<Brief Note>

Induction of regulatory T cell differentiation in the large intestine tissue of butyrate-fed mice

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Summary Regulatory T cells are responsible for immunosuppression against self antigens and suppress excessive immune responses, such as autoimmune diseases. Butyrate induces the differentiation of inducible regulatory T cells in the intestinal tract. However, it is unclear whether the expression of the *foxp3* gene, which is a master transcription factor for regulatory T cells, is differentially regulated in the large intestine tissues of mice fed butyrate or butyric acid. In addition, changes in *foxp3* gene expression upon being fed short-chain fatty acids (SCFAs) that contain butyrate produced by *Clostridium* spp. are unknown. In this study, 7-week-old mice were fed with *Clostridium beijerinckii* or a fermented liquid that contained butyrate derived from *C. beijerinckii* ad libitum for 2 weeks. The results showed that *foxp3* gene expression in the mononuclear cells isolated from the large intestine tissue of 7-week-old mice fed ad libitum with a fermented liquid not containing butyrate bacteria was significantly higher than that in the mononuclear cells isolated from the large intestine tissue of those in the control group. It is suggested that regulatory T cells differentiation may be induced in large intestine tissues of mice fed butyrate produced by *Clostridium beijerinckii*, the strain used in this study. Butyrate intake is expected to provide immunological control for many disorders related to autoimmune diseases, such as rheumatoid arthritis, and immune abnormalities, such as allergic diseases.

Key words: Butyrate, *Foxp3*, Regulatory T cells

1. Introduction

The intestinal mucosa has a large surface area, and many immune cells accumulate in the intestinal mucosa as it is constantly exposed to various bacteria and viruses and can be an invasion route of pathogens. The intestinal tract contains approximately 1×10^4 enterobacteria, affecting intestinal tract immunity[1]. Germ-free mice lacking enterobacteria have decreased immune function[2], demonstrating that enterobacteria are important in maintaining immune system homeostasis. Furthermore, metabolites produced by enterobacteria are important for establishing immune tolerance that prevents excessive immune reactions to non-self antigens[3]. Major

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Received for Publication: April 24, 2023
Accepted for Publication: August 3, 2023

enterobacterial metabolites include short-chain fatty acids (SCFAs), such as butyrate, propionate, and lactate. Among these, butyrate is produced during the anaerobic fermentation of plant fibers by enterobacteria. It inhibits histone deacetylase to promote the induction of regulatory T cells responsible for suppressing excessive immune responses[4], suppresses inflammation and the growth of pathogenic bacteria[5], and promotes the absorption of inorganic substances, such as calcium[6].

Regulatory T cells, one of the $CD4^+$ T cell subsets that express the transcription factor *foxp3*, play a role in suppressing immune responses to self and harmless foreign antigens. They suppress the activation of surrounding effector T cells by producing anti-inflammatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β, and are present in tissues throughout the body, accounting for 10% $CD4^+$ cells. The intestinal tract harbors more regulatory T cells than other tissues[7]. The human gastrointestinal tract contains bacteria that induce the differentiation of regulatory T cells,[8] like *Clostridium* spp., which produces butyrate derived from mice, and several reports have discussed the association between butyrate and induction of regulatory T cell differentiation. In this study, we focused on *Clostridium beijerinckii* and butyrate produced by *C. beijerinckii,* which have been scarcely reported, and examined their effects.

In this study, a fermented liquid obtained from fermentation bacteria, including spore-bearing *Clostridium* spp. that produce SCFAs, and natural materials, such as dried soybeans, was mixed with basic feed for experimental mice. By feeding this to mice for a certain time period, we examined its effects on the induction ability of regulatory T cells in large intestine tissue.

