cell interactions by the enhancement of intestinal barrier function [13]. *Lactobacillus salivarius* is found in the mouth and the small intestine and is believed to play a role in boosting the immune system. Therefore, to study the effect of *L. salivarius* on the intestinal immunity, we used dead *L. salivarius* to examine its effect in enhancing the intestinal barrier function in diabetes.

Diabetes is the major predisposing factor of *Klebsiella pneumoniae*-induced pyogenic liver abscesses (PLA) [14]. However, the alterations of gut microbiome, *K. pneumoniae* translocation and intestinal defense mechanisms in diabetes have not been examined in detail. The aim of this study was to investigate the dynamics of bacterial translocation (BT), changes in the enteric microbiome, expression of mucosal antibacterial proteins and the bacteria-killing activity of the intestinal mucosa in diabetic mice. Finally, the effects of reversal of diabetes-induced enteric dysbiosis with probiotic or prebiotic treatment to restore the intestinal defense mechanism and prevent *K. pneumoniae* translocation were also examined.

2. Materials and methods

2.1. Mice

We obtained specific pathogen-free (SPF) and germ-free (GF) C57BL/6J mice from the National Laboratory Breeding and Research Center (NLBRC, Taipei, Taiwan). $Ins2^{Akita}$ ($Ins2^{Akita}$ mutation mutant) mice (C57BL/6J background) were purchased from the Jackson Laboratory (Bar Harbor, ME). To develop a type 1 diabetic mouse model, male C57BL/6 mice were administered one intraperitoneal (i.p.) injection of streptozotocin (STZ; Sigma-Aldrich) to induce the death of pancreatic β cells. STZ was freshly dissolved in dilution buffer (0.1 M sodium citrate, pH 4.5, titrated with HCl and stored at 4°C) and sterilized by filtration. To induce diabetes, mice were fasted for 24 h and were then administered 0.1 ml of STZ (150 mg/kg of body weight). Mice injected with 0.1 ml of dilution buffer were used as healthy controls. The $Ins2^{Akita}$ mutation results in a single amino acid substitution in the insulin 2 gene that causes misfolding of the insulin protein [15]. Heterozygous Akita mice exhibit severe insulin deficiency. All mice had *ad libitum* access to food and water and were fed a standard laboratory diet (1324 TPF; Altromin; Lage Germany; 11.9 kJ/g, 19% crude protein, 4% crude fat and 6% crude fiber) and the blood glucose levels and body weight were measured prior to STZ injection ($n=50$ in each group). Control mice and diabetic mice after 1 month of induction of diabetes were assayed for BT to MLN, translocation of *K. pneumoniae* to MLN, intestinal permeability, intestinal bacterial content, mucosa bacteria-killing activity and endotoxin levels. The ileum was harvested and assayed for mRNA expression of Reg3β, Reg3γ, CRP-ductin, RELMβ, Defcr-rs-10, Crypt1 and Crypt4 by real-time polymerase chain reaction (RT-PCR) and for protein expression of Reg3β, CRP-ductin and RELMβ by western blotting.

2.2. FOS or dead L. salivarius feeding

To investigate the association of intestinal dysbiosis, down-regulation of antibacterial genes and protein expression with increased BT to MLN, prebiotic (FOS, 250 mg per day; Sigma-Aldrich) or dead *L. salivarius* (CECT5713, 2×10^8 CFU/ml) [16] was given in drinking water to mice for 1 week ($n=50$ in each group). Mice were given *ad libitum* access to water. The water with FOS or bacteria was refreshed every day. The dead bacteria were completely suspended in the water. There was no precipitation of bacteria in the bottle. The control group received drinking water without the addition of prebiotic or dead *L. salivarius*.

2.3. Cohousing GF mice

GF C57BL/6J mice were produced by the NLBRC and transferred to our animal facility in a transfer chamber compatible with the isolator. After arrival, a GF mouse was randomly chosen to cohouse with a wild-type (WT) mouse or $Ins2^{Akita}$ mouse and kept in a sterile lamina-flow hood for 2 weeks ($n=10$ in each group) to examine whether the microbiota of the $Ins2^{Akita}$ could change the antibacterial protein expression of the GF mice.