2. Materials and Methods

Adjustment of mouse feed (a liquids containing butyrate bacteria and fermented liquids not containing butyrate bacteria)

In this study, we used the *C. beijerinckii* NBRC SIID27451-B11 strain [butyrate bacteria strain; Japanese Patent No. 6964854 (P6964854)], which produces more butyrate and less lactate and acetate than the *C. beijerinckii* type strain (*C. beijerinckii* NBRC 109359T) anaerobic cultured for 72 h at 37°C. The strain was cultured for a specified amount of time under appropriate conditions. The culture was incubated at an average liquid temperature of 38°C, and the culture medium was changed in three stages. During the first stage, soybeans were ground into a paste and used as the culture medium. Subsequently, the solid soybean paste medium was removed, and the liquid medium from the fermentation was drained and used in the next stage. In the second stage, turmeric, wolfberry, and that plant were encapsulated in a rough mesh filter and incubated. The fermentation liquid was filtered through a filter and used for the next stage. In the third stage, a medium containing honey was used. Precipitated components were removed, and the supernatant portion was recovered. The collected culture solution was then cooled after heat treatment, and separated into a butyric acid-containing fermentation solution and a non-butyric acid-containing fermentation solution (Japanese Patent No. 6964854 (P6964854)).

The bacterial load of the liquid containing butyrate bacteria was adjusted to 5.2×10^9 cells / mL. The types and amounts of organic acids in the fermented liquids detected by high-performance liquid chromatography was shown (Table 1). Eluents were detected using a Shimadzu Organic Acid Analysis System. The system comprised a Shim-pack SCR-102(H) column measuring 300 mm × 8 mm (Shimadzu, Japan), and 5 mmol/L *p*-toluenesulfonic acid was used as the eluent. The liquids containing butyrate bacteria and fermented liquids not containing butyrate bacteria were mixed into mouse feed by the following procedure: First, distilled water and starch, which served as a diluting agent, were mixed at 2:1 weight ratio and then heated at 63°C to prepare a paste. Then, a standard solid feed for laboratory animals (standard feed; Oriental Yeast Co., Ltd. MF) was powdered. The liquids containing butyrate bacteria and fermented liquids not containing butyrate bacteria, the paste, and distilled water were added to the standard feed and mixed to homogeneity. The

Table 1 Organic acid type and content in the culture medium and fermented liquid

Organic acid type	Culture medium (mg/L)	Fermented liquid (mg/L)
n-Butyrate	※	2828
Lactate	131	※
Acetate	164	240
Succinate	163	161
Formate	30	49

※Below the lower limit of determination. Lactate: 5 mg/L, n-Butyrate: 10 mg/L

mixture was filled in a syringe for injection, pressure-formed, and then air-dried for 48 h. The feed for the control group was prepared by mixing standard feed with the paste and distilled water. The feed for the group to be fed the liquid containing butyrate bacteria (butyrate-bacteria group) was prepared by mixing standard feed with 5% (w/w) liquid containing butyrate bacteria, paste, and distilled water. The feed for the group to be fed the fermented liquid not containing butyrate bacteria (fermented-liquid group) was prepared by mixing standard feed with 5% (w/w) fermented liquid not containing butyrate bacteria, paste, and distilled water.

Animals

Five-week-old male C57BL/10 mice were purchased from Japan SLC, Inc. The weight of each mouse was measured, and the mice were divided into the control group, the butyrate-bacteria group, and the fermented-liquid group. Each group contained 10 mice each, having the same average body weight. After one week of acclimatization, the feed of the mice was changed from the standard feed to the feed adjusted for each experimental group, and the mice were reared for 14 days. All mice were housed in an environment with a light/dark cycle of 12/12 h and ad libitum access to feed and water. After 14 days, the mice were euthanized; the large intestine tissue was excised, shredded, and homogenized to collect mononuclear cells using the density gradient separation method.

This study was approved by the Institutional Animal Care and Use Committee of Saitama Medical University (approval number: 3033).

RNA isolation

The large intestine was divided into two equal parts. The cells were suspended in Hanks equilibrium salt solution (Nacalai Tesque Co., Ltd.), filtered through a Cell Strainer (Falcon), added to Separation Media (Cedarlane Laboratories Ltd.) and centrifuged at 1200 ×g for 20 min at 4°C. Subsequently, the mononuclear cell layer was removed, and the cells were washed.

Then, gDNA Eliminator (Qiagen) was added to remove unwanted DNA. After adding chloroform, the mixture was vigorously shaken, rested at room temperature for 3 min, and then centrifuged at 4°C and 12000× g for 15 min. The supernatant containing RNA was collected and mixed with the same amount of ethanol. After washing, RNA was eluted with RNase-free water.

cDNA Synthesis

cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) according to the manufacturer's protocol. A mixed solution of 10× RT buffer (Applied Biosystems), 25× dNTP (Applied Biosystems), 10× RT random primer (Applied Biosystems), Reverse Transcriptase (Applied Biosystems), and RNase inhibitor (Applied Biosystems) was prepared. Briefly, 1 μg RNA and water were added to the final reaction volume (20 μL), and the reaction was carried out at 25°C 10 min → 37°C 120 min → 85°C 5 min to synthesize the cDNA. The RNA amount was calculated from the absorbance at 260 nm.

Table 2 List of primers

Gene name		Primer Sequence
mouse *foxp3*	Forward	5′- CAGCTGCCTACAGTGCCCCTAG -3′
	Reverse	5′- CATTTGCCAGCAGTGGGTAG -3′
mouse *GAPDH*	Forward	5′- AAATGGTGAAGGTCGGTGTG -3′
	Reverse	5′- TGAAGGGGTCGTTGATGG -3′

Table 3 *foxp3* gene expression levels in the large intestine mononuclear cells of the group on normal feed, liquid containing butyrate bacteria, and fermented liquid not containing butyrate bacteria. Data are mean±SD (n=9).

	Relative quantitative value
Normal feed	1.00±0.05
Liquid containing butyrate bacteria	1.10±0.333
Fermented liquid not containing butyrate bacteria	2.62±2.02

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR analysis was performed using Step OnePlus™ Real-Time PCR System (Applied Biosystems). The template DNA extracted from large intestine tissue was added to Power Track SYBR Green Master Mixes (Applied Biosystems) containing 400 nM each primer, and PCR was carried out in a total volume of 30 μL. First, the mixture was heated at 94°C for 15 s to dissociate the double strands of the template DNA, and then it was reacted for 40 cycles of 94°C for 15 s, 60°C for 31 s, and 72°C for 30 s, followed by a final extension at 72°C for 6 min. Subsequently, the temperature was raised to 95°C for analyzing the temperature at which the double strands of the amplified product dissociated (Tm). The primers used are shown (Table 2). The $\Delta\Delta$Ct method was used for measuring the gene expression, and GAPDH was used as an endogenous control.

Statistical analysis

Data, analyzed using EasyR[9], are expressed as mean ± standard deviation. After determining the significance of the data by the Grubbs–Smirnov rejection test, a one-way analysis of variance was performed. For the combinations with a statistically significant difference, differences between groups were tested by multiple comparisons using Tukey's method, and the significance level was set at 5%.

3. Results

In this study, mice were fed with solid feed that contained a liquids containing butyrate bacteria and fermented liquids not butyrate bacteria for a certain

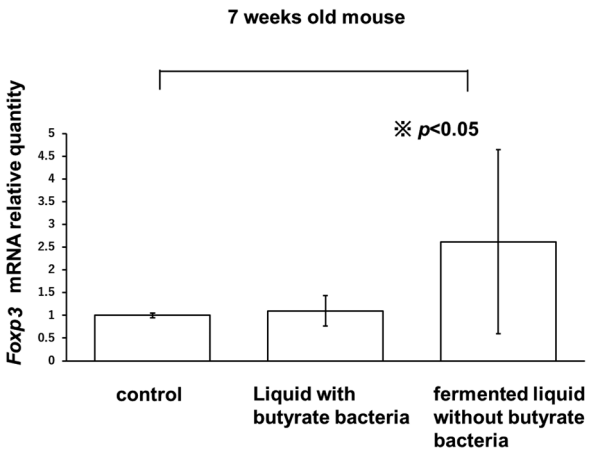

Fig. 1. *foxp3* gene expression levels in the large intestine mononuclear cells of the seven-week-old mice on normal feed, liquid containing butyrate bacteria, and fermented liquid not containing butyrate bacteria. Data are mean±SD (n=9). $p<0.05$ by Tukey's method.

time period. Then, to examine the presence of regulatory T cells in the large intestine tissue, we analyzed RNA extracted from large intestine tissue and measured the expression of the *foxp3* gene, a master transcription factor of regulatory T cells. The expression level of the *foxp3* gene in each experimental group normalized to that in the control group was shown (Table 3(a) and Fig. 1). The expression level of the *foxp3* gene in the butyrate-bacteria group was comparable to that of the control group (p=0.98). However, the expression level of the *foxp3* gene in the fermented-liquid group was 2.62 times higher than that in the control group, showing a statistically significant difference (p=0.02).