2.4. Bacterial strains

K. pneumoniae CG43 (a clinical isolate of K2 serotype) was used in this study. The optical density of a suspension of bacteria at 600 nm was determined and compared with a standard growth curve to estimate the number of bacteria per milliliter.

2.5. Bacterial content of intestinal mucosa

The intestine was opened and flushed with 2 ml normal saline. The collected mucosa from terminal ileum was weighed and homogenized in equal volume of sterile saline. Aliquots of the homogenate from each sample were diluted and 100 μl of the homogenates was plated onto different selective agar plates for identification of bacteria-specific strain. The total aerobic bacteria were cultured on tryptic soy broth (TSB) agar plates (DIFCO); the aerobic bacteria *Enterobacteriaceae* and *Enterococcus* were cultured on EMB and m-Enterococcus agar plates. The anaerobic bacteria *Bacteroides*, *Clostridium perfringens* and *Lactobacillus*/*Bifidobacterium* were cultured on BBE, TSC and BIM-25 agar plates, respectively. The plates were examined for colony forming units (CFU) after aerobic incubation at 37°C for 24 h and anaerobic incubation at 37°C for 5 days in an anaerobic chamber.

2.6. Bacterial DNA extraction and quantitative RT-PCR

Bacterial genomic DNA was extracted from terminal ileum using the Qiagen DNA stool kit according to the manufacturer's directions. The number of specific bacterial groups was determined by using StepOnePlus Real-Time PCR System (Applied Biosystems 7300). The sequences of specific bacterial primers were used as previously published [17].

2.7. Translocation of intraluminally injected K. pneumoniae

After anesthetizing the mice, two ends of a 10-cm segment of small intestine were clipped. Into this isolated intestinal segment, 500 μl of normal saline (pH 7.2) containing *K. pneumoniae* (5×10^7 CFU) was injected. After 2 h, MLN was collected, weighed and homogenized in an equal volume of sterile saline, and 100 μl of blood was withdrawn from the heart. Blood or aliquots of the homogenates were plated onto TSB agar plates with or without ampicillin (100 μg/ml). The plates were examined after aerobic incubation at 37°C for 24 h.

2.8. Determination of bacteria-killing activity of the mucosa

The mucosa of the terminal ileum was collected, weighed and thoroughly suspended in equal volume of sterile saline by incubation in a shaker (200 rpm) at room temperature for 30 min. Suspended mucosa was centrifuged at 12,000*g* for 5 min and the supernatant containing antibacterial proteins was collected. A total of 100 μl of bacterial suspension containing *Escherichia coli* (1×10^3 CFU) were added to 400 μl of supernatant and incubated at room temperature for 30 min. Finally, 100 μl of the supernatant was plated onto LB agar plates with or without ampicillin (100 μg/ml).

2.9. Endotoxin levels in portal vein

The portal plasma endotoxin levels were determined using the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GenScript, Cat. No. L00350). The blood samples (0.2–0.3 ml per mouse) were collected and centrifuged at 1500*g* for 15 min at room temperature to recover serum. One microliter of serum was diluted with Limulus Amebocyte Lysate reagent water to prepare the 0.001 EU/ml diluted solution for detection. The diluted samples were assayed for endotoxin levels using the kit according to the manufacturer's instructions, and the absorbance was measured and compared to the standard curve at 545 nm. If the mean absorbance of a sample is x, the endotoxin concentration of the sample will be $(0.2618x-0.0012)$ EU/ml. The recovery rate was calculated by comparing the measured value to the expected amount of LPS in samples. The recovery rate of endotoxin is 40% in our experiments.

2.10. Measurement of intestinal permeability

The assay of intestinal permeability was modified from the method described by Otamiri *et al.* [18]. Two ends of a 10-cm segment of small intestine were compressed with smooth end clips, 50 μl (25 mg/ml) of FITC-dextran (MW 4400; Sigma) was injected into the clipped intestinal lumen and 100 μl of blood was taken from heart at 30 min later. The sample was analyzed for FITC-dextran concentration at the excitation wavelength of 480 nm and the emission wavelength of 520 nm and the concentration of FITC-dextran in blood was calculated.