4. Discussion

From the perspective of *foxp3* gene expression, the results of our study support the previous report that butyrate enhances the induction efficiency of regulatory T cells in large intestine tissue[4]. Moreover, regulatory T cell induction by SCFAs, including butyrate produced by *C. beijerinckii* NBRC SIID27451-B11 strain, is comparable to that by existing effective butyrate.

As enterobacterial metabolites in the intestinal tract play an important role in maintaining homeostasis, probiotics, in which beneficial living bacteria are ingested to improve the intestinal environment, are expected to be a new basis for health maintenance. However, the individual intestinal environment and enterobacterial microflora vary greatly. Since anaerobic bacteria in the intestine of neonatal mice inhibit the intestinal proliferation of various bacteria[10], orally ingested live bacteria that reach the intestine may be excreted without colonizing in the intestine. The results of our study also showed that feeding a fermented liquid without butyrate bacteria is more effective in inducing *foxp3* gene expression in the mouse large intestine than feeding solid feed that contains a liquid with butyrate bacteria (Table 3 (a) and Fig. 1). While the absorption of butyrate from the intestinal tract is primarily due to spontaneous diffusion, butyrate is also a ligand for the SCFA receptor GPR41 expressed in L cells[11-12]. Interestingly, regulatory T cell differentiation through GPR41 increases in fetuses born from mice on a high-fiber diet during the gestation period exhibit[13]. In this study, we fed mice with 5% butylated starch. Therefore, the intake of butyrate itself, along with dietary fiber, probably increased *foxp3* gene expression through GPR41, promoting regulatory T cell differentiation. In addition, indigestible carbohydrate intake increases butyrate concentration in feces[14], showing that butyrate reaches the large intestine if its absorption in the small intestine is hindered by the presence of ingested food. Therefore, the hindrance of butyrate absorption by spontaneous diffusion might have increased the amount of butyrate acting on GPR41. Interestingly, the proportion of regulatory T cells in the large intestine of germ-free mice was lower than that in the large intestine of the conventional mice, whereas it was not significantly different in the small intestine of germ-free mice[15]. This suggests that the small and large intestines have different induction mechanisms of regulatory T cells, and further studies should analyze the differences in GPR41 expression between the large and small intestines.

foxp3 gene expression in the butyrate-bacteria group was comparable to that in the control group. Factors such as bacterial colonization in the intestinal tract should be considered when examining the butyrate-bacteria group. In this study, however, the butyrate concentration in the intestinal tract in the butyrate-bacteria group might have been lower than that in the fermented-liquid group. The fermentation liquid used in this study, which contained butyric acid bacteria, had a butyric acid content of only 0.12 g per 100 g of fermentation liquid. This was approximately one-fifth of the concentration of the fermentation liquid that did not contain butyric acid bacteria. It was assumed that butyric acid did not reach the large intestine due to the absence of starch. Therefore, the results of this study suggest that oral intake of butyrate for a certain time period affects the intestinal immune system even if butyrate bacteria are not expected to colonize in the intestinal tract. Owing to the abnormal composition of enterobacterial species and decreased bacterial load in patients with inflammatory bowel disease, supplementation of living

bacteria for modifying the intestinal flora has attracted attention. Thus, whether bacterial metabolite intake affects regulatory T cell induction in the colitis model mice developed by Furusawa et al.[4] should also be investigated.

Conflicts of interest

As contracted research, our study received contract and subcontract research funds from Higher Mount, Inc.

Acknowledgements

We express our sincere gratitude to the representative director, Mr. Hirotaka Takayama, for his guidance and support during this study.

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